

ABSTRACT BOOK

SSR 57th Annual Meeting

July 15 - 19, 2024

Convention Centre Dublin
Dublin, Ireland

Evolution of
Reproductive
Sciences

Where Should
We Go?



Focus Session Abstracts

FOCUS SESSION 1 - SEX CHROMOSOMES

S1.2 - Sex Chromosome Gene Regulation in the Male Germline

Julie Cocquet, Inserm, Institut Cochin Paris

In my talk, I will present our recent data on the evolution, regulation and molecular role of sex chromosome-encoded genes.

Sex chromosomes carry many genes which are specifically or highly expressed in male germ cells, and which are required during spermatogenesis. They are under different selective forces than autosomal genes and include a high number of multicopy genes. In mammals, sex chromosome-encoded genes are transcriptionally silenced during meiosis prophase I by a well-described and conserved process called Meiotic Sex Chromosome Inactivation. After meiosis, a significant proportion of sex chromosome-encoded genes is re-expressed or de novo expressed; these genes are co-regulated by the same proteins in mouse spermatids, and their deregulation results in male infertility.

In the last 15 years, we have identified several mouse-specific regulators of XY gene expression, such as the multicopy Y chromosome-encoded gene Sly (Sycp3-like-Y-linked) and its X-linked counterparts, Slx and Slxl1. We have also shown that the regulation of X and Y gene expression is at the centre of a genomic conflict involving 'selfish' genes that favour their own transmission and can affect the sex ratio of offspring.

Using multi-omic analyses, we recently discovered novel regulators of XY gene expression among SLY/SLX/SLXL1 target genes and partners, in particular the histone methyl transferase DOT1L and the family of histone readers SPIN/SSTY. We also characterized the epigenomic signature of male germ cell sex chromatin and identified distinct chromatin-based regulatory mechanisms between the X, the Y and the autosomes.

S1.3 - The Mouse T-Haplotype: Masquerading as a Sex Chromosome

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The *Mus musculus* (house mouse) t-haplotype, a variant of chromosome 17, notoriously defies Mendelian inheritance with up to 99% transmission from heterozygous males. Here, we generated the first haplotype-resolved sequence assembly of a t-haplotype. We discovered the presence of five megabase-sized inversions between the t-haplotype and wild-type chromosome 17. The t-haplotype acquired large ampliconic sequences (>8kb segmental duplications with >99% identity) harboring gene families with known and proposed roles in transmission ratio distortion. Some of the amplified sequences acquired new retrogenes expressed predominantly in spermatids. Targeted deletion of a ~1.8Mb t-ampliconic region reduces t-haplotype transmission, thus supporting a functional contribution of ampliconic gene families to transmission ratio distortion. The t-haplotype shares many similarities with mammalian sex chromosomes, including the suppression of crossing over, acquisition of ampliconic sequences, acquisition of new genes, and genes that bias sex chromosome transmission. We propose that acquiring ampliconic sequence may serve as a broad genomic signature of ongoing or past transmission ratio distortion shaping chromosome evolution.

S1.4 - Mice Sex Ratio Altered by Deletion of Y Chromosome Ssty2 Repetitions

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Preselecting the sex of laboratory rodent benefits both economically and in animal welfare by minimizing the unnecessary sacrifice of surplus males, as seen in mice where this method reduces the number of unused males. We have devised a CRISPR/Cas9 strategy aimed at the Ssty2 gene family, resulting in predominantly female offspring. Ssty2, a multicopy gene situated on the mouse Y long arm (MSYq), is reiterated over 220 times within a 500kb segment housing the acquired genes Sly, Ssty1, Ssty2, and Srsy. These genes, with homologues on the X chromosome, instigate intragenomic conflicts between sex chromosomes. Ssty2 encodes a protein expressed in post-meiotic haploid spermatids, playing a pivotal role in regulating X and Y gene expression during sperm differentiation. To scrutinize the impact of depleting Ssty2 on sex-ratio segregation, we targeted the Ssty2 mouse gene using RNA Cas9 and specific sgRNAs. Transgenic mouse lines were created by microinjecting both RNAs into B6CBAF1 (C57BL/6xCBA) zygotes. Pups underwent genotyping via qPCR to discern a noteworthy reduction in Ssty2 gene copies (Ssty2 KO) in males. From the resultant transgenic, we singled out the line displaying the most substantial decrease in Ssty2 copies, plummeting from 220 to fewer than 50. This line exhibited a sex ratio of 70 to 80% female upon in vivo fertilization (analyzed across more than 20 litters). This ratio remained consistent in IVF, although ICSI failed to induce any alteration in sex ratio. However, we noted a reduction in litter size, declining from an average of 8.5 to 6.4 pups per litter (from a pool of 20 litters analyzed). To assess sex ratio and fertility reduction, sperm samples were obtained from Ssty2 KO and WT males aged 2 to 6 months, collected by vas deferens squeezing and cauda epididymis dissection. Following a 30-minute incubation of sperm in a 0.5mL HTF medium droplet at 37°C, sperm swim-out was employed for characterization. Across each new generation, we observed consistent litter sizes and a skewed sex ratio, with 70-80% of the litter comprising females. Notably, testicle size in Ssty2 KO mice exhibited a slight enlargement compared to controls, while sperm concentration showed a minor decrease with no statistically significant differences compared to controls. However, over 80% of Ssty2 KO sperm displayed abnormal morphology, predominantly manifesting head, neck, and midpiece defects. Additionally, motility analysis (using the Integrated Semen Analysis System, ISAS®, Spain) revealed a shift, with 60.6% of sperm from Ssty2 KO mice exhibiting static motility versus 35.9% in WT controls. Motile sperm were categorized into four clusters via K-means clustering of CASA kinetic parameters, highlighting slow and non-progressive motility patterns for Ssty2 KO sperm and rapid progressive and hyperactive patterns for the majority of WT sperm. RNAseq analysis of KOs and WT testis (n=4 per group) showed deregulation of thousands of transcripts expressed in the testes, highlighting up-regulation of 4400 and down-regulation of 1400 genes in the spermatid stage in the KO, confirming the function of the Ssty and Sly gene families in chromosome-wide-level regulation of gene expression during spermiogenesis. In conclusion, with this CRISPR/Cas9 strategy targeting Ssty2, we have produced mice affected by the ability of Y chromosome-bearing sperm to fertilize both in vivo and in IVF, but not via ICSI, allowing for preferential fertilization of sperm with carriers of the X chromosome and distorting the sex ratio in favor of females.

S1.5 - Functionally Characterizing SYCP3-Related XLR3 Involvement in Male Meiosis and Mouse Spermatogenesis

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We identified the first imprinted genes on the mouse X chromosome, Xlr3b/4b/4c. These imprinted paralogs are part of the Xlr3/4/5 triad, a highly paralogous locus on the X chromosome. The Xlr locus is derived from SYCP3, an evolutionarily conserved component of the synaptonemal complex that forms between homologous chromosomes during meiosis. Each gene of the Xlr3/4/5 triad has 5 copies; 3 that are functional and 2 that are pseudogenes. Of the functional copies, only Xlr3b/4b/4c are imprinted; the rest are biallelically expressed. The imprinted paralogs are imprinted in the brain in a pattern of paternal repression and are also highly expressed in testis. Testis expression increases throughout the first wave of spermatogenesis, coinciding with the events of meiotic prophase I. We have spent the last decade investigating Xlr3's functional role in the testis during male meiosis. To that end, we have generated transgenic mice using siRNA to target the functional copies of Xlr3 in germ cells. Transgenic Xlr3 knockdown mice have sperm production reduced by 50%, possess gross morphological defects in seminiferous tubules, and display a greater degree of meocyte apoptosis compared to wild-type. Immunohistochemistry shows Xlr3 protein localizes to the sex chromosomes during Meiotic prophase I. Using quantitative Real Time-PCR we assessed the expression of several X and Y linked genes, which are normally epigenetically silenced during meiotic progression in testis via Meiotic Sex Chromosome Inactivation (MSCI). We found that Xlr3 knockdown results in disruption of MSCI and therefore inappropriate expression of X and Y-linked genes. We hypothesize that disruption of MSCI leads to the transmission of epimutated sex chromosomes via the sperm, an area we are actively investigating using Oxford Nanopore Technologies methylation sequencing. In summary, Xlr3 is highly expressed in the testis during critical events of meiosis and spermatogenesis and knockdown of it disrupts the events of meiotic sex chromosome inactivation. From these observations we hypothesize that Xlr3 is an epigenetic effector of meiotic sex chromosome inactivation.

S1.6 - Histone Demethylase Activity of UTX contributes to the Epigenetic Regulation of Sexual Spectrum

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Mammalian sex is determined by sexual chromosome and sexual differentiation of gonad is induced by Y chromosome gene Sry and sexual differentiation of other tissues are influenced by hormones secreted from differentiated gonads. In addition, sex is claimed to likely to be spectrum, which is not completely divided into two phenotypes (male/female). One of the contributing factors for sexual differentiation is histone H3 lysine 27 (H3K27) methylation. For example, the lack of M33, a chromodomain-containing protein which recognizes tri-methyl-H3K27 (H3K27me3), in mice resulted in male-to-female sex reversal (Katoh-Fukui, et al., 1998). H3K27 could be an important epigenetic regulator of not only sexual differentiation but also sexual spectrum. UTX (ubiquitously transcribed tetratricopeptide repeat X) is a H3K27me2/me3 demethylase coded on the X chromosome and its counterpart UTY (ubiquitously

transcribed tetratricopeptide repeat Y) is the Y-linked homologue. While the demethylation activity of UTX is high, that of UTY is quite low. Furthermore, Utx is a gene which escapes from X chromosome inactivation. Therefore, the demethylation activity of H3K27 in male and female cells could be different. In this study, to examine if demethylation activity of UTX contributes to sexual differentiation and/or sexual spectrum, down-regulation of demethylation activity of UTX in cells as well as in mice was accomplished by amino acid substitution using genome editing. After confirmation of amino acid substitution in vitro, mutant mice that have the amino acid substitution and the downregulation of demethylation activity of UTX were generated. As a result, sex of each delivered mouse was compatible with own sex chromosome combination, suggesting that demethylation activity of UTX could not be important for sex determination. When mutant gonads-mesonephros were observed, no morphological conversion of gonads was detected. However, RNA-Seq analyses between gonads-mesonephros isolated from 13.5 dpc control and mutant embryos revealed intriguing differences. In PCA analysis, the male mutant gonads-mesonephros was slightly approached from male control to female control gonads-mesonephros, suggesting that the demethylation activity of UTX might affect sexual spectrum. Moreover, hundreds of genes were differentially expressed by the down-regulation of UTX demethylation activity. In particular, Cyp17a1, an enzyme regulating steroid synthesis pathway, was significantly downregulated in mutant male gonads-mesonephros as compared to wildtype. These results suggest that demethylation activity of UTX could contribute to the construction of sexual spectrum in gonads-mesonephros. Sexual spectrum not only in other developing tissues but also in adult tissues constructed by the demethylation activity of UTX could be revealed by analyzing this mouse model.

S1.7 - Unraveling the Regulatory Significance of Sperm Hypomethylated Regions in Mammalian Germline Development

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Mammalian gametes exhibit significant sexual dimorphism, both morphologically and molecularly. Molecular asymmetry of the germline genome results in unequal gene expression between the sperm and the oocyte, which is the basis for the requirement of biparental genetic contributions in mammalian reproduction. DNA methylation, a key epigenetic mark, shows distinct patterns in gametes, with mature sperm being highly methylated (>90%) compared to oocytes (~40%). Curiously, the highly methylated sperm genome is interspersed with methylation valleys, termed sperm hypomethylated regions (HMRs). Sperm HMRs are systematically protected from DNA methyltransferase's access during de novo methylation period in prospermatogonia through enrichment of H3K4me3. To date, the regulatory importance of sperm HMRs remain unexplored. Tissue-specific hypomethylation signatures are known to often correlate with binding of developmentally relevant transcription factors (TFs) that drive stage-specific transcriptional milieu. We conducted motif discovery analysis using HOMER to identify potential transcription factor binding sites within sperm HMRs, genome wide. Top hits for the enriched motifs within sperm HMRs include binding sites for DMRT6, NRF1, and FOXO1; TFs that are known to be important for cell-state transition during spermatogenesis. Additionally, using whole testis mRNA samples, we have identified several sperm HMRs within gene-bodies that can serve as methylation dependent alternative transcriptional start sites (TSSs). Our results provide preliminary evidence for the functional role of sperm HMRs as non-canonical promoters, possibly used at various stages of male germline development. Our ongoing investigation involves 5'-end RNA analysis across purified spermatogonia, spermatocyte, and spermatid populations to identify differential TSSs usage at distinct

stages of spermatogenesis. By annotating these TSSs, we aim to identify stage-specific enrichment of TF binding motifs. Ultimately, our goals are to uncover novel regulators of spermatogenesis progression and ascertain the functional significance of sperm HMRs.

FOCUS SESSION 2 - JOHN EPPIG SESSION ON OOCYTE QUALITY AND FUNCTION

S2.1 - Implication Of NAD⁺ Metabolism in Ovarian Aging

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Nicotinamide adenine dinucleotide (NAD⁺), a crucial coenzyme in cellular redox reactions, is a regulator of many crucial cellular functions, including metabolism, post-translational protein modification and DNA repair, essential for maintaining tissue and metabolic homeostasis, as well as healthy aging. Ovarian aging is recognized as a natural process characterized by a decline in follicle number and function, resulting in decreased fertility and consequence on general health. Promising results are presented for strategies using NAD⁺ precursors to promote NAD⁺ biosynthesis, which could substantially improve oocyte quality and alleviate ovarian aging. Nevertheless, the specific mechanisms responsible for these effects remain unclear and necessitate further clarification. This presentation aims to summarize the current research status of NAD⁺ boosting in ovarian aging and present recent findings by my research group regarding age-related alterations of NAD⁺ biosynthetic and consuming pathways in the ovary. By employing mouse oocytes and human granulosa cells, we focused on gene networks involved in the regulation of NAD⁺ metabolism. Genes related to the control of NAD⁺ availability were significantly impacted by reproductive aging in mouse oocytes and modulated by NAD⁺ boosting agents with Sirt1 serving as a central node of a top Ingenuity Pathway Analysis (IPA)-derived molecular network. Consistently, NAD⁺ precursors were efficient in restoring NAD⁺ levels in senescent human granulosa cells and modulating SIRT1 expression. Overall, our results provide new insights in the mechanisms underlying the efficacy of NAD⁺ supplements and offer a comprehensive view of unanswered questions related to the therapeutic potential of NAD⁺ boosting in countering ovarian aging.

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S2.2 - NEDDylation: An Essential Regulatory Pathway for Oocyte Quantity and Quality

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Reproductive decline and infertility in women are largely caused by diminished oocyte quality and quantity. The mechanisms underlying this decline are not yet fully understood but are attributed to various factors, including mitochondrial dysfunction, improper oocyte-granulosa cell communication, and changes to proteostasis. Neddylation, a ubiquitin-like post-translational modification that affects protein homeostasis and quality control, is implicated in various health conditions, but its role in oocyte biology remains unknown. Using CRISPR/Cas9 gene editing, we generated a novel conditional allele of Uba3, an essential enzyme in the NEDDylation pathway, and crossed it with the oocyte-specific cre driver line, Gdf9-icre. Continuous mating for 6-months of Uba3 conditional knockout ("cKO") females failed to produce any pups, indicating that NEDDylation is required for female fertility. By histological and morphometric analysis, 12 week old Uba3 cKO ovaries are nearly devoid of follicles - severe premature aging phenotype. Prior to ovulation, GV oocytes of Uba3 cKO mice have significantly higher levels of reactive oxygen species (ROS). Transmission electron microscopy showed that mitochondrial morphology was significantly altered in Uba3 cKO oocytes. Additionally, Uba3 cKO oocytes lack well-developed transzonal projections (TZPs), structures that span the zona pellucida to allow for bidirectional communication with the oocyte and surrounding somatic cells. By transcriptomic analysis of fully grown GV oocytes, we discovered changes in genes related to cell adhesion as well as cell projection. Thus, NEDDylation appears to be essential for maintaining oocyte organelle integrity and overall female fertility. Further studies will seek to elucidate NEDDylation targets as mediators of reproductive disease and oocyte aging. These studies were supported by NIH grants T32HD098068 and R21HD109807.

S2.3 - Validating Hyper7 Sensors for Quantitative Hydrogen Peroxide Measurement in Bovine Oocytes

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Reactive Oxygen Species (ROS), particularly hydrogen peroxide (H₂O₂), are recognized for their role in inducing oxidative damage in oocytes, impacting critical biomolecules and oocyte quality. However, the physiological roles of H₂O₂ and other ROS during mammalian oogenesis are less understood, hindering advancements in fertility treatments and underscoring the need for tools to study ROS dynamics. This study aims to bridge this gap by taking advantage of a novel sensor capable of monitoring H₂O₂

fluctuations in real-time and surpassing traditional detection methods. Specifically, a novel experimental set-up employing Hyper7, an ultrasensitive genetically encoded fluorescent ratiometric sensor, was developed to directly quantify mitochondrial and cytosolic H₂O₂ in bovine oocytes. This sensor consists of a circularly permuted Yellow Fluorescence Protein (cpYFP) integrated into the OxyR domain from *Neisseria meningitidis*, which is sensitive to basal H₂O₂ concentrations. cpYFP has two excitation peaks at 405 and 499 nm and emits at 516 nm. Changes in the sensor oxidation state result in ratiometric changes with decreases in the excitation at 405 and increases at 499 nm upon oxidation. Thus, sequential excitation at 405 and 488 nm allows analysis of the F₄₈₈/F₄₀₅ ratio that serves as an index of H₂O₂ content. In our experiments, validating these sensors involved exposing bovine oocytes to a prooxidant challenge with 100 mM tert-Butyl hydroperoxide (t-BOOH). mRNAs encoding for the mitochondrial and the cytoplasmic-targeted Hyper7 sensors (Hyper7-MLS and Hyper7-NES, respectively) were obtained by in vitro transcription of the respective plasmids (Addgene). A total of 172 and 189 cumulus-oocyte complexes (COCs) were collected from abattoir-derived ovaries, freed from cumulus cells, and microinjected with Hyper7-MLS or Hyper7-NES mRNA. Diverging from the conventional In Vitro Maturation (IVM) protocol applied to COCs, a tailored protocol was adopted to align with specific research goals. These adjustments included denuding oocytes to facilitate microinjection, followed by a pre-IVM phase with 3-isobutyl-1-methyl-xanthine (IBMX) to delay meiotic resumption, critical for mRNA translation. The microinjected denuded oocytes (DO) were cocultured with intact COCs to support maturation. Finally, DOs were separated from the co-cultured COCs and imaged for 60 minutes, either under control IVM medium or ROS inducing conditions (IVM medium enriched with t-BOOH). Imaging was conducted using a Nikon ECLIPSE Ti2-E microscope with a Yokogawa W1-SoRa spinning disk, 405 and 488 nm lasers, and a multi-band filter. A high-resolution camera and a temperature-controlled CO₂ chamber enabled time-lapse imaging. Exposures were set at 3 and 2 seconds for 405 and 488 nm, respectively, capturing emissions at 516 nm. Image analysis was performed using NIS ELEMENTS AR software. Both Hyper7-MLS and Hyper7-NES sensors demonstrated their ability to detect H₂O₂ levels within oocytes, revealing distinct fluorescence patterns. A significant increase in ratio values was observed after 20 minutes for Hyper7-MLS and 25 minutes for Hyper7-NES (Friedman test). This suggests phototoxicity as an exogenous causal factor during prolonged imaging. Nevertheless, oocytes treated with tBOOH exhibited significantly higher ratio values than those in the control group (two-way ANOVA), affirming the sensors' capacity to quantify elevated H₂O₂ levels. This validation step marks the first application of Hyper7 probes in mammalian oocytes, promising a novel tool for investigating the role of H₂O₂-mediated mechanisms in oocyte biology while posing the critical need to manage phototoxicity in subsequent research. Funded by H2020 MSCA-ITN-ETN n.860960 (EUROVA) and SEED2019 UNIMI N.1250 (cROSS-Talk)

S2.4 - GDF9:BMP15 Complexes Identified in Ovarian Extracts

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GDF9 and BMP15 are oocyte-secreted growth factors (OSFs) crucial for fertility. GDF9 and BMP15 are closely related members of the TGF β superfamily and interactions between these factors regulate their bio-potency and control species-specific ovulation rates. However, the precise molecular interactions mediating these functions remain unclear; whether they function as two distinct homo-dimers or via formation of a hetero-dimer (cumulin), as occurs with some other members of the TGF β superfamily. To investigate GDF9:BMP15 interactions in vivo, specific GDF9 and BMP15 ELISAs were applied to high salt

extracts of human granulosa cells (hGC) which capture OSFs, resulting in a high correlation of GDF9 to BMP15 within samples ($r=0.83$), which was likewise observed with whole mouse ovarian extracts (mOE, $r=0.89$). A cumulin ELISA, which detects heterodimeric GDF9:BMP15, was also developed with low cross reactivity with GDF9 and BMP15 homodimers. Nonetheless, high correlations were also observed when comparing cumulin with GDF9, or cumulin with BMP15 levels, in hGC ($r=0.93$, 0.95 ; respectively) and mOE ($r=0.99$, 0.88 ; respectively), indicating the presence of complexes of GDF9:BMP15, which could be aggregates of GDF9:BMP15 and/or aggregates of GDF9 and BMP15 homodimers. Gel filtration of mOE showed highly correlated patterns of GDF9, BMP15 and cumulin immunoactivities by ELISAs, with major peaks at $>600\text{kDa}$ (postulated to be aggregates) and $40\text{-}100\text{kDa}$ forms ($r=0.86\text{-}0.91$). Western Blots on SDS-PAGE of hGC and mOE with monoclonal antibodies raised to GDF9 and BMP15, under non-reducing, and reducing alkylating conditions, revealed a range of molecular weights from 15kDa - 280kDa . Quantifying these profiles of GDF9 and BMP15 immunoactivity using densitometry across the molecular weight range showed high correlations ($r=0.86\text{-}0.95$), suggesting tight physical interactions between them. The close association between GDF9, BMP15 and GDF9:BMP15 heterodimeric immunoactivities under different fractionation conditions suggests the presence of high molecular weight heterodimeric forms (including possible tetramers and/or aggregates). These data suggest that the oocyte secretes GDF9:BMP15 complexes which can be detected across a broad molecular weight range as different precursor and processed forms of GDF9, BMP15 and cumulin. This knowledge is important for understanding the mechanisms of oocyte paracrine signalling to granulosa cells.

S2.5 - Multiple Human Induced Pluripotent Stem Cell Lines Differentiated with a Ligand-Based Approach Recapitulate Granulosa Cell Development and Express Steroidogenic Pathway Genes.

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Sex determination is a developmental process that defines the gonad as a testis or ovary, and influences sex hormone production. While key signaling events are well conserved across mammals, there can be differences between species. Therefore, there is a need to develop a human model to fully understand this critical process in humans. We have developed a ligand-based differentiation protocol for human induced pluripotent stem cells (hiPSCs) to generate granulosa-like cells (GLCs). Additionally, hiPSC-derived GLCs could become the foundation for a cell-based hormone replacement therapy that recapitulates cyclicity, physiologic levels of hormones, and production of peptide hormones that are not included in currently available hormone replacement therapies. We aim to gain further understanding of the key factors and culture conditions necessary for functional GLCs that produce ovarian hormones, which includes maintained expression of FOXL2. We used our monolayer differentiation protocol that

includes WNT-agonist CHIR99021 treatment for 2 days, followed by a combination treatment of BMP4, FST, and DKK1-inhibitor Gallocyanine for 3 days. We have tested our protocol on multiple hiPSC lines. Expression of pluripotency marker POU5F1 is reduced during differentiation, while expression of the intermediate mesoderm markers PAX2, LHX1, and WT1 and bipotential gonad marker GATA4 peak at day 2. Peak expression of the bipotential gonad marker NR5A1 occurred at day 5. Interestingly, FOXL2 expression peaks at day 2 and drops by day 5. In one line, FOXL2 protein peaks at day 2 by Jess Western while there is the highest percentage of FOXL2-positive cells at day 5 by immunofluorescence. Additionally, as FOXL2 is required for expression of aromatase and HSD17B1, we are using this model to interrogate NR5A2 upstream of FOXL2 and control hormone production. CYP19A1 and HSD17B1 along with AMH are induced during this differentiation protocol, suggesting these GLCs can be steroidogenic and produce peptide hormones. Additionally, scRNA-seq analysis of the GLCs is underway to identify types of granulosa cells produced by this protocol. We are continuing to optimize GLC culture conditions to generate hormones. Put together, the data suggests that cells differentiated with our protocol show promise as a human model of ovarian development and a foundation for a personalized cell-based hormone replacement therapy. This work is supported by the NIH/NICHD R01HD104683 (MML, HK), HuBMAP U01HD110336 (MML, RG, HK, LL), and Gesualdo Family Research Scholar (MML) and NIH/NCI training grant T32 CA009560 (HK).

FOCUS SESSION 3 - FERTILIZATION AND EMBRYOGENESIS

S3.1 - Identification of Key Embryonic Factors Facilitating Successful Reprogramming of Rabbit Induced Pluripotent Stem Cells and Production of Systemic Chimeras

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Pluripotency refers to the remarkable capability of a stem cell to generate all cell types found in mature organisms. This extraordinary property underpins the potential of induced pluripotent stem cells (iPSCs) as valuable tools in regenerative medicine, disease modeling, and organoid production. However, achieving and maintaining pluripotency is complex, as it is a metastable state influenced by various factors that impact subsequent outcomes.

Two distinct pluripotent states have been identified: a naïve state and a primed state, mirroring the pre- and post-implantation epiblast compartments in mice. Naïve iPSCs exhibit superior developmental plasticity, clonal propagation capacity, and potential for germline transmission. Notably, studies in mice have demonstrated that only naïve iPSCs are capable of forming chimeras, contributing to the development of all tissues and organs in both fetal and adult organisms following injection into blastocysts or morulae. In humans, achieving naïve pluripotency remains a challenging task compared to mouse models.

Likewise, achieving genuinely naïve pluripotent stem cell lines in rabbits remains elusive, as rabbit iPSCs (rbiPSCs) exhibit characteristics more aligned with the primed state of pluripotency. To transition rbiPSCs to a naïve pluripotent state, our initial objective was to decipher the molecular signature of naïve pluripotency in vivo. Utilizing single-cell 10x Chromium RNA-sequencing of rabbit embryos, we analyzed gene expression profiles related to pluripotency regulation, cell-cycle machinery, chromatin modification,

and metabolism. This comprehensive analysis produced a transcriptomic, epigenetic, and metabolic map of the pluripotency continuum in rabbit preimplantation embryos.

Despite the closer evolutionary relationship between rabbits and mice compared to primates, our research revealed that the transcriptome of rabbit epiblast cells exhibits similar features to both mice and primates. Additionally, we identified novel molecular markers indicative of naïve pluripotency, which could facilitate the isolation of naïve-state pluripotency in rabbit PSCs.

Building on this foundation, we conducted an unbiased screening of a cDNA library encompassing 36 pluripotency factors. Through this screening, we identified KLF2, ERAS, and PRMT6 as key factors whose overexpression enabled self-renewal in a naïve-specific culture medium. Reprogrammed cells exhibited transcriptomic and epigenetic characteristics of naïve pluripotency, including reactivation of the second X-chromosome.

Remarkably, a subset of reprogrammed cells expressing CD75 at high levels demonstrated exceptional embryonic colonization potential, with nearly all blastocyst embryos being colonized three days post-injection. Furthermore, some of these colonized embryos produced chimeric fetuses and newborns, where PSC cell contribution significantly impacted ectodermal, mesodermal, and endodermal derivatives.

These groundbreaking findings mark the successful generation of rabbit PSCs capable of producing systemic chimeras comparable to mouse PSCs, representing a significant advancement in the field. Funding acknowledgments: ANR-18-CE13-023, ORYCTOCELL; ANR-21-CE20-0018-01, CHROMNESS), FRM DEQ20170336757, ANR-11-INBS-0009, INGESTEM; CRB-Anim, ANR-11-INBS-0003, IHU-B CESAME ANR-10-IBHU-003, LabExs ANR-10-LABX-73, REVIVE; ANR-10-LABX-0061, DEVweCAN; ANR-11-LABX-0042, CORTEX, and the University of Lyon ANR-11-IDEX-0007.

S3.2 - From Mechanisms to Millions: A Complex Systems Approach to Understanding Sperm Capacitation

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Fertilization is the first step in mammalian development, and the spermatozoon that fertilizes the egg is ultimately ‘selected’ from a winnowing process that reduces hundreds of millions of candidate cells to one. Suboptimal fitness of the fertilizing sperm critically impacts developmental success through allelic ratio distortion, early embryonic mortality, or implantation failure. Mammalian spermatozoa are characterized by remarkable variation in morphology and physiological heterogeneity as they undergo ‘capacitation’—an essential, signal-dependent, maturation process involving changes in motility, post-ejaculatory lifespan, and molecular recognition patterns that facilitate locating, binding, and penetrating the egg. Though much progress has been made toward defining the molecular signaling mechanisms that control capacitation, the dynamics of these mechanisms are highly heterogeneous among populations of spermatozoa, making fitness predictions with single-cell resolution extremely difficult. A ‘rethink’ of the role of gametic variation in male fertility is essential for developing ‘high-precision’ diagnostic strategies and selection techniques for assisted reproduction. However, these advancements remain limited by a lack of quantitative multi-scale models and accompanying practical measurements capable of discerning the relative ‘fitness’ of sperm within a sample. Here we leverage tools from complex systems science to explore the fundamental relationships between sperm heterogeneity, selection, and fitness using multi-agent modeling, microscopy, and spectral flow cytometry. We explore

the heterogeneous signaling dynamics and physiological responses that underly capacitation at a scale of $\sim 10^5$ - 10^6 cells/sample and suggest mathematical/statistical methods for quantifying the effects of sperm selection. These approaches may be readily combined with computer vision and artificial intelligence for real-time assessment of sperm fitness during selection (e.g., during intracytoplasmic sperm injection), and have broad implications for clinical, agricultural, and basic research applications.

S3.3 - GATA3 Ablation Reduces Ovine Epiblast Survival and Proliferation at Post-Hatching Stages

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The first lineage differentiation events in mammalian embryos lead to the formation of the epiblast, which generates the embryonic disc (ED), and to the hypoblast and the trophoctoderm (TE), which form the extraembryonic membranes. GATA3 drives TE fate and regulates other TE genes such as CDX2, although its ablation does not abrogate blastocyst formation in mice or bovine. However, it has been reported that GATA3 deletion downregulates the core pluripotency factor NANOG in bovine blastocysts. The aim of this study was to analyze the role of GATA3 during ovine embryo development by evaluating the developmental potential of GATA3 KO embryos generated by CRISPR. In vitro matured oocytes were microinjected with Cas9 mRNA and a sgRNA against GATA3 (C+G), or with Cas9 alone as microinjection control (C). Microinjected oocytes were fertilized and cultured in vitro up to Day (D) 8 (in SOF medium) or D12 (in N2B27 medium from D6/7), when pictures were taken and embryos were fixed; or up to D6, when blastocysts from C+G group were transferred to synchronized recipient ewes and subsequently recovered at embryonic day (E) 13. Embryos were immunostained to detect GATA3 and CDX2 (TE), SOX2 and NANOG (ICM/epiblast), SOX17 and FOXA2 (hypoblast). Embryo genotyping in C+G group was performed by deep sequencing. Blastocyst rate was similar between the group containing GATA3 KO embryos (C+G) and the control group (C) ($25 \pm 1.2\%$; vs. $26.9 \pm 2.4\%$; mean $\pm$ s.e.m; 7 replicates; t-test, $p > 0.05$). In C+G group, 42/85 (49.4%) blastocysts were KO and lacked GATA3 protein. No significant differences were detected in the number of CDX2+, SOX2+, NANOG+ or total cells between KO and WT blastocysts (CDX2+: 80.9 ± 22.6 vs. 94.5 ± 18.6 ; SOX2+: 14 ± 2.1 vs. 13.5 ± 1.6 ; NANOG+: 7.8 ± 2.4 vs. 7.7 ± 1.6 ; total: 138.6 ± 16 vs. 157.7 ± 13.9 ; mean $\pm$ s.e.m; Mann-Whitney test, $p > 0.05$). Embryo survival from D6/7 to D12 in vitro was similar between C+G and C groups ($93.6 \pm 2.4\%$; vs. $85 \pm 5.4\%$; mean $\pm$ s.e.m; 5 replicates; t-test, $p > 0.05$). In C+G group, 37/86 (43%) D12 embryos were GATA3 KO. No differences were found in embryo area between KO and WT embryos (0.39 ± 0.04 vs. 0.4 ± 0.04 mm²; mean $\pm$ s.e.m; Mann-Whitney test, $p > 0.05$), but the number of embryos containing epiblast cells (13/37 [35.1%] vs. 21/34 [61.8%]) and SOX2+ cell number (8.5 ± 3 vs. 13.7 ± 3) were significantly reduced in KO embryos (Chi-square and Mann-Whitney test, respectively, $p \leq 0.05$). No significant differences were observed at E13 in conceptus length between KO (5/16 [31.2%]) and edited non-knockout (ENK; 11/16 [68.8%]) recovered from the same embryo transfer (6.1 ± 1.9 vs. 16.8 ± 3.7 mm; mean $\pm$ s.e.m; t-test, $p > 0.05$). Complete hypoblast migration was observed in 4/5 (80%) KO and 11/11 (100%) ENK conceptuses. Remarkably, the number of conceptuses developing an ED was significantly lower in the KO group (2/5 [40%] vs. 11/11 [100%]; Chi-square test, $p \leq 0.05$). In conclusion, GATA3 is dispensable for ovine blastocyst formation and extraembryonic membranes development, but plays a role in ED formation during conceptus elongation. Work supported by StG 757886-ELONGAN and PID2021-122153NA-I00.

S3.4 - Human Embryonic Mesoderm Originates from GATA6+/CDH1+ Embryonic Disc Cells

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Research on in vitro gametogenesis (IVG) aims to reconstitute germ cell development using human pluripotent stem cells (hPSCs) in culture. IVG offers a family planning tool enabling infertile individuals, same-sex couples, and cancer patients to produce biological offspring. The advancement of IVG, however, relies on the faithful recapitulation of human gametogenesis in vitro beginning first with germ cell precursors, known as human primordial germ cell-like cells (hPGCLCs). In this study, we evaluate a novel self-instructing human stem cell-based embryo model (SCBEM) that differentiates hPSCs into hPGCLCs and other key embryonic somatic lineages. We utilized a Geltrex-incorporated Medium Overlay (GiMO) model in the chemically defined media (E8) to promote a predicted and ordered series of differentiation events. Time-lapse imaging over the first 72-hours of GiMO reveals the formation of disc-like structures, that develop a cavity and initiate an epithelial to mesenchymal transition (EMT) followed by the expansion of a mesenchymal population. Using single cell RNA-Seq, our data demonstrates that these events are associated with the specification of amniotic ectoderm-like cells (AMELCs), primordial germ cell-like cells (PGCLCs), primitive-streak-like cells (PS-like cells), mesoderm-like cells (MeLCs) and endoderm-like cells. Thus, the GiMO model represents a non-integrated peri-gastrulation model of human embryo development. To spatially position the specification of major embryonic lineages relative to the EMT event, we performed immunofluorescence and time-lapse imaging. Using a TFAP2A-tdTomato human embryonic stem cell line reporter, our data demonstrates that within the first 24 hours of GiMO, a subpopulation of CDH1+ disc cells repress NANOG and up-regulate the lineage marker TFAP2A. Next, TFAP2C/SOX17/NANOG+ PGCLCs and GATA6+ MeLCs are specified within the CDH1+ discs. Following EMT, specified PGCLCs down-regulate CDH1 and migrate out of epithelial discs along with GATA6+ MeLCs and a small portion of SOX17+ endoderm-like cells. Blocking BMP4 in the first 24 hours of GiMO leads to loss of TFAP2A, and failure of the disc cells to differentiate into PGCLCs and MeLCs. In contrast, blocking EMT does not affect lineage patterning in the disc, but does prevent the expansion of GATA6+ mesoderm. In summary, the GiMO model successfully recapitulates key peri-gastrulating events that take place in the posterior developing human embryo and reveals that EMT is not necessary for the specification of PGCLCs or MeLCs.

S3.5 - Molecular Profile of Founder Primordial Germ Cells in the Bovine Embryo

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Primordial germ cell (PGC) specification is one of the earliest cell fate decisions of the mammalian embryo, occurring just before the onset of gastrulation. Despite recent advances in other species, PGC specification and development in cattle have not been described in detail. The goal of this study was to establish when and where bovine germ cells are formed, and the characteristics that differentiate PGCs from other cells in the embryo. We hypothesized that PGC specification would be underway at day 16 of

bovine embryonic development, and that founder PGCs would have a similar molecular identity to those of other mammals that form a bilaminar embryonic disc (ED), including humans, pigs, and rabbits. The experimental objectives were to investigate the presence of the founder population of PGCs in day 16 bovine embryos by immunolocalization of early germ cell markers and to establish the molecular signature of early PGCs via single-cell RNA-sequencing (scRNA-seq) at day 20-22 of embryonic development. Day-7 in vitro-produced blastocysts were transferred into recipients (6 embryos/cow) and recovered at day 16 of development by uterine flushing. Embryonic discs and bordering trophoctoderm were isolated under a stereomicroscope and prepared for cryosectioning. Serial sagittal sections of the whole ED were stained for markers of the epiblast (OCT4), including its anterior (SOX2) and posterior (TBXT) regions, and the earliest germ cell markers (SOX17, PRDM1, TFAP2C). Co-immunolocalization of proteins was analyzed using confocal microscopy. At day 16, bovine embryos spanned the pre- to early-primitive streak stages, all with anterior-posterior axes established. In line with other mammalian species, TFAP2C expression was seen in the trophoctoderm, while PRDM1 and SOX17 were expressed in the hypoblast. A founder population of PGCs was identified in the posterior epiblast (TBXT+) region in three of the five day-16 embryos analyzed, by the unique co-expression of PRDM1 and TFAP2C/SOX17 in cells of the epiblast. These cells were also positive for OCT4, but expression in the epiblast was not exclusive to PGCs at this stage. Moreover, expression of SOX2 (a PGC marker in rodents) was not observed in bovine PGCs, consistent with other ED-forming species. Next, two female bovine embryos were harvested at day 20-22 after artificial insemination for scRNA-seq. Dissociated cell suspensions were processed using the 10x Genomics platform. Data analyses were performed using the CellRanger pipeline and Seurat package of R. Of the 13,719 cells that were mapped from both samples, a subpopulation of 10 cells co-expressing OCT4, SOX17, PRDM1, and TFAP2C was identified. Further analysis of this population revealed expression of other pluripotency (NANOG, KLF4) and early germline (NANOS3, SOX15) genes, as well as markers of DNA demethylation (TET1, KDM4C) and replication (PCNA, MCM2), and onset of migration (KIT, EpCAM), with some heterogeneity. As expected, these cells did not express later migratory (DMRT1, CDH5), germline commitment (DAZL, DDX4), or meiotic (STRA8, SYCP1) genes. These findings indicate that around day 20, bovine PGCs are in the pre-early migratory stages. This work provides the earliest observation of PGCs within bovine embryos at day 16 of development and the first transcriptomic profile of day 20-22 PGCs in cattle, demonstrating a conserved program in germline emergence with other species that form an ED. Such information will be useful for the development of new assisted reproductive technologies and highlights the cow as a valuable model for human development. Financial support: USDA-NIFA Predoctoral Fellowship.

S3.6 - From Cleavage to Pregnancy Loss: Insights into the First Mitotic Division and Its Implications for Developmental Potential, Pregnancy, And Early Embryonic Loss in Horses

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The first mitotic division requires a series of orchestrated events, the success of which is pivotal for embryonic survival and development. Errors occurring during preceding steps such as asynchrony of pronuclear (PN) formation, malfunction of PN migration and apposition, and failure to unify the parental genome can lead to embryonic arrest. Additionally, chromosomal missegregation originating from the first mitotic division is a known cause of aneuploidies and mosaicism, greatly impacting embryo survival and implantation. Characterization of cleavage patterns in humans and non-primate humans has linked abnormalities during this event to a higher incidence of aneuploidies and reduced implantation

potential. In this study, we examined the cleavage patterns of in vitro produced equine embryos (IVP) by time-lapse imaging (TL) to assess abnormalities and their correlation with blastocyst formation potential (BP), pregnancy potential (PP) and early embryonic loss (EEL). The time of arrest was classified as early (EA, up to 8-cells, embryonic genome activation), mid (MA, from 16 cells to morula stage), or late (LA, after morula compaction). Statistical analysis was performed using the Chi-square comparison test with JMP®, Pro17 (SAS Institute Inc., Cary, NC, 1989–2023), with a significance threshold of $p < 0.05$. TL videos were retrospectively analyzed from transferred embryos with known pregnancy outcomes ($n=70$), as well as arrested embryos from the same ICSI session ($n=114$). Cleavage was classified as abnormal (AC) and normal (NC). The overall incidence of AC was 41%, with no difference between arrested embryos and embryos that developed to the blastocyst stage (40% and 41%; $p > 0.05$). The proportion of embryos that were arrested at different stages of culture were similar between the embryos with and without AC (EA 52% and 54%; MA 15% and 16%; LA 33% and 29% for AC and NC, respectively. $p > 0.05$). The initial pregnancy diagnosis at 14 days post-transfer showed a significant difference in pregnancy rate between embryos with AC and NC (62% and 83% respectively; $p < 0.05$). The EEL rate (before 42 days of gestation) was significantly higher in embryos with AC (53%) than the embryos with NC (20%; $p < 0.05$). Our results suggest that while abnormalities during cleavage in IVP embryos are not predictive of blastocyst formation, they are highly correlated with the likelihood of establishing a pregnancy and, more importantly, with an increased risk of EEL. Abnormal cleavage patterns do not influence the time of embryonic arrest and appear to allow for embryonic formation at a similar rate as normally cleaved embryos. The abnormal first mitotic division might be attributed to errors that do not prevent blastocyst formation but are incompatible with development after a higher degree of growth and tissue specialization, leading to EEL. Overall, we demonstrated that TL evaluation is a practical and non-invasive technique for assessing cleavage patterns in the horse readily applicable to a clinic setting.

FOCUS SESSION 4 - ENVIRONMENTAL EFFECTS

S4.1 - Heat Stress-Induced Impacts on Ovarian Steroidogenesis, Folliculogenesis and The Response to Ovotoxicant Exposures.

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Heat stress (HS) occurs when exogenous and metabolic heat accumulation exceeds heat dissipation; a thermal scenario that impairs fertility by altering steroidogenesis, impeding implantation and inducing early embryonic loss. In mammals, HS rapidly disrupts intestinal integrity and insulin is increased despite reduced feed intake. Thus, HS causes basal endocrine disruption. In pre- and post-pubertal female pigs, HS alters important ovarian molecular pathways including insulin, phosphatidylinositol-3 kinase, steroidogenic and inflammatory signaling. We also discovered altered abundance of ovarian chemical biotransformation proteins due to HS, which led to the hypothesis that HS may render females more sensitive to environmental chemical exposures. Post-pubertal female pigs were synchronized in estrus using altrenogest for 14d and maintained in thermal neutral (TN) or HS conditions and exposed to vehicle control (cookie dough) or perfluorooctanoic acid (PFOA; 70 ppt) for 29d. Five days before the end of the thermal conditions, altrenogest was withdrawn to initiate the follicular phase of the estrous cycle. Tissues were collected pre-ovulation, and PFOA content was measured in serum, liver, ovary and follicular fluid. LC-MS/MS was performed to identify both the basal proteomic impacts of HS, and those induced by PFOA exposure in TN and HS pigs. Estradiol and progesterone were quantified to determine ovarian endocrine disruption due to HS basally and in combination with PFOA. These discoveries demonstrate how HS and toxicants, independently and in combination, impact ovarian physiology.

S4.2 - Environment Matters – An Exploration of the Effects of Environment on Sexual Differentiation, Sex Determination and Reproduction

Neil J. Gemmell, University of Otago

It is well established that a variety of environmental factors affect sexual development, sexual phenotype, and reproductive function. In fishes, the focus of much of my research, these environmental effects are often pronounced, readily perturbed or manipulated, and thus amiable for study. Here, I will highlight work from my lab that is revealing the genetic underpinnings of socially controlled sex change in fish and some new insights into the genetic, epigenetic and environmental factors that influence sperm function and male reproductive success.

S4.3 - Developmental Effects of in Utero and Lactational Exposure to Perfluoroundecanoic Acid (PFUdA) in a Mouse Model

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Perfluoroundecanoic acid (PFUdA) is a long-chain perfluorocarboxylic acid (LC-PFCA). PFUdA is increasing in the environment but to date has been detected at very low frequency and low concentrations in Canadian retail food. LC-PFCAs are bioaccumulative, persistent, subject to long-range transport, and have widespread and increasing concentrations in Arctic wildlife. PFUdA has been identified in freshwater fish in the Great Lakes and the St Lawrence River. Health Canada has detected it in samples of beef, milk, french fries, marine fish, canned fish and shellfish at very low concentrations (e.g., < 0.3 ppb); in addition, PFUdA is present in elevated levels in country foods, which are essential to Indigenous food security, survival, and culture. A study of pregnant Inuit women found maternal serum levels that were three times greater than those measured in women in the overall Canadian population during the same period. Studies have shown PFUdA can cross the placental barrier to fetuses, is present in breast milk, and is associated with disruption of hormone metabolism in utero. A previous study using rats found decreased body weight and decreased weight gain in pups exposed to maternally toxic doses during gestation. However, several markers of development classically affected by endocrine disrupting chemicals (EDCs) were not assessed in this study. Therefore, this study aims to assess these developmental milestones, which could be affected by EDCs, in a mouse model of human health exposure and effects to PFUdA. Twenty breeding pairs of CD-1 mice per dose group (0, 0.3, 1.0, 3.0, 10.0 mg/kg BW/d) were fed AIN 93G diet ad libitum 2 weeks prior to mating, during mating, gestation, lactation, and to a total of 90 days on dose. To assess endocrine disrupting effects in the F1 generation due solely to in utero and lactational exposure, pups were put on control diet upon weaning at PND21. The F0 animals in the 10.0 mg/kg Bw/d dose group exhibited signs of systemic toxicity after 14 days on diet and were removed from the study. Histopathologic analysis determined that the females were no longer cycling and males had increased testicular degeneration, macrovacuolation, and apoptotic bodies. Pups were assessed at PND4, 7, 21, 42, and 90 for developmental effects including body weight (BW), anogenital distance (AGD), crown rump length (CRL), mammary gland development, vaginal opening (VO), and preputial separation (PPS). A dose-related response to in utero and lactational exposure to PFUdA was evident. Pups exposed to PFUdA had lower BWs and inhibited weight gain in the 1.0 and 3.0 mg/kg BW/d dose groups starting at PND4, which persisted and became more apparent as the pups aged. Only male pups exhibited lower BW at the 0.3 mg/kg BW/d dose group, beginning at PND4. The

CRL in female pups was increased in the 1.0 and 3.0 mg/kg BW/d dose groups; whereas the CRL was significantly increased in male pups at all doses at PND4. AGD was decreased in both male and female pups. Both VO and PPS were delayed in pups exposed to PFUDa. Our findings provide evidence that PFUDa has endocrine disrupting potential in offspring exposed in utero and during lactation at doses where no maternal systemic toxicity is observed in a mouse model.

S4.4 - Reproductive Seasonality Influences Follicle Dynamics and the Ovarian Extracellular Matrix Structural Properties in Ewes

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Successful ovarian function, which includes follicular development, ovulation, and corpus luteum formation and regression, requires extensive cyclical tissue remodeling provided by its extracellular matrix (ECM). The ovarian ECM was long considered to be a passive structure that provides anchoring and mechanical support to the cells, but is now increasingly recognized as a pivotal element in the multi-directional communication that occurs between components of the ECM, stroma, theca, granulosa cells, and the oocyte. As new roles for the ECM emerge, in-depth examination of its changes across suspension and resumption of ovarian activity during seasonal breeding may reveal key aspects for improving oocyte yield and embryo production for agricultural purposes or restoring ovarian function in women. The aim of the present study was to investigate the influence of reproductive seasonality on follicle dynamics and the ovarian ECM properties. Ovaries from ewes were obtained from the local abattoir, with eight of them collected during the breeding season (November/December) and eight during anoestrus (April/May). Ovaries were weighed and measured, and ovarian morphometric parameters as well as ECM-related proteins expression (collagen, fibronectin, laminin) were quantified using histological and immunohistological analysis. Ovarian weight and volume were higher during the breeding season compared to the non-breeding season ($P < 0.001$). No difference was observed in the proportion of pre-antral follicles and earlier stages of development, but the percentage of antral follicles was significantly increased during anoestrus compared to oestrus ($P = 0.028$) while 6 out of the 8 ovaries (75%) collected during oestrus contained a corpus luteum, compared to none in those retrieved during anoestrus ($P = 0.010$). In addition, the tunica albuginea, the connective tissue layer located beneath the surface epithelium, thickened during the anoestrus season, rising from $110.3 \mu\text{m} \pm 14.9 \mu\text{m}$ during the breeding season to $121.3 \mu\text{m} \pm 18.8 \mu\text{m}$ during anoestrus ($P = 0.215$). Stromal collagen, fibronectin and laminin content were also quantified. Both collagen and fibronectin peaked during anoestrus compared to oestrus (collagen: $87.0\% \pm 3.3\%$ positive area versus $72.4\% \pm 5.4\%$, respectively [mean $\pm$ SD], $P < 0.0001$; fibronectin: $33.5\% \pm 4.3\%$ positive area versus $25.4\% \pm 6.0\%$, $P = 0.007$), while laminin slightly dropped ($12.4\% \pm 2.8\%$ positive area versus $16.1\% \pm 3.4\%$, $P = 0.032$). Collagen fibres thickness and packing density were further assessed and showed a significant increase of both thick and mid-sized fibres during anoestrus compared to the breeding season ($P = 0.003$ and $P < 0.001$, respectively), while the proportion of thin fibres dropped ($P < 0.001$). Given that collagen confers tissues with rigidity, these results suggest a stiffer microenvironment during anoestrus, corroborated with a thicker tunica albuginea. Similar features have been reported in patients with polycystic ovary syndrome and are likely to prevent ovulation. Ongoing experiments are assessing the mechanical properties of the ECM at each reproductive phase to clarify the consequences of such structural changes. These results offer a first look at the dynamic remodeling that occurs across the reproductive season, modulating the microenvironment to potentially regulate follicle growth and ovulation. They also reinforce the use of

the ovine model, that share physiological characteristics with women, as a model to provide comparative insights into ovarian biology. This study was supported by a grant from the Medical Research Council (MR/T025654/1 to E.E.T.).

S4.5 - Early Preimplantation Alcohol Exposure: Developmental Effects and Persistent Epigenomic and Cognitive Implications

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Prenatal alcohol exposure is intricately linked to the Developmental Origins of Health and Disease (DOHAD) hypothesis, positing that early-life environmental factors, including maternal alcohol consumption, can influence fetal development and contribute to long-term health outcomes. Prenatal alcohol exposure is known to alter the epigenetic profiles of cells during development and is a key factor in the molecular basis of Fetal Alcohol Spectrum Disorder (FASD) etiology. However, the impact of unique prenatal alcohol exposure occurring early in pregnancy, specifically during the preimplantation period, remains poorly investigated. To address this gap in knowledge, we have developed a preclinical mouse model involving a single binge-like prenatal alcohol exposure during the preimplantation development stage at the 8-cell stage (embryonic day 2.5; E2.5). This particular period is critical for reprogramming DNA methylation marks needed for proper embryonic development. We hypothesize that early embryonic alcohol exposure will introduce long-lasting errors in DNA methylation and transcriptomic profiles, leading to adverse effects on both the future development of the embryo and placenta. In our study, we found that following early embryonic alcohol exposure, mid- and late-gestation embryos showed increased morphological defects, such as growth delays or brain malformations. Using Methyl-Seq and RNA-seq methodologies, we discerned sex-specific genome-wide DNA methylation dysregulations and identified changes in gene expression within the forebrain of ethanol-exposed embryos at both E10.5 and E18.5. These gene expression changes affected crucial genes associated with brain development and function, such as *Arx*, *Dcl2*, *Dlx2*, *Lhx6*, and *Sox6*. Additionally, we observed delayed migration of GABAergic interneurons in the embryonic brain at E15.5, along with altered gene expression in these specific cells. When we investigated the long-term impact of preimplantation alcohol exposure on cognitive functions, we discovered sex-specific behavioral alterations related to sociability and short-term memory in post-natal mice. Furthermore, our genome-wide analysis of the placenta's methylome and transcriptome at late gestation revealed significant sex-specific dysregulations in regions or genes associated with placental functions directly affecting embryo development, such as the regulation of growth (e.g., *Clstn3*, *Socs5*, *Tro*) and serotonin uptake (e.g., *Itgb3*, *Slc22a2*, *Snca*). Using a computational machine learning approach, we successfully used placental DNA methylation profiles as a molecular signature for early prenatal alcohol exposure, effectively distinguishing between control and ethanol-exposed embryos. In summary, our findings provide valuable insights into the epigenetic mechanisms underlying FASD phenotypes resulting from prenatal alcohol exposure during early embryonic development. Additionally, we have demonstrated the potential utility of the placenta as a molecular diagnostic tool for the early detection of FASD at birth. This progress serves as a proof of

concept for molecular detection of alcohol exposure in newborns with invisible phenotypes during gestation, aiming to support and improve outcomes for diagnosed individuals.

S4.6 - Altered DNA Methylation Causes Transgenerational Effects on Female Fertility and Reproductive Development Following Exposure to an Estrogenic Endocrine Disruptor

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University of Melbourne

Prenatal exposure to the estrogenic endocrine disruptor Diethylstilbestrol (DES), a prescribed drug given to millions of pregnant women worldwide, led to multiple reproductive effects in the exposed offspring. We have previously shown that DES exposure causes transgenerational impacts on female mice, affecting fertility, timing of puberty and anogenital distance. To determine the mechanism behind these transgenerational impacts, we investigated DNA methylation following DES exposure and sought to correlate methylation changes to the phenotypes previously described. Female mice were exposed to DES during gestation and oocytes collected from control, F1, F2 and F3 DES exposed generations. Bisulfite converted libraries were generated using the post bisulfite adaptor tagging (PBAT) method. Whole genome bisulfite sequencing analysis was conducted to identify differentially methylated regions (DMRs) between treatment and control oocytes. We identified 140 DMRs that were common across F1, F2 and F3 oocytes exposed to a high dose of DES, and 80 DMRs that were common in F1, F2 and F3 oocytes when exposed to a low dose. Genes associated with these DMRs are involved in female infertility, breast cancer, ovarian disorders and metabolic function, phenotypes all previously described both in our DES mouse model and in human DES descendants. These results reveal a direct correlation between prenatal DES exposure, transgenerational changes in the female germ line methylome and phenotypes observed in DES descendants. These results are significant for the more than 50 million DES descendants who may still be at risk of developing reproductive abnormalities and provide insights into the mechanisms through which estrogenic endocrine disruptors are impacting human fertility and reproductive health.

FOCUS SESSION 5 - EVOLUTION

S5.1 - Pervasive Relaxed Selection on Spermatogenesis Genes Coincident with the Evolution of Polygyny in Gorillas

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Gorillas have a polygynous social system in which the highest-ranking male has almost exclusive access to females and sires most of the offspring in the troop. Such behavior results in a dramatic reduction of sperm competition, which is ultimately associated with numerous traits that cause low efficacy of gorilla spermatogenesis. However, the molecular basis behind the remarkable erosion of the gorilla male reproductive system remains unknown. Here, we explored the genetic consequences of the polygynous social system in gorillas by testing for altered selection intensity across 13,310 orthologous protein-coding genes from 261 Eutherian mammals. We identified 578 genes with relaxed purifying selection in the gorilla lineage, compared with only 96 that were positively selected. Genes under relaxed purifying selection in gorillas have accumulated numerous deleterious amino acid substitutions, their expression is biased towards male germ cells, and are enriched in functions related to meiosis and sperm biology. We tested the function of gorilla relaxed genes previously not implicated in sperm biology using the *Drosophila* model system and identified 41 novel spermatogenesis genes required for normal fertility. Furthermore, by exploring exome/genome sequencing data of infertile men with severe spermatogenic

impairment, we found that the human orthologs of the gorilla relaxed genes are enriched for loss-of-function variants in infertile men. These data provide compelling evidence that reduced sperm competition in gorillas is associated with relaxed purifying selection on genes related to male reproductive function. The accumulation of deleterious mutations in these genes likely provides the mechanistic basis behind the low efficacy of gorilla spermatogenesis and uncovers new candidate genes for human male infertility.

S5.2 - How Meiosis Can Shape Sex Chromosome Evolution and Beyond

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The generation of haploid gametes is a hallmark of sexual reproduction. And this is accomplished by a complex, albeit tightly regulated, reductional cell division called meiosis. Although meiosis has been extensively studied in eutherian mammal model species, our understanding of the mechanisms regulating chromosome synapsis, recombination and segregation during meiosis progression is still incomplete especially in non-eutherian mammals. And this is especially relevant for genome evolution as a whole and sex chromosomes, in particular. Thus, exploring the similarities and differences of chromatin folding across evolutionary lineages is central to developing an appreciation of the dynamics of genome function. I will discuss on the dynamics of chromatin remodelling during meiosis and how the evolution of heteromorphic sex chromosomes (mainly the Y) has been shaped by the intricacies of the meiotic programme. Understanding the meiotic programme in vertebrate and invertebrate species will be crucial to further understand the plasticity of the rise and fall of heteromorphic sex chromosomes.

S5.3 - Oviductin Plays a Crucial Role in Establishing the Species-Specificity of Mammalian Fertilization

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The zona pellucida (ZP) is a thick extracellular coat present on all mammalian eggs, composed of three or four glycoproteins. It plays a crucial role in facilitating the binding of sperm to the oocyte and determining species-specific fertilization. However, we have previously observed that bovine ovarian oocytes can be fertilized by spermatozoa from another species of the same order Artiodactyla (sheep, goats, and dolphins). In this study, we aimed to determine if bovine oocytes can be fertilized by spermatozoa from other mammalian orders and explore the underlying reasons for this phenomenon. Our objectives were: firstly, to analyze whether spermatozoa from Carnivora (cats), Primates (humans), and Rodentia (mice) could penetrate intact bovine oocytes; secondly, we investigated the role of oviductal fluid and bovine, murine, or human oviduct-specific glycoprotein (known as OVGP1 or oviductin) in modulating the species-specificity of bovine and murine oocytes. In order to quantify the capacity of sperm from heterologous species to bind and penetrate the bovine or murine ZPs, we developed the "empty zona penetration test" (EZPT), where the cytoplasm of oocytes was removed by micromanipulation, and empty ZPs were used to assess the mentioned parameters. Our findings

revealed that spermatozoa from all the mammalian species tested were capable of penetrating the ZP-intact of bovine or murine ovarian oocytes, resulting in the formation of hybrid embryos. However, upon contact with bovine oviductal fluid or bovine OVGP1, the bovine ZP acquired its species-specificity, allowing only bovine sperm to penetrate. The same effect was observed in mice with oocytes obtained from the ovary (not species-specific) or from the oviduct (species-specific). Similar results were also found by using EZPT analysis. Using bovine and murine ZPs obtained from IVM oocytes, as well as sperm and OVGP1 from bovine, murine, and human sources, we conducted incubations involving various combinations. These included combinations of ZP and OVGP1 from the same species with sperm from different species, ZP and sperm from the same species with OVGP1 from different species, or OVGP1 and sperm from the same species with ZP from different species. We observed that when ZP and OVGP1 are from the same species and did not match the sperm species, penetration was completely blocked. However, when ZP and sperm (excluding OVGP1) or OVGP1 and sperm (excluding ZP) were from the same species, penetration and binding were reduced by approximately half, though not completely blocked, achieving better results when OVGP1 and sperm came from the same species. Microstructural analyses using a scanning electron microscope revealed that the appearance of ZP changes substantially when OVGP1 is added to it, with OVGP1 observed to bind to the ZP, showing a relevant reduction of the porous structure. To investigate the involvement of sialic acid in the interaction between bovine sperm, OVGP1, and ZPs, we subjected both ZPs obtained from ovarian oocytes and same ZPs after exposure to OVGP1 to neuraminidase digestion before EZPT. Our findings revealed that sperm penetration was completely blocked in both cases. Additionally, the binding of sperm to the ZP was reduced by 70%. This highlights the role of sialic acid in mediating both non-specific and species-specific sperm binding to ZP or ZP-OVGP1 respectively. These results suggest that mammalian fertilization is a non-specific process until the oocyte acquires its species-specific fertilization capacity within the oviduct through the incorporation of specific OVGP1 to the ZP.

S5.4 - A Newly Identified Cilium in Meiosis Mechanically Controls Chromosomal Pairing and Germ Cell Morphogenesis in Zebrafish, Mouse, And Human Fetal Ovaries

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Developing eggs and sperm must accurately organize their chromosomes for successful fertilization through the program of meiosis. A hallmark of meiosis is chromosomal pairing, which requires telomere tethering and rotation on the nuclear envelope via microtubules, driving chromosome homology searches. Telomere pulling towards the centrosome forms the “zygotene chromosomal bouquet” configuration, which is conserved from yeast to mammals. However, despite its discovery in 1900, how the cytoplasmic counterparts of bouquet formation are mechanically regulated was unknown. We

discovered the “zygotene cilium” in zebrafish - a previously unrecognized cilium in oocytes that completes the bouquet cytoplasmic machinery of chromosomal pairing and extends through the germline cyst, a conserved cellular hub of developing oocytes. Using ciliary mutants and live manipulations, we demonstrated that the cilium anchors the centrosome to counterbalance telomere pulling. The cilium is essential for bouquet and synaptonemal complex formation, cyst morphogenesis, ovarian development, and fertility. Here, we demonstrate that the cilium is conserved in zebrafish male gametogenesis, and also in mice and human meiosis. First, using ciliary mutants, we found that the cilium is essential for seminiferous tubule morphogenesis, as well as male meiosis and fertility in the zebrafish. Next, by conditional genetics in mice we induced ciliary loss in the germline of fetal ovaries and testes. Ciliary loss resulted in failure of synaptonemal complex assembly as well as infertility, demonstrating that the zygotene cilium is essential for meiosis in mammals, consistent with our findings with the zebrafish. Strikingly, we found that the cilium is also conserved in prophase oocytes within human fetal ovaries at 16 weeks post-fertilization, identified by specific markers labelling ciliary structures, and substantiated by expression of signature ciliary genes in female fetal germ cell single-cell transcriptomics data. Our work has uncovered the novel concept of ciliary functions in meiosis that appears to be conserved across vertebrates, as well as a new cellular paradigm that cilia can control chromosomal dynamics. In humans, early oogenesis in the developing fetus determines by birth the number and quality of eggs for the entire lifespan. Defects in chromosome organization and in early oocyte and ovarian development are the leading cause for miscarriages and infertility, but the mechanistic defects are unknown because we lack a fundamental understanding of these early processes. We propose that the zygotene cilium could provide a major advance in our understanding of the mechanisms of human germ cell development and offers a new platform to explain overlooked mechanisms of infertility. Funding: ERC CoG ZygoCiliaAct – 101089065.

S5.5 - Rewired Regulatory Pathways Involving Retrotransposons Impact Reproduction and Preimplantation Development

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Throughout evolution, viruses and mobile elements have hijacked cellular systems to copy/paste themselves throughout mammalian genomes, giving rise to Retrotransposons. While 2% of the human genome encodes proteins, ~48% is so-called “Junk DNA” that originates from these ancient insertions. Rare cases where insertions disturb important gene functions has led to an “US versus THEM” model. However, this model may be an over-simplification, failing to reflect symbiotic relationships developed through co-evolution. Most retrotransposons are inactive through constant epigenetic surveillance or degraded through mutation, but a subset retains partial function and can influence nearby gene expression and function by acting as enhancers, repressors, lncRNAs and in some cases, fusion with a nearby protein coding gene called “Chimeric Transcripts” that completely change the original function. Disruption of retrotransposons results in embryonic lethality, suggesting unknown but essential functions. The "ModzLab" studies the phenomenon of Retrotransposon Reactivation that is essential and intentional in early embryonic development. While silenced in most tissues, unintentional retrotransposon reactivation is observed in cases of epigenetic breakdown associated with aging, disease, and cancer. Using novel bioinformatics tools to map these extremely repetitive elements and a

highly efficient electroporation-based embryo editing method we call CRISPR-EZ, we generated 7 retrotransposon deletions mouse lines, each impacting different aspects of development and reproduction, ranging from fertilization failure, loss of totipotency, embryonic metabolism defects (impact in vitro culture) and failed implantation, often leading to both embryonic and maternal death. Once regarded as parasitic DNA, we are now realizing retrotransposons can be co-opted by the host as transient but essential gene regulatory elements in development, reproduction, and disease.

S5.6 - Dampened HPG Axis Activity and Improved Reproductive Lifespan in Dummerstorf Superfertile Mouse Lines

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Research in the field of reproductive biology is largely based on informative mouse models. Almost all of these mouse models (~99%) exhibit an unfavourable reproductive phenotype - these mouse models are subfertile or infertile in one or possibly both sexes. In contrast, the number of animal models that show an enhanced reproductive phenotype is extremely rare. Nevertheless, these mouse models could be very informative in identifying mechanisms and pathways associated with enhanced fertility. We analyse two Dummerstorf outbred mouse lines that have been selected for high fertility for 200 generations (fertility lines 1 and 2; FL1, FL2). In both mouse lines, the number of offspring per litter almost doubled (from 11.5 (ctrl) to 20.6 (FL1) and 21.4 (FL2)), without the offspring showing any signs of growth retardation compared to animals from a non-selected control line. We analysed the lifetime fecundity of all three lines and found significant differences. Control and FL1 females gave birth to up to 10 litters, whereas FL2 only gave birth to a maximum of six litters per female per lifetime. In a first set of experiments, we analysed the ovarian phenotype of FL1 females. We noticed a reduced concentration of FSH, both on serum hormone levels (-60%) as well as in pituitary gene transcription (-90%). In line with this decreased FSH levels also hypothalamic GnRH transcription was decreased by 70% compared to ctrl. Gene expression analysis in ovaries revealed that multiple genes associated with follicular development, such as *Lhcgr*, *Esr1*, *Kit* or *Foxl2* are differentially expressed in FL1 mice. Holistic gene expression analysis in ovarian granulosa cells further identified a differential regulation of multiple genes involved in follicular atresia. These data suggest that the phenotype of FL1 is at least partly due to attenuated follicular atresia leading to increased follicular survival. More successful folliculogenesis is in turn associated with higher ovulation rates (from 13 (ctrl) to 24 (FL1) oocytes per cycle). There is evidence that the complex interplay of endocrine and molecular changes leading to enhanced follicular development in FL1 females is associated with good reproductive longevity and health. Several genes associated with reproductive lifespan, such as *Per2*, *Kl*, *Tex14* and *Rora*, are differentially expressed in the ovaries and granulosa cells of FL1 mice. These data suggest that the unique ovarian phenotype of FL1 females selected for high fertility provides valuable approaches for the search for molecular mechanisms predisposing to increased fertility.

FOCUS SESSION 6 - EPIGENETICS

S6.1 - The Role of Early Life Nutrition on the Physiological and Molecular Control of Sexual Development in Calves

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Improved nutrition during early life can advance the ontogeny of sexual development in cattle. While the precise molecular mechanisms are unknown it is likely that epigenetic modifications such as DNA methylation induced through prevailing dietary regimen modulate gene expression in key metabolic, neural and reproductive tissues. As such, epigenetics can provide valuable insights into the effects of environmental stimuli, such as nutritional or disease management in livestock. Moreover, the integration of epigenomic information with other 'omics' data (transcriptomic/proteomic) can deliver a more holistic overview of the molecular response to specific environmental conditions. For example, an examination of differential methylated regions in the testes parenchyma of bull calves fed either an enhanced or moderate diet between two and either 12 or 18 weeks of age highlighted an effect on methylation sites located near the RPS6KA2 gene. Moreover, co-regulatory network analysis conducted on the same tissue using proteomic (12 weeks) and transcriptomic (18 weeks) data indicated the importance of the RPS6KA2 gene as a hub node in both analyses. Specifically, a co-regulatory network of the testes proteome highlighted interactions between the RPS6KA2 protein with other proteins involved in relevant biological pathways, including MAPK family signalling cascade, TCA cycle, fatty acid degradation, oxidative phosphorylation, and insulin signalling pathway. Whilst a cluster of co-expressed genes containing RPS6KA2 derived from the testes transcriptomic data revealed an association between the cluster with stage of spermatogenesis, suggesting a clear influence of dietary intake on the abundance and interactions of this protein. Further insights into diet induced modulation of the underlying molecular mechanisms leading to advanced sexual development will be discussed.

S6.2 - Epigenetic Dynamics in the Mammalian Germline

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The Stewart-Morgan lab studies epigenetic dynamics in mammalian germ cells. A cell's epigenome is composed of its cell type-specific chromatin landscape and acts to preserve cell identity. In mammals, primordial germ cells (PGCs), the precursors to male and female gametes, experience extensive epigenetic reprogramming. This reprogramming erases the chromatin landscape that defines somatic cells and establishes an epigenetic "blank slate" on which the sperm and oocyte epigenomes are later established. We are interested in the mechanistic basis for reprogramming and its function in gametogenesis. Here we present our recent work in these areas, focusing on the dynamics of histone post-translational modifications and DNA methylation.

S6.3 - Parallel Genotyping and DNA Methylation Profiling from Trophoblast Biopsies of Bovine IVP Embryos

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Elite bovine genotypes are increasingly selected using single nucleotide polymorphisms (SNP) markers from microsurgical biopsies of in vitro produced (IVP) embryos. Compared to in vivo-derived embryos, IVP is associated with higher pregnancy loss and large offspring syndrome (LOS) in ~10% of calves, especially females. We hypothesized that DNA methylation changes in trophoblast biopsies can predict LOS. To identify LOS biomarkers, we developed a protocol for analysing genome-wide DNA methylation without compromising SNP-based genomic selection. First, we quantified the number of trophoblast cells from grade 1-2 blastocysts on D7, using either a microblade or a micropipette, by staining DNA with Hoechst 33342. Next, we established a protocol for parallel processing of genomic DNA for SNP detection and genome-wide DNA methylation from biopsies. Biopsies were collected in DLB lysis buffer (Qiagen) and either whole genome amplified (WGA) from half or whole samples for SNP analysis on a 50k microarray or processed as half-biopsies for whole genome bisulfite sequencing (WGBS). From a single WGA embryo, we produced both methylated and unmethylated controls (MCO and UCO) by treatment with or without M.SssI, respectively. Four half biopsies served as treatment samples. Samples were sequenced using DNBSEQ-G400 PE100 at ~1X coverage and aligned to the Bos Taurus genome (ARS-UCD1.2-bosTau9) using BWA-meth. Methylation was determined (MethylDackel), differentially methylated regions (DMRs) analysed (Metilene) and overlapped (bedtools) for 37,191 CpG islands and 69 candidate DMRs from our own meta-analysis. The average number of cells per blade and needle biopsy was 15 ± 6 (n=46 biopsies) and 19 ± 3 (n=26), respectively. There was no loss in average SNP call rates or the proportion of samples meeting the quality threshold of 0.85 (Q85) from half vs whole biopsies, using either blade (0.93 vs 0.91, with 89% vs 90% >Q85, respectively) or needle (0.94 vs 0.95, with 95% vs 94% >Q85, respectively) sampling. The average call rate from needle-biopsied commercial embryos was 0.94 ± 0.09 with 95% >Q85 (n=279 half biopsies). From an average 36 ± 3 million reads obtained (1.3X coverage), both controls (MCO: 98.5%; UCO: 87.0%) and treatments (87.7%; 86.7%; 87.4%, 87.1%) efficiently aligned to the reference genome. The sequenced regions covered 55% of CpG islands for MCO, 48.4% for UCO, and 32.8-43.5% for treatment samples. Coverage was similar between all different autosomes and the X chromosome. Global CpG methylation levels were higher in MCO vs UCO (81.3% vs 14.7%), while cytosine methylation in different contexts was similar (CHG 14.2% vs 10.2%, CHH 15.5% vs 10.6%). The average methylation across the four half biopsies was CpGs $68.9 \pm 6.7\%$, CHG $34.2 \pm 15.1\%$, CHH $37.0 \pm 15.8\%$, indicating that we can identify variation in global CpG and non-CpG methylation. From 69 candidate DMRs selected as potential LOS biomarkers, 57 (83%) were detected in at least one biopsy and 26 (38%) in all four biopsies. DMRs were identified in all biopsies, ranging from 12-36 hypomethylated regions vs MCO and 4-71 hypermethylated regions vs UCO. Most DMRs (12-100%) resided in the mitochondrial genome. We conclude that it is feasible to coextract meaningful genomic and epigenetic information from trophoblast half-biopsies for accurate SNP genotyping and WGBS analysis, respectively. We retrospectively identified half-biopsies from four embryos that resulted in LOS (2 male, 2 female), as well as four sex-matched controls of normal weight. These biopsies will be screened for DNA methylation changes by NovaSeq 30X coverage to find potential biomarkers for LOS.

S6.4 - Obesity in Mice Affects the Occurrence of Alternative Splicing Events in Oocytes and Early Embryo Development

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Reproductive success is largely dependent on maternal metabolic performance. Reports indicate that the risk of infertility in obese women is three times higher than in non-obese women. Contributory factors may include defects in oocyte maturation, impaired meiosis and abnormal mitochondrial dynamics, which decrease fertilisation rates and lead to a defective development of the preimplantation embryo. However, despite extensive research, the exact molecular mechanisms underpinning reduced fertility in obese women are still unclear. Research suggests that anomalies in alternative splicing during embryonic development might be linked to decreased oocyte quality. Therefore, we hypothesise that maternal obesity impacts the occurrence of differential alternative splicing events (DASs) in oocytes and the early stages of embryo development. We used C57BL/6 (B6) mice fed a standard diet (CD) and a high-fat diet (HFD) for 4 and 16 weeks. MII oocytes and 2-cell embryos were recovered and processed for single-cell RNA-seq, and blastocysts used for low-input bulk RNA-seq analysis. To compare the trajectories of alternative splicing from the oocyte to the developing embryos, longitudinal analysis was first performed in CD-fed animals, and then the dietary effect was determined by comparing CD progression in comparison to HFD. BRIE2-based analysis of 292 acquired cells that passed quality control (read number > 1×10⁵; and mapped gene number > 2.5×10³) revealed differences between the proportions of spliced transcriptional variants (among which skipping exons were identified). Among consecutive cell groups in the progression MII 4 wks → MII 16 wks → 2-cells → blastocysts, analysis revealed 70, 956, and 881 significant DASs for CD (FDR < 0.05) and 68, 1026, 1082 for HFD (FDR < 0.05), respectively. Importantly, in several comparisons we observed the occurrence of DASs within the genes *Srsf3*, *Srsf7* and *Akap17b*, whose protein products are directly involved in mRNA splicing, indicating the autoregulatory mechanism of their activity. Comparison of dietary effects revealed 12, 62 and 61 DASs across the three transitions. Gene Ontology (GO) analysis performed for DAS-containing genes revealed 98 significant terms (adjusted *p* < 0.05) associated exclusively with CD and 59 with HFD. In the CD, DASs were involved in unique processes such as “telomere maintenance via telomerase”, “positive regulation of organelle organisation”, “cytoskeletal organisation”, “regulation of chromosome organisation” and “protein ubiquitination”. On the other hand, HFD DASs were characterised by terms such as “histone-modifying activity”, “negative regulation of mitotic cell cycle”, “positive regulation of RNA metabolic process”, “vesicle organization” and “cellular response to stress”. The detected changes in alternative splicing indicate the occurrence of modifications of sophisticated intracellular mechanisms that might affect the quality of early-stage embryos and the success of fetal development. This research was supported by the Polish National Science Centre (2019/34/E/NZ4/00349) and the UK Biotechnology and Biological Sciences Research Council (BBS/E/B/000C0423) and Medical Research Council (MR/K011332/1, MR/S000437/1).

S6.5 - Molecular Profile of Brains from IVF Offspring Using a Mouse Model

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Assisted Reproductive Technologies (ART) are procedures that help achieve a successful pregnancy, with in vitro fertilization (IVF) being one of the most used ART today. While IVF procedures are safe, they have been associated with adverse adult outcomes in the offspring, including cardiovascular, neurological, and metabolic changes. We hypothesized that IVF procedures alter the expression of aging related genes in the brains of ART offspring, with consequences for behavior. To address this, brains from naturally-conceived and IVF offspring were collected at E18.5, 12-weeks, and 39-weeks, and 10-12-weeks old offspring were tested in a number of behavioral assays, including the elevated zero maze (EZM) and forced swim test (FST). Expression of genes involved in brain aging were measured by RT-qPCR. We observed an upregulation of *Ogg1*, *Hdac1*, and *Mecp2* in IVF offspring in the cerebellum, cortex, and hippocampus. Interestingly, preliminary data suggests male IVF offspring display increased anxiety-like behavior, and female IVF offspring display increased anxiety- and depressive-like behavior, compared to naturally-conceived offspring. Taken together, these results suggest an aging phenotype in the brains of IVF offspring, which may be linked to behavior changes.

S6.6 - Maternal Obesity During Pregnancy Impacts Pancreatic DNA Methylation and Protein Expression in the Offspring

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It has been postulated that DNA methylation is one of the mechanisms by which developmental programming causes long term, multi-generational impacts on the developing offspring. However, the role this molecular mechanism has in mediating the effect of maternal obesity on the offspring's pancreas is limited. We hypothesize that maternal obesity during pregnancy will result in changes to offspring pancreas DNA methylation that would be reflected as corresponding changes in protein expression. C57BL/6J (N=6) female mice were fed a high fat diet (HFD; n = 3; TD.07011 Pellet Envigo, Somerset, NJ) to induce obesity or a control diet (CON; n = 3) for fourteen weeks prior to breeding. These animals also remained on their treatment diets for the duration of pregnancy and lactation. Pups were euthanized at 8 wks of age and pancreas tissue was collected. Pancreas tissue DNA was isolated and whole genome bisulfite sequencing was performed at Genewiz (Illumina Hi-Seq; Azenta, Cambridge, MA). Data were analyzed in conjunction with CD genomics (New York, NY). Read alignment to the *Mus musculus* reference genome (mm39) were performed using Bismark (v0.24.2; Babraham Bioinformatics). Differentially methylated regions (DMR) and the associated locations were determined using methylKit v3.18 (Bioconductor; $q \leq 0.05$). Proteomics analyses were performed via ultra-performance liquid chromatography-tandem mass spectrometry (ThermoFisher Scientific Q Exactive HF mass spectrometer; University of Connecticut Proteomics and Metabolomics Facility, Storrs, CT). The number and identity of differentially expressed proteins were determined using the average precursor intensity in Scaffold v5.3.2 (Proteome Software, Inc; Portland OR). From our analyses we were able to identify 41,515 and 44,958 DMR for CON vs HFD males and females respectively ($q \leq 0.05$). 30.23% of the top 25% of DMR were hypomethylated whereas 69.76% were hypermethylated in CON vs HFD females. Likewise, 39.61% of the DMR identified were hypomethylated and 60.39% of DMR were hypermethylated in CON vs HFD males. We were able to identify a subset of proteins in our treatment

comparisons whose change in expression match the change in methylation status for that DMR. 3 targets (heparan sulfate proteoglycan 2, collagen type VI alpha 2 chain, and collagen type IV alpha 2) exhibited a reduction in DNA methylation that corresponded with an increase in protein expression in CON vs HFD males. 31 targets in the CON vs HFD males exhibited a reduction in protein expression that corresponded with increased DNA methylation of the DMR. For CON vs HFD females, Cathepsin A expression was reduced while there was increased DNA methylation at an associated DMR. Three proteins (immunoglobulin heavy constant gamma 1, isopentenyl-diphosphate delta isomerase 1, and glutamic-oxaloacetic transaminase 2) exhibited increased expression while having reduced DNA methylation at an associated DMR. In conclusion, we have demonstrated that there is a relationship between the DMR identified and changes in the pancreatic protein expression of the offspring from HFD-fed dams. This multi-omic approach has provided valuable insight into the interplay between epigenetics, maternal diet, and the pancreas tissue of the offspring.

S6.7 Investigating the Impact of Topoisomerase II Inhibition on Aneuploidy Levels in Human Oocytes

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Human eggs show a high level of segregation errors in the form of both whole chromosome aneuploidies and very large copy number variants (vL-CNVs; >10Mbp). Abnormal chromosome connections, including cohesion deterioration and chromosomal threads, have been suggested as the primary cause of these missegregation events. We previously found evidence of chromosomal threads in mature human eggs, potentially representing unresolved crossover intermediates or DNA catenanes. To assess the connection between aneuploidy formation and the resolution and dissolution of these catenanes, we disrupted topoisomerase II (TOP2) in prometaphase of both mouse and human oocytes. We identified a significant increase in vL-CNVs in our treatment groups, indicative of an accumulation of unresolved double strand breaks. These findings highlight the importance of TOP2 activity during meiosis I in both mouse and human.

FOCUS SESSION 7 - SPERM QUALITY AND FUNCTION

S7.1 - Multiple Evaluation of Sperm Function: From Motility Assessment to Advanced Chromatin Analyses

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Male gametes undergo a very important cellular differentiation to reach their mature state, leading to a highly specialized sperm cell with very particular characteristics that are designed to leave the male genital tract to carry paternal components, mainly genetic material, to the oocyte.

The evaluation of sperm quality and function has always been on the focus of researchers to understand the male fertilizing capacity and generate biomarkers that help to predict and improve fertilization and pregnancy rates. This is timely important as assisted reproductive technologies are broadly applied not only to treat human infertility but also in livestock animals, in which artificial inseminations are regularly used to generate offspring from the individuals with a major interest.

In my talk, I review multiple analyses that may be used for the assessment of sperm function, focusing on sperm kinetics, sperm membrane status, acrosome integrity, mitochondrial function, oxidative stress and other molecular biomarkers. Some of these assays have led to biomarkers that are commonly applied in livestock animals but with more limited information regarding their clinical value in human assisted reproduction. After, given that sperm DNA contributes to half of the embryo genetic material, a special section will be dedicated to discuss how the evaluation of sperm chromatin integrity provided valuable information to understand species-specific differences, to comprehend the mechanisms through which sperm cells are degraded and lose their function, and to figure out paternal causes of fertilization failure, embryo arrest and pregnancy loss.

S7.2 - Simultaneous High-Resolution Live Imaging of Cytoplasmic and Intracellular Calcium Store Dynamics in Progesterone-Induced Capacitated Mouse Sperm Acrosomal Exocytosis

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During capacitation, sperm acquire the ability to undergo acrosomal exocytosis, an absolute requisite for mammalian fertilization. Progesterone stimulates acrosomal exocytosis by increasing intracellular calcium. The regulation of intracellular calcium levels in sperm is controlled by plasma membrane channels, pumps and transporters and by intracellular stores. In mouse, the main calcium channel reported in sperm, CatSper is not stimulated by progesterone. We previously showed progesterone-triggered acrosomal exocytosis via a transient intracellular calcium rise in the anterior sperm head. However, it is still unknown whether this increase in intracellular calcium is due to an influx of extracellular calcium and/or the release of this ion from intracellular calcium reservoirs. Recent findings under non-physiological conditions revealed acrosomal calcium release as acrosomal exocytosis trigger, yet source and dynamics of calcium needed for inducing acrosomal exocytosis in physiological conditions remain unclear. In this study, we evaluated for the first time simultaneously the cytoplasmic and intracellular calcium store dynamics in progesterone-induced acrosomal exocytosis in capacitated mouse sperm. Our novel experimental strategy was to use simultaneously two calcium dyes (Fluo-5N AM and Calbryte 590 AM) with distinct affinities (K_d s of 90 μ M and 1.4 μ M respectively) and wavelengths

enabling us to visualize calcium subcellular locations and fluxes promoted by progesterone. To determine the occurrence of acrosomal exocytosis, we combined them with CellTrace Violet, which labels sperm acrosome. Employing high-resolution live cell imaging, we found that the intracellular calcium dyes fluoresce in different subcellular locations according to their affinities and that their fluorescence changes in distribution and intensity with sperm capacitation. While Calbryte fluorescence increases uniformly throughout the entire sperm structure after capacitation, Fluo-5 changes from a compartmentalized localization restricted to the reservoirs (the acrosome and mitochondria) in the non-capacitated condition, to a homogeneous distribution throughout the sperm structure (n=5). These results not only demonstrate that capacitation generates an intracellular calcium increase but also a redistribution of this cation. Both calcium dyes revealed that only capacitated sperm present an intracellular calcium increase in response to progesterone, mostly with an oscillatory pattern (n=5, $p < 0.001$). Interestingly, while Calbryte displays global oscillations in the entire sperm head, Fluo-5 intracellular calcium oscillations are confined to a specific localization within the sperm head, suggesting the presence of a calcium store in that region. When we evaluated the spatiotemporal correlation between cytoplasmic and calcium reservoir changes, and acrosomal exocytosis in response to progesterone, we found that without extracellular calcium acrosomal exocytosis still occurs (n=6). This result suggests that calcium released from intracellular stores participates in the acrosomal exocytosis induction by progesterone. Moreover, in sperm from CatSper KO mice, progesterone was able to induce an intracellular calcium increase and acrosomal exocytosis (n=4), indicating that the channel is not needed for progesterone-induced acrosomal exocytosis. Our findings highlight live-cell nanoscopy's significance to unveil the molecular mechanisms involved in acrosomal exocytosis regulation. This information could be helpful for infertility etiology comprehension and contraceptive strategy development. Research supported by NIH-P-10776, CONAHCYT CF-2023-I-291 and PICT 2020-00988.

S7.3 - Electrophysiological Insights into Ionic Conductivity of Noncapacitated versus Capacitated Mice Spermatozoa

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Objectives: Ejaculated sperm cells undergo terminal biochemical and physiological changes in the female genital tract known as capacitation to acquire fertilizing competence. Precise, time-dependent changes in sperm plasma membrane polarization lead to the activation of principal sperm-specific calcium channel CatSper and result in the influx of Ca^{2+} into sperm cells followed by hyperactivated motility. Capacitation is not a uniform event, and at any given point only a subset of sperm population is considered capacitated. It is possible that variability in sperm membrane conductance plays a role in this process, and can be explored using sperm patch clamp. Direct electrophysiological recording of murine sperm ion permeability was limited to non-capacitated sperm cells, mainly due to difficulties in studying capacitated spermatozoa with a patch clamp method. Using a sperm patch clamp method, ionic conductance was studied in capacitated spermatozoa for the first time and was compared to non-capacitated spermatozoa to bring new insights into understanding the mechanisms of sub-populations of spermatozoa undergoing capacitation. **Methods:** Here, we report the first-ever systematic evaluation of the ionic permeability of capacitated murine spermatozoa and compare their conductance to the non-capacitated sperm cells using a whole-cell sperm patch clamp configuration. We explored the differences between cationic conductance at various time points of capacitation, such as early (first 30 minutes), mid

(between 30 to 60 minutes), and late (more than 60 minutes). Results: Capacitated spermatozoa showed reduced permeability for cesium (Cs⁺) and sodium (Na⁺) with reduction in current by 12.5 ± 1.7 % and 64.6 ± 2.6 %, respectively, compared to noncapacitated sperm cells at negative membrane potentials. However, 34.8 ± 3.6 % and 12.9 ± 1.8 % increase in outward conductance of Cs⁺ and Na⁺, respectively, was observed at positive membrane potentials (+80 mV). On the other hand, inward permeability, and selectivity for divalent cations (Ba²⁺) increased for CatSper in capacitated spermatozoa by 208.6 ± 2.5 % compared to noncapacitated CatSper. Non-CatSper potassium (K⁺) permeability increased with capacitation by 24.9 ± 2.7 %, compared to noncapacitated spermatozoa, particularly at mid-stage of capacitation. However, with the advanced stage of capacitation, K⁺ permeability gradually decreased. Conclusions: These results decipher the conductivity changes in spermatozoa as they undergo capacitation and adjust to the changing extracellular environment. The enhanced divalent cation selectivity of the CatSper channel over monovalent ions with capacitation allows spermatozoa to effectively import calcium, triggering hyperactivation and achieving superior fertilization competence. Our findings provide new insights into sperm membrane ion selectivity for the sub-populations of spermatozoa undergoing the capacitation process and compare this to non-capacitated sperm cells.

S7.4 - Spermatogenesis and Sperm Quality Protections under Nutritional Stress – Novel Roles of Melanoma Antigens

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Spermatogenesis, a vital process for reproductive success, likely underwent adaptations to cope with limited resources. However, the molecular effects of nutritional stress on the germline and the activated protective measures remain poorly understood. Our previous research revealed the contribution of Magea genes, which are normally expressed in male germ cells but aberrantly activated in cancer, to robust spermatogenesis under nutritional stress. In this study, we aimed to investigate the underlying mechanisms involved. To achieve this, we exposed both wild-type and Magea knockout mice to a 3-month nutrient restriction regimen, maintaining them at 80% of their initial body weight. The effects were assessed at both the organismal and single-cell levels. Our findings indicated a significant increase in cortisone levels in all food-restricted animals. Notably, transcriptome changes were only observed in diverse testicular cells of the knockout mice, including in spermatogonia, spermatocytes, haploid spermatids, and Sertoli cells. This suggests that the Magea genes orchestrate protective mechanisms in wild-type mice under stress which are breached in Magea KO animals leading to significant changes in transcriptomes. Intriguingly, Magea genes are expressed in spermatogonia, but the impact of their loss is increasing during the differentiation process and is the highest in haploid spermatids, highlighting the importance of Magea-regulated pathways in the development of robust male gametes during the early stages of differentiation. Among differentially regulated pathways metabolic pathways, including gluconeogenesis, AMPK signaling, and lipid metabolism ubiquitination, signaling pathways, and adhesion molecules, are all critical for robust spermatogenesis. We are now following with in-depth molecular and functional analysis. Our discoveries hold promise for developing effective strategies to mitigate the adverse effects of stress on male fertility, progeny health, and advancements in cancer treatment.

S7.5 - Sperm Motility In Mice With Oligo-Astheno-Teratozoospermia Restored By in Vivo Injection And Electroporation Of Naked mRNA

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Oligo-astheno-teratozoospermia (OAT), a recurrent cause of male infertility, is the most frequent disorder of spermatogenesis with a probable genetic cause. Patients and mice bearing mutations in the ARMC2 gene have a decreased sperm concentration, and individual sperm show multiple morphological defects and a lack of motility – a canonical OAT phenotype. Intra Cellular Sperm Injection (ICSI) is required to treat such a condition but it has limited efficacy and was associated with a small increase in birth defects. Consequently, new targeted treatments are needed to restore spermatogenesis. Here, a combination of in vivo injection and electroporation of capped and poly-A-tailed naked mRNA is tested as a strategy to treat ARMC2-related infertility in mouse. mRNAs coding for several reporter genes are tested and the efficiency and the kinetic of expression are assessed using in vivo and in vitro 2D and 3D imaging experiments. We show that mRNA-coded reporter proteins are detected for up to 3 weeks mostly in germ cells, making the use of mRNA possible to treat infertility. Consequently, injection and electroporation of naked mRNA-Armc2 into the testes of Armc2-deficient males were performed and we show the presence of normal and motile sperm in the epididymis. This study shows for the first time that mRNA-Armc2 efficiently restores spermatogenesis and opens new paths for male infertility treatment.

FOCUS SESSION 8 - OVARY/OOCYTE AND TECHNOLOGIES

S8.1 - Reconstructing Human Oocytes

Shoukhrat Mitalipov, Oregon Health & Science University, Center for Embryonic Cell and Gene Therapy
Infertility affects millions of women worldwide and often caused by absence of functional oocytes due to age. However, management of infertility for these patients remains a challenge, even with advanced IVF treatments that often resort to using donor oocytes. My laboratory developed the concept of cytoplasmic/mitochondrial replacement therapy (MRT) to restore development competence of poor-quality oocytes for infertile patients. Initially conceived and tested in a nonhuman primate model, MRT

has been recently employed in clinical trials resulting in birth of healthy children for infertile couples. MRT has been also used as a germ line gene therapy to replace mutant mtDNA in oocytes from women carrying pathogenic mutations. Our program is also actively investigating a strategy for generating functional haploid oocytes from somatic cells by direct reprogramming using modified somatic cell nuclear transfer (SCNT). Current research is focused on landmark oocyte features that somatic cells must acquire including: 1) epigenetic identity of mature oocytes; 2) cytoplasmic maternal factors of oocytes critical for induction of totipotency and preimplantation embryo development; 3) recombination; 4) haploidy; 5) imprinting reset.

S8.2 - Mechanisms of Spindle Positioning in Oocytes and Their Developmental Implications (2023 Investigator Awardee)

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Chromosome mis-segregation frequently occurs during the completion of oocyte meiosis I (known as oocyte maturation), leading to aneuploidy, the major cause of infertility and congenital abnormalities. Why female meiosis I is error-prone is largely unknown. In mouse oocytes, spindle positioning is a dynamic process. The spindle is assembled and positioned at the oocyte center until metaphase I. Later, the spindle undergoes a dramatic migration towards the cortex, in a time-sensitive manner, a critical process for the highly asymmetric division. The biological significance of the initial spindle positioning at the oocyte center remains unknown. We employed time-lapse super-resolution microscopy, laser ablation, chemical tools, and genetic approaches to unveil a new model for meiotic spindle positioning and to demonstrate that abnormal spindle positioning is a risk factor for aneuploidy.

S8.3 - A Tug-Of-War Between Germ Cell Motility and Stable Intercellular Bridges Determines Cyst Size in Mice

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Oocytes are one of the largest cell types in animals, stockpiling materials in their cytoplasm that will support the earliest stages of embryonic development. In many species part of this volume is acquired through a unique developmental strategy: oocytes are generated in germline cysts, which form as germ cells undergo divisions with incomplete cytokinesis and remain connected through intercellular bridges, which in turn will enable material transfer to enrich the future oocyte. In mice, oocytes emerge from cysts of variable sizes, which are thought to result from cyst fractures. Previous studies, however, relied exclusively on the analysis of fixed samples that failed to capture fracture events, and therefore, the mechanism of cyst fractures remained elusive. Here we use a novel labeling strategy and high-resolution live imaging to directly visualize germline cyst dynamics for the first time and show that motile germ cell behavior during cyst formation underlies fracture events. We found that germ cells display a surprising protrusion-driven motility phenotype for several days after their arrival in the embryonic ovary, which gradually decreases until germ cells enter meiosis. We show that motility is randomly oriented in individual cells, even as they remain connected in cysts, and that this activity can fracture intercellular connections. On the other hand, we show that stabilized intercellular connections act to resist excessive fracturing. Our results reveal the mechanism of cyst fracture and suggest a tug-of-war model where the

opposing activities of cell motility and intercellular bridge stability determine the rate of fractures and ultimately the size of cysts. Understanding how variable cysts form in a mammalian species is an important starting point for further studies aimed at investigating how oocytes are selected by enrichment within such variable structures. We also anticipate that our live imaging approach will enable novel avenues of inquiry into the unique mechanisms of mammalian germ cell biology.

S8.4 - The cGAS-STING Pathway is Functional in Ovarian Secondary Follicles and May Serve as an Active Surveillance Mechanism

Prianka H. Hashim, Aubrey K. Converse, Jennifer L. Gerton, Francesca E. Duncan

The follicle is the functional unit of the ovary comprised of an oocyte surrounded by somatic granulosa and theca cells. Individuals are born with a non-renewable source of follicles, indicating the need for tight regulation of follicle activation and development to prioritize the removal of poor-quality oocytes and ensure fertilization of high-quality oocytes. In multiple cell types, the cGAS-STING pathway serves as a surveillance mechanism. cGAS (cyclic GMP-AMP synthase) is a cytosolic double-stranded DNA (dsDNA) sensor and after activation, forms 2'3'-cGAMP which functions as a second messenger to activate STING (Stimulator of Interferon Genes). STING then initiates an inflammatory response. Our overarching hypothesis is that the cGAS-STING pathway functions as a surveillance mechanism in follicles to detect and respond to dsDNA leading to inflammation and removal of poor quality follicles from the growing pool. To test this, we examined the expression of cGAS and STING in ovarian follicles, and then utilized an ex vivo follicle growth assay to determine the effect of pharmacological activation of the cGAS-STING pathway on follicle survival and growth. The treatments used included: Q-VD-OPH & ABT-737 which release mitochondrial DNA (mtDNA) into the cytosol, G3-YSD which is a cGAS agonist, and diABZI which is a STING agonist. RNA in situ hybridization in ovarian sections demonstrated that cGAS transcript expression was predominantly in the oocyte and STING transcript expression was enriched in granulosa and theca cells. This expression was consistent at the protein level when assessed by Western blot. Importantly, there was a dramatic increase in cGAS transcript expression in the oocyte between the primary and secondary follicle transition. To assess the function of the cGAS-STING pathway, early secondary follicles were isolated from CD-1 mice (12-16 days) and treated with either Q-VD-OPH & ABT-737, G3-YSD, diABZI, or vehicle alone. Short-term follicle treatment with the three pharmacological compounds ranging from 3-24 hours resulted in increased expression of phospho-IRF3 and phospho-TBK1 compared to controls via western blots, indicating pathway activation. Staining for cell viability with Calcein AM to detect live cells and ethidium homodimer-1 to detect dead cells after short-term treatment with the three compounds demonstrated that these compounds were not toxic to follicles. Long-term 10-day treatment of follicles encapsulated and cultured in alginate hydrogels with all compounds showed decreased follicle survival and altered growth compared to controls by day 8 of culture. These results demonstrate that the cGAS-STING pathway machinery is expressed in early secondary ovarian follicles and can be pharmacologically activated. Additionally, long-term treatment with pathway activators leads to decreased follicle survival, indicating that activation of the cGAS-STING pathway can contribute to removal of follicles from the growing pool. Future studies will assess the time frame of pathway activation and responsiveness and determine the physiologic function of this pathway in ovarian follicles and how it changes with age. Funding: This work was supported by the Eunice Kennedy Shriver National Institute of Child Health & Human Development (R01HD105752).

FOCUS SESSION 9 – SRB EXCHANGE SESSION ON PLACENTA

S9.1 - The Soluble Prorenin Receptor in Preeclampsia: A Potential Therapeutic Target

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Preeclampsia affects 5-7% of all pregnancies and is responsible for more than 7,000 maternal and 500,000 fetal deaths worldwide each year. Preeclampsia is associated with maternal hypertension, vascular injury, and multi-organ dysfunction, it also increases the risk of fetal growth restriction and preterm birth. A better understanding of the pathophysiology of the disease, and the development of new effective therapies is therefore essential.

We have identified a new circulating factor, soluble prorenin receptor (sPRR), that is likely to be contributing to the pathogenesis of preeclampsia. Maternal sPRR levels are elevated in patients with preeclampsia and gestational hypertension and have been shown to cause hypertension and renal injury in non-pregnant animals.

Our recent findings have demonstrated that sPRR produces both endothelial dysfunction in vitro, and renal arterial dysfunction with hypertension in pregnant rats in vivo. Further, we have demonstrated that the sPRR antagonist, PRO20, is able to ameliorate sPRR-induced endothelial dysfunction as well as endothelial dysfunction induced by preeclamptic serum. We have also begun exploring the potential mechanisms of action of sPRR in driving endothelial dysfunction and have demonstrated that its effects are at least partly driven by its interactions with the Angiotensin 1 type II receptor.

Together, our data provide strong evidence that sPRR plays a casual role in the pathogenesis of preeclampsia and shows that inhibiting sPRR could be an effective therapeutic option.

S9.2 - Trophoblast Stem Cell Models to Understand the Pathogenic Drivers of Fetal Growth Restriction

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Fetal growth restriction (FGR) affects ~5% of pregnancies worldwide, leading to an increased risk of perinatal morbidity and mortality, developmental delay in childhood, and cardiovascular disease later in life. Placentas from FGR pregnancies are smaller overall, with impaired blood vessel development and trophoblast differentiation/function, which together reduces exchange capacity and limits oxygen and nutrient delivery to the fetus. We aim to better understand why placentation is impaired in FGR by combining in vitro experiments, ex vivo imaging, and in silico modelling, with a vision to develop novel tools that better predict, detect, and treat this disorder to reduce its pathological impact. As part of this, we employ trophoblast stem cell models to better understand why trophoblast function is dysregulated in FGR. We have previously used the side-population technique to isolate trophoblast stem cells directly from first and third-trimester placentae across gestation, showing that these cells are depleted 10-fold in FGR, and providing novel insight into their role in the pathology of this disorder. This talk will present recent functional data that point to the stem cell apex of the trophoblast differentiation pathway as a

source of multiple aspects of placental dysfunction, including impaired trophoblast proliferation and differentiation, and increased susceptibility to apoptosis. In addition, work utilising trophoblast stem cells in novel 3D 'placentoid' models of the trophoblast bilayer to better understand the impact of mechanical cues on impaired syncytiotrophoblast function and villous development in FGR will be highlighted.

S9.3 - High-Fat, High Sugar Feeding Interrupts Placental Lactogen Adaptation Signaling in a Murine Model of Gestational Diabetes Mellitus

Kathryn M. Storey, Ashley Monksa, Kathleen A. Penningtonb, Kylie H. Cataldoc, Rene Cortesec and Laura C. Schulza,da
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During the late second and third trimesters of pregnancy, maternal and placental hormones induce insulin resistance in maternal muscle, liver, and fat. As a result, these tissues take longer to utilize available glucose, and post-prandial blood glucose levels in the mother remain elevated. This diverts glucose to the fetoplacental unit for fetal growth, as glucose is a main fetal nutrient, and placental glucose uptake is insulin-independent. Thus, normal pregnancy is a natural state of insulin resistance, and the maternal metabolic system must compensate to prevent prolonged maternal hyperglycemia and fetal macrosomia, which is done through beta cell augmentation. This compensation is mediated in part by lactogenic hormones coming from both the maternal pituitary (prolactin, PRL) and the placenta (placental lactogen(s), PL), which act through the beta cell prolactin receptor (PrLr). Gestational diabetes mellitus (GDM) is characterized by elevated blood glucose levels specific to pregnancy and is the most common complication of pregnancy. It can lead to future health complications in the mother and baby, such as metabolic and cardiovascular disease. About 27% of women with GDM have a pancreatic beta cell insulin secretion defect. We hypothesize that impaired placenta-beta cell communications that regulate maternal compensation to pregnancy can result in GDM. Using an acute high fat, high sugar (HFHS)-induced mouse model of GDM, we profiled placenta and pancreatic islet transcriptomes to characterize potential crosstalk in pregnancy, and test whether these signals are disrupted in GDM. Transcriptomes were analyzed at d0 or baseline in the islets, and at d7.5-17.5, or throughout elevated placental secretion exposure in both tissues. This time period also encompasses the onset (d9) and peak (d12-15) of gestational beta cell proliferation, which ceases at d18. Additionally, we tested whether placental secretions from HFHS and control-fed (CD) dams differentially stimulate proliferation in a murine beta cell line, NIT-1. Of note, the d10.5 samples proved to be particularly intriguing, as there were the highest number of significant differentially expressed genes (DEGs) in placental samples when compared by diet and the sex/diet interaction, and the least number of significant DEGs in islet samples when compared by diet at this timepoint. At d7.5 when compared by diet alone, two placental lactogen I isoforms (PrL3d3 and PrL3d2) that signal through the PRLR on islet beta cells to stimulate beta cell proliferation were significantly downregulated in HFHS samples. Interestingly, later timepoints such as d10.5 and d17.5 HFHS placenta samples had more mapped reads for placental lactogen II, suggesting an attempted hormone compensation response in HFHS placenta at mid-pregnancy for the lack of placental lactogen I in early pregnancy. In tandem with the observed changes in placental lactogen expression, islet PrLr is significantly downregulated in HFHS islets at d7.5. Lactogenic hormones have been shown to stimulate PrLr expression in insulin-producing cells in vitro. This suggests lower PrLr expression in early

pregnancy could contribute to the observed impaired insulin secretion and glucose intolerance in later HFHS pregnancy, ultimately resulting in GDM. Taking the placenta and islet data together, a new overall hypothesis emerges about the impact of HFHS feeding on temporal changes in placenta and islet adaptations to pregnancy. With placental lactogens and PRLR downregulated after HFHS exposure in early-mid pregnancy, and upregulated in mid-late pregnancy, HFHS may interrupt early placental hormone signaling, which leads to compensation in late pregnancy to stimulate beta cell proliferation.

S9.4 - Maternal Obesity is Associated with Global Reductions in Gene Expression including mRNAs that Contribute to Cell Migration and Glucocorticoid Metabolism in the Mid-Gestation Mouse Placenta

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The rising epidemic of maternal obesity has contributed to increased incidence of miscarriage, preeclampsia, and abnormal fetal growth. We hypothesized that these negative outcomes are due in part to abnormal placental development. Here we performed RNA sequencing of embryonic day (e) 12.5 placentas from satiety suppressed obesity (Lethal Yellow, LY) mouse dams compared to metabolically normal lean (C57BL6/J, B6) dams. This unique paradigm allowed us to examine the effects of maternal obesity on placental gene expression without a change in maternal diet composition. We chose e12.5 because all trophoblast lineages in the mouse placenta are established at this timepoint. Furthermore, it corresponds to week 10 of human placental development. Using standardized analyses, 1,185 differentially expressed genes (>2-fold difference, corrected P-value ≤ 0.05) were identified in placentas from LY compared to B6 dams. Interestingly, 94% of the differentially expressed genes were downregulated. To identify pathways that were altered in placentas from satiety-suppressed dams, Gene Ontology analysis was performed. Among the top categories were inflammation, cholesterol and steroid metabolism, morphogenesis, and cell migration. Furthermore, 30 of the genes with decreased expression in placentas from satiety suppressed dams have also been reported as decreased in pre-eclamptic human placenta. Because satiety suppression in LY mice is the consequence of a naturally occurring mutation on the C57BL6/J background, our next objective was to determine if there were similar gene expression changes in e12.5 placenta from C57BL6/J female mice fed a high fat, high sugar diet (HFHS). Specifically, 13-week-old dams with proven fertility were fed HFHS or normal rodent chow (ND) for 8 weeks prior to breeding and subsequent collection of fetus-placenta pairs at gestational day e12.5. As expected, dam weight ($P = 0.0033$), fasting glucose ($P = 0.0001$), and inguinal fat mass ($P = 0.0003$) were increased whereas placenta weight ($P = 0.0009$) was decreased in HFHS compared to ND dams. However, there were no significant differences in fetal weight ($P = 0.071$) or fetal-to-placental weight ratio ($P = 0.0675$) between treatment groups. Quantitative PCR analyses were performed using RNA isolated from whole placentas collected from e12.5 HFHS ($n=7$) and ND ($n=11$) dams and primers against mRNAs associated with cell migration (*Twist1* and *Snail1*), lipid metabolism (*Nr1h3*, *Nr1h4*, and *Cebpa*), and glucocorticoid metabolism (*Hsd11b1*, *Hsd11b2*, and *Pparg*). These analyses detected decreased abundance of *Twist1* (-2.7-fold, $P = 0.0284$), *Snail1* (-3-fold, $P = 0.0012$), *Nr1h4* (aka *Fxra*, -2.3-fold, $P = 0.0061$), *Nr1h3* (aka *Lxra*, -6-fold, $P = 0.0069$), *Cebpa* (-2.3-fold, $P = 0.020$), *Pparg* (-2.1-fold, $P = 0.0154$), *Hsd11b1* (-6.26 fold, $P = 0.0128$), and *Hsd11b2* (-2.38 fold, $P = 0.0103$) which was consistent with the reported sequencing data. Cell migration and glucocorticoid signaling are essential for successful placentation. Furthermore, poor cell invasion has been proposed as a mechanism of preeclampsia.

Deconstructing these collective pathways and identifying the mechanistic details by which maternal obesity is causing these changes could be critical in our understanding of how to rescue the maternal obesity-dependent effects on placentation and potentially reduce the incidence of preeclampsia. The overall decreases in mRNA abundance in placenta from obese dams could also suggest global effects of maternal obesity on synthesis, stability, and/or mRNA translation and represents a novel target for reversing abnormal placentation associated with obesity.

FOCUS SESSION 10 - ENDOMETRIUM

S10.1 - Endometriosis: New Insights and Opportunities for Relief of Symptoms

Philippa T. Saunders, The University of Edinburgh

Endometriosis is a common, chronic neuroinflammatory disorder believed to impact on wellbeing of more than 190 million women and people assigned female at birth. The defining hallmark of endometriosis is the growth of endometrial-like tissue as 'lesions' outside the uterus. Most endometriosis lesions are found in the pelvis and commonly referred to as peritoneal (superficial), ovarian (endometrioma) or deep depending on location. Patients often suffer from persistent pelvic pain which can be worse during menstruation as well as other symptoms including fatigue, gastro-intestinal and urinary symptoms which impact on quality of life. It is estimated 30-50% of patients with endometriosis may have problems conceiving. Diagnostic delay is ~7-9 years after first symptoms. There is currently no reliable biomarker, advances in imaging have helped with diagnosis of ovarian and deep subtypes but definitive diagnosis may still require invasive laparoscopic surgery. Standard treatment options include surgery (which may need to be repeated) as well as drugs that suppress ovarian hormones which have unwanted side effects.

Recent progress, including new approaches to symptom management, have been informed by the reframing of endometriosis as a multisystem disease which shares genetic risk factors with conditions such as migraine and IBS. Studies that have identified alterations in hormonal, metabolic and inflammatory pathways in tissue and fluid samples from endometriosis patients have opened up new avenues for medical therapy including drug repurposing. The delay in diagnosis and limited range of current therapies has also increased interest in self-management strategies such as dietary modification that can impact on the gut-microbiome-brain axis the importance of which in regulation of symptoms in pain and neurological disorders has only recently been fully appreciated.

In this talk I will review the latest insights into the mechanisms that may predispose some individuals to develop symptomatic endometriosis and results from studies in our own Centre focused on management of pain symptoms.

S10.2 - Investigation of Receptor Tyrosine Kinases as Novel Drug Targets for Treatment of Endometriosis

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Endometriosis is a common and debilitating disease affecting 10% of reproductive age women with limited therapeutic options. It is characterized by the presence of benign endometrial glands and stroma outside of the uterus. Infertility, pelvic pain, and dysmenorrhea are common presenting symptoms and can cause significant distress in those affected. Receptor tyrosine kinases (RTKs) are a class of cellular membrane proteins that are important for cell metabolism, growth, and survival. Because of their importance in cellular function, we sought to identify RTKs that might serve as novel targets for endometriosis therapeutic options. To identify RTK's of interest, we used the published databases that evaluate gene expression in endometriosis lesions compared to control endometrium. From this we identified two RTKs with increased expression in lesions compared to controls: Macrophage colony-stimulating factor 1-receptor (CSF1R) and Mast/stem cell growth factor receptor (KIT). To validate CSF1R and KIT protein expression in collected primary endometriosis lesions, we completed immunohistochemistry (IHC) staining. We found that KIT stained primarily in epithelial cells and CSF1R stained primarily in stromal cells and macrophages. Surface area of positive staining was measured using ImageJ software and demonstrated that pelvic endometriosis lesions showed significantly increased expression of CSF1R when compared to eutopic midsecretory phase endometrium sections. A similar upward trend was observed in the lesions stained for KIT though statistical significance was not obtained. CSF1R and KIT expression were also validated in the immortalized endometriosis epithelial cell line, 12Z, via western blot analysis. Finally, mRNA expression of our identified RTKs in stromal and epithelial cells was confirmed via quantitative PCR (qPCR). Pexidartinib (PLX3393) is an FDA-approved dual inhibitor of CSF1R and KIT used in the treatment of tenosynovial giant-cell tumors. We sought to evaluate if pexidartinib could be used to inhibit pro-survival and pro-inflammatory signaling pathways in 12Z cells via inhibition of two of our identified RTKs. Cells were treated with and without KIT-Ligand (KITL) and Macrophage colony-stimulating factor 1 (MCSF) - activators of KIT and CSF1R respectively. Cells were then treated with pexidartinib at either a 3 μ M or 10 μ M concentration. Gene expression of the proliferative and inflammatory markers cyclin D1 (CCND1) and interleukin 8 (IL8) was measured using qPCR. With MCSF stimulation, mRNA expression of CCND1 was reduced at the 3 μ M and 10 μ M concentrations of pexidartinib. With both MCSF and KITL stimulation, expression of IL8 was reduced at the 10 μ M concentration. However, without first stimulating the cells with MCSF or KITL, significant downregulation of the inflammatory markers was not achieved indicating that ligand-receptor binding produces effective pexidartinib-mediated inhibition of CSF1R and KIT. Thus, our results reveal that our identified RTKs have increased expression in endometriosis lesions at the level of mRNA and protein and that up-regulation of such proteins may serve as a potential therapeutic target for treatment of this disease. Supported (in part) by an Alpha Omega Alpha Carolyn L. Kuckein Student Research Fellowship (DC). Eunice Kennedy Shriver National Institute of Child Health and Human Development grants R01-HD105800 (DM) and R01-HD110038 (MMM). Burroughs Wellcome Fund Next Gen Pregnancy Award (NGP10125, DM).

S10.3 - Progenitor Cells Responsible for Endometrial Regeneration Reside in the Uterus

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Our objective was to determine the origin of cell types that comprise the endometrium in a human model of uterus transplant. Endometrial biopsies were obtained from uterus transplant (UTx) recipients in the secretory phase of the menstrual cycle at two clinical sites (University of Pennsylvania [Penn], Philadelphia, PA and Baylor University Medical Center [BUMC], Dallas, TX). Three individuals were biopsied multiple times at different post-transplant intervals. All UTx recipients were on immunosuppressive agents at the time of biopsy, however the regimens differed slightly by center. Endometrial biopsies were also obtained from ovulatory, nulliparous healthy control subjects (HC, n=3) in the secretory phase of the menstrual cycle. A total of 12 biopsies were obtained: nine from the four UTx recipients and three from HCs. Samples were dissociated, and 5' scRNA-Sequencing (scRNA-seq) was performed using the 10X Chromium platform. Semi-automatic cell type annotation was performed using Single R and select reference datasets to annotate cell clusters. Whole exome sequencing on each UTx donor and recipient pair was performed, variant call files were generated and bioinformatic pipeline demuxlet was used to determine if cells originated from tissue-resident (donor-derived) or extrauterine (recipient-derived) precursors. Cell proportions were compared in the groups by performing one-way ANOVA followed by Tukey's multiple comparisons test in GraphPad Prism version 10.0.3. After excluding cells with >10% mitochondrial content and < 500K features, 76,886 cells were included in the final analysis; 48,132 and 28,753 cells derived from UTx recipients and healthy controls respectively. scRNA seq revealed 11 cell clusters. Stromal and epithelial cells comprised 69% and 15% of the cells represented in the data set respectively. The proportion of lymphocytes present in the BUMC samples ($43\% \pm 15\%$) was significantly higher than that of the Penn UTx ($10\% \pm 8\%$) and healthy control samples ($11\% \pm 7\%$) ($p < 0.01$). The proportion of epithelial cells in the Penn UTx ($13\% \pm 4\%$) and healthy control samples ($13\% \pm 5\%$) were significantly higher than that of the BUMC samples ($3\% \pm 2\%$) ($p < 0.01$). In every sample, >98% and >95% of stromal and epithelial cells were donor-derived, suggesting that these cell types originate from tissue resident stem/progenitor cells located in the uterus. In contrast, most immune cells identified were recipient in origin. On average 92% (range 85-97%) and 95% (range 51-99%) of myeloid and lymphoid cells respectively were recipient in origin, suggesting that immune cells migrate to the endometrium from the systemic circulation. Of note, after reiterative clustering was performed on immune cells, one macrophage subcluster that was identified had a higher proportion of donor-derived cells than other subclusters (range 5-30%). Our findings support the theory that tissue-resident progenitor cells are responsible for endometrial regeneration. Immune cells in the endometrium are the notable exception as the majority of these cells migrate to the endometrium from the periphery. In our data there does appear to be a small subset of myeloid cells that are donor-derived which warrants further investigation.

S10.4 - Paternal MiR-146a Regulates Female Immune Receptivity to Embryo Implantation and Fetal Viability in Mice

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In mammals, seminal fluid delivered at mating exerts a female reproductive tract response that influences the trajectory of fetal and placental development. This is mediated through promoting maternal immune tolerance, as well as direct effects of seminal fluid-induced cytokines on preimplantation embryos. Permissive signalling factors in seminal fluid include TGFB and CD38. Inhibitory signalling factors in semen including interferon- γ that impair maternal tolerance can arise under inflammatory circumstances, but other inhibitory signals remain to be characterised. Male reproductive tract miR-146a is upregulated in response to inflammatory challenge and is another candidate inhibitory factor. In this study we evaluated the hypothesis that paternal miR-146a is an inhibitory regulator of the female reproductive tract immune response, with consequences for embryo implantation and pregnancy outcomes. The impact of seminal fluid miR-146a on the uterine response to semen was examined by qPCR analysis of endometrial gene expression in BALB/c females 8 hours following mating with wild-type miR-146a $^{+/+}$ or miR-146a $^{-/-}$ males, and compared with unmated estrous BALB/c controls. To assess the impact of paternal miR-146a on maternal immune tolerance, uterine T cell populations at the peri-implantation phase were assessed by flow cytometry on day (d) 3.5 post-coitum (pc). Pregnancy outcomes were measured on d5.5pc and d17.5pc. Neonatal outcomes were assessed at birth and offspring weight was measured fortnightly until week 16 post-partum. Sperm parameters in miR-146a $^{+/+}$ and miR-146a $^{-/-}$ males were assessed at 6-7 months old. Compared to females mated with miR-146a $^{+/+}$ males, females mated with miR-146a $^{-/-}$ males exhibited impaired seminal fluid-mediated induction of endometrial cytokines. Notably, there was a 50% decrease in endometrial expression of Cxcl2 and Il6 ($n=7-11$ dams/group, $P < 0.05$). This correlated with a 40% reduction in uterine CD3 $^{+}$ T cells in females mated with miR-146a $^{-/-}$ males ($n=10-16$ /group, $P=0.02$). Despite a 10-12% reduction in sperm motility and morphology ($n=18-19$ /group, $P < 0.05$), miR-146a $^{-/-}$ males sired litters containing 19% more viable pups on d17.5pc ($n=28-30$ /group, $P < 0.05$). There was no change in fetal weight in litters sired by miR-146a $^{-/-}$ males, but an 8.0% decrease in mean placental weight suggesting improved placental efficiency. Analysis of a second cohort on the day following implantation (d5.5pc) showed a similar increase in litter size ($n=14-18$ /group, $P=0.03$). However in a third cohort analysed at birth, litter size changes were no longer evident, corresponding to a 21% loss of pups in late gestation or early neonatal period in litters sired by miR-146a $^{-/-}$ males, compared with $< 5\%$ loss in litters sired by miR-146a $^{+/+}$ males ($n=10-17$ /group). Gestation length and offspring survival and growth trajectory to 16 weeks were not affected by paternal genotype. Collectively, these findings indicate that paternal miR-146a attenuates uterine receptivity to effectively modulate the number of embryos permitted to implant and develop. In part, this may be mediated through influencing either the composition of seminal fluid signals or the female immune response to seminal fluid. Additional experiments are required to further elucidate the changes to endometrial receptivity and the immune and/or non-immune mechanisms by which paternal miR-146a influences female control of embryo implantation and pregnancy progression.

S10.5 - From Ovary to Endometrium: Mapping Gene Expression Across Female Reproductive Tissues and Cells in Humans and Mice

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Despite considerable progress made—over multiple decades and rounds of technological innovation—in identifying male reproductive tract specific genes, the identification of genes predominantly expressed within the various tissues and cells of the female reproductive tract—through any high-throughput approach—has lagged notably behind. To bridge this significant knowledge gap, our research leveraged an extensive collection of recently published RNA-seq datasets, comprised of 431 human and 322 mouse datasets, which span both reproductive and non-reproductive tissue and cells. Key tissue and cell types include ovary, oocytes, follicles, uterus, endometrium, fallopian tubes, placenta, and embryonic cells like blastocysts and zygotes. The objective was to identify genes that could have potential implications for women's reproductive health. Our reanalysis identified 4,326 human and 2,424 mouse genes that are significantly enriched in the female reproductive tract. These genes encompass a broad range of protein coding and noncoding genes, including lncRNAs, miRNAs, snRNAs, and snoRNAs. Interestingly, 253 human and 241 mouse genes exhibited expression specific to the reproductive tract, with several genes demonstrating exclusive expression in particular reproductive tissues, such as MIR28 in the endometrium. Our study identified an astonishing total of 2,095 mouse genes—and 304 human genes with corresponding mouse orthologs—that currently lack a documented mouse model. Among these, a total of 47 human genes—38 protein-coding and 9 noncoding genes—that currently lack a corresponding mouse model show congruent enriched expression in both species. Our analysis also includes several comparisons across datasets. Notably, we compared human endometrial tissue and epithelium between early proliferative and mid-secretory phases, which showed that MIR450B (miRNA) had significantly higher expression ($p \leq 0.01$) in the early secretory phase compared to mid-secretory and proliferative phases; this gene is also enriched in the endometrium according to our findings. In the context of reproductive tract enrich or specific expression, other comparisons we performed included age-related comparisons between young women (21-31 years) and postmenopausal women (57-68 years) for ovary, uterus, and vagina tissue datasets; comparison across developmental stages of oocytes (GV, MI, MII) and zygotes (1-, 2-, 4-, 8-cell); and, comparison of different pregnancy timepoints of uterus and myometrial tissue. While our analyses—the first of its kind for female reproductive tissues and cells—identify novel genes that are enriched or specific in the female reproductive tract, our findings offer promising candidates for addressing female reproductive tract-related disorders as well as novel candidates for the development of a non-hormonal female contraceptive. The substantial number of genes that are currently lacking a documented mouse model and are enriched or specific to the female reproductive tract could serve as key points for further investigations in both reproductive biology and applied translational research. Furthermore, the genes newly identified in this study could improve the precision of diagnosis and treatment approaches for unexplained infertility in women, marking a significant advancement towards personalized reproductive healthcare. This work was funded by the Male Contraceptive Initiative and NICHD grants (R01HD095341 and R01HD106056) to TXG.

FOCUS SESSION 11 - EMBRYO EPIGENETICS

S11.1 - Inter- and Intra-Specific Variation in One Carbon Metabolism: Implications for Epigenetic Programming of Long-Term Development

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One-carbon (1C) metabolism comprises a series of interlinking metabolic pathways that provide methyl groups for the synthesis and/or methylation of DNA, RNA, biogenic amines, proteins (including histones), and phospholipids. It follows that 1C metabolism is intricately linked to a diverse range of anabolic and catabolic pathways within the cell. However, although it operates to a varying degree in all cell types within the body, our understanding of 1C metabolic pathways pertains mostly to the (rat) liver.

Given inter-specific variation in size, digestive metabolism, reproductive and metabolic rates, (and related timing of senescence), one might reasonably expect species divergence in the nature and extent to which 1C metabolism functions in germ/embryonic cells and somatic cells of the gonad. In turn, this could go some way to explaining inter-specific variation in epigenetic and developmental responses to environmental stimuli (e.g., parental diet, environmental chemicals and procedures (including media composition) employed in assisted reproduction). Emerging data is beginning to support this proposition. A case in point relates to the enzyme betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5), involved in the methylation of homocysteine to methionine. This enzyme appears functional in the inner-cell mass (ICM) but not trophectoderm (TE) of the mouse blastocyst [PMID: 25466894], but is absent in all somatic cell lineages of the bovine ovary, oocyte and pre-hatching embryo [PMID: 33673278]. Mathematical model predictions indicate that this would render germ/embryonic cells particularly sensitive to maternal dietary or culture-induced deficiencies in 1C-related metabolites (e.g., methionine), thus affecting S-adenosyl-methionine (SAM) mediated transmethylation. This we confirmed in bovine embryos, demonstrating genome-wide alterations to DNA methylation in both ICM and TE lineages, including a cohort of imprinted genes associated with naturally occurring developmental anomalies in humans and farm animals.

Intra-specific variation in 1C metabolism can arise due to polymorphic variants in genes encoding enzymes involved in these interlinking pathways. Such variants likely account for strain-specific responses to dietary interventions in mice. Ongoing work in sheep, employing new approaches for genome-wide association analyses [PMID: 36223768], are seeking to identify single-nucleotide polymorphisms (SNPs) in genes encoding 1C enzymes and associated SAM dependent epigenetic regulators in this species. Ultimately, this work is seeking to identify the extent to which these SNPs account for within and between breed (strain) variation in epigenetic responses to dietary deficiencies in methionine/vitamin B12 in this outbred species; thus greatly extending the limited range of genetic variants investigated to date in humans. This could open up the prospect for the development of personalized prevention and treatment strategies for intending parents and their offspring.

Funding: BB/K017810/1/BB (Biotechnology and Biological Sciences Research Council, UK).

S11.2 - Feeding Rumen-Protected Choline During the Peri-Conceptional Period Programs Postnatal Phenotype of Beef Calves

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It has been shown previously that addition of the methyl donor choline to embryo culture medium increased DNA methylation of blastocysts and programmed development to increase birth and weaning weights of calves born after embryo transfer. The current objective was to evaluate whether feeding rumen-protected choline (RPC) during the peri-conceptual period also programs fetal development. A total of 211 postpartum first service, suckled beef cows were blocked by breed and body weight and assigned randomly to RPC or vehicle group. Cows were synchronized with a 7-day Cosynch-CIDR protocol for timed artificial insemination (TAI) and individually fed 60 g RPC in 454 g ground corn gluten or 454 g ground corn gluten (vehicle) through the Super SmartFeed® machine (C-Lock, Rapid City, SD, USA) from one day before until 7 days after TAI. Pregnancy diagnosis was performed with ultrasonography at days 28 and 42 post-TAI, and calves were followed until weaning. There was no effect ($P > 0.05$) of RPC supplementation on pregnancy rate and pregnancy loss. The calving rate was 44% (44/99) for vehicle and 41% (49/119) for the RPC group ($P = 0.41$). The mean gestation length did not differ ($P = 0.8743$) between vehicle and RPC (278.7 ± 0.9 vs 278.6 ± 0.9 days). Similarly, RPC did not affect ($P = 0.5853$) calf birth weight [vehicle; 32 ± 1.0 , RPC; 31 ± 0.9 kg]. At an average age of 118 days (range 75-150 days; age included in the statistical model), vehicle calves were heavier ($P=0.0534$) than RPC (150 ± 0.9 vs 143 ± 2.7 kg). There was no difference in withers height, so the weight/height ratio was greater ($P < 0.05$) for vehicle than RPC (1.6 ± 0.03 vs 1.5 ± 0.03 kg/cm). Paired testes weight measured at castration at an average age of 118 days (range 75-150 days) was similar ($P = 0.66$) between vehicle and RPC (47 ± 4 vs 45 ± 4 g). Differences in body weight persisted at weaning (248 days, range 205-280 days) with vehicle being heavier ($P=0.0300$) than RPC (231 ± 3.8 vs 221 ± 3.7 kg). Vehicle calves also tended ($P=0.0624$) to be taller at weaning (114 ± 0.8 vs 112 ± 0.8 cm) but weight/height ratio was greater ($P=0.0269$) for vehicle (2.2 ± 0.03 vs 2.1 ± 0.03 kg/cm). In conclusion, supplementation of RPC during the periconceptual period programmed development to alter calf phenotype in the postnatal period. The net result, reduced body weight, was the opposite of the phenotype caused by addition of choline to culture medium. Thus, choline administered to cows as RPC exerts different actions on the embryo than choline provided to embryo culture medium. The reasons for these differences need to be evaluated. (USDA AFRI 2020-67015-30821). Key Words: choline, programming, cattle

S11.3 - TET2 and TET3 Methylcytosine Dioxygenases Are Required for Blastocyst Implantation and Endometrial Decidualization

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In human endometria, changes in DNA methylation correlate positively with gene expression dynamics during the transition from pre-receptive to receptive phase for blastocyst implantation. In aged murine endometria, DNA methylation fidelity is also lost at a number of CpG islands (CGIs), leading to CGI hypermethylation of key genes required for endometrial stromal cell decidualization. Concomitantly, the ten-eleven translocation (TET) enzyme family (TET1, TET2 and TET3), responsible for DNA demethylation via iterative oxidization of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), is downregulated in the endometrium of both physiologically aged (PA; C57BL/6 wildtype, 45-48 weeks of age) and genetically aged (GA; *Pgrcre/+Sirt1f/f*, 6-8 weeks of age) mouse models. However, the enzymatic and/or non-enzymatic roles of Tet genes in regulation of

blastocyst implantation and endometrium decidualization remain largely unknown. Given the distinct expression levels of the three Tet genes (Tet2 > Tet3 > Tet1) in both human and murine endometria, we hypothesize that Tet2 and Tet3, being highly expressed, are critical for maintaining DNA methylation fidelity required for blastocyst implantation and endometrial decidualization during pregnancy. Thus, mice carrying the Tet2f/f or Tet3f/f allele were bred to Pgrcre/+ mice to generate Pgrcre/+Tet2f/f (Tet2d/d) and Pgrcre/+Tet3f/f (Tet3d/d) mice. During the 6-month breeding trial, both Tet2d/d and Tet3d/d mothers exhibited subfertility with significant reductions ($P < 0.01$) in pups per litter and number of litters as compared to Tet2f/f and Tet3f/f controls ($n=6$). Notably, Tet3d/d mothers had further reduced litter frequency ($P < 0.01$) when compared to Tet2d/d mothers. Although the number of implantation sites was not affected by Tet2 or Tet3 deletion, these females displayed decreases ($P < 0.01$) in the decidual bulb weight as well as the size of the labyrinth and junctional zone at gestational day (GD) 9.5, suggesting decidualization defects. In addition, the ability of the endometrial stromal cells to undergo an artificially induced decidualization was severely compromised ($P < 0.01$) in Tet2d/d uteri. At GD 3.5, Tet2d/d uteri exhibited dysregulated progesterone and estrogen signaling and heightened DNA methylation. At GD 5.5, Tet2d/d females displayed abnormal cytoplasmic expression of FOXO1 in uterine luminal epithelium, indicating that the yin-and-yang balance of FOXO1 and PR had been disrupted. To compensate, Tet1 and Tet3 expression levels rise in the Tet2d/d uteri. Thus, we generated a Tet2-Tet3 double-deletion mouse, i.e., Pgrcre/+Tet2f/fTet3f/f (Tet2d/dTet3d/d), to further explore the relationship between the members of the Tet family. The Tet2d/dTet3d/d females were infertile with failed blastocyst implantation and heightened DNA methylation. These novel findings suggest that TET deficiency impairs endometrial receptivity and stromal cell decidualization, leading to defective placentation and pregnancy loss. Future studies will investigate the epigenetic mechanisms regulating uterine aging via both enzymatic and non-enzymatic function of TETs

S11.4 - Epigenomic Crosstalk in H3K27Ac Regulation and RNAseq: An Integrative Approach Assessing Metabolically Modulated Bovine Embryos

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In the intricate embryonic development dynamics, the chromatin landscape and the expression patterns are tightly coordinated responding to metabolic cues that shapes the fate of the implantation embryo. This study delves into the molecular choreography of bovine embryos, elucidating the interplay between the H3K27ac epigenetic marker and gene expression profiles under the influence of metabolic modulators - DCA (dichloroacetate, a pyruvate analog that increases the pyruvate to acetyl-CoA conversion) and IA (iodoacetate, which inhibits the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), leading to glycolysis inhibition), from day 5 of culture. Both modulations were reported previously as affecting targeted metabolic pathways and acetylation levels for H3K27. The goal of this study was to integrate results from RNAseq and H3K27ac Cut&Tag (Cleavage Under Targets and Tagmentation) of inner cell masses from metabolically modulated bovine embryos and find crucial relationships between the datasets. For this, in vitro produced bovine embryos were cultured from day 5 in 3 experimental groups [synthetic oviductal fluid with amino acids (SOFaa) + 4% bovine serum

albumin]: Control (CO - no additional supplementation), DCA (addition of 2mM of DCA) or IA (addition of 2μl of IA). Blastocysts were collected on Day 7 and their inner cell masses were submitted to RNASeq and Cut&Tag analysis. 3 ICM per group per replicate (3 replicates) were used for RNA extraction or for the Cut&Tag procedure. Samples were sequenced by the Illumina Genome Analyzer II (NextSeq PE40 high output). Results from both datasets were submitted to quality control with FastQC, genome alignment (Bos_taurus_UMD_3.1.1, NCBI) was done by Bowtie2, normalization and differential expression measurement of RNASeq data was performed by Deseq2. Peak-calling was done with MACS2 and the genomic annotation by ChipSeeker. Regarding the RNASeq dataset, comparing to CO, 855 transcripts were found differentially expressed in DCA, while 659 were found in IA. Assuming that H3K27ac is described as a permissive mark, the subsequent step was to consider unique peaks from each group confronted with DEGs, to retrieve the regulatory behavior among group comparisons. Therefore, for CO vs. DCA, 126 matches were found between CO peaks and CO upregulated DEGs, mainly associated with translation activity, mRNA processing and protein folding, while 184 matches were present between DCA peaks and DCA upregulated DEGs, linking to glucose homeostasis, chromatin organization, cell differentiation and positive regulation of transcription from RNA polymerase, making up to 36% of complementary IDs across datasets. Concerning the contrast between CO vs. IA, 118 IDs were found upregulated in IA together with their mirroring peaks, aligned with molecular functions such as DNA topological change, mitochondrial ATP synthesis, positive regulation of DNA replication and translation. Along with 119 matches between upregulated DEGs in CO and their counterpart peaks pointing to in utero embryonic development, positive regulation of embryonic development and regulation of transcription from RNA polymerase II promoter, equally accounting for 36% of compatible IDs. This integrative approach revealed the important role H3K27ac in controlling gene expression and reinforces the metabolism as a key regulator of epigenomic remodeling during the early embryogenesis. Acknowledgements: Federal University of ABC (UFABC), Sao Paulo Research Foundation (FAPESP, grant numbers: 2019/25982-7 and 2022/14472-0)

S11.5 - Developmental Patterns and Gene Expression of Bovine Blastocysts Exposed to Imbalanced Energetic Levels

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The Developmental Origins of Health and Disease (DOHaD) states that an imbalanced maternal diet has long-term effects on the health of the offspring. In humans and animal models, an inadequate nutrient supply during pregnancy may impact post-natal life, predisposing to cardiovascular and kidney diseases, metabolic disorders, and poor female fertility in the absence of overt developmental defects. Given the extensive genome and epigenome reprogramming during the preimplantation embryo development, in this study we asked if exposure to nutritional imbalance during this window triggers changes in developmental patterns and gene expression in the blastocyst. We modelled this stage using in vitro bovine embryo production to strictly control the environmental conditions and reduce the number of animals used for experimentation. Furthermore, the amount of in vitro produced bovine embryos has increased exponentially in the last decades, surpassing the number of embryos produced in vitro since 2016, posing the need to carefully evaluate the long-term safety of embryo culture. Bovine blastocysts

were produced in vitro from abattoir-derived oocytes using X-sorted semen. The zygotes were cultured for 8 days in a modified, serum-free culture medium. The composition of the medium was changed to contain either 0.5 (energetic restriction) or 1.5-fold (energetic excess) of the standard content of energetic substrates. RNA was extracted from a pool of 5 expanded blastocysts per treatment and amount and quality were assessed by BioAnalyzer. Three biological replicates per experimental group with a minimum RNA amount of 289 pg/μl and RNA integrity number 8.6-10 were used in downstream procedures. Libraries were prepared and sequenced on NovaSeq 6000 generating 150 bp paired-end reads. Sequenced reads were mapped and quantified using Salmon or HISAT2/HTSEQ. Differential gene expression analysis was performed in R using DESeq2 package. Genes with adjusted $p < 0.1$ were considered differentially expressed. Markers of cell lineage specification, SOX2, CDX2, and NANOG, were traced by immunofluorescence. The obtained blastocysts were also characterized morphologically by recording the overall blastocyst rate and morphology, and nuclei counting. Principal component analysis evidenced that increased levels of energetic substrates induced a clear clustering compared to the control, in contrast to energetic restriction. Furthermore, only a few genes were differentially expressed, without substantial differences between the two bioinformatic pipelines, when conducting paired comparisons. When considering gene expression patterns across the 3 treatments, 2 patterns were generated: increasing and decreasing expression along with increased energy substrates. Consistent with these findings, the blastocyst rate, comprising the expansion and hatching rates, the number of cells, and the expression of the first cell lineage markers were comparable. A lack of major disruption of the expression profile and normal development patterns seem in line with previous observations made in vivo that a mild nutrient restriction in the mother does not prevent the development to term and the birth of healthy-looking offspring, which will only be affected later in life. We are therefore tempted to speculate that at this stage of development embryo plasticity allows to adjust in response to energetic imbalance. However, such adjustments might come at the cost of heritable alterations of the epigenetic profiles that will impact cells and organ functions later in life. For instance, overexpression of MUC13, as consistently observed in the energetic excess treatment, has been linked with changes in the methylation levels of its promoter and with higher glucose uptake.

FOCUS SESSION 12 - EXOTIC ANIMALS

S12.1 - Advanced Assisted Reproduction Technologies in Large Endangered Mammals.

Thomas Bernd Hildebrandt, Head Department, Reproduction Management

Studying reproduction in captive and wild terrestrial megavertebrates is not only challenging in regards to their exceptional size and defensiveness it also represents a challenge due to the time scale of the reproductive events such as onset of puberty, sexual cycle and pregnancy length. Zoo animals can be often successfully trained for certain manipulations like frequent blood sampling for hormonal analyses, ultrasound examinations or even semen collections. The wild counterparts require general anaesthesia for any kind of intervention. Besides these general challenges, there are several new advanced assisted reproduction techniques (aART) specifically tailored to e.g. the northern white rhino rescue program. The current portfolio of aART in this mammalian taxon is ranging from sexual cycle characterisation and manipulation, semen collection and cryopreservation, artificial insemination, oocyte collection, In Vitro Fertilisation (IVF) and embryo production, embryo transfer as well as to the first attempts of stem cell derived In Vitro Gametogenesis for generating gametes. The presentation will cover several of these new aART techniques in white rhinoceroses, elephants as well as other critically endangered mammals such as giant panda, okapi and reptiles such as komodo dragon.

S12.2 - Challenges and Joys of Working with Monotreme Reproduction

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The short-beaked echidna (*Tachyglossus aculeatus*) is one of only five extant species of egg-laying mammals (Monotremata: four echidnas and one platypus). Despite its common and ubiquitous distribution throughout Australia, detailed information on its reproductive biology is limited. In conjunction with a captive breeding colony of echidnas in Currumbin Wildlife Sanctuary (Queensland, Australia) we have been investigating and discovering new insights in echidna reproduction and development. The reproductive biology of the echidna is anatomically and behaviourally distinct in comparison with both marsupial and eutherian mammals. After a short gestation of 16-17 days, the egg is incubated in the pouch for a further 10 days and once hatched, the young puggle continues its development while residing in a temporary pouch, sucking milk from teatless mammary patches. The development of the hatched pouch young is remarkably similar to that of the marsupial neonate, but has a number of distinct differences, particularly with regards to their sexual differentiation. The adult male echidna has a unique phallus terminating in four glans penises but only 2 are engorged and used during ejaculation. This phallus morphology is present at hatching as a distinct protuberant structure in both male and female young. Interestingly, the overall morphology of this structure is also retained in the adult clitoris. Examination of the adult female echidna reproductive tract has identified a number of other anatomically distinct features. These features highlight the unique adaptations that monotremes have made to support their unique form of oviparous mode of reproduction, representative of their evolutionary link between ancestral and modern mammals.

S12.3 - Indefinitely Preserving Mammalian Biodiversity Using Induced Pluripotent Stem Cells: Progress and Perspectives

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The current pace of global biodiversity loss is unsustainable, marking what is widely termed as the sixth mass extinction event. This alarming trend, exacerbated by climate change and a complex political and economic landscape, has made large-scale in situ conservation efforts increasingly unfeasible. Approaching this impasse, programs have continued to focus on captive breeding and assisted reproduction over the years, albeit with very limited success even in flagship species. Recently, focus has shifted to genome resource banks (GRBs) to secure future genetic rescue attempts. However, the scale and scope of this approach remains inconsequential with preserved material limited to nonviable DNA, gametogenic tissues, or haploid gametes from captive collections. Hence, we pursued an in vitro live cell system applicable across mammals, versatile for research and reproduction, and easily collectable under field conditions. Through dissecting systems biology in the early bovine embryo, we previously developed a method to generate and sustain naïve bovine induced pluripotent stem cells (iPSCs). Unlike other iPSC models, cells reprogrammed using this approach were similar to a totipotent 16-cell stage

embryo (doi: 10.1242/bio.058756). Recently, we applied this same method on mice, and obtained totipotent iPSCs that could contribute to both the embryo and placenta after injection into early murine blastocysts. This highlighted a conserved mechanism for deriving a primordial cell type that would allow us to broadly translate iPSC technology for use in GRBs. The ability to generate iPSCs from animals irrespective of age and sex together with its characteristics of indefinite self-renewal, genomic stability, and the potential to efficiently generate embryos, make this cell type a versatile inclusion for GRBs. To demonstrate that our approach can be applied to non-model species, we generated iPSCs from fibroblasts collected from adult American Bison (*Bison bison*). By using lentiviral transduction of pluripotency genes Oct4, Sox2, cMyc, Klf4, and the simian vacuolating virus 40 large T antigen in bison fibroblasts cultured from the ovarian mesentery, we were able to reprogram and select bison iPSC colonies. Bison iPSCs were sustained in a defined serum-free medium with small molecules that inhibit GSK3 β , MEK1/2 and TGF β /activin/nodal signaling. This indicated that our optimized reprogramming approach can work across genera and enable preservation of self-renewing diploid living genomes for a diverse range of mammals. Beyond the technical challenges of immobilization, a key barrier to collecting living tissues from free-ranging populations is the serious risk of microbial contaminations. As aseptic peripheral blood collection using vacuum sealed tubes is a procedure that can be readily performed at all sites, we sought to grow “fibrocytes”, a rare mesenchymal stem cell type in circulation that mediates tissue repair. We optimized fibrocyte growth conditions by experimenting with bovine and ovine blood samples. We found that from 10 ml blood collections, we could generate highly proliferative fibrocytes (>1E6 cells) within seven days in culture. This approach provides a novel path to viable aseptic cell collections irrespective of the environment. Preserving an inexhaustible diploid cell type that also has embryonic totipotent qualities offers GRBs a valuable asset for future reproductive and regenerative technologies. Our findings highlight the first steps towards implementing a path to preserve existing population structures as stem cells before their entry into extinction vortices. At the present time, implementing such an approach is crucial for rescuing invaluable genetics for research, management and future population resuscitation attempts.

S12.4 - Luteinisation of Granulosa Cells In 3D Cell Culture from Southern White Rhinoceros

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With only 20% of the captive female white rhinoceroses reproducing successfully, breeding of rhinoceroses in captivity remains challenging. This low ratio is partly associated with corpus luteum (CL) dysfunctions, which compromises embryo implantation and pregnancy maintenance. In the white rhinoceros, CL activity after ovulation may last 4 or 10 weeks. Many captive females however show anovulatory cycles without luteal activity or cycles with persistent luteal activity. Moreover, in aged females, CL insufficiency may lead to pregnancy failure due to inadequate progestagen production. The reasons for variations in cycle length, anovulatory cycles and CL insufficiency are not fully understood. To support captive breeding programs, including the fine-tuning of assisted reproductive technologies, we urgently need to acquire detailed knowledge about those factors regulating the CL lifecycle in rhinoceroses. CL/luteal cells from rhinoceroses are not randomly accessible. The aim of current study

was therefore to test if Southern white rhino granulosa cells (progenitors of large luteal cells), collected after ovum pickup, can be luteinized in vitro, providing an in vitro model for future functional studies identifying factors regulating CL physiology. For the luteinisation, we adapted an in-house protocol successfully validated for the domestic cat. In brief, the collected cells were enzymatically separated and were subjected to culture on ultra-low attachment plates for 19 days. Such plates facilitate spheroid formation and the applied media composition (containing luteinizing hormone, forskolin, insulin) supports luteinization. Subsequently, the differentiation success of granulosa into luteal cells was characterized by morphological analysis of HE stained cells, immunohistochemical analysis of protein expression for CYP11A1, progesterone analysis in medium by EIA (for different progesterone metabolites) and semi-quantitative PCR analysis for HSD3B1, CYP11A1, SRD5A1 – genes coding for enzymes involved in steroid hormone synthesis/metabolism. These techniques were applied for samples of various days of the culture period. Cell size increased during luteinization and culture period (day 0 – $7.2 \pm 1.8 \mu\text{m}$; day 8 – $16.5 \pm 3.9 \mu\text{m}$; day 13 – $18.1 \pm 4.6 \mu\text{m}$; day 19 – $21.9 \pm 5.5 \mu\text{m}$). Luteinized cells appeared to exhibit a greater cytoplasm to nucleus ratio, and numerous vacuoles were visible within the cytoplasm, indicating active steroid metabolism. The IH-staining signal for CYP11A1 was localized in the cytoplasm of the luteinized cells (d8, d14 and d19) and was moderate to strong, contrasting with weak or undetectable signals in isolated granulosa cells (d0). Progesterone was detected throughout the culture period, with a strong increase during the first week of luteinization. Semiquantitative PCR analysis revealed clear signals for isolated granulosa cells (d0) and luteinized cells (d14, d19) for HSD3B1 on the samples of the analyzed days. Faint or no signals were present for isolated granulosa cells for CYP11A1 and SRD5A1, while strong bands were observed in samples of luteinized cells. Controls (BACT and GAPDH) showed in all samples signals of equal strength expressions. This case report describes the first luteinization of granulosa cells from the Southern white rhinoceros in vitro. The luteinized cells were morphologically corresponding to typical luteal cells and exhibited high steroidogenic capabilities, shown by the increased progesterone production during transformation. The progesterone production seems to be regulated by CYP11A1 and/or SRD5A1. Current study results strongly point towards a successful development of an innovative 3D cell culture model based on granulosa cell luteinisation, facilitating future studies focusing on enhancing our understanding of CL functionality in rhinoceroses.

FOCUS SESSION 13 - SPERMATOGENESIS

S13.1 - Germ Granule-Mediated RNA Regulation during Spermatogenesis

Noora Kotaja, Institute of Biomedicine, University of Turku, Finland

Differentiating male germ cells face several challenges in terms of transcriptome regulation during spermatogenesis. First, meiotic and early post-meiotic cells need to cope with broad and pervasive genome expression resulting in an exceptionally diverse transcriptomes. This is followed by near to complete transcriptional silencing during later steps of spermatogenesis, which creates high demand for mRNA storage and translational regulation. A complex set of RNAs is also retained in transcriptionally inactive sperm, where they serve important functions in transmitting epigenetic information on father's acquired conditions to the offspring. Our research focuses on the functions of germ granules in coordinating RNA regulation during spermatogenesis. Germ granules are germline-specific large cytoplasmic ribonucleoprotein (RNP) granules that are particularly prominent in spermatocytes and round spermatids at the time of the broad genome expression. We have actively characterized two types of germ granules, the intermitochondrial cement (IMC) and the chromatoid body (CB), and their contribution to the PIWI-interacting RNA (piRNA) pathway and transcriptome regulation in male germ

cells. Altogether, our research highlights the central function of germ granules as molecular platforms that coordinates RNA regulatory pathways required for normal sperm production and fertility.

S13.2 - Activin A Modulation of Reproductive Development and Pathology Through Impacts on Steroids and Lipids

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The growth factor activin A [AA; dimer of inhibin α subunits] influences both somatic and germline cells in the mammalian testis throughout life. AA signals through receptors and intracellular signalling machinery shared with other TGF β superfamily ligands (e.g. BMPs, TGF β s). We have been investigating how modulated AA levels, including its elevation in pre-eclamptic pregnancies, infections and cancers, may affect male fertility, and have unearthed clues to how it may more broadly influence organ growth and function. Our studies of fetal and neonatal testes have employed both *Inhba*KO (lacking activin A) and *Inha*KO (lacking the inhibin α subunit; has unopposed AA bioactivity) mice. Importantly, testicular *Inhba* (encoding AA subunits) levels normally increase in the testis, but not ovary, during the key window of fetal masculinization. Either higher or lower AA levels reduces testosterone in fetal testes, by directly affecting transcription of different genes encoding steroidogenic enzymes. RNAseq analyses identified Leydig cell progenitors are affected by elevated AA (*Inha*KO) at embryonic day [E]13.5, while Sertoli cells are predominantly affected at E15.5 by AA absence (*Inhba*KO). These *Inhba*KO testes have an abnormal abundance of lipid droplets inside cords, while E15.5 *Inha*KO testes exhibit multinucleated germ cells, similar to the effects of endocrine disruptor chemical exposure. To learn more about how altered AA levels influence cellular metabolism and steroid production, here we investigated *Inha*KO mice (45-56 days). Unopposed AA results in somatic cell tumours, first in adult *Inha*KO testes and ovaries, and then in adrenal glands; we examined both normal and tumour-bearing regions in gonads. In pfa-fixed tissue sections, Oil-Red-O staining identified gonadal and adrenal lipid droplets (n=3 WT, 5 KO), and mass spectrometry imaging (MSI) profiled testicular lipids (n=5-7). Transcript analyses immunohistochemistry, and liquid chromatography-mass spectrometry (LC-MS) (n=6-7) defined alterations in steroid production capacity, while tumour-microenvironment effects (testis tumour, associated seminiferous tubules, and morphologically normal regions) were investigated by RNA-sequencing following microdissection. In *Inha*KO mice. lipid droplet size and number close to testis and ovarian tumours are grossly larger and more abundant, with no affect apparent in the adrenal cortex. LC-MS and MSI identified lipids differentially present in both abundance and distribution, respectively (~90; fold-change >1.5, p< 0.05), in *Inha*KO testes, while significant changes in steroidogenic enzyme transcript levels indicate abnormal metabolism of cholesterol to steroids (testes: reduced *Star*, *Cyp17a1*, *Hsd17b3*, elevated *Hsd3b1*, *Cyp19a1*; ovary: reduced *Cyp11a1*, *Srd5a1*, elevated *Hsd17b1*; adrenal: *Cyp11a1* elevated); local tumour

microenvironment effects were detected in transcript and protein levels for HSD3B1 and CYP11A1. LC-MS confirmed abnormal steroid production in InhaKO gonads, including reduced testicular testosterone (< 0.0001) and DHT (< 0.0255). These outcomes emphasize the imperative to study AA biology with organ- and cell-specific optics, and to address the lifelong vulnerability to excess or reduced AA bioactivity.- Supported by NHMRC GNT1181516

S13.3 - Investigation of Undifferentiated Spermatogonial Population Dynamics in an Experimentally Tractable System

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Biological actions of the undifferentiated spermatogonial population are the foundation for maintenance and regeneration of the spermatogenic lineage during steady-state conditions and following damage or transplantation. While most undifferentiated spermatogonia are progenitors poised to initiate differentiation, a minor portion of the population functions as stem cells (SSCs) to provide continuity and regeneration. At present, the cellular dynamics and molecular mechanisms that underly undifferentiated spermatogonial biology are undefined due in part to challenges with sophisticated investigation of the population within a complex in vivo environment. Previous studies established methodology for long-term maintenance of primary mouse undifferentiated spermatogonia that grow as clumps comprised of progenitors and a minor portion that possesses SSC capacity, thus reflecting the in vivo population. In addition, we previously engineered a transgenic mouse model in which a subset of undifferentiated spermatogonia express an Id4-eGfp transgene and demonstrated that the level of EGFP demarcates SSCs from early progenitor spermatogonia. The SSC pool in primary cultures of undifferentiated spermatogonia derived from this model was shown to label as ID4-EGFP+, thus validating utility to serve as a defined and experimentally tractable platform for addressing gaps in knowledge about dynamics of SSC and progenitor fate. Here, we performed fluorescent live cell imaging on cultures derived from Id4-eGfp mice to visualize the behavior of SSCs over a period of 6 days following subculture which is known to encompass a self-renewal period. The resulting observations revealed constant changes in germ cell clump composition and a variety of cell division dynamics by ID4-EGFP+ cells including both asymmetric and symmetric fates. Further investigation of clump formation and composition was carried out by mixing cultured spermatogonia derived from Id4-eGfp mice and mice harboring a constitutively expressed Rfp transgene in equal proportions. Daily imaging of these cultures revealed that greater than 50% of clumps are composed of cells from mixed backgrounds and, therefore, not clonal in nature. Next, ID4-EGFP+ SSCs and ID4-EGFP- progenitors were separated by fluorescent activated cell sorting and cultured independently to investigate the dynamics of undifferentiated spermatogonial population regeneration. Outcomes of flow cytometric analysis and fluorescent imaging to track changes in the ratio of ID4-EGFP+ and ID4-EGFP- over the culture period suggest that a quorum sensing mechanism occurs to establish and maintain a consistent SSC pool either through self-renewing divisions or resurgence of the stem cell program in initial progenitors. Ongoing studies are aimed at assessing functional properties of biomarker-defined SSC pools in undifferentiated spermatogonial populations that have been subjected to different situations of in vitro regeneration. Together, these findings generated from a defined and experimentally tractable in vitro system advance our understanding of SSC dynamics which may prove vital to identifying potential causes of male infertility and developing strategies for correcting heritable deleterious mutations through direct germline editing.

S13.4 - Two Mouse Spag6 Genes Coordinate to Control Sperm Formation and Male Fertility

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Sperm-associated antigen 6 (SPAG6) is the mammalian orthologue of Chlamydomonas PF16, a protein localizing within the central pair of the axoneme and involved in flagellar motility of the algae. In mice, two Spag6 genes have been identified. The ancestral gene, located on mouse chromosome 2, is named Spag6. A related gene, located on mouse chromosome 16 and evolved from the ancient Spag6 gene, has been named Spag6-like (Spag6l). Global Spag6 knockout mice (C57 background) are grossly normal, and fertility was not shown to be affected in both males and females. In particular, the homozygous males had normal sperm parameters, including sperm count, motility, and morphology. Examination of testis histology also revealed normal spermatogenesis. In contrast, the global Spag6l knockout mouse model (C57 background) was previously generated and showed lethality. Most homozygous Spag6l knockout mice died before 4-weeks of age; the few males which survived to sexual maturity were infertile, and the fertility of the females was also affected. However, fertility of the heterozygous Spag6l knockout mice was normal. We have crossed the two knockout lines to generate Spag6/Spag6l double knockout mice. The compound heterozygous (Spag6+/-; Spag6l+/-) mice showed normal fertility, and all the mice carrying Spag6l homozygous mutation (Spag6l-/-), either with heterozygous (Spag6+/-) or homozygous (Spag6-/-) Spag6 mutation died before 4-weeks of age. However, even though all Spag6-/-; Spag6l+/- mice were grossly normal, all the males were completely infertile. Compared to the controls (Spag6-/-; Spag6l+/+ or Spag6+/-; Spag6l+/-) males, the Spag6-/-; Spag6l+/- males had significantly reduced sperm number, and the sperm that were formed were morphologically abnormal and motility was significantly reduced. Histologic examination of the testes by light microscopy also revealed impaired spermiogenesis. Analysis by Transmission Electron Microscopy revealed abnormal sperm ultrastructure with both head/chromatin and tail formation defects. The Spag6-/-; Spag6l+/- females sired normal number of pups compared to the control females but the time from breeding to delivery was longer than normal, suggesting gestation defects. Overall, our findings indicate that in mouse, both genes, Spag6 and Spag6l, coordinate to modulate sperm formation and male fertility and are also likely required for proper female fertility.

S13.5 - A New Device for Seminiferous Tubule Culture

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Spermatogenesis in mice takes approximately 35 days, during which time various events occur spatiotemporally, including proliferation and differentiation of spermatogonial stem cells, meiosis, and morphological changes with flagellum formation, the mechanisms of which are not fully understood.

Seminiferous tubule culture is an effective technique for understanding spermatogenesis in vitro. In the conventional cultural method, a piece of testis was placed on agarose. However, it is difficult to perform the sequential imaging at high magnification while culturing because agarose prevents using an inverted microscope. In the present study, a novel culture device was developed in which the tubules are sandwiched between oxygen-permeable plastic and a porous membrane, and spermatogenesis was successfully observed at higher resolution than previously possible while culturing. This device could contribute to further understanding spermatogenesis by culturing seminiferous tubules derived from various transgenic and knockout mice.

S13.6 - Towards a Map of Promoter Interactions During Male Germ Cell Development

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Spermatogenesis is a complex process of cell differentiation that requires an extremely tight regulation at different levels. The chromatin of developing male germ cells is extensively remodeled across the three phases of spermatogenesis, namely mitosis, meiosis, and spermiogenesis. These three phases present very different gene expression programs, which are accompanied by a very dynamic chromatin environment in terms of histone post-translational modifications (PTMs). Three-dimensional (3D) genome structure is an additional layer of regulation during spermatogenesis, as described by several groups which used chromosome conformation capture experiments followed by high-throughput sequencing (Hi-C) on male germ cells in recent years. Spermatogonia show a somatic-cell-like arrangement, with well-defined compartment patterns and topological domains, which are dramatically rearranged in primary spermatocytes, where these features are lost due to the mitotic chromosomal conformation. Compartment patterns re-emerge in round spermatids, with lower but still present topological domains. However, despite the fast recent advances in the characterization of 3D genome dynamics during spermatogenesis, at the gene level, the resolution achieved by Hi-C (50kbp) is not enough to resolve promoter-enhancer(s) interactions, thus limiting our understanding of gene expression regulation.

To address this question, we are developing a promoter capture Hi-C (PCHi-C) approach in cell types representing each spermatogenic phase (germinal stem cells as a mitotic point, primary spermatocytes as meiotic point, and round spermatids as post-meiotic point). PCHi-C goes one step further than regular Hi-C by capturing those Hi-C genomic interactions involving promoter sequences using probes against genomic regions of interest. This strategy simplifies the complexity of the library and allows a resolution below 5kbp, to assign chromatin contacts between promoters and distal regulatory regions at the whole genome level.

Promoter capture data will be integrated with the occupancy of different chromatin binding proteins known to mediate the genomic interactions (i.e. CTCF, BORIS, cohesins) and with the profile of histone PTMs on different histone H3 residues obtained in developing male germ cells from published datasets and from our group. Thus, we will provide an integrative analysis of different histone marks at promoters and distal enhancers, to explore their association with gene expression dynamics and chromatin environment. Our results will provide deeper insights into regulatory mechanism driving male germ cell differentiation, which rely on much more sophisticated combinatorial patterns than formerly imagined.

Funded by: Agence Nationale de la Recherche (ANR-21-CE44-0035 to J.C., D.P., and C.B.), the Fondation pour la Recherche Médicale (SPF201909009274 to A.I.), the LABEX Who am I (to M.C., ANR-11-LABX-0071 and Idex ANR-18-IDEX-0001).

FOCUS SESSION 14 - SRF FOCUS SESSION ON GAMETES

S14.1 - Actin-dependent Mechanisms of Chromosome Segregation in Mammalian Oocytes

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Chromosome segregation is conserved throughout eukaryotes. Meiotic chromosome segregation in oocytes generates fertilizable eggs. Chromosome segregation errors are remarkably high in oocytes and give rise to aneuploidy, a leading cause of embryo deaths – it accounts for nearly 35% of miscarriages. When compatible with life, aneuploidy in embryos frequently leads to genetic disorders such as Down's syndrome, which affects about 1 in 1,100 live births worldwide. A major goal of our research is to understand the mechanisms that safeguard accurate chromosome segregation in mammalian oocytes for successful fertilization and healthy embryogenesis. We recently showed that a unique mechanism underpins chromosome segregation in mammalian eggs: actin filaments that are organized in the form of a spindle promote accurate chromosome segregation by bundling spindle microtubules into functional kinetochore-fibers. Here, I will discuss our recent discovery that spindle-associated actin structures prevent oocyte aneuploidy during female reproductive aging.

S14.2 - Application of Imaging Mass Cytometry to Reveal Protein Networks in Competent and Non-Competent Meiosis I Oocytes

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Human oocytes are highly prone to chromosome segregation errors in meiosis I. These errors are the primary genetic cause of failed zygotes, early miscarriages, and IVF failures.

When studying why these errors occur, compared to highly abundant mitotic cells or meiotic spermatogonia, a major limiting factor in oocyte research is material availability.

Limited numbers of appropriately staged oocytes can be recovered from mammalian model organisms, and human oocyte research is hampered even further by significant and legitimate ethical implications. This material bottleneck means many biochemical techniques are off limits and instead we heavily rely on low through-put fluorescence-based imaging methods. This approach generates high-resolution, beautiful images, reporting protein dynamics, but can only analyse a small number of proteins simultaneously. Consequently, we have a poor understanding of the overall temporospatial regulation of key cell cycle events driving female meiosis, and how these protein networks differ between competent and non-competent oocytes.

To address this shortfall, we have developed a protocol to apply Imaging Mass Cytometry (IMC) to mammalian oocytes. IMC utilises heavy-metal conjugated antibodies and sequential laser-ablation to build up a 1 μ M2 pixel image of up to 40 channels simultaneously. Our current panel of 30 antibodies already allows us to begin building a highly-multidimensional model of the cell-cycle proteome in mouse oocytes throughout meiosis, capturing protein abundance, location, and post-translational modifications states. The data generated by this method is novel in format and requires the building of new data-analysis pipeline. Here we present early results examining protein networks in meiosis I mouse oocytes.

S14.3 - Diminished Autophagy Activation Contributes to Weakened DNA Damage Repair in Full-grown Oocytes

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The DNA damage repair (DDR) mechanism is not robust in full-grown mammalian oocytes, and the underlying reasons remain largely unknown. In this study, we used three different methods to induce DNA breaks, hereafter referred to as DNA damage. Treating full-grown germinal vesicle (GV) oocytes, incubated with milrinone to prevent meiotic resumption, with etoposide (50 mg/ml for 3h), ultraviolet radiation (302nm for 30 seconds) or ionizing irradiation (1Gy) induced DNA damage as evidenced by the significant increase in gH2AX and pATM levels, sensitive markers for DNA damage in GV oocytes, as well the increase in DNA tail length and moment, as assessed by the alkaline comet assay, when compared to controls. Despite the significant increase in pATM and gH2AX following DNA damage, we did not observe a significant increase in the localization of RAD51 or Ku80— critical DDR proteins for homologous recombination and non-homologous end-joining DNA repair pathways, respectively— to the damaged DNA compared to control oocytes. This suggests that the DDR mechanism is not fully functional in full-grown GV oocytes. RAD51-chromatin binding assay in the extracted chromatin confirmed that RAD51 fails to localize to the DNA in DNA-damaged oocytes. To explore the accessibility of DNA-damaged sites to RAD51, we assessed chromatin structure in DNA-damaged oocytes. Using the DNase sensitivity assay and H3K9/14ac marker, we found that DNA-damaged oocytes exhibited a relatively “closed” chromatin structure compared to control oocytes, likely contributing to defective RAD51 localization to the damaged DNA. In somatic cells, exposure to DNA damage activates both Poly [ADP-ribose] Polymerase-1 (PARP-1) and autophagy, which play critical roles in promoting an “open” chromatin status, thereby rendering DNA-damaged sites accessible to DDR proteins. Strikingly, in contrast to somatic granulosa cells, full-grown murine and porcine GV oocytes failed to activate autophagy (assayed by LC3I/II western blot analysis and CytoID autophagy detection kit) and PARP-1 (examined by western blot). We found that PARP-1 acts downstream of autophagy activation because autophagy induction restored PARP-1 activity in DNA-damaged oocytes. Decreasing autophagy levels using ATG5 knockout oocytes or its inhibition by spautin-1 increased the sensitivity of GV oocytes to DNA damage. Importantly, inducing autophagy by rapamycin or MG132 treatments increased open chromatin structure, increased DDR protein localization to the DNA and decreased gH2AX and pATM in DNA-damaged oocytes. The addition of spautin-1, a specific autophagy inhibitor, abolished the beneficial effects of rapamycin on chromatin status, DDR protein localization and DNA damage levels, indicating that rapamycin effects are specifically mediated through autophagy activation and not other mTOR-dependent effects. Upon oocyte maturation in milrinone-free medium, DNA-damaged oocytes exhibited a significant increase in the incidence of chromosome fragmentation, lagging chromosomes (assessed by time-lapse imaging) and aneuploidy (assayed by in situ chromosome counting) compared to controls. Importantly, treatment with rapamycin, but not with rapamycin+spautin-1, significantly decreased the incidence of chromosome fragmentation, lagging chromosomes and aneuploidy in DNA-damaged oocytes. Taken together, our findings reveal molecular mechanisms underlying the response of full-grown oocytes to DNA damage and provide evidence that diminished autophagy activation contributes to weakened DDR function in oocytes, which are notoriously prone to errors, resulting in aneuploidy— the leading genetic cause of infertility and developmental disorders. Statistical analysis was performed using one-way ANOVA followed by the Tukey

post-hoc test, p-value less than 0.05 was considered statistically significant. This research was supported by the ASRM and NIH-R35GM142537 to A.Z.B.

S14.4 - PHB/JAK Axis Regulates Meiotic Recombination via the Modulation of Histone Modifications in Spermatogenesis

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Previously, we have shown that human sperm Prohibitin (PHB) expression is significantly negatively correlated with mitochondrial ROS levels but positively correlated with mitochondrial membrane potential and motility. However, the possible role of PHB in mammalian spermatogenesis has not been investigated. Here we document the presence of PHB in spermatocytes and its functional roles in meiosis by generating the first male germ cell-specific Phb-CKO mouse. Loss of PHB in spermatocytes resulted in complete male infertility, associated with not only meiotic pachytene arrest with accompanying apoptosis, but also apoptosis resulting from mitochondrial morphology and function impairment. Our mechanistic studies show that PHB in spermatocytes regulates the expression of STAG3, a key component of the meiotic cohesin complex, via a non-canonical JAK/STAT pathway, and consequently promotes meiotic DSB repair and homologous recombination. Furthermore, the PHB/JAK2 axis was found as a novel mechanism in the maintenance of stabilization of meiotic STAG3 cohesin complex and the modulation of heterochromatin formation in spermatocytes during meiosis. The observed JAK2-mediated epigenetic changes in histone modifications, reflected in a reduction of histone 3 tyrosine 41 phosphorylation (H3Y41ph) and a retention of H3K9me3 at the Stag3 locus, could be responsible for Stag3 dysregulation in spermatocytes with the loss of PHB.

S14.5 - Single-Cell and Bulk Transcriptional Profiling of Mouse Ovaries Reveals Novel Genes and Pathways Associated with DNA Damage Response in Oocytes

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Females are born with a limited number of primordial follicles stored in their ovaries, which can last for years or decades until they are recruited for growth and ovulation. Throughout a female's life, the immature oocytes within these follicles are constantly threatened by DNA damage from endogenous and exogenous sources. Our previous research has shown that CHEK2 regulates oocyte survival in response to DNA damage resulting from failed meiotic recombination or induced by radiation and chemotherapy. However, the mechanisms regulating DNA damage response (DDR) and survival of immature oocytes are poorly understood. We used single-cell and bulk RNA sequencing to profile the ovarian response to DNA damage induced by a low but oocyte-lethal radiation dose. We compared DDR between Chek2^{-/-} and wildtype ovaries to identify DDR signaling downstream of CHEK2. We found that radiation-induced damage triggers DDR in the ovary, which is solely dependent on CHEK2 and is mediated by TAp63 and p53 transcription factors. DNA damage activates multiple response pathways in the ovary, including apoptosis, p53, interferon signaling, inflammation, cell adhesion, and intercellular communication. Analysis of DDR at the single-cell level revealed that different ovarian cell types differentially employ these pathways in response to DNA damage. Moreover, radiation disproportionately affected oocytes, which had the highest number and functional diversity of differentially expressed genes. We identified novel genes and pathways induced by radiation, specifically in oocytes, shedding light on the mechanisms of DDR and oocyte sensitivity to DNA damage. Several newly identified radiation-response genes in oocytes are linked to plasma membrane, cell junctions, and signaling, suggesting a coordinated response between oocytes and pre-granulosa cells within the primordial follicle. These findings provide a foundation for future studies on the specific mechanisms regulating oocyte survival in the context of DNA damage arising during aging, therapeutic, and environmental genotoxic exposures.

S14.6 - Identification of New Targets for Non-Hormonal Contraceptive Drug Development Using a Phenotypic Assay of Murine Oocyte Meiotic Maturation

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Oocyte meiotic competence refers to the ability of the oocyte to exit the prophase I arrest and resume meiosis. During this process of meiotic maturation, the oocyte undergoes breakdown of its intact nucleus, or germinal vesicle (GV), and extrusion of the first polar body. The biological process of oocyte meiotic maturation is essential to produce a fertilizable egg arrested at metaphase of meiosis II and is thus considered a target for non-hormonal contraceptive development. Oocyte meiotic maturation in vivo occurs in response to the luteinizing hormone surge which stimulates ovulation, but it can also be recapitulated in vitro and key hallmarks of germinal vesicle breakdown and polar body extrusion can be visualized by transmitted light microscopy. Therefore, the goal of this study was to screen a compound library to identify positive hits that can block meiotic maturation. We selected the Selleckchem Bioactive Compound library of 5,309 bioactive, cell-permeable, and structurally diverse compounds. For the initial screen, we prioritized a subset of 800 compounds based on calculated chemical properties and known targets. The phenotypic assay used oocytes at the GV stage collected from gonadotropin-stimulated mice, and spontaneous in vitro maturation (IVM) was performed in the presence of a test compound (10 μ M) or DMSO (vehicle). The positive control was milrinone (10 μ M), a Pde3a inhibitor known to inhibit meiotic maturation by elevating cAMP levels. Brightfield images were taken before and after IVM for

analysis of the maturation status. We defined a positive hit as one that reduced maturation rate by $\geq 60\%$ in 10 oocytes in two replicate assays. The hits were categorized into two tiers: Tier 1 (80-100% rate reduction) and Tier 2 (60% rate reduction). Tier 1 compounds that completely blocked meiosis at the prophase I stage (all GV) were prioritized over those that permitted a degree of meiotic resumption. In the 800-compound screen, we identified 21 positive hits (19 Tier 1 and 2 Tier 2). Thirteen compounds caused oocyte degeneration or severely abnormal morphology and were deprioritized. Several positive hits were annotated to target proteins known to inhibit meiotic resumption or progression, including Pde3, Kif11, and Cacna1h, validating our phenotypic screening platform. Other hits were annotated for activity on targets with no previously known function in meiotic maturation, including Galr3 and Scn9A. Hit validation and target identification experiments are ongoing to elucidate mechanisms of action on oocyte function and fertility. Hit validation includes concentration-response testing of fresh compound stocks, screening of structural analogs, and testing of additional compounds with the same annotated targets. Target identification experiments are focused on in vitro and in vivo gene modulation studies. Overall, these findings reveal a powerful screening pipeline to identify potential targets for non-hormonal female contraceptive development. This study was part of the Ovarian Contraceptive Discovery Initiative (INV-003385) funded by the Bill & Melinda Gates Foundation.

FOCUS SESSION 15 - PREGNANCY - IMPLANTATION TO PARTURITION

S15.1 - Assisted Reproduction Technologies: Friend or Foe for Conceptus Attachment and Pregnancy Maintenance?

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The combined use of genomics and Assisted Reproduction Technologies holds great promise to accelerate genetic progress in ruminants. In the pursuit of strategies to improve the sustainability of milk and beef production, Stephen T. Butler from Teagasc, Moorepark, Patrick Lonergan from the School of Agriculture and Food Science at University College Dublin and PhD student Alan D. Crowe collaborated to address this issue.

The research compared the timing of pregnancy loss in lactating dairy cows bred using artificial insemination versus transfer of fresh or frozen-thawed in vitro produced embryos. In addition, the effect of timing of conceptus attachment was evaluated in cows bred using artificial insemination with conventional semen versus cows bred using sex-sorted semen and cows that received a frozen-thawed in vitro produced embryo. The findings present evidence that initial pregnancy establishment, conceptus attachment and pregnancy maintenance to term is affected by the choice of reproductive technology used to breed cows.

Funding is gratefully acknowledged from the Teagasc Walsh Scholarships Program, The Department of Agriculture, Food and the Marine (Dublin, Ireland) Research Stimulus Fund (Grant 2021R665), as well as funding from a research grant from Science Foundation Ireland and the Department of Agriculture, Food and Marine (Grant 16/RC/3835; VistaMilk).

S15.2 - Single Cell Multiome Analysis of the Bovine Placenta Identifies Gene Regulatory Networks in Trophoblast Differentiation

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A central determinant of pregnancy success is proper development of the conceptus (embryo/fetus and associated extraembryonic membranes including the placenta). Pregnancy loss in cattle is a significant challenge and can be caused by inadequate development and differentiation of the placenta. Of note, the cellular and molecular mechanisms regulating bovine placenta development and trophoblast differentiation and function remain essentially undefined. While previous single-cell RNA-seq analyses have revealed dynamic changes in cell populations and gene expression patterns during bovine placental development, the specific contributions of genetic regulatory regions to these changes and their impact on successful pregnancy remain unclear. Therefore, the chromatin accessibility landscape across diverse cell populations was determined in the developing (day 40) and mature (day 170) bovine placenta using the 10X Genomics single-nuclei multiome (snRNA-seq and snATAC-seq) platform. The data were analyzed using Cell Ranger ARC v2.0.2, Seurat v5.0, Signac v1.12.0, Monocle3, and TFBSTools. Cell population defining genes were localized in the placenta using in situ hybridization. These analyses revealed distinct trophoblast, mesenchyme, endothelial, immune, and epithelial cell populations characterized by unique gene expression and chromatin accessibility signatures across both time points. ATAC-seq peaks defined open chromatin regions, facilitating the identification of transcription factor binding sites and gene regulatory networks involved with trophoblast differentiation. Transcription factors (TEAD4, GATA3, TFAP2A, TFAP2C), known for their involvement in trophoblast differentiation in other mammalian species, were identified as drivers of uninucleate (UNC) to binucleate (BNC) trophoblast differentiation. This study establishes a foundational understanding of gene regulation and expression in the placenta, offering insights into the mechanisms governing pregnancy loss in cattle. Supported by USDA NIFA AFRI Grant 2019-67015-28998.

S15.3 - Transcriptomic Profiling Identifies Key Players in Myometrial Activation During Chronic Equine Placentitis

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Equine ascending placentitis, an inflammation of the placenta typically caused by an infectious agent, remains the leading cause of abortions, stillbirths, and perinatal losses in several countries. The key event in placentitis-induced preterm labor is the loss of myometrial quiescence with subsequent initiation of labor; however, little is known about the molecular mechanisms underlying myometrial activation in the horse. To uncover these mechanisms, we characterized the transcriptome of the equine myometrium during ascending placentitis in comparison to gestationally matched normal pregnancy. In brief, myometrial samples were collected after euthanasia from mares with induced placentitis at approximately 290 days of gestation (placentitis group; n=6) and un-inoculated mares (control group; n=6). RNA sequencing (RNA-Seq) was conducted with the Illumina NovaSeq6000 platform and raw reads were trimmed with TrimGalore 0.4.3 before mapping to EquCab3.0 using STAR 2.4.3a. Mapped reads were quantified using Cufflinks 2.2.1 with the NCBI annotation. Differentially expressed genes (DEGs)

were evaluated using Cuffdiff 2.2.1 based upon a FDR < 0.05. Our study identified 263 DEGs in the myometrium during placentitis as compared to the control including the discovery of seven genes (APOBEC3Z1B, PLA2G2D, CXCL17, MMP8, MMP13, S100A8, and S100A9) that are uniquely upregulated (exclusively expressed) in the myometrium during placentitis. Gene groups of connexin (GJC2, also known as connexin-47), matrix metalloproteinases (MMP1, MMP8, MMP13, and MMP25), prostaglandin signaling (PTGS2, and PTGER3), calcium channel regulation (TRPM2), and calcium ion transport (RAMP2, CACNA1D, PRKCB, COR1A, and DEFB1), suggest a clear pathway for myometrial activation during equine placentitis. In addition, we identified CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL3, CXCL16, and CXCL17 as potential chemoattractant factors in the myometrium during placentitis. This finding was supported by the marked leukocytic infiltration observed in myometrial samples from the placentitis group compared to the control group. It is well known that leukocytic infiltration is the hallmark of myometrial activation. To identify genes linked with this inflammatory response, we utilized weighted gene co-expression analysis (WGCNA). The WGCNA resulted in identification of nine gene modules and revealed that the orange module retained the highest module-trait relationship ($r=0.92$, $P < 0.0001$) with inflammation score trait. This module was enriched for toll-like receptor (TLR) signaling, chemotaxis and inflammatory signaling. To identify potential upstream regulators relevant to myometrial activation, upstream regulator analysis was performed using Ingenuity pathway analysis, revealing 12 potential regulators: TLR2, STAT1, IRF7, SPI1, PTGS2, S100A8, S100A9, ICAM1, NRG1, HIF1A, PRKCB, and TYROBP. Additionally, the immunolabeling of TLR2, PTGS2, S100A8, S100A9, and HIF1A showed a significant positive correlation with their corresponding mRNA expression. Overall, the current study improves our understanding of key regulators and mechanisms underlying myometrial activation during equine placentitis, which may lead to the development of therapies for the prevention of preterm labor and/or treatment of placentitis. For instance, our results revealed the central role of TLR2 in triggering inflammatory signaling in the equine myometrium during placentitis, leading to the upregulation of several contraction-associated transcripts such as PTGS2, PTGER3, MMP1, MMP8, and GJC2. Therefore, strategies to block TLR2 and associated pathways (such as TLR antagonists) hold potential for therapies to prevent placentitis-induced preterm birth. Beyond the myometrial activation events described in the current study, this study provides the cornerstone for the construction of new hypotheses to be tested in the future.

S15.4 - Estradiol-Induced Labor Initiation: Changes on Gene Expression of Maternal Uterine and Cervical Tissues in Periparturient Ewes.

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The onset of labor is a complex process involving various hormonal and physiological pathways that remain to be completely understood. Herein, we investigated the influence of estradiol (E2) in the initiation of parturition of periparturient Rambouillet ewes. To this end, two experiments evaluated the relationship between E2 circulating levels, birth timing (Exp.1), and gene expression (Exp.2) on the myometrium, endometrium, cervix, and caruncle tissues. Both experiments utilized the same approach but with varying E2 dosages. Multiparous ewes

between 139 to 142 days of gestation were randomly assigned to either the E2 group, which received six Silastic implants of 50 mg of E2 in Exp.1 (300 mg/ewe; n=12) and four in Exp.2 (200 mg/ewe; n=6); or the control (C) group, which received 6 Silastic empty implants in Exp.1 (n=12) and 4 in Exp.2 (n=6). The implants were inserted subcutaneously in the axillary region of the ewe and removed two days after parturition (Exp.1) or at the time of slaughter, 26h after treatment (Exp.2). Maternal blood samples were drawn one day before treatment initiation in Exp.2 and 8h (Exp.1) or 26h (Exp.2) from the beginning of treatment to measure circulating levels of E2 and progesterone (P4) using immunoassay. Cross sections of the uterus, caruncles, and cervix were collected for RNA-Seq analysis. After RNA-Seq data quality control and mapping, differentially expressed genes (DEGs) were identified using DESeq2. Results indicated that before treatment, E2 levels between groups were similar. However, at 8h (Exp.1) and 26h (Exp.2) after treatment, E2 concentration was significantly higher in the E2 group (192.91 ± 17.32 vs. 10.97 ± 20.47 pg/ml at 8h $p=0.01$, and 149.21 ± 55.93 vs. 30.61 ± 11.73 at 26h $p=0.01$). No differences on the P4 level between groups were found at any point. Exp.1 showed a significant difference in days to deliver after treatment: 2.84 ± 1.02 in the E2 group vs 7.18 ± 1.11 in the C group ($p=0.01$). Furthermore, the E2 treatment significantly impacted gene expression in all the tissues tested ($p\text{-value} \leq 0.1$ and $\log_2$ fold change $|0.5|$). The cervix exhibited the strongest effect, with 8,073 DEGs, including E2 upregulation of PTGS-2, PTGES2, ESR2, and IL-6, and downregulation of IL-10, ACTA2, MYH11, GJA1, and TIMPs. The endometrium also showed significant changes, with 722 DEGs, including E2 upregulation of OXTR and ESR1 and downregulation of SULT1E1 and TIMPs. The caruncles had a more modest response, with 109 DEGs, while the myometrium was less responsive to the treatment, with 90 DEGs, including E2 upregulation of PTGS-2. Our findings suggest that E2 causes significant changes in gene expression in the maternal reproductive tissues, leading to parturition without a decline in circulating levels of P4. These effects appear to be primarily driven by the alterations in the cervix, where E2 upregulated genes associated with inflammation and cervical ripening and downregulated genes associated with smooth muscle contraction and tissue integrity. Supported by USDA-NIFA-AFRI grant 2021-67015-34277 to LPR and AJC

S15.5 - Embryo Developmental Potential Is Sensed by Receptivity-Related Transcriptional Networks in the Endometrial Epithelium

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Background The endometrium exhibits receptivity to embryo implantation in response to ovarian progesterone mid-way through the secretory phase of the menstrual cycle. Previous studies have suggested that embryo signals such as microRNAs may augment receptivity in the endometrial epithelium, while a selective quality mediated by underlying stromal fibroblasts has been proposed to prevent the implantation of non-viable embryos. **Experimental design and methods** Here, we used permeable transwell-separated cultures of endometrial epithelial organoids and stromal cells (derived

from a fertile donor) to explore endometrial responses to spent embryo culture media from morphologically comparable blastocysts that resulted in either live birth (n=4) or failed implantation (n=5) as part of assisted reproduction treatments. Cultures were primed with estrogen and stimulated with progesterone to model the mid secretory phase before treatment with embryo culture media for 24h, in parallel with cultures treated with blank embryo culture media (n=3). Organoids and stromal cells were separately analysed by RNA sequencing and their transcriptomes were profiled using EdgeR, Webgestalt and Ingenuity Pathway Analysis. Results Differential expression analysis between the groups identified 116 differentially expressed genes (DEG) in stromal cells in response to non-viable embryo culture media when compared to untreated samples (112 upregulated, 4 downregulated; padj< 0.05, >1 log2 fold change), though these were not mirrored in the comparison with samples treated with viable embryo culture media as no DEG were found. Organoids presented upregulation of 116 and 124 genes in response to developmentally competent embryo culture media when compared to untreated organoids and those treated with non-viable embryo culture media, respectively (padj< 0.05, >1 log2FC, 39% shared genes). Gene ontology analysis revealed that only 1 biological process was over-represented among the stromal cell DEG (FDR< 0.05), whereas functional linkages among the organoid DEG were highlighted by enrichment of 45 overlapping biological processes including cell adhesion and extracellular structure reorganization (FDR< 0.05). Interestingly, several developmental biological processes were also enriched within these DEG and consistent among these were the developmental patterning transcription factors HOXA10, HOXA11 and HOXD10, which are also well established as regulators of endometrial receptivity. Upstream regulators of HOX genes in this organoid dataset were predicted to be Hippo, PI3K and Relaxin signalling pathways (FDR< 10⁻⁷). Conclusions This is the first study, to our knowledge, that has investigated endometrial responses to clinically transferred blastocysts with known outcomes. Though a small sample size, we detected a robust and functionally enriched transcriptomic response in the endometrial epithelial cells only to developmentally competent embryos. Developmentally competent embryo culture media increased the expression of transcriptional regulators of receptivity in the epithelium, suggesting that these embryo quality cues augment receptivity likely by stimulating transcription rather than regulating post-transcriptional events. No evidence of stromal cell sensitivity to embryo developmental potential was found. Identifying pre-implantation embryo signals that reflect developmental competence and stimulate endometrial receptivity will advance both embryo selection in assisted reproduction treatments as well as our understanding of endometrial preparation for pregnancy.

S15.6 - ACLY Regulates the Metabolic Control of Histone Acetylation to Promote Human Trophoblast Stemness

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Expansion of the human trophoblast stem cells (hTSCs) pool is highly active during the first-trimester of pregnancy but declines as hTSCs differentiate into their specialised lineages over the course of

pregnancy. We previously showed that hTSCs differentiating into either syncytiotrophoblast or extravillous trophoblast results in decreased glycolysis leading to reduced acetyl-coA levels. Histone acetylation depends on acetyl-coA availability and thus on the cellular metabolic state. Histone acetylation is an important epigenetic process that supports trophoblast stemness by providing a permissive chromatin environment that promotes the transcription of stemness genes. However, the underlying mechanisms linking acetyl-coA availability with trophoblast stemness are unclear. We hypothesize that the metabolic enzyme ATP-citrate lyase (ACLY) which converts citrate to acetyl-coA regulates trophoblast stemness by promoting acetyl-coA availability for histone acetylation. Human TSCs (hTSCs, N>4) were transfected with siRNAs targeting ACLY or a non-targeting control and their effects on bioenergetics, histone acetylation, trophoblast stemness/differentiation and organoid development determined. hTSCs bioenergetics was quantified by extracellular flux analysis using the Seahorse bioanalyzer or LC-MS analysis of glycolytic and tricarboxylic acid (TCA) cycle intermediates. Global histone acetylation was measured by Western blot or CUT&RUN sequencing of H3K27Ac, a major mark for active enhancers and promoters regulating stemness genes. Trophoblast stemness and differentiation were determined by single-nucleus RNA-sequencing (sNuc-RNA-Seq). Lastly the ability of hTSCs to form trophoblast organoids was assessed as a proxy for placental development. In hTSCs, ACLY is nuclear localized but translocates to the cytosol upon differentiation. ACLY knockdown (KD) led to decreased H3K27 acetylation ($P < 0.01$), and reduced mRNA of trophoblast stemness genes (ELF5, TEAD4, TP63; $P < 0.01$) but an increase in syncytiotrophoblast markers (CYP19A1, HERVW; $P < 0.05$) suggesting precocious differentiation. Moreover, sNuc-RNA-Seq demonstrated reductions in populations associated with stemness markers but a gain in the syncytiotrophoblast population ($P_{adj} < 0.05$). Interestingly, ACLY-KD did not alter glycolytic or TCA cycle intermediates (except citrate and acetyl-coA) or mitochondrial respiration, suggesting that the effects were not due to altered bioenergetics. ACLY-KD in hTSCs resulted in decreased organoid size and number ($P < 0.05$) demonstrating a reduced capacity to form organoids. ACLY maintains trophoblast stemness by regulating the nuclear availability of acetyl-coA for histone acetylation. Loss of ACLY decreased stemness and resulted in premature differentiation into syncytiotrophoblast, which may lead to impaired placental formation. ACLY functions as a critical link between metabolism and epigenetics in the regulation of human trophoblast stemness.

FOCUS SESSION 16 - TROPHOBLAST

S16.1 - Bovine Beginnings: From Trophoblast Stem Cells to Placental Development

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In early embryogenesis, the development of the trophoblast into a functional placenta is vital for the success of pregnancy in all eutherian mammals. Prior to implantation, embryos are highly vulnerable and entirely reliant on the biotic and abiotic factors present in the uterine fluid. This crucial period, which is relatively prolonged in Ruminantia, also witnesses an exponential proliferation of trophoblast stem cells (TSCs) as embryos undergo elongation. In this clade, embryo elongation is akin to an essential race to maximize numerical access to uterine caruncles (the restricted endometrial sites that permit implantation) before placental differentiation. In recent systems biology investigations that integrate the embryo and the dynamic uterine environment in cattle, we uncovered the fundamental basis of TSC self-renewal and differentiation, along with key support provided in the uterine lumen for preimplantation development. Distinctively, suppressing the Ras homolog family member A (RhoA) supports an

autoregulated TSC self-renewal and accelerates proliferation, leading to unprecedented increases in TSC numbers and blastocyst sizes. In vivo, we found that a quasi-anoxic uterine lumen is conducive to increased glycolytic flux (anaerobic glycolysis), a mechanism that ensures the availability of carbon equivalents necessary for anabolism during embryo elongation. By analyzing the transcriptional basis of spontaneous TSC differentiation, we uncovered a core role for TGF β signaling in placental development. In vitro differentiation of TSCs by TGF β -RhoA directed downstream effectors recapitulates the range of terminally differentiated trophoblast cell types observed in the mature in vivo placenta. These mechanisms enrich crucial aspects of comparative and translational placental biology.

S16.2 - Rethinking Pregnancy Recognition in Ruminants: Initial Insights on Pregnancy in Type I Interferon Receptor (IFNAR2) Knockout Ewes

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The accepted paradigm for pregnancy recognition in sheep and other ruminants is that interferon-tau (IFNT), which sheep trophoblast cells secrete between days 10 and 21 of pregnancy, blocks endometrial epithelial cell expression of estrogen and oxytocin receptors (ESR1 & OXTR, respectively) and prevents the pulsatile release of prostaglandin-F2 α (PGF2 α) thereby preventing regression of the corpus luteum (CL). The effects of IFNT are mediated by the type I interferon receptor (IFNAR), a heterodimer of IFNAR1 and IFNAR2. We used CRISPR/Cas9 to disrupt IFNAR1 and/or IFNAR2 in sheep fetal fibroblasts and produced knockout lambs by somatic cell nuclear transfer. Subsequently, a herd of IFNAR2+/- ewes was developed and bred to IFNAR2+/- or IFNAR2-/- rams to produce IFNAR2-/- sheep. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) for interferon-stimulated gene (ISG) expression confirmed that IFNAR deficient sheep fail to respond to interferon-alpha (IFNA) and IFNT. Because IFNT is believed to provide the pregnancy recognition signal in ruminants, we hypothesized that IFNAR2-/- ewes would be infertile. However, two approximately nine-month-old IFNAR2-/- ewes were bred to an IFNAR2+/- ram in December 2022 and both ewes became pregnant. Pregnancy in these ewes was confirmed by progesterone profiling, pregnancy specific protein B (PSPB) enzyme linked immunosorbent assay (ELISA), and ultrasound exams. The ultrasound exams demonstrated that both ewes had live fetuses with a heartbeat. Both ewes delivered healthy lambs at term, one had an IFNAR2+/- lamb and the other had an IFNAR2-/- lamb. Establishment of pregnancy in two IFNAR2-/- ewes proves that the accepted mechanism of pregnancy recognition in ruminants, which involves IFNT interacting with IFNAR, is not required for establishment of pregnancy. Either there is a second receptor for IFNT, which seems unlikely, or there is an alternative, possibly redundant, mechanism for pregnancy recognition. Both IFNAR2-/- ewes were bred again in the fall of 2023, and both became pregnant for a second time. One ewe was euthanized on day 16 of pregnancy and had a filamentous conceptus, morphologically normal embryo, and prominent CL. Single cell suspensions were prepared from the chorion, uterine epithelium, and CL for single cell RNA sequencing (scRNA-seq). Analysis is ongoing, but thus far the most notable finding is that endometrial epithelial cell expression of the genes for the progesterone receptor (PGR) and estrogen receptor one (ESR1) were high, but oxytocin receptor (OXTR) gene expression was low. The accepted model for pregnancy recognition in ruminants implies that expression of all three receptors should be low on day 16 in a pregnant ewe. However, low expression of

the OXTR should be sufficient to block pulsatile release of PGF2 α and regression of the CL. Since IFNAR1 $^{-/-}$ and IFNAR2 $^{-/-}$ fetuses survive to term and are healthy at birth, and both IFNAR2 $^{-/-}$ rams and ewes are fertile, evidently IFNT signaling via IFNAR is not required for pregnancy recognition or the development of a healthy ruminant fetus. Hence, our working hypothesis is that something produced by trophoblast cells, other than IFNT, suppresses OXTR expression by endometrial epithelial cells and that this is the primary signal for recognition of pregnancy. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2021-67016-33504 from the USDA National Institute of Food and Agriculture.

S16.3 - Cell-Type Specific Transcriptomic Changes Associated with Maternal Obesity in Term Human Placenta

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One third of women in the United States are obese at the start of pregnancy. Maternal obesity (MO) increases the risk of adverse pregnancy outcomes, as well as the offspring's risk of neurodevelopmental and metabolic disorders later in life, but the mechanisms linking MO to offspring disease are poorly understood. The human placenta, located at the maternal-fetal interface, is thought to contribute to fetal programming of maternal exposures. Its transcriptome may reflect mechanisms underlying offspring disease risk. In this study, we examine cell-type specific changes associated with maternal obesity in the human placenta using single-nucleus RNA sequencing (snRNA-seq), with improved recovery of multinucleated syncytiotrophoblast (SCT) nuclei compared to single-cell sequencing. Term placentas from pregnancies with maternal BMI 35+ (MO condition) and 18.5-25 (control condition) were obtained from the Women's and Infant Health Specimen Consortium. Nuclei were extracted from each of 10 decidual samples and 10 villous tissue samples of the placenta and subject to 10X snRNA-seq. Nuclei expression were filtered, clustered, and annotated by cell type using Seurat, after removing noise from empty droplets using CellBender. Differentially expressed genes (DEGs) for each cell type were calculated using the Nebula3 package in R, accounting for the covariates fetal sex, maternal age, batch, and delivery mode. Pathway analysis was conducted using fast gene set enrichment analysis (fgSEA) and decoupleR. After quality control filtering, 62,864 nuclei remained. Trophoblast, myeloid, lymphoid, endothelial, and fibroblast cell clusters were identified using markers from previous placenta single-cell RNA-seq studies. In both decidua and villous tissues, fetal macrophages (FM), which make up 6% of all nuclei, and SCTs, the most abundant cell type at 61% of all nuclei, had the highest number of DEGs. >80% of DEGs which were downregulated in MO in each cell type and placental face. DEGs in SCTs were enriched for targets of transcription factors HMGA1 and HOXB1 in the villous tissue, and MYCN, FOXD1, ARID3B, and ETV4 on the decidual side. Several trophoblast sub-types showed enrichment for hypoxia-induced genes. FM from MO in the villous tissue were enriched for increased allograft rejection and graft versus host disease pathways. To further characterize this inflammatory process, we compared DEGs to signatures from immune signaling pathways and found that JAK-STAT signaling was increased in numerous cell types in villous tissue, and NF κ B perturbation was found in stromal cells on both sides of the placenta. MO disrupts vascularization and inflammatory processes in the placenta. DEGs in fetal macrophages show enrichment for inflammatory pathways, potentially relevant for neurodevelopment, as placental macrophages are thought to reflect fetal microglia due to a shared developmental lineage.

Consistent perturbations across cell types suggests that cell-cell communication should be further investigated.

S16.4 - Exposure Of Trophoblast Cells to the Antiretroviral Drug Dolutegravir Impairs MMP Activity and Cytokine Release

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Exposure of Trophoblast Cells to the Antiretroviral Drug Dolutegravir Impairs MMP Activity and Cytokine ReleaseMona H Al-Mugotir¹, Guojuan Li¹, Micah Summerlin², Claire Wing¹, Aditya N. Bade², Lynda K. Harris¹.1. Department of Obstetrics & Gynecology, University of Nebraska Medical Center, Omaha, USA2. Department of Pharmacology & Experimental Neuroscience, University of Nebraska Medical Center, Omaha, USA

Pregnant individuals with human immunodeficiency virus type one (HIV-1) infection are recommended to continue antiretroviral therapy (ART) throughout gestation, to prevent vertical transmission of HIV-1 from mother to child. However, limited safety data exist for ARVs, especially the potential detrimental effects on fetal or placental development. The aim of this study is to assess the effects of ARV therapy on placental function and identify potential interventions to mitigate adverse responses. Dolutegravir (DTG) is a preferred ARV for pregnant women and those of child-bearing age, despite being associated with poor pregnancy outcomes and neurodevelopmental deficits in exposed offspring. We have previously reported that DTG inhibits trophoblast migration and matrix metalloproteinase (MMP) activity in HTR-8/SVneo trophoblast cells. In this study, we hypothesized that placental exposure to DTG elicited an inflammatory response, which resulted in altered cytokine secretion and reduced MMP activity. Aspirin is an effective anti-inflammatory compound, but its use after 20 weeks' gestation can cause fetal renal dysfunction and premature closure of the ductus arteriosus. Thus, we also assessed whether free aspirin, or aspirin-loaded into placental targeting liposomes, could protect trophoblasts from the detrimental effects of DTG. HTR-8/SVneo cells representing first trimester extravillous trophoblast, and BeWo choriocarcinoma cells representing term villous trophoblasts were treated with clinically relevant concentrations of DTG (10 μ M) in the presence or absence of aspirin (100 μ M). Targeted liposomes containing aspirin or PBS were prepared by the thin film method, using cholesterol, DSPC, and DSPE-PEG. The placental targeting peptide TAMRA-CGGGCGKRK was covalently coupled to maleimide groups on the liposomal surface, producing nanoparticles ~200 nm in diameter. MMP-2 activity and proinflammatory cytokine release were measured by gelatin zymography and ELISA, respectively. Exposure of BeWo cells to DTG significantly reduced MMP-2 activity after 48h and co-treatment with aspirin did not restore MMP-2 activity. Release of the pro-inflammatory cytokine interleukin-6 (IL-6) from BeWo cells was modestly decreased by DTG exposure and was not rescued by aspirin co-treatment. Interleukin-1 β (IL-1 β), interleukin-1 receptor antagonist (IL-1Ra) and IL-10 were undetectable in BeWo cell culture medium. In HTR-8 cells, DTG treatment did not significantly alter IL-1Ra and IL-10 release, but dramatically reduced levels of IL-6. Co-treatment of HTR-8 cells with free- or liposomally encapsulated aspirin did not rescue DTG suppression of IL-6. Levels of IL-1 β were below the level of detection. In contrast to our hypothesis, DTG treatment decreased IL-6 release from both trophoblast cell lines and neither freely administered, nor liposomally-encapsulated aspirin ameliorated this response. As observed in HTR-8 cells, DTG significantly inhibited MMP-2 activity in BeWo, which could not be rescued by aspirin co-treatment. Given the important roles

of trophoblast-derived MMP-2 and IL-6 in mediating uterine spiral artery remodeling, and regulating extracellular matrix turnover and placental hormone synthesis, DTG may impair placental function by multiple mechanisms. As DTG continues to be recommended in pregnancy worldwide, effective strategies to define and mitigate its detrimental effects are urgently needed.

S16.5 - Microbial-Derived Tryptophan Metabolites Alter Trophoblast Differentiation and Metabolism Through G-Protein Coupled Receptors (GPCRs)

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Impaired placental development accounts for many pregnancy complications and contributes to nearly 5 million maternal and infant deaths annually. Optimal placental and hence trophoblast development depends on the fine balance between the renewal of the cytotrophoblast stem cells and their differentiation into the invasive extravillous trophoblast and the multinucleated syncytiotrophoblast (ST) epithelial cells. While pregnancy is punctuated by finely timed fluctuations in various hormones, some of which regulate trophoblast differentiation, the reasons for the concomitant changes in the gut microbiome with advancing gestation are unclear. The gut microbiome is confined to the gastrointestinal tract, biologically active metabolites derived from it do reach the systemic circulation. These may mediate microbial effects on the host during pregnancy. We aimed to investigate effects of microbial-derived tryptophan metabolites (Tryptamine (Tryp), Indole-3-acetylaldehyde (IAAID) and Indole-3-acetic acid (IAA)) on trophoblast function and differentiation. For all assays, primary differentiated trophoblast cells isolated from term human placentas from participants who had given consent were used. We assessed dose dependent effects of the metabolites on energy metabolism by treating the cells with the metabolites for 24 hrs. An hour before energy metabolism assessment (Mito stress test), the metabolites were removed and replaced with normal media without the metabolites. Effects of Tryp and IAA on trophoblast differentiation, were assessed by measuring Beta-human chorionic gonadotropin (β -hCG), (a marker of trophoblast differentiation) released into the media over four days using immuno assays. To assess whether the metabolites act through G-protein coupled receptors (GPCR) cells were also treated with either 50 μ M Tryp alone or in the presence of forskolin (1.25, 2.5, 5 or 10 μ M for 10 minutes) and analysed using the cyclic adenosine monophosphate (cAMP) Glo assay. Forskolin is an inducer of cyclic adenosine monophosphate (cAMP) production and is commonly used to assess effects of ligands on inhibitory GPCR signalling. We demonstrate that microbial-derived tryptophan metabolites Tryp (n=10), IAAID (n=5), and IAA (n=7) confer differential effects on metabolism. Tryp reduced basal respiration ($p=0.039$) and maximal respiration ($p=0.004$) and IAAID reduced maximal respiration ($p=0.025$) but IAA had no effect on energy metabolism. Tryp dose dependently reduced β -hCG production at 12.6 μ M ($p=0.043$, n=5) and 50 μ M ($p\leq 0.0001$, n=5). At similar doses IAA had no effect on trophoblast differentiation. Tryp reduced cAMP production by 20% ($p=0.035$, n=4) and shifted the EC₅₀ of forskolin induced cAMP production dose response from 2.6 μ M to 4.0 μ M. This suggests that Tryp may act through inhibitory GPCR signalling. Consistent with our suggestion above, microbial metabolites altered both trophoblast energy metabolism and differentiation and this may occur through GPCR signalling. As GPCR signalling is important for the regulation of stem cell pluripotency, differentiation, and cell polarity in embryogenesis, these metabolites may also affect other developmental processes. These findings

have implications for microbial community and metabolite changes during pregnancy which may influence placental development, function and potentially pregnancy outcomes.

S16.6 - 3D-Cultured Human Blastoids Model Trophoblast Expansion, Diversification, and Invasion During Post-Implantation Development

Rowan M. Karvas, Joey Zemke, Eric W. Upton, Eshan Sane, Laura A. Fischer, Kyoung-mi Park, Chen Dong, Brian Chew, Brittany Meyer, Andrew M. Kelleher, Thomas E. Spencer, Sabine Dietmann, and Thorold W. Theunissen

Since access to human embryos is limited, our understanding of the factors governing blastocyst implantation, trophoblast differentiation, and invasion into the maternal endometrium remains rudimentary. Stem-cell-derived blastoids provide a model of human pre-implantation development, but their capacity for post-implantation development beyond 11-12 days is yet to be explored. The objective of this study was to determine whether blastoids allow for the study of the emergence of placental structures, which includes primary and secondary villi, beyond 14 days. Blastoids were derived from Si/L/A naïve stem cells obtained from multiple independent genetic backgrounds. Pre-implantation blastoids were generated using a newly optimized two-step protocol for a total of 7 days. Pre-implantation blastoids were transferred to a thick, 3D commercially available extracellular matrix and allowed to undergo apposition, attachment, nidation, and invasion for an additional 7-11 days. Single cell transcriptome profiling reveals lineage segregation and an absence of off-target cell types in pre-implantation blastoids, while extended blastoid culture is accompanied by epiblast maturation and rapid expansion and diversification of trophoblast lineages by day 14. 3D-cultured human blastoids undergo lumenogenesis of the epiblast and sprout primary villus-derived invasive trophoblasts that interact with human endometrial organoids. Extended blastoid culture for an 4 additional days results in the localized activation of gastrulation marker T within the epiblast and the migration of VIM+ extraembryonic mesoderm cells into the trophoblast compartment. We also provide evidence that modulation of WNT signaling alters cell fate decisions in post-implantation blastoids. These results indicate that 3D-cultured human blastoids model trophoblast expansion, diversification, and invasion during early post-implantation development. Implantation failure is implicated in approximately half of all pregnancy losses and is a major cause of idiopathic infertility. Consequently, our work provides a robust model system to probe a previously inaccessible but critical stage of human development.

FOCUS SESSION 17 - TOXICOLOGY

S17.1 - Bovine and Porcine In Vitro Embryo Production (IVEP) and Confocal Laser Scanning Microscopy of Blastocysts in Toxicity Testing

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In vitro models offer the potential to predict in vivo outcomes. Within reproduction, a narrow yet critical timeframe surrounds fertilization, encompassing the final maturation of the oocyte, fertilization itself, and the initial stages of embryo development. This temporal window can be simulated in vitro. Environmental contaminants and other chemicals can reach the follicular fluid and affect the maturation of the oocyte. Through collaboration between human reproduction clinics and our animal IVEP unit, we have collected and analyzed follicular fluid (FF) from women undergoing reproductive treatments. We then utilized bovine and porcine IVEP to test the effects of some of the anthropogenic chemicals

identified. Since our aim was to simulate the final oocyte maturation within the follicle environment, our treatments lasted only for the duration of the in vitro maturation.

Our primary focus has been perfluoroalkyl substances (PFASs), a large group of synthetic chemicals used in a wide range of products, from everyday use non-stick surfaces to firefighting foam. Possible health effects of exposure to PFASs include liver damage, alteration in immune responses, and cancer. On the reproductive side, PFASs is suspected to be endocrine disruptors and could be linked to disturbances in cyclicity, follicle growth and pregnancies, but there is limited evidence supporting this. PFASs are considered “forever chemicals” will persist in the environment long after a possible ban of these products is in place.

In a first study, perfluorononanoic acid (PFNA) was investigated in the concentrations 10000 ng/mL and 100 ng/mL during 22 h of final bovine oocyte maturation. The higher concentration had a negative effect on cumulus cell expansion as well as on blastocyst formation on day 8 after fertilization and was not investigated further. The lower concentration showed no effect on blastocyst percentage but in confocal laser scanning microscopy the lipid droplet distribution was significantly altered in the blastocysts. In FF from women undergoing reproductive treatments, PFNA was found in concentrations around 0.3 ng/mL. Subsequently, perfluorohexane sulfonate (PFHxS) was examined in the same system at concentrations ranging from 10 ng/mL to 100000 ng/mL. Oocyte developmental competence decreased at concentrations ≥ 40000 ng/mL, with observed altered lipid distribution in blastocysts exposed to 1000-10000 ng/mL PFHxS. In human FF, PFHxS concentrations were around 1.1 ng/mL.

Perfluorooctane sulfonate (PFOS) was then investigated at concentrations of 2 ng/mL and 53 ng/mL in the same setting. The higher concentration led to delayed cleavage and blastocyst development, with fewer blastomeres in blastocysts and altered lipid distribution observed compared to controls. Human follicular fluid contained PFOS concentrations around 4.5 ng/mL.

In a subsequent experiment, porcine in vitro maturation (45 hours) was utilized. PFHxS at 40000 ng/mL and PFOS at 100 ng/mL were employed. PFOS showed no discernible effect, while PFHxS resulted in higher blastocyst rates and blastomere numbers on day 6 post-fertilization. However, there was a tendency for higher apoptosis rates in blastocysts following PFHxS treatment during oocyte maturation. In conclusion, we utilized bovine and porcine IVEP along with confocal microscopy to assess PFASs effects at concentrations reflective of both human exposure and with safety margins to account for the short treatment time and possible species differences. Effects on embryo development and blastocyst phenotype in both species suggest the potential utility of this model for screening chemicals suspected of reproductive effects.

S17.2 - Ovarian Impact of Forever Chemical Per- and Polyfluoroalkyl Substances

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The extensive use of forever chemical per- and polyfluoroalkyl substances (PFAS) has led to environmental contamination and bioaccumulation. Previous research linked exposure to PFAS and female reproductive disorders, but the mechanism remains elusive. Further, most studies focused on legacy long-chain PFAS, yet the reproductive impacts of other long-chain PFAS and short-chain alternatives are rarely explored. In this talk, we will introduce our research regarding the effects and mechanisms of long- and short-chain PFAS on the ovary and associated ovarian functions and reproduction. The research models we use include a 3D ex vivo ovarian follicle culture system, an in vivo long-term oral exposure mouse model, a human cohort epidemiological study, and other molecular, computational, and analytical approaches. Our research demonstrates that long-chain PFAS acts as an

agonist of peroxisome proliferator-activated receptor gamma (PPAR γ) in granulosa cells to interfere with gonadotropin-dependent ovarian follicle maturation, hormone secretion, and ovulation, posing a non-negligible risk to women's reproductive health by heightening the probability of anovulation, irregular menstrual cycles, and infertility.

S17.3 - Understanding the Impact of Estrogen and Endocrine-Disrupting Chemicals (EDCs) On the Mammary Gland Metabolism Using Mammary Epithelial Cell in Two- and Three-Dimensional Cultures

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Due to its sensitivity to hormonal signaling, the mammary gland is often referred to as a sentinel organ for the study of endocrine-disrupting chemicals (EDCs), environmental pollutants that can interfere with the estrogen signaling pathway and induce mammary developmental defects. If and how EDCs impact mammary epithelial cell metabolism has not yet been documented. Herein, to study how estrogens and EDCs modulate mammary gland metabolism, we performed bioenergetic flux analyses using mouse mammary epithelial organoids compared to cells grown in monolayer culture. Several EDCs were tested, including bisphenol A (BPA), its close derivative BPS, a new BPA replacement copolyester called TritanTM, and the herbicide glyphosate. We report that estrogens reprogrammed mammary epithelial cell metabolism differently when grown in two- and three-dimensional models. Specific EDCs were also demonstrated to alter bioenergetic fluxes, thus identifying a new potential adverse effect of these molecules. Notably, organoids were more sensitive to low EDC concentrations, highlighting them as a key model for screening the impact of various environmental pollutants. Mechanistically, transcriptomic analyses revealed that EDCs interfered with the regulation of estrogen target genes and the expression of metabolic genes in organoids. Furthermore, co-treatment with the anti-estrogen fulvestrant blocked these metabolic impacts of EDCs, suggesting that, at least partially, they act through modulation of the estrogen receptor activity. Finally, we demonstrate that mammary organoids can be used for long-term studies on EDC exposure to study alterations in organogenesis/morphogenesis and that past pregnancies can modulate the sensitivity of mammary epithelial organoids to specific EDCs. Overall, this study demonstrates that estrogens and EDCs modulate mammary epithelial cell metabolism in monolayer and organoid cultures. A better understanding of the metabolic impacts of EDCs will allow a better appreciation of their adverse effects on mammary gland development and function.

S17.4 - Cannabis Consumption Impacts Human Embryo Euploid Rates

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In recent decades, cannabis consumption and legalization has been increasing globally. It is the third most used substance by reproductive aged people and our group demonstrated that tetrahydrocannabinol (THC) reaches the ovarian follicle. Using animal models, there have been reports of altered oocyte maturation and embryo development, but the results were conflicting. In humans, previous studies investigating the impact of cannabis consumption on in vitro fertilization (IVF) outcomes were based on self-reporting, introducing significant bias, and the effects of cannabis are inconclusive. Thus, the objective of this study was to unbiasedly measure THC in follicular fluid (FF) from patients undergoing IVF treatment and investigate the impact of this drug on IVF outcomes. Follicular aspirate was obtained from consented patients on the day of their oocyte pick-up (OPU) by our biobank (Jan 2018-Dec 2023), processed to remove cells and debris, and the resulting follicular fluid (FF) was snap-frozen and stored in liquid nitrogen. In 40 sample batches with appropriate positive and negative controls, 1059 FF samples were assessed for Δ^9 -THC, and two of its metabolites 11-OH- and 11-COOH-THC by LC-MS/MS. Calibration curves (0.001-200ng) were generated to permit absolute quantification and samples were considered positive when the concentration was above the lower limit of detection. For a subset of patients, matched serum and FF were assessed for THC. Positive samples were matched to negative samples (2:1 control:case) for demographic and stimulation parameters. Of the 1059 cycles, 62 were positive for THC or its metabolites resulting in 5.9% positivity rate, with mean concentrations of 27.1nM for Δ^9 -THC, 8.1nM for 11-OH-THC and 53.0nM for 11-COOH-THC. There was no significant difference between THC levels in serum compared to matched FF. There was no significant difference in the demographics of cases vs. controls (age, BMI, AMH levels, etc.). Further, there was no significant difference in maturation rate (cases: 74.6%, controls: 71.7%), fertilization rate (cases: 79.3%, controls: 80.8%), or blastocyst rate (cases: 53.3%, controls: 55.5%). When assessing the euploidy rate, there was a significant decrease (cases: 57.2%, controls: 63.9%, $p < 0.01$). Altogether, these results show that the presence of THC in the ovarian follicle did not alter oocyte maturation or embryo development, but was associated with a modest, but statistically significant, decrease in the IVF embryo euploidy rate. This is the first study, to our knowledge, to objectively assess the impact of THC on IVF outcomes. Cannabis legalization and use is increasing worldwide and understanding its role in female fertility and folliculogenesis is critical.

S17.5 - Environmentally Relevant Concentrations of Individual Per- And Polyfluoroalkyl Substances (PFAS) and a PFAS Mixture Impacts Proliferation and Gene Transcription in a Human Myometrial Cell Line.

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Per- and Polyfluoroalkyl substances (PFAS) are a group of environmentally persistent synthetic chemicals. PFAS are found in many consumer products including non-stick cookware, food packaging materials, upholstery, and personal care products. Accordingly, PFAS are a major source of water and soil contamination. In women, many adverse reproductive effects have been associated with PFAS exposure including reduced fertility, changes in the menstrual cycle, reproductive hormone disorders, and reproductive tract diseases. Epidemiological studies have linked forever chemicals with increased risk in uterine diseases including fibroids however, the mechanisms involved remain to be elucidated. This study investigated the effects of individual PFAS, including long-chain “legacy” PFAS [perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS)] and short-chain “alternative” PFAS compounds [undecafluoro-2-methyl-3-oxahexanoic acid (GenX/HFPO-DA), perfluorobutanesulfonic acid (PFBS)], as well as a mixture of these chemicals on the function and transcriptome of an immortalized human myometrial cell line (UT-TERT), hypothesizing that exposure to various PFAS would promote cell growth and alter gene expression. The impact on PFAS exposure on UT-TERT cell proliferation and cell viability was evaluated by the MTT assay. Cells were cultured with different environmentally relevant concentrations (0.1, 1, 10, 10, 1000 ng/mL), PFAS mixture (1 ng/mL each of PFOS/PFOS/GenX/PFBS), or vehicle control (Methanol < 0.01% final concentration) for 96h. Increased cell proliferation was observed at all concentrations of individual PFAS and the PFAS mixture ($P < 0.05$). Cell cycle analysis via flow cytometry further supported the proliferative action of the individual PFAS (1 ng/mL) and PFAS mixture via increases ($P < 0.05$) in the percentage of cells in S-phase. To identify genes altered after exposure to a PFAS in myometrial cells, RNA-sequencing was performed followed by bioinformatic analysis. The significantly upregulated and downregulated genes were chosen based on a log2foldchange value of 1.5 and ($P < 0.05$). Relative to control treated cells, UT-TERT cells exposed to legacy PFAS (PFOA and PFOS), 114 genes increased and 96 genes decreased after exposure to PFOA, while exposure to PFOS increased 128 and decreased 122 genes. UT-TERT cells treated with alternative PFAS GenX and PFBS had 172 and 116 genes upregulated, respectively, while 120 and 98 genes were downregulated after 96 h exposure compared to control treated cells. Additionally, the PFAS mixture upregulated 134 genes and downregulated expression of 91 genes. Among the genes altered, 33 genes were shared between all the PFAS treatments, indicating the existence of shared and distinct transcriptomic profile and pathways. Interestingly, genes involved in cell proliferation and differentiation, Homeodomain-Only Protein Homeobox (HOPX) and WW And C2 Domain Containing 1 (WWC1, also known as KIBRA) were upregulated by 4.8 and 1.5-fold, respectively. Additionally, KEGG pathway analysis indicated enrichment in TNF signaling and Hippo signaling pathways. Overall, this study demonstrates functional and transcriptomic changes in myometrial cells after exposure to PFAS, improving our knowledge about the impacts of PFAS exposures and female reproductive health. These findings suggest that PFAS compounds have the potential to disrupt normal myometrial cell function with possible long-term consequences on overall reproductive health.

S17.6 - Influence of Gut Microbiome and Diisononyl Phthalate (DiNP) Exposure on Uterine Development in Mice

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Diisononyl phthalate (DiNP) is a high-molecular weight phthalate used to make plastics more flexible and durable. DiNP usage is increasing as it is replacing another similar phthalate, di(2-ethylhexyl) phthalate (DEHP). However, DiNP exposure has been shown to have negative effects on female reproductive health due to its endocrine disrupting chemical properties. Ingestion is the most common route of DiNP exposure, making the gastrointestinal tract and its metabolizing and hormone secreting gut-microbiome directly exposed to DiNP. The gut-microbiome has been shown to influence female reproductive health and conditions. This study examined the effect of absence and presence of a gut microbiome on uterine development as well as the effects of acute DiNP exposure either in the absence or presence of a gut microbiome on uterine development. Over a 3-day period, 18 female C57Bl/6 germ-free (-microbiome) one-month old mice were orally dosed with either sterile PBS (n=8) to remain germ free (-microbiome) or dosed with colon contents (n=10) to develop a gut-microbiome (+microbiome). This was followed by a 10-day period where half of the -microbiome and +microbiome mice were orally dosed with corn oil and half were orally dosed with 200 µg/kg/day of DiNP. Mice used as a control group were specific pathogen free (SPF) (born with microbiome). Mice were euthanized in diestrus. Uteri were collected, processed, and fixed in paraffin for histological analyses. Tissue sections were stained with hematoxylin and eosin for morphological measurements. Immunohistochemistry was done for specific markers, estrogen receptor alpha (ESR-1), FOXA2 (uterine gland marker), Ki67 (proliferation), and alpha smooth muscle actin (smooth muscle cells). Slides were scanned and imaged using NDP.scan 3.2.15 software and Hamamatsu NanoZoomer 2.0 HT. Statistical analyses were performed using Graph Pad Prism 9.4.0 and significance levels were set at $p < 0.05$. Uterine development appeared to be markedly delayed by the initial absence of a gut-microbiome in all germ free mice. In comparison to the control specific pathogen free mice, there was a significant reduction in uterine size, stroma area, and numbers of uterine glands in all the treatment groups. Analyses of myometrium thickness showed that the two myometrial layers were substantially underdeveloped in all of the germ-free mice, regardless of whether a microbiome was established subsequently in the treatment protocol. Proliferation of endometrial stromal cells was reduced in the germ-free mice. Notably, the number of uterine glands was greatly reduced in all the germ-free mice groups though ESR-1 and FOXA2 expression appeared normal. DiNP treatment had no significant effects on these parameters. Peripheral estradiol levels did not differ between groups and the ovaries had similar numbers of follicles at the different stages of development. These results reveal that the initial absence of the gut-microbiome has a crucial effect on uterine development, resulting in smaller uterine size, a thinner, underdeveloped myometrium, and many fewer uterine glands. Whether establishing a microbiome in germ free mice at an earlier age might overcome the initial negative impact of no microbiome deserve further investigation. Although DiNP exposure had no effect in either the presence or absence of a gut-microbiome, this suggests examining alternative pathways that DiNP exposure can affect uterine development.

FOCUS SESSION 18 - MALE FERTILITY

S18.1 - Sperm Transport Across the Ovine Cervix; Do Norwegian Sheep Hold the Answer?

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Cervical/vaginal artificial insemination (AI) with frozen-thawed semen in sheep is limited worldwide due to poor pregnancy rates. The exception is in Norway where vaginal AI to a natural oestrus yields non-return rates in excess of 60% and work by our group has demonstrated that this is due to the ewe breed used in Norway. Here, we used a novel sheep model consisting of four European ewe breeds, namely, Suffolk, Belclare, Fur and Norwegian White Sheep (NWS) with known differences in cervical sperm transport following cervical AI with frozen-thawed semen to interrogate the components in cervical mucus and cervical tissue that could explain these differences. By combining chemical analytical techniques, we identified higher levels of sialic acid in the low fertility Suffolk breed compared to high fertility ewe breeds (NWS and Fur). This is supported by higher expression of sialic acid related genes in the cervical epithelium of the Suffolk. Using RNA-sequencing, we also detected a more active immune response in Suffolk. This molecular signature was reflected in the metabolomic composition of cervical mucus, represented by reduced levels of lipids involved in the resolution of inflammation and a large number of amino acids derived from mixed anaerobic bacteria in the Suffolk breed. Using 16S rRNA sequencing we also identified higher microbial abundance and diversity in the low fertility Suffolk breed. In conclusion, these results indicate a suboptimal environment in the cervix of some ewe breeds which is likely to have knock-on negative consequences for sperm transport.

S18.2 - Hyper-7 Sensor: A Novel Tool for Unveiling the Role of Hydrogen Peroxide in Bovine Zygotes

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Despite being viewed as detrimental to cellular processes, hydrogen peroxide is an essential signaling molecule in biological processes, including early embryogenesis in *Xenopus*, where the interplay between calcium and mitochondrially produced H₂O₂ (mt-H₂O₂) mediates early cleavage. However, the dynamics of H₂O₂ fluctuation and its role in the mammalian zygote remain largely unexplored owing to the absence of imaging techniques to monitor H₂O₂ fluctuation in living embryos and the challenge of experimentally suppressing its production during culture. To address this issue, we first optimized a protocol to detect basal mt-H₂O₂ levels in bovine zygotes using a novel ratiometric sensor consisting of a mitochondrially targeted fluorescent protein called HyPer7 (Hyper7-MLS). This sensor utilizes sequential excitation to 405 and 488 nm wavelengths and emission peaks at 516 nm. As HyPer7 undergoes oxidation by H₂O₂, F405 intensity diminishes while F488 intensifies, resulting in an F488/F405 ratio that serves as an index of H₂O₂ content. The mRNA encoding Hyper7-MLS was produced by *in vitro* transcription of the pCS2+MLS-HyPer7 plasmid (Addgene). Cumulus oocyte complexes (COCs) were

collected from abattoir-derived ovaries, cultured under meiotic arrest conditions for 3 hours to synchronize the onset of meiotic resumption, and then matured for 22 hours. In vitro matured oocytes were then freed of cumulus cells and microinjected with Hyper7-MLS mRNA. Oocytes were co-incubated with sperm for 10 hours in the presence of intact in vitro matured COCs. Zygotes were imaged for up to 1 hour using a Nikon-Eclipse Ti2-E with Yokogawa W1-SoRa spinning disk microscope equipped with a CO2 chamber using the appropriate Hyper7 settings. Subsequently, the ratio of fluorescent signal intensities was calculated after background subtraction using the NIS-Elements software (Nikon). The sensor's ability to detect increasing H2O2 levels was initially confirmed by comparing the Hyper7-MLS signal ratio in 154 zygotes, divided into control and tert-butyl hydroperoxide (t-BOOH)-treated groups. t-BOOH served as a pro-oxidant stimulus to induce mt-H2O2 production. Then, we tested the hypothesis that increasing concentrations of an antioxidant cocktail could serve as an experimental model to suppress mt-H2O2, thus providing a tool to assess the role of mt-H2O2 in zygotes. A total of 370 in vitro matured oocytes were fertilized, separated from the co-cultured COCs, and exposed for 6 hours to standard culture conditions or a mix of acetyl-L-carnitine, N-acetyl-L-cysteine, and α -lipoic acid at 10, 100 and 1000 times the concentration previously reported to reduce oxidative stress. The zygotes were then cultured for 7 days. Data analysis revealed that increasing antioxidant concentrations impaired blastocyst development with significant effects starting at 100X concentration (One-way ANOVA followed by Tukey's test, $p < 0.05$). To further assess whether the 100X antioxidant mix reduced mt-H2O2, 35 and 30 zygotes expressing Hyper7-MLS were cultured under control or 100X antioxidant condition, respectively, and imaged. Contrary to our hypothesis, increasing the concentration of antioxidants did not reduce mt-H2O2 but significantly increased its production (Two-way ANOVA followed by Šidák's test). Hence, the reduced embryonic developmental competence is not due to suppression of mt-H2O2, but rather to some paradoxical effect of antioxidants in high concentrations. In conclusion, we established a method to quantitatively measure mt-H2O2 in bovine zygotes that could serve as a tool to further elucidate its function during early embryogenesis. Additional efforts are needed to optimize methods to modulate its production. Funded by SEED2019 UNIMI No.1250 (cROSs-Talk) and RL PSR2014-2020 No.202102146691 (R-INNOVA).

S18.3 - Investigating the Role of the Sperm Centrioles in Rabbit Reproduction

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Centrioles are subcellular organelles often defined by their barrel shape and triplet microtubule blades. They nucleate astral microtubules or cilia and orient the spindle pole during cell division. Regularly dividing cells have precisely two centrioles that are duplicated during cell division so that each daughter cell also has two centrioles. Surprisingly, the sperm of non-murine mammals appeared only to have one recognizable centriole to contribute to the zygote, and the egg had no centrioles. Our lab discovered that the sperm of humans, cattle, and rabbits do have two centrioles, the previously known typical centriole, the proximal centriole (PC), and one with an atypical structure, the distal centriole (DC), which is attached to the tail axoneme (Ax). The distal centriole is composed of two asymmetrical novel rod-like structures. These sperm centrioles are part of a multi-component dynamic basal complex (DBC) that coordinates sperm head and tail movement. As part of this movement, the distal centriole rods move relative to each other, but the essential role of the rod is unclear. To gain insight into this question, we generated a genetic model where we mutated one of the rod components, POC1B, which is essential for sperm morphology and motility. We generated the mutation in rabbits because their sperm closely

resembles human and bovine, unlike mice which have a different sperm morphology and no sperm centrioles. Severe POC1B loss of function resulted in spermatozoa with abnormal morphology, therefore we created a rabbit mutant with partial POC1B loss of function. We found that rabbits homozygous for a POC1B mutation that results in a 78 amino acid truncation, produced motile sperm with normal morphology, demonstrating that the POC1B C-terminus is dispensable for spermatozoa morphology. In the homozygote Poc1B rabbit mutant, POC1B does not localize to either the PC or the DC, demonstrating that the POC1B C-terminus is essential for POC1B localization to the distal centriole rods. We also found that the POC1B C-terminus is essential for the normal structure of the DC rods, and the rods are fused and unable to slide during sperm tail beating. Furthermore, it appears that the POC1B C-terminus mutant may be less competitive than wild-type sperm. Overall, these studies provide first insight about Poc1 function in centriole remodeling and DBC function.

S18.4 - Structural Snapshots of the Sperm Acrosomal Matrix and Its Disassembly

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The mammalian sperm acrosome is not simply a bag of enzymes but is an organized compartment with a carefully constructed detergent-resistant matrix. Release of acrosomal contents by exocytosis is an absolute requirement for mammalian fertilization, yet we do not fully understand the molecular architecture of the acrosomal matrix, nor how it is disassembled during acrosomal exocytosis. To address these knowledge gaps, we leveraged the specialized acrosomal matrices of guinea pig sperm, which are large and can be readily isolated in low pH (5.2) buffer in the presence of protease inhibitors. Proteomic analysis of isolated acrosomal matrices detected all known guinea pig acrosomal matrix proteins (e.g. ACR/proacrosin, ACRBP, NPTX2, and ZP3R) and identified about 40 additional proteins involved in known acrosomal functions, including enzymatic/proteolytic processes (several PRSS family members), egg interactions (ZPBP 1 & 2, ARSA/Arylsulfatase A), and other structural or regulatory functions (ACRV1, GRN/Progranulin). To observe the molecular structure of the acrosomal matrix in a native fully-hydrated state, purified matrices were plunge-frozen and imaged by cryo-electron microscopy (cryo-EM) and cryo-electron tomography (cryo-ET). Initial observation revealed that the periphery of the acrosomal matrix did not contain paracrystalline patches as has been observed in the acrosome of some species (e.g. bovine, porcine), but is instead an amorphous densely-packed assembly. To mimic the conditions of acrosomal exocytosis in vitro, matrices were incubated in buffer at physiological pH (7.2) without protease inhibitors and plunge-frozen. Cryo-EM revealed noticeable decondensation of the matrices, particularly at their peripheries, manifesting as thinning and spreading of matrix material. Within decondensed regions, the matrix appeared to have fragmented into smaller protein complexes. We report our attempts to characterize these acrosomal matrix protein complexes by single particle analysis. Future work will synergize structural information from cryo-EM with proteomics in order to build a molecular atlas of the mammalian sperm acrosome and how it disassembles in preparation for fertilization.

S18.5 - Generation of a Rat Nanos2 Knockout Model for Spermatogonial Stem Cell Transplantation and Xenotransplantation

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Spermatogonial stem cell (SSC) transplantation has been a critical tool for identifying functional germline stem cells, and with advances in gene-editing technologies, culture systems, and xenografting approaches, SSC transplantation has broad potential to support generation of gene-edited animals through direct germline modification. Germline transplantation relies on robust depletion or elimination of endogenous germ cells. Treatment with gonadotoxic alkylating agents such as busulfan may be used but can cause widespread toxicity and interference from eventual recovery of endogenous spermatogenesis. Genetic modifications that lead to loss of postnatal germ cells, such as ablation of the *Nanos2* gene, produce ideal recipients for germline transplantation, as demonstrated in multiple mammalian species. Here, we applied CRISPR-Cas9-mediated gene editing to successfully generate a 353bp deletion of *Nanos2* coding sequence in rats to produce a model of male germline ablation. Briefly, female Sprague Dawley embryo donors were superovulated and paired with intact males overnight, and 2-cell embryos were collected and microinjected with Cas9 mRNA and two sgRNA's targeting the single exon of *Nanos2*. Seventeen 2-cell embryos were transferred to the oviduct of a pseudopregnant recipient female, and 8 pups were produced. Four of the 8 offspring (3 males, 1 female) carried at least one modified *Nanos2* allele, and the wild-type allele was undetectable in 2 males. These *Nanos2* null male founders did not produce pups after being paired with a wild-type female for at least 16 weeks. The female founder was bred with a wild-type male to produce 2 litters, with 57% of the pups carrying the targeted *Nanos2* deletion. The line is currently being expanded for analysis of testis histology, to carry out formal breeding trials, and to generate germ cell-less males to serve as recipients for SSC transplantation assays. We expect *Nanos2* knockout rats to be a valuable tool for generation of additional genetically modified rat lines through direct germline editing and allotransplantation, as a refinement to eliminate the need for vasectomy surgery when generating pseudopregnant recipients for embryo transfer, and to test xenografting approaches toward improvements in agriculture, animal gene editing, and biomedical research.

FOCUS SESSION 19 - WILLIAM HANSEL SESSION ON OVARIAN BIOLOGY

S19.1 - Decoding the Transcription Circuitry When the Life Begins

Wei Xie, Tsinghua University

Zygotic genome activation (ZGA) marks the first transcription event in development. Deciphering key regulators of ZGA is crucial for understanding how life begins and how a totipotent embryo arises from terminally differentiated gametes. Probing these questions in mammals was long hindered by the scarce experimental materials that are available from early embryos. By developing a set of ultra-sensitive chromatin analysis technologies, we previously investigated chromatin accessibility, epigenetic modifications, 3D chromatin architecture, and RNA Pol II engagement during mammalian ZGA. These studies unveiled highly dynamic and non-canonical transcription and chromatin regulation during the maternal-to-zygotic transition. However, how ZGA is kickstarted and how the early development program is progressively driven by transcription factors (TFs) remain enigmatic. Recently, we sought to identify key TFs that act at the onset of ZGA, and those that connect ZGA to the first cell fate commitment. In this talk, I will discuss how these findings help illuminate the core transcription circuitry underlying the beginning of the life.

S19.2 - Understanding The Functional Impact of Modulating Luteinizing Hormone Receptor (LHR) Oligomerisation in the Murine Ovary.

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The gonadotrophin hormone receptor, LHR, is essential for reproductive functions. LHR and its cognate ligand, LH, mediate pleiotrophic functions within the ovary, including steroidogenesis, ovulation and corpus luteum progesterone production. LHR is a G protein-coupled receptor (GPCR), primarily thought to signal via the Gαq/adenylyl cyclase/cAMP pathway. However, studies have shown that in conditions of heightened LH concentration and LHR expression, such as those observed in the peri-ovulatory follicle, LHR can activate Gαq/11, triggering intracellular calcium release, with proposed roles in ovulatory cascades. Yet, how LHR mediates such signal, functional and physiological diversity remains unknown. Our previous work has shown that LHR can self-associate to form homomers, providing a means to fine-tune signal specificity and magnitude of signal activation. The aim of this study was therefore to determine the effect of modulating LHR homomerization on LH/LHR ovarian functions. To modulate LHR oligomerisation, synthetic peptides designed to target specific receptor transmembrane domains (TM1, TM2, TM4, TM5 and TM6) were employed to disrupt LHR oligomerisation. To determine the effects of disrupting LHR oligomerisation on LH/LHR-dependent response gene expression, mouse ovarian fragment cultures were performed, which were inclusive of all ovarian cell types and follicles at differing developmental stages. Ovarian fragments were first pre-treated with $\pm 4\mu\text{M}$ of individual TMs (1 or 5) disrupting peptides, or in co-incubation of TMs (1, 2, 4, 5 and 6 or 4, 1 and 5) for 4 hours. These combinations were chosen as they were shown to inhibit LH-dependent cAMP production and modulate IP1 production. After pre-treatment, fragments were then cultured $\pm$ LH (10nM) for 24 hours. Culture of mouse ovarian fragments in the presence of the disrupting peptides highlighted trends for distinct LHR disrupting peptides in differentially modulating several LH response genes. Fragments treated with TM1 alone highlighted trends towards a reduction in LH-induced expression of the EGF mediated pathways, Egfr, Areg, Ereg compared to culturing with other peptides alone and in combination. This contrasted with the peptide targeting combination of TM1, 4, and 5, which decreased expression of steroidogenic pathways including StAR expression. Moreover, culturing with all TMs indicated an increase in Ptg2 production whereas decreasing expression with just TM1 alone. Together these data suggest that specific targeting of different LHR homomeric forms can modulate distinct LH-dependent physiological functions within the ovary, supporting the concept that different LHR oligomeric complexes may specify individual physiological functions.

S19.3 - LRH-1: The Master Regulator of Lipid Metabolism in Luteal Cells.

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The corpus luteum (CL) functions as a transient endocrine gland, responsible for producing the progesterone vital for embryo implantation and maintenance of early pregnancy. To ensure rapid and consistent progesterone production, luteal cells accumulate lipids in the form of lipid droplets. In mice, conditional depletion of the orphan nuclear receptor liver receptor homolog-1 (LRH-1) in granulosa cell

from the onset of luteinization onward (via *Pgr-cre Lrh-1^{flox/flox}*; LRH-1-cKO) leads to infertility. Despite the formation of corpora lutea in LRH-1-cKO, the secretion of progesterone, the main function of luteal cells, is compromised. We hypothesized that LRH-1 is essential for progesterone synthesis by regulating lipid metabolism in luteal cells. We first confirmed loss of LRH-1 in corpora lutea relative to control (CON, *Lrh-1^{flox/flox}*) by immunofluorescence on superstimulated CL-enriched ovaries at 48h after the ovulatory signal (n=3-4 per genotype, $P < 0.05$). To delineate the LRH-1 transcriptional program, we conducted mRNA-seq analysis on CON (n=4) and cKO (n=5) ovaries at 48h hCG to identify differentially expressed genes (DEGs) in CL-enriched ovaries. Transcriptomic analysis with DESeq2 revealed 1115 DEGs ($\log_2$ fold change $> |0.5|$; adjusted p value < 0.05) associated with the loss of LRH-1 in luteal cells. Then, we determined by LRH-1 ChIP-seq analysis of wild type ovaries treated for 48h hCG (n=2) that 40% (n=446) of these dysregulated genes had nearby (± 300 kb) LRH-1 binding sites indicating direct regulation of these genes by LRH-1. Indeed, the majority (75.3%, n=336) of these genes were downregulated, and gene ontology analysis revealed enrichment in biological pathways of steroid metabolism, organic hydroxy compound metabolic processes, and regulation of lipid metabolism. To determine whether the LRH-1 target genes we identified were specific to luteal cells, we reanalyzed single-cell RNA sequencing published from the ovaries of cycling mice. An impressive 38% (n=57) of the top 150 luteal markers were LRH-1 target genes, including *Star*, *Scarb1*, *Scap*, *Plin4*, *Elovl5*, and *Idh1*, all of which are involved in steroid and lipid metabolic processes. Finally, to assess the effects of LRH-1 depletion on lipid metabolism, neutral lipid staining (Nile red) was performed on CL-enriched ovaries from CON and cKO mice (n=3-4 per genotype), revealing a significant decrease in lipid droplets in the corpus luteum of cKO mice ($P < 0.01$). In conclusion, the LRH-1 cistrome identified in luteal cells in this study is responsible for the most significant ovarian phenotype observed in the cKO mouse model, namely the impaired secretion of progesterone. Through this integrative genomics approach, we demonstrate that LRH-1 orchestrates gene networks associated with lipid metabolism in the corpus luteum. Therefore, it is an essential player in ovarian regulation of gestation. Supported by PJC 166020 from the Canadian Institutes of Health Research to BDM and NG.

S19.4 - AMH Induces a Reversible Quiescence-Associated Secretory Phenotype in Preantral Follicles

Marie-Charlotte Meinsohn, Maeva Chauvin, Josephine Cleverdon, Phoebe May, Aki Kashiwagi, Rhian Stavely, Nicholas Nagykeri, David Pepin

Women are born with a limited number of primordial follicles forming the ovarian reserve, whose depletion triggers menopause. The mechanisms involved in maintaining the quiescence of the primordial follicles remain mostly unknown. The anti-Mullerian hormone (AMH), produced by granulosa cells of growing follicles, is the only hormone known to provide negative feedback to primordial follicle activation through its unique receptor AMHR2. We have previously demonstrated that administration of supraphysiological levels of exogenous AMH in mice elicits a profound suppression of follicle development. When mice were treated with AAV9-AMH at postnatal day 1 (PND1), their ovaries presented significantly less activated follicles than their AAV9-empty treated counterparts by PND6. AMH-treated ovaries also contained a unique population of abnormally activated follicles, with an enlarged oocyte, surrounded by a single layer of flattened pregranulosa cells. To understand the mechanisms involved in the establishment of this phenotype, we performed single cell RNA sequencing (scRNAseq) of PND6 control and AMH-treated ovaries and identified several pathways dysregulated in granulosa cells including the Wnt/ β -catenin pathway and hypoxia, which were consistent with the upregulation of immediate early genes we previously reported. We hypothesize that AMH maintains

pregranulosa cells in a deep quiescence state by preventing their activation, differentiation, and proliferation through Wnt and Cdk inhibitor pathways. We confirmed the downregulation of Wnt6 in the AMH treated samples in our scRNAseq dataset by qPCR and in situ RNA hybridization (RNAish). We also found that Wnt6 expression was upregulated in AMHR2KO mice, and incubated prepubertal mice ovaries with Wnt inhibitor do phenocopy the Amh-induced phenotype. We observed significantly fewer activated follicles with inhibitors, while reactivation of the Wnt pathway following co-treatment with AMH and GSK3 inhibitor rescued this phenotype with an increase in follicle activation and proliferation. These findings support the hypothesis that Wnt downregulation by AMH regulates primordial follicles activation. To characterize the deep quiescence phenotype, we evaluated expression of Senescence Associated Secretory Phenotype (SASP) related genes by qPCR and found a significant activation of a subset of canonical markers yet did not observe a significant change in the expression of β -galactosidase between control and AMH treated ovaries suggesting that AMH-induced hyperquiescence recapitulates only some aspects of senescence. In particular Cdkn1a was strongly upregulated in AMH treated ovaries. We noted that in prepubertal ovaries Cdkn1a expression was specific to AMH-treated primordial follicles oocytes, and pregranulosa cells. Interestingly, the partial activation phenotype could be reversed by treating prepubertal mouse with Cdkn1a inhibitors. This treatment resulted in an increase in abnormal activation of primordial follicles characterized by an activated enlarged oocyte surrounded by pregranulosa cells. We concluded that Cdkn1a upregulation in AMH treated ovaries contributed to the block of activation of primordial follicles, by acting on the oocytes. Finally, to study the reversibility of AMH on induced hyperquiescence we treated with recombinant AMH from PND3 to PND8, PND11, or PND13 and examined ovaries at PND14. We showed that the arrest in primordial follicles activation could be reversed, with an increase in granulosa cell differentiation (upregulation in Nr5a2, Amh), proliferation (upregulation in Ki67, Ccnd2, Top2a) but also a significant downregulation of Cdkn1a. Moreover, induction of Amh as seen by RNAish suggests that the pregranulosa cells of stalled follicles were able to progress following the release from AMH suppression.

S19.5 - In Vivo Dynamics of Mouse Ovulation and Oocyte Transport

Kohei Umezumi¹ and Irina V. Larina¹

1. Baylor College of Medicine

Ovulation is a highly dynamic reproductive event where mature oocytes are released from the ovary into the periovarian space, followed by their transfer to the oviduct. This process is critical to human fertility: ovulation-related disorders are the leading causes of female infertility, accounting for 32% of cases. Therefore, much effort has been put into understanding the genetic and endocrine regulations of ovulatory processes. However, limited imaging access to the site of ovulation has left unclear how oocytes behave within the ovulating follicle and how they travel to the site of fertilization after ovulation. The objective of this study was to investigate the dynamic regulation of ovulation and subsequent oocyte transport using mouse models. Toward this aim, we develop a novel approach for in vivo 3D imaging of ovarian tissues using optical coherence tomography that allows spatiotemporal analysis of mouse ovulation within the periovarian space. With this approach, we volumetrically and quantitatively capture both the process of ovulation as well as the transfer of ovulated oocytes through the periovarian space into the infundibulum, the section of the oviduct closest to the ovary. We observed a slow, gradual release of the oocyte surrounded by the cumulus cell mass after follicular rupture, which coincides with the reduction in follicular volume. The oocyte shows the highest velocity and becomes stretched into an oval shape as it exits the follicle, suggesting that the oocyte may be squeezed through a follicular

opening. After ovulation, spatial tracking of the oocyte reveals that the ovulated oocyte remains at the ovulation site for approximately 25 minutes. After leaving the site of ovulation, the oocyte shows directional movement into the infundibulum and reaches the ampulla within a few minutes. In vivo volumetric imaging reveals additional intriguing observations which provide new insight into oocyte transport mechanisms. During ovulation, the infundibulum repeatedly pumps oviductal fluid into the periovarian space. This finding aligns with micro-computed tomography analysis of the ovarian bursa, a membrane surrounding the periovarian space, showing dramatic expansion during ovulation. Another intriguing finding is that spatial tracking of scatters such as loose cumulus cells and mucus demonstrated bidirectional periovarian flow inside the ovarian bursa. Interestingly, the velocity of periovarian flow was significantly higher during ovulation than before ovulation. These results suggest that the oviductal pumping may cause the ovarian bursa to expand during ovulation and potentially generates the periovarian flow. To investigate whether the oviductal pumping plays a role in ovulated oocyte transport, we inhibited oviductal smooth muscle contraction during ovulation. The mice treated with the inhibitor showed a lower number of oocytes successfully transported to the ampulla and a higher number of oocytes remaining inside the ovarian bursa, indicating that inhibition of oviductal contraction caused retention of ovulated oocytes in the ovarian bursa. Together, our findings demonstrate that pumping induced by oviductal contraction contributes to oocyte transport from the site of ovulation to the site of fertilization. In conclusion, this study reveals the physiological dynamics of ovulation, captures never-before-seen oocyte behaviors, and proposes a potential mechanism for ovulated oocyte transport through the periovarian space into the oviduct.

FOCUS SESSION 20 - IMMUNOLOGY

S20.1 - Dispelling Misconceptions Regarding Pregnancy Pathophysiology Using Insights from the Horse

Amanda M de Mestre¹; Shebl E. Salem¹; Joanna Jaworska²; Dawid Tobolski²; Jessica M Lawson³, Don Miller¹

Horse pregnancy provides a unique model organism to explore some of the fundamental questions of reproductive pathophysiology. Similar to humans, they produce chorionic gonadotropin, have comparable gestation lengths and the single greatest risk factor for early miscarriage is advancing maternal age. Further, due to the late implantation of the embryo combined with intense gynecological care, the products of conception and the pregnant endometrium are both easily accessible over key periods of early development. Our laboratory leverages these features to investigate the underlying molecular and cellular events required for a successful pregnancy as well as those that lead to miscarriage.

Using genetic approaches applied to clinical miscarriage tissue, one of the primary goals of the laboratory is to define the intolerance of early mammalian development, that is key regions, and dosage, of chromosomes and genes critical to progression through early in utero life. In our recent work we have identified a high prevalence of chromosomal copy number aberrations in products of conception following natural miscarriage, including variants highly enriched over the period of Embryonic Carnegie Stages 7-23, a unique window for any species. MicroCT combined with gross and histological assessment of fetuses possessing these variants revealed abnormalities of the central nervous system and asymmetrical growth disorders inconsistent with life. Collectively, our findings suggest genomic disruption in the developing embryo is the primary cause of miscarriage in the mare, with prevalence similar to that observed in women.

Horse pregnancy is known for an enrichment of CD4 and CD8 T cells at the maternal-fetal interface with minimal attention given to other immune cell populations and their role. Understanding the phenotype and function of these cells is key to unravelling novel pathways that regulate interactions between maternal and fetal tissues. We have recently performed single cell RNAseq on endometrial tissue before and after both implantation and eCG exposure. Surprisingly and contradictory to previous reports, we identified several large subpopulations of uterine NK cells which were similarly abundant to CD3+ lymphocytes. Other interesting clusters included those assigned as MAIT T cells, and a unique population of tissue resident cells that expressed high levels of ITGA1 (CD49a) as well as genes characteristics of interferon and IL-15 signaling. The most dynamic changes in gene expression between the two time points in development were found in the cluster assigned as monocytes and their direct derivatives. Collectively, this data shows that despite many important anatomical and physiological differences previously reported between horses and humans, the two species also have incredible similarities including the shared greatest impediment to progression through early development. Further, use of single cell transcriptomics has revealed novel immune populations at the maternal-fetal interface including an abundance of several uterine NK cell subpopulations previously not described in equine pregnancy and poorly defined in epitheliochorial placentation.

S20.2 - Repeated Seminal Fluid Exposures Progressively Expand the Female Reproductive Tract Regulatory T Cell Pool in Mice

Sarah A. Robertson, Lachlan M. Moldenhauer, John E. Schjenken, Bihong Zhang, Hanan H. Wahid, Simon C. Barry, Michael Garrett

Delivery of seminal fluid factors to the female at insemination elicits an immune response in the female reproductive tract. Under favourable circumstances, this promotes activation and proliferation of regulatory T (Treg) cells that increase the likelihood of successful embryo implantation and pregnancy. In several species, including humans, there is evidence that past reproductive history affects future reproductive outcomes - but it is unclear whether an immune mechanism involving seminal fluid factors is involved. In this study, we aimed to determine whether prior exposure to seminal fluid affects reproductive tract Treg cell populations in subsequent reproductive cycles. Female C57Bl/6 mice (H-2b) were mated once, twice or four times in allogeneic matings with Balb/c (H-2d) males, and pregnancy was curtailed on day 3.5 post coitum (pc) after each mating by administration of RU486. On day 3.5 pc on the last mating, the uterus and uterus-draining para-aortic lymph nodes (dLN) were collected and analysed by immunohistochemistry (IHC) and flow cytometry. IHC showed a >4-fold increase in the density of Foxp3+ Treg cells in the uterine endometrium in mice mated 4 times, compared to mice mated once (n=12 mice per group, $P < 0.01$). Similar changes were seen in endometrial MHCII+ activated macrophages and dendritic cells, indicating that greater antigen presenting capacity accompanies the T cell changes. By flow cytometry analysis (n=10-12 mice per group), both the uterus and dLN showed a progressive increase in Treg cell number with each seminal fluid exposure. Both thymic-derived neuropilin-1 (NRP1)+ tTreg and peripherally-generated NRP1- pTreg cell pools expanded, but the ratio of NRP1+ to NRP1- cells increased with number of matings (all $P < 0.05$). pTreg cells showed a notable increase in expression of the suppressive marker CLTA4 and analysis by unbiased tSNE analysis confirmed that compared to other CD4+ T cell subsets, highly suppressive NRP1+Foxp3+Ki67+CD4+ T cells were most impacted by repeated seminal fluid contact. Ex vivo suppression assays demonstrated that the change in Treg cell phenotype was accompanied by a greater capacity to suppress generation of potentially detrimental immune activation towards male partner MHC antigens (n=4 replicates, $P < 0.01$).

Experiments in which C57Bl/6 females were mated with Balb/c males from which the seminal vesicle was surgically excised, or intact syngeneic C57Bl/6 or congenic Balb/b males (both H-2b, the same MHC haplotype as the C57Bl/6 females), showed that expansion of the Treg cell pool upon repeated mating depended on both female contact with seminal plasma and male-female disparity in MHC antigens (n=8-10 per group, $P < 0.05$). Collectively this data demonstrate that exposure to seminal fluid by mating over sequential reproductive cycles can progressively increase Treg cell number, alter Treg cell phenotype, and promote suppressive function. We speculate that if similar effects occur in humans, strengthening of the Treg cell response over time might provide a mechanistic explanation for reduced rates of pregnancy complications after extended sexual cohabitation with a conceiving partner.

S20.3 - Effects of Heat Stress on the Immune Response of Bovine Endometrial Epithelial Cells in Dairy Cows

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Increasing environmental temperatures pose significant challenges to food security and animal welfare, with summers exacerbating the incidence and persistence of postpartum uterine diseases in dairy cows. The immune system is important in the development and progression of disease, and chronic heat stress alters peripheral immune cell function of dairy cows; however, the effect of heat stress on the innate immune capacity of endometrial epithelial cells has not been determined. Here, we investigated the effects of in vitro heat shock or chronic whole animal heat stress on the innate immune function of bovine endometrial epithelial cells. The effect of heat shock was evaluated in cultured endometrial epithelial cells collected from ten healthy, cooled cows. Cells underwent two treatment periods; first they were exposed to either heat shock at 40°C or thermoneutral conditions at 38.5°C for 24 hours and then rested at 38.5°C for 12 hours. During the second treatment period, cells were exposed to either heat shock at 40°C or thermoneutral conditions at 38.5°C in the presence of 1 µg/mL of lipopolysaccharide or medium alone. Culture at 40°C in the second period increased the expression of HSPA1A in endometrial epithelial cells, confirming a cellular heat shock response. Exposure to lipopolysaccharide increased the expression of proinflammatory mediators IL1B, IL6 and CXCL8 compared with medium alone. However, culture at 40°C in the second period significantly reduced the lipopolysaccharide induced expression of CXCL8 and IL6 compared with the cells cultured at 38.5°C. Culture temperature in the first period did not affect the expression of proinflammatory mediators. The chronic effect of heat stress was evaluated in cows during the summer months that were either cooled (CL) by soakers, fans, and shade (n=21) or heat stressed (HS) with shade only (n=23) for 45 d prior to expected calving. Cows exposed to HS experienced elevated respiration rates and rectal temperatures relative to the CL cows during the treatment period, indicative of heat stress. After calving, all cows were housed under cooling conditions. Endometrial epithelial cells were collected on d 21 ± 1 postpartum by cytobrush and cultured at 38.5°C in the presence of 1 µg/mL of lipopolysaccharide or medium alone for 24 h. Expression of proinflammatory mediators CXCL8, IL1B, IL6, TNF and PTGS2 were increased in response to lipopolysaccharide exposure compared to the medium alone. However, the lipopolysaccharide response of endometrial epithelial cells was not affected by prepartum heat stress applied to the cows. In conclusion, we found no evidence for long-term effects of prepartum heat stress on the innate immune capacity of endometrial epithelial cells. Alternatively, acute heat shock of cultured endometrial epithelial cells alters the innate immune response to lipopolysaccharide which could affect

an animal's capacity to respond to bacterial infection of the uterus. Acute effects of heat shock on endometrial immune function may contribute to the increased incidence and persistence of uterine diseases in cows under heat stress conditions.

S20.4 - Single Cell RNA Sequencing and Multiplex Flow Cytometry Reveal Dynamic Changes in Immune Cell Composition and Function During Menstruation

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Endometrial immune cells are essential for normal reproductive function with important roles in tissue remodelling, foetal tolerance, and protection from infection. The composition of immune cells in the endometrium changes in response to hormonal cues as part of the normal menstrual cycle and in pregnancy. Menstruation is an inflammatory event, but we lack detailed understanding how immune cell composition and function changes during this process. This study performed detailed phenotypic characterisation of uterine immune cells during menstruation. A mouse model of simulated menstruation was utilised to investigate uterine immune cell dynamics during endometrial tissue breakdown and repair. Briefly, mice were ovariectomised to deplete endogenous hormone production and then received exogenous estradiol and progesterone followed by induction of decidualisation via intrauterine oil injection. Uterine tissues were collected from cycling control mice or menstruating mice 24- and 48-hours after withdrawal of progesterone. Uterine tissues were digested and CD45+ immune cells isolated by FACS from each experimental condition. Isolated cells were subjected to single cell RNA sequencing analysis (n=4-8 per group) or 14-plex multi-parameter flow cytometry analysis (n=10-11 per group) to determine the transcriptional profile and phenotype of uterine immune cells. Unbiased clustering of single cell RNA sequencing data generated from isolated CD45+ immune cells identified 8 distinct clusters. Transcriptomic analysis of differentially expressed genes and canonical markers allowed annotation of the 8 distinct immune cell subsets as T cells, B cells, natural killer cells, neutrophils, eosinophils, monocytes, macrophages, and dendritic cells. Comparison of single cell RNA sequencing data generated from cells isolated from cycling, 24- and 48-hour tissues demonstrated dramatic changes in immune cell composition during simulated menstruation. Notably, endometrial breakdown (24-hour) was characterised by an increase in the abundance of neutrophils compared to both the 48-hour timepoint (endometrial repair) and cycling controls. Detailed phenotyping of uterine immune cells using flow cytometry confirmed compositional changes in Ly6G+ neutrophils which were significantly increased during endometrial breakdown (24-hour timepoint) when compared to endometrial repair (48-hour timepoint; $p < 0.001$) and cycling controls ($p < 0.0001$). In contrast, the proportion of CD11c+MHCII+ dendritic cells were significantly decreased during endometrial breakdown (24-hour timepoint) compared to endometrial repair (48-hour; $p < 0.01$) and controls ($p < 0.05$). Endometrial repair was associated with a significant increase in CD64+F4/80+ macrophages (48-hour timepoint; $p < 0.05$). Gene ontology biological process analysis identified functional processes associated with the transcriptomes of distinct immune cell subsets during menstruation. Neutrophils were associated with cytokine-mediated signalling and leukocyte migration; Macrophages were associated with activation and regulation of the immune response, whilst dendritic cells were associated with leukocyte adhesion and antigen processing and presenting. These results provide a comprehensive profile of uterine immune cells during menstruation and demonstrate that dynamic changes in immune cell composition and function underpin this unique physiological process.

S20.5 - Seminal Fluid Histocompatibility Antigens Contribute to T Cell Priming After Mating in Mice.

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At conception, the female reproductive tract is exposed to factors in the male partner's seminal fluid. Immunogenic and immune regulatory factors then act to prime a female T cell response that initiates maternal immune tolerance towards the ensuing genetically disparate fetus. Amongst the most antigenic proteins in the seminal fluid are male major and minor histocompatibility antigens (MHC and MiHC, respectively). The degree of disparity between maternal and paternal histocompatibility genes is thought to influence T cell proliferation and hence affect the quality of the immune response to impact subsequent fetal development, but this has not been formally evaluated. Our study tests the hypothesis that disparate MHC and MiHC antigens in seminal fluid both contribute to stimulating the T cell repertoire after mating, leading to optimal reproductive outcomes. Using C57BL/6 female mice, we assessed fetal and placental development during late gestation at 17.5 days post-coitum (dpc) following mating with males of three genotypes: BALB/c (MHC and MiHC disparate), BALB/b (MHC matched and MiHC disparate), or C57BL/6 (MHC and MiHC matched). To comprehensively analyze the T cell repertoire, we sequenced T cell receptor (TCR) gDNA (Adaptive Biotechnologies) from uterine-draining lymph nodes (udLNs) at 3.5 dpc from females in each mating group, with unmated C56BL/6 females as a comparison. Using the same mating combinations, we further analyzed the T cell phenotype by flow cytometry to examine the proportion of permissive regulatory T cells (Treg) and inflammatory conventional T cells (Tconv). We found that pregnancies sired by BALB/c males had an 18% increase in fetal weight and an 8% increase in fetal to placental weight ratio compared to pregnancies sired by C57BL/6 males, and 4% and 16% increase, respectively, compared to those sired by BALB/b males (n=14-16/group), indicating greater maternal resource investment and placental efficiency when females were exposed to both disparate MHC and MiHC. This was accompanied by ~2.5-fold increased udLNs and splenic weight in females mated to BALB/c males (n=12-15/group), indicating increased T cell activation and proliferation. Genomic sequencing of TCRs revealed that while all mated mice showed evidence of clonal T cell proliferation, mice mated with BALB/c males exhibited increased proliferation of specific T cell clones compared to other groups (n=3-5/group). Additionally, the TCR repertoire of BALB/c mated mice was the most distinct from the unmated group, suggesting MHC and MiHC antigens in seminal fluid both drive the activation and expansion of specific T cell populations. Flow cytometry of udLNs revealed that pregnancies sired by BALB/c males had the highest Treg:Tconv ratio compared to other matings, indicative of a more tolerogenic immune response compared to other mating groups. These findings demonstrate that disparate seminal fluid histocompatibility antigens, especially MHC, contribute to the activation and expansion of a paternal antigen-specific T cell repertoire to support optimal placental development and function. Further research is underway to investigate the underlying mechanisms and define these interactions at higher resolution with single-cell RNA-seq. We expect that the capacity to predict immune compatibility and potential reproductive success based on histocompatibility genes will improve reproductive efficiency and reduce poor outcomes in endangered species conservation and livestock.

FOCUS SESSION 21 - REPRODUCTIVE TRACT ORGANOID

S21.1 - Mechanisms of Uterine Epithelial Specification: Insights from Organoid Culture

Andrew Kelleher, University of Missouri

S21.2 - Melatonin and Resveratrol Alleviate Molecular and Metabolic Toxicity Induced by Bisphenol A In Endometrial Organoids

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Endometrial organoids, three-dimensional models mimicking endometrium, serve as crucial tools for unraveling the impact of environmental factors on reproductive health. Among these factors, Bisphenol A (BPA), a widespread environmental contaminant, poses concerns due to its disruptive effects on endometrial function. In contrast, melatonin (MT) and Resveratrol (RSV) are under scrutiny for their potential protective roles against BPA-induced damage. This study aimed to comprehensively characterize the morphological, molecular, and metabolic responses of endometrial organoids to BPA and assess the potential protective effects of MT and RSV. To investigate the environmental impact on reproductive health, endometrial organoids were treated with BPA, MT, and/or RSV. Molecular screening, utilizing mRNA and miRNA-specific primers, revealed treatment effects on oxidative stress, inflammation, and apoptosis. Additionally, gene expression of steroidogenesis and monoacylglycerol lipase in the endocannabinoid system was assessed. Western blotting provided insights into altered epithelial and mesenchymal profiles. Immunofluorescence staining assessed cytoskeletal proteins. Metabolic profiling for glucose, galactose (using ion chromatography), and amino acids (by LC-MS/MS) was performed. As expected, BPA induced structural and molecular disruptions in organoids, affecting cytoskeletal proteins, Wnt/ β -catenin signaling, and epithelial/mesenchymal markers. It triggered oxidative stress and apoptotic pathways, altered miRNA expression, and disrupted the endocannabinoid system. Glucose and galactose levels were elevated in BPA-treated groups, indicating metabolic changes. MT and RSV exhibited protective effects, mitigating BPA-induced disruptions across multiple pathways. For the first time, our study models endometrial organoids, advancing our understanding of environmental impacts on reproductive health.

S21.3 - Evaluation of Extracellular Vesicles Secreted by Mouse Oviductal Organoids for Loading With CRISPR-Cas9 Ribonucleoproteins

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Extracellular vesicles (EVs) are nanoparticles secreted by cells for intercellular communication via transfer of bioactive molecules, such as lipids, proteins, and nucleic acids. Organoids are 3D, spherical cell clusters formed in vitro that are capable of long-term proliferation and self-organization with similar function to their tissue of origin. EVs produced by organoids are more similar to those produced in vivo because organoids retain more in vivo-like properties than traditional 2D cell culture models. Our objectives were to i) generate EVs from mouse oviductal organoids and ii) investigate their suitability for loading with GFP-conjugated CRISPR-Cas9 ribonucleoproteins (RNPs). Our hypothesis was that fluorescent channels on ZetaView QUATT4 nanoparticle tracking analysis instrument (NTA; Particle Metrix) can be utilized for evaluation of EV loading with GFP-tagged cas9. Mouse oviductal cells (n=3 mice) were used to generate organoids which were cultured for up to 60 days (passaged every 7 days), and spent culture medium was collected for isolation of EVs via differential ultracentrifugation. Organoids were assessed via brightfield microscopy for maintenance of phenotype throughout the culture period. EVs were assessed for i) size and concentration using NTA and ii) the presence of EV-associated protein markers using a Jess Automated Western Blot system (Bio-technique). EVs or EV-free PBS (control) were then treated with Lipofectamine CRISPRMAX in the presence of GFP-tagged RNPs to initiate EV loading and then purified using size exclusion chromatography (SEC; Exo-Spin; Cell Guidance Systems). EVs and EV-free PBS control subsequently were analyzed using an anti-cas9 antibody with the Jess system and using the 488 nm laser on the NTA. Brightfield imaging of mouse oviductal organoids demonstrated round cellular clusters with a central lumen, which is consistent with previous mouse oviductal organoid reports. EVs assessed by the NTA measured an average of 134 nm diameter with a concentration of 9.4×10^9 particles/mL. Additionally, Jess analysis demonstrated the presence of EV-specific proteins TSG101, FLOT1, and ANXA5. After Lipofectamine treatment and SEC, EVs demonstrated the presence of cas9 on Jess analysis and positive GFP-cas9 fluorescence on NTA. However, cas9 also was detected in the control sample after analysis with both the Jess and NTA. Data demonstrated that i) mouse oviductal organoids produce EVs, ii) western blot and NTA can be utilized for evaluation of EV loading with GFP-tagged cas9, and iii) protocols for removal of unloaded RNPs must be optimized to determine EV loading efficiency. RNP-loaded EVs have the potential for in vivo gene editing applications such as in utero gene therapy for fetal genetic disorders and as a novel contraceptive. Research reported here was supported by the Office of the Director, National Institutes of Health under award number K01OD032455. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

S21.4 - Establishment of In Vitro Implantation Model Using Newly Developed Endometrial Organoid

Taishi Fujimura, Isao Tamura, Amon Shiroshita, Norihiro Sugino

Implantation is the process in which a blastocyst attaches to the uterine epithelium and invades the underlying uterine stroma. Although this process is essential for a successful pregnancy, detailed mechanisms of implantation have not been clarified. This is due to the lack of techniques for constructing an organized endometrium-like structure in vitro. In previous studies using in vitro implantation model, a two-dimensional culture of endometrial cells has been used. Even though endometrial organoids were recently established, they consisted of only endometrial epithelial cells without endometrial stromal cells. Furthermore, endometrial organoids require an ECM, which causes

the apical surface of the epithelium to face inward (apical-in organoid). As blastocysts attach to the apical surface of the epithelium, these models cannot observe the attachment of blastocysts to the epithelium. Considering the process of implantation, it is essential to generate a 3D structure including both epithelial cells and stromal cells, and reverse the polarity of epithelial cells (apical-out organoid). In this study, we developed newly developed apical-out endometrial organoid with endometrial epithelial and stromal cells. Furthermore, we examined its potential to recapitulate the implantation process when it is co-cultured with a mouse blastocyst. Estradiol was subcutaneously injected into eight-weeks female B6 mice for 3 days. Mouse uteri were obtained and digested with collagenase. The digested tissues were suspension cultured in the monolayer. In 7 days, self-organized aggregations were observed. These aggregates were picked up onto low attachment 96 well plates to perform 3D culture for 9 days. Their structure, cell components, and cellular polarity were examined by hematoxylin and eosin (HE) and immunofluorescence staining, respectively. They consisted of a single layer of E-cadherin-positive epithelial cells lining the outside and vimentin-positive stromal cells inside. In addition, signals of mucin-1, a protein that colocalizes in the apical side of epithelial cells were observed in the lateral side of epithelial cells of the endometrial organoid, which enables blastocyst to attach to the endometrial organoid. To establish the in vitro implantation model, they were stimulated with estradiol, medroxyprogesterone acetate, and cyclic AMP for 2 days to reproduce the receptive endometrium of a mouse. Then, they were co-cultured with blastocysts obtained from EGFP transgenic mice for 5 days. Time-lapse imaging was used to observe the implantation process in real time. It confirmed the major four steps of implantation; attachment of blastocysts to the epithelium, invagination of the epithelium, entosis (opening of the epithelium), and invasion of blastocysts into the stroma. After the coculture, the differentiation markers for trophoblasts and decidual cells were examined by immunofluorescence staining. The invaded cells expressed proliferin, which is a marker for trophoblast giant cells. Their surrounding stromal cells expressed cyclooxygenase-2, which is a marker for decidualized cells. We newly established the endometrial organoid, which was formed by apical-out epithelial cells outside and stromal cells inside, which is similar to the normal endometrium. Furthermore, it could recapitulate the implantation process of mice. This organoid is a promising model to investigate the implantation process in vitro.

S21.5 - Transcriptomic Characterization and Effect of Reproductive Cycle on Equine Endometrial Organoids: A Tool for Exploring Embryo-Endometrium Crosstalk

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Endometrial organoids represent invaluable models for studying embryo-endometrium interaction. However, insights into the transcriptomic signature of these organoids and the influence of the reproductive cycle stage at the time of sampling on the development and molecular profile of endometrial organoids remain limited. In the present study, we generated cytokeratin+, vimentin+, desmin-, alpha-smooth muscle actin-, and PECAM-1- equine endometrial organoids (ORG) from mares in estrus (E) and diestrus (D). The organoids were cultured in a progesterone-supplemented [7ng/ml]

condition to closely mimic the progesterone phase of the endometrium. No differences were observed in the development, growth rate, morphology, size, cell number, or viability of organoids derived during estrus (E-ORG) or diestrus (D-ORG). The transcriptome of the progesterone-conditioned ORG was compared with their respective endometrial tissue samples (END) from which they were derived. We identified 7,962 differentially expressed genes (DEGs; $FDR < 0.05$) between D-ORG and D-END, and 9,173 DEGs between E-ORG and E-END. When comparing the DEGs from these two sets, 48% were shared, indicating a commonality in their transcriptome that is independent of the cycle stage at which the samples were collected. The genes with higher expression in ORG were involved in cell cycle and metabolic processes, and those genes with lower expression with blood vessel and tissue development. We also investigated the effect of the estrous cycle stage (E versus D) of the donor mares on the resulting ORG. The 1,563-identified DEGs between E-ORG and D-ORG were associated with metabolic processes. Only 6.5% of the DEGs were shared between E-END/D-END and E-ORG/D-ORG, highlighting the minimal influence of the cycle stage on the resulting ORG. Overall, we characterized the transcriptomic profile of equine endometrial organoids. The results indicated that, in our culture system, the stage of the estrous cycle of the donor mare exerts minor effects on ORG development and transcriptome profile. This work was supported by The Foundation for the Horse (91321), UC Davis Center for Equine Health with funds provided by the State of California satellite wagering fund and contributions by private donors (CEH-22), Boehringer Ingelheim, the Belgian American Educational Foundation fellowship (2020-FELLOW-E075), and the L'Oréal-UNESCO Fellowship/Fonds Wetenschappelijk Onderzoek (11L3822N).

FOCUS SESSION 22 - ARTIFICIAL INTELLIGENCE

S22.1 - Application of Artificial Intelligence in the IVF Laboratory

Charles Bormann, Massachusetts General Hospital

The increasing global reliance on in vitro fertilization (IVF) highlights the urgent need for more precise and efficient practices within embryology laboratories. Conventional manual assessments of embryos are subjective and often result in variable IVF outcomes. This lecture highlights the significant impact of artificial intelligence (AI) across various aspects of the IVF laboratory, with an emphasis on novel advancements in embryo and sperm analysis.

AI technologies, particularly machine learning models such as convolutional neural networks (CNNs), have been transformative, offering more objective and accurate assessments. These AI-driven systems enhance embryo selection processes by significantly reducing subjective interpretation and increasing the predictability of successful implantations. Furthermore, AI applications in sperm analysis have led to groundbreaking improvements in sperm viability assessments and selection, which are critical for successful fertilization.

The adoption of AI tools in IVF practices worldwide has shown promising results, including higher consistency in embryo selection and improved pregnancy rates. By integrating AI, clinics can achieve a more standardized approach, reducing the incidence of high-risk multiple pregnancies and improving overall treatment outcomes.

The presentation will discuss these technological advancements and their implications for future reproductive medicine, illustrating how AI is poised to redefine standard practices in embryology laboratories globally, making successful outcomes more accessible to a broader population of patients seeking fertility treatments.

S22.2 - 3D Approximation of the Expression of Lineage-Specific Markers of Inner Cell Mass (SOX2, OCT4) And Trophectoderm (CDX2) In Mouse Blastocysts.

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The study of embryo structure in real dimensions is important to develop predictive methods of implantation in embryos produced in vivo or in vitro and even designed in silico. The integration of biological strategies with the bioinformatics tools could help to achieve this aim. Numerous biological events have not yet been elucidated such as the study of embryo development, polarization and exclusion of blastomeres, cell-cell communication and differentiation of inner cell mass into epiblast and primitive endoderm. We have shown the preliminary results of the development of a non-invasive structural graphical model to evaluate the expression of markers usually used to investigate the inner cell mass (SOX2 and OCT4) and trophectoderm (CDX2) development in mouse blastocysts. We showed quantitative data such as area (um²), ellipticity (oblate), volume (um³), minimum and maximum intensity, of blastomere morphology and structure inside a mouse blastocyst in a three-dimensional study using Imaris 10.1 software. We obtained a spatial approximation of the surfaces of the blastomere that allowed us to identify and count them, obtaining single-cell data, determining the place of evaluation and the stage of development of the blastocyst. These advances could be applied to a large number of experiments in embryology and basic science, as they could help to understand processes such as chromatin conformational changes and different protein expression patterns in blastomeres without biopsies. The expectations of these models are high, however, the substantial amount of numerical data may require the use of Interpretable Machine Learning (IML) and Deep Learning (DL), since the interpretation of a single variable could lead to errors and premeditated conclusions. Further studies are needed to determine the algorithms necessary for the creation and refinement of the model, however, our data will serve as a precedent for the advancement of these technologies in the future. Key words: Embryo, Blastocyst, Inner cell mass, Trophectoderm, Blastomere Financial support: Grant from NIHR21 ES026388

S22.3 - Deep Learning, Label-Free Sperm Plasma Membrane Integrity Diagnostics

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The conventional method for evaluating semen quality involves assessing sperm concentration, viability, motility, and morphology. However, these parameters may not detect altered or non-functional spermatozoa within boar ejaculates, potentially leading to lower reproductive efficacy despite good motility and morphology. Biomarkers offer deeper insights into sperm cell fertility potential, indicating alterations from defective spermatogenesis, epididymal maturation, dysfunctional capacitation, sperm handling, and more. The adoption of fluorescent biomarkers in the production field has been limited by the need for costly equipment, highly trained labor, significant time investment per assay, and the low throughput of standard epi-fluorescent microscopes. Our goal is to translate sperm health-reflecting biomarkers to livestock semen diagnostics by establishing label-free sperm quality analysis. We hypothesized that artificial intelligence (AI), deep learning convolution neural network analysis methods could detect subtle micro-morphology differences, trained by sperm health-reflecting biomarkers, with increased F1-scores (a measure of predictive performance) by increasing magnification objective and the number of cell images used for training. We utilized high-throughput, image-based flow cytometry to acquire 250,000 images over three magnification objectives, 20x, 40x, and 60x magnification, from each

of the 20 boar samples. Each cell had two brightfield, side scatter, and fluorescent images acquired to gather data on propidium iodide (PI) incorporation. Additionally, we tested extended depth of field (EDF) optics and computational algorithms at a 40x objective to observe if EDF optics had higher label-free F1-scores. All together this resulted in a comprehensive dataset of 20,000,000 images. Models were trained with a specific number of cells for each of the PI positive and negative reflecting populations across all brightfield conditions: 1,000; 2,000; 5,000; 10,000; 20,000, 50,000; and 100,000. As the cell number increased across all objectives, F1-scores also increased. This was also true of the objective, as magnification increased, so did the F1-score. The F1-scores for the population trained with 100,000 cells had the highest scores across all objectives, with 20x at 77.26%, 40x at 89.1%, and 60x at 91.26%. The incorporation of EDF at 40x did not see an increase in F1-score, remaining at 84.24% at 100,000 cells. Upon analyzing 1,000,000 cells with a 60x objective, we were able to achieve an F1-score of 97.5%. This exceeds the traditional morphology industry analysis threshold of a human technicians which is typically 95%. These results support the integration of label-free algorithms into economically viable microscopes without the need for expensive fluorescent biomarker setups, advancing andrology in both livestock and human-assisted reproductive therapies. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2022-67015-36298 from the USDA National Institute of Food and Agriculture and National Pork Board Award no. 026357-00001.

S22.4 - Puberty Classifications In Beef Heifers Are Moderate To Highly Heritable And Associated With Single Nucleotide Polymorphisms From Candidate Genes Related To Cyclicity And Timing Of Puberty

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A heifer's ability to attain puberty and maintain cyclicity early in development is critical to her reproductive longevity. We have identified heifer pubertal classifications using progesterone concentrations greater than 1 ng/ml with continuous cyclicity in blood plasma collected from weaning to breeding (Oct to May). Initially four pubertal classifications were identified: 1) Early (E); 2) Typical (T); 3) Start-Stop (SS) and 4) Non-Cycling (NC) with E and T heifers initiating and maintaining cyclicity, SS starting and then stopping cyclicity and NC never initiating cyclicity. Start-Stop heifers segregated into Start-Stop-Discontinuous (SSD) or Start-Stop-Start (SSS) with SSD having similar reproductive traits to NC, and SSS being similar to T heifers. If genomic regions associated with puberty attainment using these puberty classifications could be elucidated, tools could be developed to cull and retain females based on their genetic potential. We hypothesized that these heifer pubertal classifications are heritable and single nucleotide polymorphisms (SNPs) identified by genome wide association studies (GWAS) could be associated with puberty in females. For the current trial, we utilized heifers (n=532) born from 2017 to 2021 that were genotyped on the Illumina Bovine SNP50 v2 or GGP Bovine 100K SNP panels for variant component estimation and GWAS. Heritability estimates were developed using a univariate animal model within a Bayesian framework. When considering all 5 pubertal classifications (ALL): E, T, SSS, SSD, and NC, pubertal class was determined to be moderately heritable (0.38 ± 0.08). If pubertal classifications that initiated and maintained cyclicity in the trial were compared to those that did not cycle (E + T vs. SSD+NC groups; ETvDN) the heritability estimate was greater (0.59 ± 0.19). Analysis of E vs T females as a method to determine heritability of pubertal timing (EvT) resulted in reduced estimated heritability (0.19 ± 0.14). A GWAS was performed to identify loci associated with pubertal classification

(ALL), cyclicity during the trial (ETvDN) or pubertal timing (EvT). However, no SNPs were significantly associated with pubertal attainment, indicating puberty is a polygenic trait. To leverage information from literature, a candidate gene approach was subsequently used, which fitted SNPs within or nearby a set of 72 genes that had previously been associated with puberty or polycystic ovary syndrome (which often has delayed or precocious puberty; PCOS) in multiple species. These SNPs were fitted as fixed effects with one SNP analyzed at a time. Eight genes/regions were associated with the five-level trait of ALL including Anti-Mullerian Hormone Receptor (AMHR2), and Synaptotagmin 1 (SYT1). Twenty-two genes/regions were associated with whether puberty was attained during the trial (ETvDN), including MOB Kinase Activator 3B (MOB3B), Follicle Stimulating Hormone Receptor (FSHR), and Mitogen-Activated Protein Kinase Kinase Kinase 1 (MAP3K1). Fifteen genes/regions were associated with the timing of pubertal attainment during the trial (EvT) including Suppression of Tumorigenicity 7 (ST7), and Disco-Interacting Protein Homolog 2 (DIP2B). Taken together, this study demonstrated that these pubertal classifications are moderately to highly heritable depending on the definition of the trait. While no significant SNPs were identified with the GWAS, when considering candidate genes, several SNPs were associated with the 5 puberty classifications, pubertal attainment during the trial, or timing of pubertal attainment, which will allow for future investigation into genomic tools that may be used to cull or retain heifers in the herd.

S22.5 - OoCount: A Deep-Learning Based Approach To Mouse Ovarian Follicle Counting And Classification

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The number and distribution of ovarian follicles in each growth stage provides a reliable readout of ovarian health and function. However, the total reported number of follicles present in the ovaries at any given time in life varies greatly from study to study. As most of the field uses histological sections of the ovary to extrapolate total follicle counts, we have been limited in our ability to obtain accurate assessments of the ovarian reserve. The development of techniques for three-dimensional (3D) imaging of ovaries in toto have significantly advanced our understanding of ovarian morphogenesis and female reproduction. The advent of deep-learning algorithms has allowed the rapid development of ultra-fast, automated methods to analyze microscopy images. In recent years, these pipelines have become more user-friendly and accessible to non-specialists. We leveraged these tools to create OoCount, a high-throughput, open-source method for automatic oocyte segmentation and classification from fluorescent 3D microscopy images of whole mouse ovaries using a deep-learning based approach. We developed a fast clearing and spinning disk confocal-based imaging protocol to obtain 3D images of whole mount perinatal and adult mouse ovaries. Then, fluorescently labeled oocytes from 3D images of ovaries were manually annotated to develop a machine learning training dataset. This dataset was used to train a deep-learning neural network to automatically label all oocytes in the ovary. In a second phase, we trained another deep-learning network to classify labeled oocytes and sort them into growth stages. Using OoCount, we can obtain accurate counts of oocytes in each growth stage in the perinatal and adult ovary, improving our ability to study ovarian function and fertility. We expect OoCount to become a major resource for the field of ovarian biology and female fertility. This work was funded by NIH #K99HD103778 and #R00HD103778.

FOCUS SESSION 23 – YANAGIMACHI SESSION ON ASSISTED REPRODUCTION TECHNOLOGIES

S23.1 - Assisted Reproductive Techniques in Mice and Hamsters - Past, Present, and Future

Atsuo Ogura, Deputy Center Director, RIKEN BioResource Research Center

Mice and Syrian (golden) hamsters have contributed to the development of assisted reproductive technologies for decades, but in different roles. The first mammalian IVF and ICSI were successfully performed in hamsters (Yanagimachi and Chang 1963; Uehara and Yanagimachi 1976) because of the ease of sperm capacitation (or hyperactivation) and acrosomal reaction observations in vitro, as well as that of oocyte injection. The leading role since the 1980s has shifted to mice, which enabled easier manipulation of embryos and production of offspring. This was also true of the research at Yana's laboratory, where they established a mouse Piezo-assisted ICSI in 1995 (Kimura and Yanagimachi). I was fortunate to work at Yana's laboratory from 1991 to 1992 and participate in hamster and mouse experiments. Since then, my primary focus has been on ICSI, ROSI (round spermatid injection), and SCNT (somatic cell nuclear transfer) in mice, techniques that have provided invaluable models to decipher developmental genetics and epigenetics. We recently successfully performed intra-oviductal genome editing (i-GONAD) in hamsters, which avoids the developmental blocks of hamster embryos, since it does not require in vitro embryo manipulation. The genetic knockout hamsters produced thus far have provided significant information on reproductive biology, such as the indispensable role of sperm acrosin in fertilization, that could not have been achieved with mice. Thus, 60 years after Yana first used hamsters for fertilization research, we are back to working with hamsters using new assisted reproductive technologies.

S23.2 - I Aim to Be a Researcher Who Inherited Dr. Yanagimachi's Genes

Teruhiko Wakayama, Advanced Biotechnology Centre, University of Yamanashi, Yamanashi, 400-8510, Japan

I studied research attitude in Dr. Yanagimachi as a post-doctoral fellow or assistant professor for three years from 1996 to 1999. However, since the first paper I read (1990) was by Dr. Yanagimachi, his name was imprinted on me, and since then I have been studying mammalian fertilization through his papers. Therefore, the period during which I think I studied under Dr. Yanagimachi spanned about 10 years. Of course, even after I left Dr. Yanagimachi's lab, he has remained my greatest mentor until now. Probably many scientists may have thought of Dr. Yanagimachi as a steady and serious researcher. However, Dr. Yanagimachi always conducted unique researches that would have been unthinkable by conventional wisdom. For example, no one would have believed at that time that "if sperm were freeze-dried, it might be possible to preserve sperm at room temperature, like instant coffee." I worked with Dr. Yanagimachi on this experiment and, as he predicted, we finally succeeded in producing offspring from freeze-dried mouse sperm preserved at room temperature for one month. This technology is attracting attention as a low-cost method for permanent preservation of mammalian genetic resources without the use of liquid nitrogen. Dr. Yanagimachi always had such foresight.

I was fascinated by Dr. Yanagimachi's research attitude and tried to conduct my own unique research, but even if I came up with something new idea, he had already published it as a paper long time ago. I sometimes worried that there was nothing left to experiment with new ideas that Dr. Yanagimachi had not already come up with. However, when I reported to Dr. Yanagimachi on my ICSI experiment using formalin-fixed sperm, he was overjoyed, and said, "You are just like me". I was so pleased and from this

point on, the purpose of my research became twofold: one was scientific inquiry, and the other was to surprise Dr. Yanagimachi.

Although I have since become independent, my research policy has been strongly influenced by Dr. Yanagimachi, and there is no doubt that I have inherited his genes. For example, Dr. Yanagimachi was pleased with the experiments conducted in my laboratory to preserve freeze-dried sperm on the International Space Station (ISS) or to produce cloned mice from cells in urine. Sadly, we can no longer please Dr. Yanagimachi, but we would like to repay him by passing on his research genes to the next generation.

In this talk, I will introduce Dr. Yanagimachi's passion for research, and the latest results of various experiments that began in Dr. Yanagimachi's laboratory, such as the transportation of sperm by attaching to postcards, experiment to culture mouse embryos in the zero gravity of the ISS, or production of cloned mice from freeze-dried somatic cell. I am very bad English and it is much more difficult for me to speak English than to produce a lot of clone mice. However, Dr. Yanagimachi told me that if my research is good, people will listen to me no matter how bad my English is. He taught me to do research rather than study English. Therefore, I will do my best to give a good talk, so please bear with me even though my English is terrible.

S23.3 - SPACE SPERM – The Importance of Gravity in Sperm Navigation, Fertilisation and Early Embryo Formation.

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Space and space exploration attracts curiosity, excitement, and innovation nationally and internationally, with growing interest in Mars settlement, deep space exploration and Moon mining. However, effective long-term human settlement of celestial bodies and exploration of deep space poses many challenges, including but not limited to the requirement for mammalian reproduction in micro-gravity environments. It is crucial to understand the impact of low gravity on the earliest stages of space-born life, including the viability of conception and healthy fetal development in mammals, processes of reproductive success which are normally dependant on Earth's gravity. Utilising a gamete and embryo culture suitable dual-axis 3D clinostat to simulate microgravity we aimed to determine if exposure of mammalian sperm and embryos to simulated micro-gravity altered functional and developmental capacities. In this study non-capacitated mouse (N&#3f10) and human (N&#3f6) sperm were challenged to swim through an Ibidi 6-channel μ -slide for 1-6 h at either normal gravity (NG) or stimulated micro-gravity (OG), in conditions that mimic the female reproductive tract (media – G-IVF PLUS, 37oC, 6% CO₂, 5% O₂). Sperm recovery and motility kinetics were assessed by computer-assisted semen analyser (CASA). Additionally, non-capacitated mouse sperm (N&#3f7) underwent in vitro fertilisation (IVF) for either a 4 or 24 h fertilisation window at NG or OG and zygotes subsequently cultured in GT-L at NG for 5-days. 2-cell cleavage rates were assessed at 24 h and on-time blastocyst development and cell numbers/allocation (Oct4 – inner cell mass and Nanog – epiblast fetal cells) at 96 h post-insemination. A subset of embryos underwent time-lapse culture to assess timing of PN fading, PN to 2-cell cleavage, and

PN to blastocyst development. Additionally, porcine sperm (N&#3f3) underwent in vitro fertilisation for a 6 h fertilisation window at NG or OG, and the zygotes subsequently cultured in NCSU23-plg and NCSU-G at NG for 7-days at 38.5°C, 6% CO₂, 5% O₂. Porcine 2-cell cleavage rates were assessed at 96 h and on-time blastocyst development at 144 h post-insemination. Exposure of both human and mouse sperm to OG reduced its ability to navigate a channel slide, with a 40% reduction in sperm recovery after 1 hour (both $P < 0.05$) and a reduction in both amplitude lateral head movement and curve linear velocities in the mouse (-33%, $P < 0.05$), although total motility was unchanged in both species. Fertilisation rates in the mouse were reduced by 33% following 4 h fertilisation at OG ($P=0.003$), although unchanged during a 24 h fertilisation window. On-time blastocyst rates were reduced by 22% (all $P < 0.05$) following any fertilisation period at OG. Interestingly, in those embryos that did make it to blastocyst, exposure to OG increased epiblast fetal cells (+30%, $P < 0.05$), although, blastocyst total cell numbers were unaffected. Similar effects were observed in the pig, where there was a 20% reduction in fertilisation rate ($P=0.067$) and a 23% reduction in on-time blastocyst rate ($P=0.046$). This study indicates that microgravity can negatively influence sperm's ability to navigate and fertilise an egg. Even after returning to normal gravity, embryos exposed to microgravity during fertilisation have reduced on-time blastocyst rates, and an increased fetal cell cellular allocation. The ability of mammals to reproduce in micro-gravity environments currently seems limited.

S23.4 - Calcium Oscillations: The Egg's Elevator Pitch at Fertilization

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In mammals, the initiation of new life involves repetitive changes in the egg's intracellular calcium level, called calcium oscillations. These oscillations last only for a short time (about 5 h in mice) but encode essential instructions for egg activation and subsequent embryo development. As a unique, concise, and critical discourse, calcium oscillations resemble an elevator pitch. Despite their important role, they are susceptible to the environment and can be altered in vitro by changing the ionic composition of culture media, or in vivo by medical conditions such as obesity or inflammation. In mice, abnormal calcium oscillations at fertilization have a long-term impact on offspring growth and health. These calcium-dependent long-term outcomes raise concerns that there could be similar consequences for children conceived in vitro or from women with conditions affecting the egg's ability to handle calcium. However, it remains unclear what mechanism links changes in calcium homeostasis in the zygote with offspring health. To identify this link, we developed a mouse model of dramatically increased calcium exposure at fertilization in vivo by targeting two plasma membrane calcium pumps (PMCA). PMCA extrude excess calcium from the cytosol to maintain calcium homeostasis. Using CRISPR technology, we knocked out the two main PMCA, PMCA1 and PMCA3, in mouse oocytes. PMCA1/PMCA3-null eggs (dKO) had extended elevated calcium at fertilization (~10 times more than controls, $p < 0.0001$). When mated to wild type males, dKO dams had fewer pups per litter than controls (5 vs 8 pups, $n=9$ dKO and 7 control dams; $p < 0.0001$). Moreover, preliminary data suggest that dKO derived pups are heavier than genotype-matched controls obtained after physiological calcium oscillations at fertilization. To determine when elevated calcium affects fertility, dKO and control eggs were fertilized in vitro and monitored for development. dKO eggs exhibited fertilization rates and cleavage rates comparable to those of controls. However, only 26 out of 108 dKO fertilized eggs reached the blastocyst stage compared to 56 of 136 controls ($p < 0.007$). Similarly, in vivo fertilized eggs were flushed from dKO or control dams mated to WT males, and cultured in vitro. While no difference was observed at the 2C stage, only 44 out of 110 dKO fertilized eggs reached

the blastocyst stage compared to 97 of 108 controls ($p < 0.0001$), which aligns with the ~40% reduction the litter size. To identify the link between calcium oscillations and preimplantation development, the redox status of the fertilized eggs was monitored by imaging FAD+ production during calcium oscillations. FAD+ is a cofactor involved in various metabolic reactions, cellular respiration, and ATP production. Interestingly, FAD+ production followed the calcium oscillatory pattern, and therefore was abnormally elevated in dKO eggs. Moreover, by using the Click-iT EU assay, we found that dKO derived embryos show dramatically low global transcription at the mid-1cell stage (60% less than controls $p < 0.0001$). According to our data, a tenfold increase in calcium levels at fertilization negatively affects the redox status and global transcription shortly after fertilization, resulting in a ~40% reduction in blastocyst formation and a 40% decrease in litter size. Our findings intimately connect calcium homeostasis at fertilization, the redox status of the zygote, early activation of gene expression, and raise awareness of the impact of abnormal calcium exposure on embryonic developmental potential and offspring health.

S23.5 - Neurodevelopmental (ND) Changes in Mice Obtained Following In Vitro Culture of Embryos Conceived at Advanced Paternal Age (APA).

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Children conceived at advanced paternal age (APA) are at increased risk of neurodevelopmental (ND) disorders. The use of assisted reproduction technologies (ART) has been also associated with increased incidence of ND alterations in offspring. Recent studies indicated that APA negatively affects early embryo development and pregnancy obtained by ART. Possible long-term risks of ND pathologies were not investigated in those pregnancies. We wanted to know whether the in vitro culture (IVC) of mouse embryos obtained at APA increases the degree of ND changes observed in APA. Sixty-eight C57BL/6 3-4 months old females were mated with 12-14 months old males. From fifty-two females, one day following mating embryos were collected, cultured in vitro until the blastocyst stage, and transferred to pseudopregnant recipients for further development. Offspring developed from in vitro cultured APA embryos (IVC APA, $n=10$), conceived naturally by APA males (APA, $n=11$), and control offspring obtained from the mating of young 3-4 months old parents (CTR, $n=12$) were subjected to social interaction and anxiety-like behaviour evaluation using three-chamber and light/dark box tests. Subsequently, brains (hippocampus) collected from 4-month-old males were used for global proteome profiling (Student's t-test, $p < 0.05$), while the prefrontal cortex was used for expression analysis of selected genes involved in the pathophysiology of ND disorders by qPCR. IVC APA offspring showed anxiolytic response by reduced latency time to enter open-lit areas (IVC APA: 4.43 ± 4.43 vs. APA: 34.7 ± 51.1 , $p < 0.05$ and vs. CTR: 25.77 ± 13.34 , $p < 0.0001$). This aberrant behaviour is symptomatic of schizophrenia and other ND disorders. There were no additional effects of IVC in reference to social interaction impairment. Furthermore, IVC APA exhibited an impaired preference for novel social stimuli (IVC APA: 0.13 ± 0.44 vs. APA: 0.31 ± 0.32 , and vs CTR: 0.34 ± 0.34 , $p < 0.05$). Proteomic analysis revealed 240 differentially expressed proteins (DEPs) between IVC APA and CTR brains and 178 DEPs between APA and CTR, indicating that IVC further alters brain homeostasis in APA offspring. In IVC APA brains, DEPs mainly refer to abnormal behaviour and abnormal nervous system. In particular, a decreased expression of proteins involved in the regulation of neurotransmitter receptor activity and postsynaptic organization was observed in IVC APA brains. Furthermore, the expression of two genes associated with autistic-spectrum-disorders,

Cntnap2 (IVC APA: 0.40 ± 0.11 vs. CTR: 1.02 ± 0.22 , $p < 0.0001$) and Mapk3 (IVC APA: 0.44 ± 0.08 vs. CTR: 1.02 ± 0.21 , $p < 0.0001$) was markedly changed in IVC APA prefrontal cortex when compared to CTR. We demonstrated that IVC induces ND and behavioural alterations that are additional to those observed in offspring conceived at advanced paternal age that developed naturally. It indicates that the cumulation of two pregnancy risk factors, such as ART and APA may more deeply affect the ND health of offspring. Our study suggests caution when considering ART treatment for couples where the male partner is at an advanced age. This study was supported by the National Science Centre, contract no. 2019/35/B/NZ4/03547 and 2021/41/B/NZ3/03507, Ministry of Education and Science, contract no. 0175/DIA/2019/28.

S23.6 - Novel Therapies to Protect Sperm Quality in ART

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The manipulation of sperm for assisted reproductive technology (ART) results in a significant decrease in sperm quality, especially higher levels of DNA damage. Elevated levels of DNA damage in sperm have been linked to poor-quality embryos and increased pregnancy loss after ART. Utilizing the novel therapeutic BGP-15, we have previously shown that treating the whole ejaculate prior to and during cryopreservation increases sperm quality compared with frozen/thawed untreated samples. In this study, we investigated the potential of this BGP-15 therapy to preserve sperm quality during semen washing prior to IVF/ICSI. Donated human semen specimens excess to ART (N=41), were treated with 10 μ M BGP-15, and sperm motility and DNA damage (DNA fragmentation and oxidation) were first examined to understand the effect of incubation time in whole semen. Samples were then washed using different clinical sperm washing methods: simple wash, swim-up and density gradient centrifugation (DGC); and purified sperm underwent assessment for motility, vitality, ROS levels, mitochondrial membrane integrity, mitochondrial membrane potential, and DNA damage. Statistical analysis was two-tailed paired Student's T test when comparing between two groups, or repeated measures Two-Way ANOVA when comparing between three groups. Post-hoc pairwise comparisons were two-tailed paired Student's t-tests to analyze matching data pairs for each patient. Semen samples incubated with BGP-15 demonstrated 15% improved sperm motility ($p=0.002$) and 57% reduced oxidative DNA damage levels ($p=0.03$). Comparison between the untreated groups for each washing method, showed that simple washed samples had the highest sperm recovery rate, which was 55% and 21% higher than DGC ($p < 0.005$) and swim-up ($p < 0.0005$), respectively. Additionally, sperm recovered by swim-up had 18% and 27% increased vitality compared to DGC ($p < 0.001$) and simple wash ($p < 0.0001$), respectively. Swim-up sperm also presented DNA oxidation levels that were reduced by 40% and 76% when compared to simple wash ($p=0.01$) and DGC ($p < 0.0001$), respectively. However, swim-up sperm also had the lowest mitochondrial membrane potential, that was 28% lower than simple wash and DGC ($p < 0.03$). Comparison between untreated and BGP-15 treated groups for each sperm washing method showed that DGC sperm had 11% increased mitochondrial membrane potential after BGP-15 treatment ($p=0.0006$). Moreover, BGP-15 treatment only reduced sperm DNA fragmentation in washed samples (22% reduction, $p=0.03$), but it lowered DNA oxidation by 48% in washed ($p=0.002$), by 42% in swim-up ($p=0.04$) and by 29% in DGC ($p < 0.0001$) sperm. We further tested the safety of sperm treatment with

BGP-15 in a mouse model and analyzed its effect on IVF, pre-implantation embryo development, embryo cryopreservation and thawing. After surgical embryo transfer, surrogate female mice were assessed for pregnancy success, gestation length and litter size. Litters were also assessed for growth trajectory, righting reflex and survival to weaning at 21 days old. There were no differences between untreated and BGP-15 groups in any measurement analyzed, indicating there was no detectable effect of in vitro BGP-15 treatment of sperm on embryo, pregnancy or offspring outcomes (n=6 males/pools of embryos/surrogate females/litters per treatment group; $p > 0.1$). Our findings indicate that the utilization of BGP-15 during semen preparation can protect and improve on its quality. Our preclinical models further highlight that the addition of BGP-15 could be used clinically to improve the outcomes of patients undergoing ART.

FOCUS SESSION 24 - OVARY AND OOCYTE

S24.1 - Lipid Profile Changes in the Egg – Causes and Consequences

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Oxidative stress (OS) in the cell leads to changes in lipid composition which is reflected by changes in intracellular lipid droplets (LDs) profile. LDs profile can be evaluated in single cells using, Coherent anti-stokes Raman scattering (CARS) microscopy.

Under the influence of OS, the molecules that make up the cell, such as lipids, proteins, carbohydrates and nucleic acids, may undergo modifications. Between all these molecules, lipids are the first target of oxidative changes. Because OS is associated with conditions affecting egg quality, such as aging, evaluation of LDs profile may represent sensible method of oocytes and embryo evaluation.

We investigated lipidomic compartment of the egg by various spectrometric methods such as Fourier transform infrared spectroscopy, Matrix-assisted laser desorption/ionization mass spectrometry, and with CARS, which is clinically relevant live-cell diagnostic method. Furthermore, we examined if modifications of lipids in the egg affect embryo developmental competence.

We show lipid profile changes in human and mouse oocytes from females and advanced age and in in vitro cultured mouse embryos.

In conclusion, we provide evidence of female-age-dependent lipid profile changes in oocytes from human and mouse that are maintained throughout embryo development. Additionally, we shown that in vitro culture may also induce lipid profile changes in the embryos. Lipid changes in the egg may affect other cellular compartments, including DNA. This study provide basis for further investigations aimed to explore how changes in the profile of lipids influence embryo development, implantation and pregnancy. Study supported by National Science Centre, Poland GA 2022/44/C/NZ4/00076; 2021/41/B/NZ3/03507; 2019/35/B/NZ4/03547.

S24.2 - Effect of Highly Expressed Intrauterine MicroRNAs in Low-Fertility Cows on Embryo Gene Expression

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Embryonic death is a significant cause of pregnancy failure in cows, often happening before implantation. Embryo survival and development pre-implantation is dependent on the uterine luminal

fluid. The fluid component is believed to play a role in early embryonic death and infertility in cows. Exosomes, found in uterine luminal fluid, contain microRNAs (miRNAs), which are crucial intercellular communication mediators. miRNAs regulate conceptus–maternal interactions and participate in embryonic development by suppressing gene expression. Therefore, our hypothesis posits that miRNAs within intrauterine exosomes of low-fertility cows may hinder embryonic survival and development by affecting embryonic cells. Exosomes were gathered from the bovine uterine luminal fluid of both normal and low-fertility cows seven days after estrus. Small RNA-sequencing analysis utilizing miRNAs isolated from intrauterine exosomes revealed that eight miRNAs exhibited high expression in normal-fertility cows (Normal8miRs, $p < 0.05$), while eight miRNAs showed elevated expression in low-fertility cows (Low8miRs, $p < 0.05$). Subsequently, fluorescent-labeled intrauterine exosomes were exposed to bovine-hatched blastocysts to confirm exosome uptake by embryos. Green fluorescence emitted from labeled exosomes was observed in the inner cell mass and trophoblast after 6 h of co-culture. Following this, Normal8miRs and Low8miRs were transfected into hatched blastocysts via lipofection, and RNA-sequencing analysis was conducted to examine gene expression changes. Low8miRs treatment resulted in 424 differentially expressed genes (DEGs, $p < 0.05$) compared to the control. Only seven genes were detected as DEGs in the Normal8miRs treatment group. Analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways revealed remarkable enrichment of TNF signaling pathway- and MAPK signaling pathway-related genes among DEGs between Low8miRs and control. These findings indicate that at least eight highly expressed miRNAs in low-fertility cows directly influence the pre-implantation embryo, altering gene expression associated with embryonic survival and development. The increased expression of miRNAs in low-fertility cows possibly induces infertility since the embryonic TNF and MAPK signaling pathways in embryos are crucial for their proliferative and differentiation potential.

S24.3 - RGD-Modified Dextran Hydrogel Promotes Follicle Growth and Theca Cell Migration Through Integrins $\alpha\beta3/\alpha\beta5$ in Three-Dimensional Culture

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Due to ovarian damage that might result from chemotherapy and/or radiation treatment, cancer patients run the risk of losing their fertility. In vitro follicle culture is a promising technology to preserve the fertility of cancer patients. We recently established a three-dimensional (3-D) ovarian tissue culture system supported by a biochemically defined dextran hydrogel. To functionalize the dextran hydrogel, extracellular matrix-derived triple Arg-Gly-Asp (RGD) peptide was supplemented in the gel. Murine ovarian tissues at 14 days old cultured in the RGD-modified dextran hydrogel significantly promoted antral follicle development and oocyte quality compared with those in dextran hydrogel alone. Next, we determined the RGD-binding target integrins expressed in the murine ovary. In mammals, the family of integrins is composed of 24 $\alpha\beta$ subtypes, and 8 integrin subtypes recognize and bind to the RGD motif. Using qPCR and immunohistochemical analyses, we found that integrins $\alpha\beta3$ and $\alpha\beta5$ are the main targets of RGD in the immature ovary. To examine the effect of RGD-integrin interaction on follicle development, we used Cilengitide (Ci), an inhibitor for integrins $\alpha\beta3/\alpha\beta5$. Ovarian tissues were cultured in dextran hydrogels under three different conditions: RGD–, RGD+, and RGD+Ci. In the RGD+

condition, follicle development and theca cell differentiation were significantly enhanced in the ovarian tissues. However, in the presence of Ci (RGD+Ci), RGD-induced follicle differentiation was dramatically suppressed, similar to the RGD– condition. Next, we examined the direct effect of RGD on ovarian interstitial cells. The aggregates of ovarian interstitial cells were 3-D cultured under the three conditions. The RGD+ condition significantly enhanced cell migration and the expression levels of theca cell marker genes in the aggregates. The RGD- induced cell activity was totally suppressed in the RGD+Ci condition, similar to those in the RGD– condition. To examine theca layer formation, interstitial cell aggregates were isolated from transgenic Gli1ERcre/td tomato (tdT) mice. Since Gli1 is a known marker for theca cells, tamoxifen was used to inject Gli1ERcre/tdT pups. This caused cre-mediated recombination and the expression of tdT fluorescence in cells that expressed Gli1 and all their offspring. The transgenic-derived cell aggregates were co-cultured with the wild-type (WT) secondary follicles under the three conditions. In the RGD+ aggregates, tdT-positive cells migrated to form the cell layers around follicles. In contrast, cell migration and cell layer formation were absent in both RGD+Ci and RGD– aggregates co-cultured with follicles. In conclusion, these results suggest that RGD peptide promotes follicle growth and theca cell differentiation via binding integrins $\alpha\beta3/\alpha\beta5$ expressed in the ovarian interstitial compartment in our 3-D culture system.

S24.4 - Like Mother, Like Daughter: Multigenerational Effect of Early-Life Malnutrition on Female Mice Phenotype and Oocyte Energetic Capacity

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Parents play a critical role in shaping the health of their offspring. Nutritional deficiencies during early life can have long-lasting effects on health, significantly influencing phenotypic trajectories. While several studies have demonstrated that fetal growth restriction increases the risk of adult diseases, there remains limited understanding of how postnatal nutritional experiences program reproductive functions across generations. In this study, our objective was to investigate the effects of transient undernutrition of mothers (F0) during the nursing period on the phenotype and gametes of F1 and F2 females. Newborn pups (F1) were assigned to either the control group (CON), where mothers were fed ad libitum, or the lactation undernutrition group (LUN), where mothers were provided with 50% of the daily portion of chow consumed by control dams. After weaning (postnatal day 21 = PND21), F1 offspring was fed ad libitum. Sexually mature F1 mice were bred in three different combinations: CON female x CON male (CC), LUN female x CON male (LC) and LUN female x LUN male (LL), to generate F2 females not directly exposed to undernutrition. In both generations, body weight, composition, and timing of puberty were assessed. Ovaries collected at PND21 underwent RNA-seq and/or RT-qPCR analysis. Metaphase II (MII) oocytes collected from sexually mature F1 and F2 females were assayed for mtDNA copy number, mitochondrial membrane potential, lipid droplet content, and levels of reactive oxygen species (ROS). The optical redox ratio (ORR; $FAD/[NAD(P)H + FAD]$) within individual oocytes was calculated in the presence of different doses of pyruvate. PND21 LUN and LL females exhibited decreased body fat content and adiposity index compared to CON and CC, respectively. Delayed puberty was observed in LUN and LL females. RNA-seq analysis of ovaries unveiled numerous differentially expressed genes related to lipid metabolism and lipid transport between CON and LUN females, which was later confirmed by RT-qPCR in both generations. Despite no difference in mtDNA copy number, LUN and LL oocytes displayed more

active mitochondria compared to respective controls. Interestingly, LUN oocytes had elevated levels of lipids and this effect persisted in the F2 LL and LC groups. Consistent with these findings, LUN and LL oocytes demonstrated a higher ORR compared to controls under basic conditions. However, only CON oocytes exhibited an increased ORR in response to low pyruvate doses (0.8 mM), while high doses (10 mM) led to higher ORR in LUN compared to CON. Surprisingly, both LC and LL oocytes showed reduced ROS levels compared to the CC group. In summary, despite experiencing transient energy deficits during early life, LUN females with lower adiposity had oocytes with increased lipid content and mitochondrial activity. The effects observed in the second generation align with the principles of nutritional programming theory. Even though the insult is no longer present, oocytes retain the memory of scarce nutrient availability, likely to prepare for future increased demands for cellular components and constituents essential for reproductive success. Research supported by Polish National Science Centre [2018/31/B/NZ4/03527].

S24.5 - Defining The Function of Tubulin Post-Translational Modifications in Mammalian Oocyte Meiosis

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Female meiosis is a specialized form of cell division that gives rise to fertilizable eggs. Oocytes, the progenitors of eggs, are held in prophase of meiosis I for remarkably long periods (up to decades in humans). When released from this prolonged arrest during their maturation into eggs, oocytes segregate the chromosomes using a meiotic spindle machinery that is assembled from microtubules. Fidelity of oocyte chromosome segregation by this meiotic machinery is critical for healthy embryogenesis as its inaccuracy can lead to aneuploidy (incorrect number of chromosomes) in eggs, a major cause of miscarriages and genetic conditions. Oocytes face unique cell division challenges that are not present in most cell types. These include building a robust meiotic spindle in the absence of canonical centrosomes, transporting it across an unusually large cellular volume (~80µm) and positioning it extremely close to the cell surface to preserve cytoplasm needed for embryo development. We hypothesize that oocytes overcome these challenges by employing post-translational modifications (PTMs) of tubulins (the building blocks of microtubules) to diversify microtubule properties and function (a concept known as the 'tubulin code'). To investigate the role of tubulin PTMs in oocyte cytoskeletal organization and chromosome segregation, we are implementing quantitative immunofluorescence microscopy for identification and characterization of microtubule PTMs at various stages of mouse oocyte meiosis. By combining these with high- and super-resolution live imaging assays and wild-type/mutant PTM enzyme overexpression approaches, we are able to override the tubulin code and address tubulin PTM function in oocyte meiosis. We are also leveraging targeted protein degradation tools to destroy modified tubulin proteins rapidly and specifically at any desired stage of meiosis. These integrated approaches have revealed exciting new functions of tubulin PTMs in meiotic microtubule organization and chromosome segregation that we are currently following up with mechanistic experiments. We anticipate that previously unknown chromosome segregation mechanisms emerging from these studies could be exploited in future to innovate preventive medicines against infertility.

S24.6 - Investigating Ribosome Dynamics in the Aging Mammalian Oocyte

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The female reproductive system is the first system to experience age-dependent functional decline in mammals. In humans, advanced maternal age severely compromises the likelihood of successful conception and pregnancy, due to decreased fertility and increased risks of pre-eclampsia, miscarriage, birth defects, and pre-term birth. This age-dependent decline has become a significant concern to society, as more women than ever are delaying childbearing until their advanced maternal years. Oocyte quality is the greatest barrier to fertility at advanced maternal age. Though the impact of oocyte quality is appreciated, our understanding of its molecular culprits is limited. One aspect of oocyte quality that remains understudied is translation, the production of proteins by macromolecular machines called ribosomes. Oocytes are unique in that they rely entirely upon ribosomes to modulate gene-regulation during meiotic maturation and the oocyte-to-embryo transition. Furthermore, the oocyte ribosomes provide for the zygote until zygotic ribosome biogenesis becomes active. Ribosomes are therefore an important - but poorly characterized - factor of oocyte quality. If ribosome production or activity is perturbed with age, oocyte quality would be significantly compromised. To address this gap in knowledge, we conducted single-cell RNA sequencing from oocytes isolated from reproductively young and old female mice. The pathways identified to be the most enriched with age were those associated with ribosome biogenesis and translation. We then measured the levels of ribosomal RNA (rRNA), the rate limiting factor of ribosome biogenesis, within oocytes. As expected, we found that rRNA also significantly increases in oocytes with age. This observation compliments the finding that cytoplasmic ribosome populations in oocytes significantly increase with age. To determine the functional output of these ribosomes, global translation assays were conducted in germinal vesicle oocytes from reproductively young and old mice. The results revealed significantly lower translation levels in old oocytes. Taken together, we surmise that ribosome numbers increase while translation decreases. These paradoxical findings point to translation perturbations within the aging oocyte that warrant further investigation. We hypothesize that as oocytes age, the regulation of ribosome production and translation are compromised. Continued investigation of age-associated changes in ribosome production and translation activity in oocytes will provide new insight to oocyte quality and how it changes with age.

S24.7 - Differential Expression of Mitochondrial Function-Related Genes After In Vivo And In Vitro Maturation of Bovine Cumulus-Oocyte Complexes.

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In vitro maturation (IVM) of cumulus-oocyte complexes (COCs) is routine practice in producing bovine embryos in vitro, and its application in human infertility treatment is increasing. However, COCs

subjected to IVM show inferior developmental competence compared to those matured in vivo (IVO). We hypothesize that the maturation environment alters the mRNA profile of both oocytes and cumulus cells. Therefore, we characterized the global mRNA expression of oocytes and their respective cumulus cells obtained following either IVM or IVO using a bovine model. Cows (n=5) were superovulated with 180 mg of FSH (Folltropin, Vetoquinol, Canada) administered twice daily for four days in decreasing doses, and LH surge was induced with 2.5 mL GnRH (Receptal, Intervet, Germany). Follicles >8 mm were aspirated by ovum pick-up 24h post-GnRH injection to retrieve IVO COCs. The aspirated follicular fluid was collected in a 50 ml tube, and the COCs recovered were individually denuded using 0.1% hyaluronidase (Sigma) in a 0.3% PVP medium. Denuded oocytes were classified as mature or immature based on the presence or absence of the polar body, respectively. Oocytes (n=23) were washed three times in PVP medium, treated with pronase to dissolve the zona pellucida (0.1% protease from *S. griseus* in TCM-199), and stored individually in Buffer RLT (Qiagen). Cumulus cell samples from mature IVO COCs (n=4) were pooled based on the cow of origin and stored in lysis buffer. After a resting cycle, the same animals were monitored for follicle growth to aspirate follicles ≤ 8 mm and collect immature COCs. Collected COCs underwent IVM in groups in 500µl bicarbonate-buffered TCM199 medium supplemented with gentamicin (50 µg/mL) and epidermal growth factor (20 ng/mL). After 22h, COCs were denuded, and IVM oocytes (n=30) and their respective cumulus cell samples (n=3) were processed and stored as described before. Single-cell RNA sequencing of 53 oocytes was performed, and 19,744 transcripts were identified. Principal component analysis (PCA) of global gene expression segregated the oocytes according to their maturation state, clustering IVM and IVO oocytes in two groups. A total of 100 differentially expressed genes (DEG; 72 up- and 28 down-regulated in IVO) were identified in IVO versus IVM ($P < 0.05$). GO pathway analysis showed involvement in the electron transport chain, mitochondrial respiratory chain, mitochondria respiratory, and protein-to-membrane docking. Similarly, RNA sequencing of the corresponding cumulus cells (n=7) identified 5898 features, and PCA analysis segregated the IVM and IVO cumulus cell samples into two separate clusters. We identified 844 DEG (454 up- and 390 down-regulated in IVO; $P < 0.05$) by comparing IVM and IVO cumulus cells. These genes were associated with biological processes, including RNA processing, ovarian cumulus expansion, protein processing in endoplasmic reticulum, and oxidative phosphorylation. DBSCAN clustering of DEG from single-cell oocytes and corresponding cumulus cells, using STRING database v12, revealed a shared cluster of genes regulating mitochondrial electron transport and ATP synthesis. These results suggest that IVM alters mRNA expression related to mitochondrial function in both oocytes and cumulus cells. Mitochondria's ability to balance ATP supply and demand is crucial for correct oocyte maturation and competence acquisition. Therefore, the present study highlights potential molecular mechanisms underlying the developmental competence disparities between in vivo and in vitro matured oocytes. This project is funded by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 860960.

FOCUS SESSION 25 - EMBRYO MODELS

S25.1 - Synthetic Ex Utero Embryogenesis: From Naive Stem Cells to Complete Embryo Models

Jacob H. Hanna

The identity of somatic and pluripotent cells can be epigenetically reprogrammed and forced to adapt a new functional cell state by different methods and distinct combinations of exogenous factors. The aspiration to utilize such in vitro reprogrammed pluripotent and somatic cells for therapeutic purposes necessitates understanding of the mechanisms of reprogramming and differentiation and elucidating the

extent of equivalence of the in vitro derived cells to their in vivo counterparts. In my presentation, I will present my group's recent advances toward understanding these fundamental questions and further detail our ongoing efforts to generate developmentally unrestricted human naive pluripotent cells with embryonic and extra-embryonic developmental potential. I will expand on new avenues for utilizing custom made electronically controlled ex utero platforms and optimized conditions for growing natural mammalian embryos ex utero for extended periods capturing development from pre-gastrulation until advanced organogenesis, for better studying of stem cell transitions during embryogenesis and organogenesis. I will detail how the latter platforms offered an exclusive technical platform to demonstrate and unleash the self-organizing capacity of mouse naive PSCs to generate post-gastrulation synthetic Bona Fide and organ-filled, synthetic embryo models with both embryonic and extraembryonic compartment ex utero, as well as our ability to extend these findings with naive human PSCs and generate complete structured day 14 human embryo models. Collectively, I will be highlighting prospects for new platforms for advancing human disease and developmental modelling.

S25.2 - Bioengineering Human Embryo and Organ Models

Jianping Fu, University of Michigan

Recent advances in developmental biology, stem cell biology, and bioengineering have spurred interest in constructing controllable, stem cell-based models of human embryos and organs. These models offer enhanced controllability and reproducibility, along with the ability to genetically modify stem cell lines, manipulate culture conditions, and perform live imaging. They provide robust systems to investigate cellular behaviors and signaling interactions driving human development. In this presentation, I will discuss our work using human pluripotent stem cells (hPSCs) and bioengineering tools to develop controllable models of peri-implantation embryonic and early neural development. The peri-implantation human embryo models accurately mimic key developmental landmarks, including amniotic cavity formation, ectoderm-epiblast patterning, primordial germ cell specification, primitive streak development, and yolk sac formation. Additionally, we have created microfluidic neural tube-like structures from hPSCs, which recapitulate critical aspects of neural patterning in brain and spinal cord regions along both rostral-caudal and dorsal-ventral axes. These structures enable the study of neuronal lineage development and reveal insights into axial identities of neural crest progenitors and the role of genes like CDX2 in spinal cord and trunk neural crest development. Moreover, we have developed microfluidic forebrain-like structures that mimic human embryonic brain development in pallium and subpallium areas, with spatially segregated dorsal and ventral regions and layered cellular organizations. Together, these hPSC-based neural development models offer 3D tissue architectures with in vivo-like cell differentiation and organization, providing valuable models for studying human neurodevelopment and disease.

S25.3 - Mechanical YAP1 Function Safeguards Amnion Fate from the Early Human Embryonic Lineages

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The formation of the amniotic membrane plays a crucial role in fetal development and provides protection to the embryo throughout pregnancy. Despite its importance in successful pregnancy, the

molecular mechanisms that regulate amnion formation are not well understood. During human embryonic development, amniogenesis is triggered in a collection of pluripotent epiblast cells as the human embryo implants. Utilizing a human pluripotent stem cell (hPSC)-based model of amniogenesis (called Gel-3D), our previous studies showed that amniogenesis is sensitive to substrate rigidity (Shao et al., Nature Materials 2017). In this system, soft gel substrate triggers the activation of endogenous BMP signaling, followed by the BMP-dependent amniogenic transcriptional cascade, and 3-dimensional cysts with a central lumen uniformly surrounded by squamous amnion cells are seen by day 4. While recent studies have shed light on these amniogenic machineries, mechanisms that mediate this mechanosensitive amniogenic process is not known. Here, we show that YAP1 (Yes-associated protein 1), a well-established mechanosensitive transcriptional activator, promotes amniotic fate progression during this mechanically triggered event. In the Gel-3D system, YAP1 translocates into nucleus starting at day 2, suggesting a potential role of YAP1 in promoting amniogenesis. Using a mini auxin-inducible degron (mAID) technology to inducibly degrade endogenous YAP1 during amniogenesis in hPSC, we found that the loss of YAP1 in Gel-3D leads to the formation of cysts that are smaller, and not all cells acquire amniotic lineage. Transcriptomic analyses show that, in the absence of YAP1, the expression of markers associated with primordial germ cell (PGC) and advanced mesodermal lineages is increased. Importantly, this change in fate progression is not seen in a culture condition that bypasses the mechanically triggered BMP signaling (Glass-3D+BMP, Sekulovski et al., eLife 2023). These results provide a novel role for YAP1 in promoting amniogenesis by suppressing embryonic lineages. This research is supported by NIH grant R01-HD098231 as well as by MCW CBNA Start-up funds.

S25.4 - TET2-Mediated ENPEP Activation Is Required for Trophoblast Cell Differentiation

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Proper trophoblast differentiation is one of the determinants for successful embryo implantation, placentation and fetal development. Dynamic changes in DNA methylation occur during preimplantation development and are critical for cell fate determination. However, the underlying regulatory mechanism remains unclear. Recently, we established morula-like expanded potential stem cells from human preimplantation embryos (hEPSC-em), providing a tool for studying early trophoblast differentiation. In this study, we aimed at identifying epigenetic regulators in controlling the earliest trophoblast differentiation. Data analysis on published datasets demonstrated differential expressions of enzymes mediating DNA methylation during early trophoblast differentiation in both human embryos and hEPSC-em derived trophoblastic spheroids (BAP-EBs). Among the analyzed ten-eleven translocation methylcytosine dioxygenases (TETs), a family of DNA demethylase, TET2 was substantially induced during early trophoblast differentiation. With the use of integrative analysis on published datasets and confirmation by Chromatin immunoprecipitations (ChIP)-qPCR, TET2 was found to be involved in demethylation and activation of glutamyl aminopeptidase (ENPEP) during trophoblast differentiation. CRISPR-CAS9 knockout of TET2 or ENPEP in hEPSC-em compromised trophoblast differentiation potency and reduced attachment and invasion potential of the derived BAP-EBs. Collectively, our results

illustrated the crucial role of ENPEP and TET2 in trophoblast fate commitment and the unprecedented TET2-mediated demethylation of ENPEP. This study sheds light on the role of DNA demethylation in trophoblast differentiation, aiding our understanding of early human embryo development and potential interventions for placenta-related pregnancy complications.

S25.5 - Single-Cell RNA-Seq Reveals the Earliest Lineage Specification and X Chromosome Dosage Compensation in Bovine Preimplantation Embryos

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Lineage specification and X chromosome dosage compensation are two crucial biological processes that occur during preimplantation embryonic development. Although extensively studied in mice, the timing and regulation of these processes remain elusive in other species, including human and bovine. We therefore aim to provide fundamental insights into these programs and the regulation using a bovine embryo model, which suggested conserved principles of human early development, by employing single-cell transcriptomics and genome editing approaches. In silico analysis of 286 individual cells from morula to late blastocyst reveals that the transcriptomes of trophectoderm (TE)/inner cell mass (ICM) do not diverge until early blastocyst stage and differential expression genes at this stage are mainly belongs to TE markers, suggesting the first lineage specification occurs after cavitation with TE fate specified earlier in bovine embryos. In order to investigate the emergence of pluripotent cells, this study identifies novel transcriptomic markers and depicts dynamic metabolic change associated with pluripotency transition in the bovine embryo. To unravel the molecular circuitry governing pluripotency, we focus on the transcriptional factor SOX2, which is considered as the most notable marker restricted into ICM cells with indispensable function in the second cell fate decision in bovine embryos, by combining base editing and single-cell transcriptomic technology. In particular, our study shows that SOX2 is required for the initiation of ICM fate and TE commitment as early as the morula stage, likely mediated through a metabolic mechanism. Moreover, our results suggest that the dosage compensation of X-linked genes initiates from the morula stage accompanied by the partial inactivation of the X chromosome and the progress of X chromosome dosage compensation is more extensive within TE lineage. In summary, this comprehensive analysis depicts molecular landmarks and regulation mechanism underline the first cell fate decision program and X chromosome dosage compensation during bovine early embryonic development. Furthermore, the comparison of differences and similarities among bovine, mouse, and human embryos in these biological events, providing valuable resources for studying mammalian preimplantation development.

S25.6 - Pcgf5 Transcribed from Endogenous Retroviral Element Has Important Functions in Mouse Preimplantation Embryos

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Long terminal repeat (LTR) of murine endogenous retrovirus with leucine tRNA primer (MERVL) generates chimeric transcripts with host genes during zygotic genome activation (ZGA) in mice. Recent studies have shown that the LTR of MERVL expresses chimeric mRNAs involving adjacent genes, but the function of MERVL chimeric mRNAs remains unclear. Because MERVL has been reported to be one of the earliest transcribed genomic sequences in preimplantation embryos, we hypothesized that MERVL

chimeric transcripts contribute to the gene expression after ZGA. In our laboratory, reanalysis of previously reported RNA-sequencing data revealed that polycomb group ring finger 5 (Pcgf5) is expressed as a chimeric transcript with MT2C_Mm, one of the LTR of MERVL, in mouse preimplantation embryos. Pcgf5 is one of the components of polycomb repressive complex 1 (PRC1) which has high catalytic activity for lysine 119–monoubiquitinated histone H2A (H2AK119ub1) deposition. Previous reports have shown that the H2AK119ub1 modification co-localized with the H3K27me3 modification from the fully grown oocytes to the preimplantation embryos after fertilization. However, the function of Pcgf5 in preimplantation embryos and the significance of the chimeric Pcgf5 transcript remain unknown. To address these questions, we compared the abundance of the Pcgf5 transcript variants and examined the role of Pcgf5 in mouse preimplantation embryos. Relative quantification of mRNA at each developmental stage was performed using primers for each Pcgf5 variant including chimeric transcript (Pcgf5MT2C_Mm) and primers for amplifying the consensus sequence of all Pcgf5 variants (total Pcgf5). As a result, the expression level of total Pcgf5 and Pcgf5MT2C_Mm were highest in late two-cell stage embryos. Absolute quantitative PCR for estimating the copy number of each Pcgf5 variants showed that most of the Pcgf5 transcripts in late two-cell embryos were Pcgf5MT2C_Mm. PCGF5 immunofluorescence (IF) at each developmental stage showed PCGF5 protein expression increased from zygotes to two-cell embryos and maintained the highest expression level from two-cell embryos to morulae. PCGF5 IF showed PCGF5 spots in some morulae. Since the localization patterns of these spots were similar to those of Xist in RNA-FISH, it is suggested that PCGF5 accumulate in Xist RNA region, which is involved in X chromosome inactivation. A half of Pcgf5 knockdown (KD) embryos produced by electroporation of siRNA into one-cell embryos did not reach the blastocyst stage. This data shows Pcgf5 is important for proper embryo development. Decrease of H2AK119ub1 and H3K27me3 in Pcgf5 KD embryos indicated that Pcgf5 adds or maintains both modifications from the two-cell to four-cell stage. The expression levels of imprinted genes were measured by RT-qPCR in Pcgf5 KD embryos. Then we performed ChIP-seq analysis of H3K27me3 in Pcgf5 KD morulae obtained from mating between different strains and investigated the female allele specific H3K27me3 modifications in noncanonical imprinting genes. The results showed that Pcgf5 is important for the regulation of maternal Xist expression. This is the first report to find that chimeric transcripts may regulate the expression of Xist. The MERVL insertion upstream of Pcgf5 may have contributed to mammalian evolution via regulating genomic imprinting and placentation in embryonic development.

FOCUS SESSION 26 - UTERINE BIOLOGY

S26.1 - Müllerian Duct Biology in Development and Reproduction

Richard R. Behringer

The Müllerian ducts are paired epithelial tubes that are surrounded by a basement membrane, embedded within the mesenchyme of the mesonephroi. The Müllerian ducts and their adjacent mesenchyme are the progenitor tissues of the uterus and Fallopian tubes. The Müllerian ducts also form in males but in contrast to females subsequently disintegrate during male differentiation. The disintegration of the Müllerian ducts in male embryos is induced by anti-Müllerian hormone (AMH), secreted by the fetal testes. In female embryos, once the two Müllerian ducts have elongated and connected to the urogenital sinus, the posterior regions migrate to the midline and fuse to create the bipartite uterus in the mouse and simplex uterus in human. Alterations in these processes are thought to lead to defects in uterine morphogenesis, associated with infertility, high risk pregnancy, and miscarriage. Our studies investigate the molecular and cellular mechanisms of uterine morphogenesis

and the cell regulatory networks that lead to the disintegration of the Müllerian ducts during male differentiation. Defects in these processes can lead to alternations in sex development that can compromise reproduction. Supported by NIH HD30284.

S26.2 - Dysregulation of Insulin Signaling in the Uterine Stroma Impacts Pregnancy Establishment and Maintenance

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Insulin receptor signaling in the uterus is required for implantation and successful pregnancy. Insulin receptors (INSR and IGF1R) promote cell differentiation, proliferation, and growth through the PI3K/Akt, MAPK, and STAT3 pathways. These processes are essential for oocyte maturation, embryo implantation, endometrial decidualization, and placentation. To investigate insulin signaling in the uterine endometrial stroma, the site of embryo implantation and growth, we utilized the Cre/LoxP system to target the tissue-specific conditional ablation (cKO) of insulin receptor (Insr) and insulin-like growth factor-1 Receptor (Igfr1r). Anti-müllerian hormone receptor 2 (Amhr2) Cre-driven ablation of receptors occurs in the granulosa cells and uterine stroma. Puberty occurred with normal timing in all cKO models. Estrous cycles progressed normally in Insr-cKO females but were briefly stalled in diestrus in Igfr1r-cKO and double receptor (DKO) mice. The expression of vital ovulatory genes (Lhcgr, Pgr, Ptgs2) was lower but not significantly different in Igfr1r-cKO and DKO in 12 h post-hCG superovulated ovaries. Antral follicles exhibited elevated apoptosis of granulosa cells in cKO females. However, the distribution of ovarian follicle subtypes and subsequent ovulations was normal in all insulin receptor mutants compared to littermate controls. This contrasts with our published findings ablating Insr and Igfr1 with the stronger Pgr-Cre that exhibited 50% diminished ovulation, even in hyperstimulated conditions. While ovulation was normal, all cKO lines are subfertile suggesting loss of insulin receptor signaling in the stroma elicits implantation and decidualization defects. INSR and IGF1R showed significantly reduced protein localization in the stroma of Insr-cKO, Igfr1r-cKO, and DKO mice, but normal receptor expression in adjacent epithelium. PGR and ESR1 were unaltered in receptor localization and intensity in the uterine stroma and their relative mRNA expression levels at 5.5 DPC were unaltered suggesting insulin receptors act downstream of these critical implantation driving hormones. Igfr1r-cKO and DKO dams exhibited altered embryo spacing, evaluated through embryo-to-embryo length (EE) and embryo-to-oviduct (EO) length. The decidualization response was reduced in Igfr1r-cKO and DKO. These defects culminated in a greater incidence of embryo loss. Longitudinal fertility trials showed normal mean gestation length but a significant reduction in litter size for Insr-cKO, Igfr1r-cKO, and DKO pairs. The severity varied between dams, which may stem from inconsistent ablation using Amhr2-Cre, the best available stromal-acting Cre which has well-established issues of off-target and incomplete penetrance. To gain a greater insight into the stromal-specific actions of insulin receptors, we have repeated our conditional knockout scheme and analyses using a novel Foxl2-Cre that escapes the pitfalls of Amhr2-Cre. INSR and IGF1R ablation in the uterine stroma leads to reduced decidualization, implantation inefficiency, altered embryo spacing, and increased adverse pregnancy outcomes. Profiling of insulin receptor-regulated gene dysregulation that underlies the observed phenotype is underway.

S26.3 - $\gamma\delta$ T Cells Are a Major Constituent of the Uterine Immune Response to Seminal Fluid Exposure in Mice

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Major changes to the uterine immune response are induced after seminal fluid exposure, including cytokine and immune cell changes that initiate maternal immune tolerance for pregnancy. However, the full extent of these immune system changes, their dependence on seminal fluid components, and their contribution to endometrial receptivity to embryo implantation is not fully defined. Therefore, we aimed to identify genes and pathways regulated by seminal fluid components in the endometrium. We utilized high-throughput RNA-sequencing of the endometrium of C57Bl/6 female mice 3.5 days post-coitum (dpc) after mating with intact (INT), vasectomized (VAS), seminal-vesicle-deficient (SVX), or SVX/VAS BALB/c males (n=3-4/group). Flow cytometry was then used to assess uterine $\gamma\delta$ T cells on 3.5dpc, with virgin females as estrous controls (n=11-12/group). RNA-sequencing and Ingenuity Pathway Analysis revealed that compared to SVX/VAS-mated females, the endometrium of INT-mated females strongly activated the T Cell Receptor (TCR) Signalling Pathway (Z-score=3.2) and other pathways associated with T cell recruitment and function. Interestingly, top differentially regulated genes included $\gamma\delta$ TCR genes *Trdc* and *Tcrg-C1* (2.0- and 2.5-fold increase) and genes associated with $\gamma\delta$ T cell function including *Il7r* and *Blk* (2.5- and 2.8-fold increase). Seminal plasma was the main driver of these changes, as these genes were similarly increased in VAS-matings. Flow cytometry confirmed dramatic expansion of uterine $\gamma\delta$ T cells after INT or VAS mating by 8.3- and 10-fold compared to virgin females, with 22.4- and 21.9-fold more $\gamma\delta$ T cells expressing the proliferation marker Ki67. These changes were not evident after mating with SVX or SVX/VAS males, affirming the role of seminal plasma in eliciting these responses. These findings demonstrate expansion of resident $\gamma\delta$ T cells as a dominant element of uterine immune response effects regulated by seminal fluid. Increased $\gamma\delta$ T cells in response to semen has potential to influence uterine homeostasis and modulate receptivity to embryo implantation. Further investigation is underway to define its exact contribution to reproductive success.

S26.4 - The Impact of Negative Energy Balance Mediated by Extracellular Vesicles on Uterine Environment in Dairy Cows

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Dairy cows usually present negative energy balance (NEB) during post-calving period, which has significant economic impact for the farmer. Extracellular vesicles (EVs) are mediators of intercellular communication and can modulate several reproductive processes. Thus our aims were: 1) evaluate if NEB intensity can modify uterine EVs (UF-EVs) miRNA contents at 30 and 60 days post-calving (DPC); 2) to investigate if endometrial naïve cells can be modulated by UF-EVs from Low and High NEB animals. First, blood samples from individual cows at weeks 1 and 2 post-calving were subjected to metabolite analysis in order to determine the NEB intensity (Low NEB: NEFAs 0.3 to 0.8 mmol/L and BHB 0.55 to 1.1 mmol/L; High NEB: NEFAs $\geq$ 0.9 mmol/L and BHB $\geq$ 1.2 mmol/L). At 30 and 60 DPC (30 DPC n=7, being 3 Low and 4 High NEB; 60 DPC n= 6, being 3 Low and 3 High NEB) selected cows had the estrous cycle synchronized and at Day 5 after artificial insemination (AI) the collection of uterine fluid (UF) was performed. EVs isolated were analyzed for particle size and concentration by nanoparticle tracking analysis. To collect epithelial uterine naïve cells, the oestrous cycles of dairy heifers (n = 9) were synchronized. Four days after oestrous, luminal epithelial cells from the ipsilateral horn to the CL were collected in vivo (n=4) with a cytology brush. Cells were processed and plated in multiple wells of 6-well plates until reaching the confluence in a humidified atmosphere of 5% CO₂ at 38.5 oC. The cell culture medium consisted of DMEM/F-12 supplemented with 10% FBS, 2% penicillin/streptomycin, and 1% fungizone. For the treatment, UF-EVs isolated from 1 mL of either Low or High NEB at 30 or 60 DPC groups were resuspended in 1 mL of culture medium without FBS and maintained during 6 h in a culture medium. Total RNA from UF-EVs and uterine naïve cells treated with UF-EVs, were isolated and analyzed for the relative expression levels of 382 miRNAs. Differences between treatments were assessed by Student's t test. No differences were identified in EVs mean particle concentration isolated from uterine fluid or mean size between the groups. At 30 DPC, UF-EVs presented 299 miRNAs in common between the groups, 19 miRNAs were up-regulated in UF-EVs from High NEB group. MiRNA increased in High NEB are predicted to regulate metabolism related genes. EVs isolated from uterine fluid 60 DPC presented 316 miRNAs in common between the groups and one (miR-369-3p) exclusive to Low NEB group. A total of 28 miRNAs were differently expressed being 27 upregulated in Low NEB and 1 upregulated in High NEB. The miRNAs increased are involved with cell remodeling. The miR-369-3p, which is exclusive in Low NEB, is related with decrease inflammatory response, which may suggest that animals with low NEB have more favorable uterine environment to receive an embryo at 60 DPC. In the epithelial uterine naïve cells treated with UF-EVs from dairy cows with Low and High NEB, the UF-EVs were able to modulate uterine naïve cells inducing the expected phenotypes (altered cell metabolism and tissue remodeling, respectively). In conclusion, the high NEB modifies the UF-EVs miRNA content and modulates endometrial naïve cells generating an unfavorable uterine environment at 30 and 60 DPC. Financial support: FAPESP grants 2020/13075-2, 2023/15072-9 and 2021/06645-0.

S26.5 - Investigating The Impact Of Experimentally-Induced Endometriosis On Ovarian Function And Fertility

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Endometriosis is a chronic gynaecological condition where endometrial-like tissue (lesions) grows in other parts of the body. Endometriosis is associated with pelvic pain, heavy periods, and infertility. Between 30-50% of people with endometriosis experience infertility, however there is little understanding of how different subtypes of endometriosis affect fertility. Although the literature is conflicting, it is commonly believed anti-Müllerian hormone (AMH) is decreased in patients with ovarian

endometriosis (endometrioma), particularly after surgical treatment. In contrast, the impact of superficial peritoneal endometriosis (SPE) on fertility has been largely unexplored. SPE is the most common subtype of endometriosis and is associated with inflammation within the peritoneal microenvironment but our understanding of how this impacts fertility is limited. To understand the association between SPE and infertility, a mouse model of endometriosis was utilised to investigate the impact of induction of lesions on peritoneal inflammation and ovarian function. Donor mice underwent a simulated menses event to collect menstrual-like tissue for ectopic transfer into recipient endometriosis mice. Endometriosis-like lesions were induced by intraperitoneal injection of donor material and left to establish for twenty-one days, and compared to age-matched control mice where no lesions were induced (n=5-10/group). At the time of tissue collection, a peritoneal flushing was collected for flow cytometry analysis of the peritoneal immune cell environment. One ovary was fixed for total follicle estimation and histological analysis including PicroSirius red to assess collagen deposition, immunohistochemistry for immune cell infiltration, and TUNEL to assess apoptosis. The contralateral ovary was snap frozen for gene expression analysis. Serum AMH levels were determined by ELISA. Data normality was determined by Shapiro-Wilk test followed by appropriate statistical test (unpaired t-test or Mann Whitney U test) with significance set at $p < 0.05$. Induction of endometriosis was associated with changes in T cell and myeloid populations in the peritoneal microenvironment. This was characterised by a significant decrease in small peritoneal macrophages ($p < 0.05$) in endometriosis compared to controls. Strikingly, endometriosis was associated with a decrease in CD4+ ($p < 0.01$) and CD8+ T cells ($p < 0.05$) as well as CD62L+CD44- resting T cells ($p < 0.001$) and CD62L-CD44+ effector T cells ($p < 0.05$). To investigate whether these inflammatory changes associated with endometriosis could affect ovarian function serum AMH was measured by ELISA to estimate the ovarian reserve and showed no change between control and endometriosis groups. Intraovarian levels of inflammatory cytokines (Tgfb1, Ifng, Tnf, Il10) or hormone receptors (Esr1, Pgr, Ar) were measured by qPCR but were not different between treatment groups. However, mRNA levels of Il6 were greater in the endometriosis group ($p=0.06$). These data provide detailed analyses of inflammatory changes in the peritoneal microenvironment as a consequence of experimentally induced endometriosis and explore the potential impact this has on ovarian function. Future work will focus on quantifying changes to the ovarian reserve and assessing reproductive function.

S26.6 - Defining the Human Endometrial Stem/Progenitor Cell Atlas

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Rare adult stem cells present in human endometrium; N-cadherin+ (CDH2) epithelial progenitors and SUSD2+ mesenchymal stem cells (eMSC) are shed during menstruation and refluxed in small numbers into the peritoneal cavity. SSEA1+ basalis epithelial cells resurface the denuded endometrium to generate the luminal epithelium. Investigations into alterations in the gene expression of these cells may provide essential information regarding the pathogenesis of reproductive pathologies such as during

endometriotic lesion formation and endometriosis progression. Our aim was to determine the gene expression atlas of single endometrial stem/progenitor cell populations and identify their stem cell state signalling pathways and the niche cells regulating their function. Human hysterectomy endometrium (n=5) was digested to single cells for 6-way FACS sorting to collect stem/progenitor cell subpopulations and their differentiated progeny using N-Cadherin+/-, SSEA1+/- and SUSD2+/- . For single cell RNA sequencing (scRNA seq), the 6 cell fractions were combined in proportions to enrich for the stem/progenitor subpopulations, processed using 10X Genomics, libraries generated, and analysed using Cell Ranger and Seurat pipelines. Immunofluorescence on full thickness endometrium examined the localisation of thyrotropin releasing hormone (TRH), Indian Hedgehog (IHH) and co-localisation of SSEA-1, CDH2 and IHH, and BOC and SSEA-1. scRNA seq identified 16 cell clusters; 10 of epithelial and 6 of mesenchymal origin. SUSD2 expression was localised to two main clusters, representing eMSC with high MYH11 and MUSTN1 expression, transitioning to more mature stromal fibroblast clusters. CDH2+SOX9+ cells localised to two clusters suggesting a basalis glandular epithelial population, with high expression of TRH and IHH. TRH localised to the basalis endometrial glandular epithelia and IHH to the glandular epithelia, stroma and myometrium. CDH2, SSEA-1 and IHH co-localised in the basalis endometrial glandular epithelia. Ligand-receptor analysis indicated key interactions between IHH in the basalis epithelial progenitor cells with its co-receptors BOC and CDON in the basalis stromal and smooth muscle cells respectively, reflective of a potential new stem cell niche environment in the human endometrium. BOC and SSEA-1 co-localised in the glandular and luminal epithelia, indicating their possible involvement in epithelial migration and re-epithelialisation of the endometrium at the onset of menstruation. Characterising gene expression profiles of endometrial stem/progenitor cell types at the single cell level and further examining their niche cells will enable future investigations into their role in regenerating the endometrium each menstrual cycle and in the pathogenesis of endometrial proliferative disorders such as endometriosis, adenomyosis, Asherman's disease and thin endometrium, possibly identifying new targets for developing treatments for these diseases.

FOCUS SESSION 27 - CHA HEALTH SYSTEM SPONSORED SESSION ON NEW TECHNOLOGIES

S27.1 - Dynamic In Vivo Investigation Of The Fallopian Tube Physiology: Eggs, Sperm, And Cilia

Irina V. Larina

The processes within the fallopian tube are highly complex and dynamic to support the reproductive function. However, our understanding of this complexity is limited by restricted imaging access to the mammalian reproductive system. This report will present an in vivo imaging study of reproductive events within the mouse fallopian tube using an intravital dynamic optical coherence tomography, focusing on egg/embryo movements, spermatozoa tracking, and motile cilia analysis. A novel functional method for studying cilia coordination within the fallopian tube will be introduced. The method is based on spatiotemporal mapping of the phase of ciliary beat calculated based on intensity fluctuations in OCT images. Novel dynamic imaging methods uncover unexpected findings about the physiological dynamics of eggs, sperm, and motile cilia within the fallopian tube, setting a platform for a variety of future investigations of normal physiological reproductive function and reproductive disorders.

S27.2 - Fluidics and AI for Fertility

Reza Nosrati

Microfluidic methods offer numerous advantages over their traditional macro-scale counterparts by extending the possibility of biomedical research through miniaturization. These miniaturized platforms

enable the manipulation of cells and biological processes at the single-cell level, fostering the development of nature-inspired technologies for diagnostic and therapeutic applications. In the context of fertility, microfluidics can match the geometry of micro-confined regions within the female reproductive tract, thus, presenting opportunities for fundamental understanding of the event of fertilization and biomimicry-based selection of sperm that reflect the in vivo process. In this talk, I will provide an overview of our work in developing microfluidic technologies in combination with machine learning strategies for sperm analysis and selection in assisted reproduction.

S27.3 - Disruption of Reproductive Function In Vivo and Ex Vivo Using a Novel CRISPR/Cas13d Mouse Model

Stephanie Tanis¹; Carolline Ascenção²; Leah E. Simon¹; Daryl Phuong¹; Anna Wood¹; Audrey Xie¹; Roni Ben-Shlomo¹; Jelena Lujic¹; John Schimenti¹; Marcus Smolka²; Paula E. Cohen¹

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Genome editing technologies, like CRISPR/Cas9, have revolutionized genetic engineering in recent years by allowing precise modifications to DNA sequences, offering immense potential for both scientific research and therapeutic applications. However, as CRISPR/Cas9 technology targets DNA sequences within the genome in a permanent manner, this method is limited in its ability to study the transient loss of gene expression. Furthermore, the permanent nature of genome editing makes for a relatively expensive and cumbersome method, as a whole animal is required to study a single modification. Revolutionizing the next generation of genome editing methods is Cas13d, which has recently emerged as a valuable tool to investigate the depletion of target messenger RNA (mRNAs). Unlike CRISPR/Cas9, Cas13d enables the reversible disruption of gene expression without altering the underlying genetic code. While previous studies utilizing Cas13d have been performed on various in vitro models, here, we generated a novel mouse model in which Cas13d is expressed ubiquitously. This will, for the first time, enable Cas13d-mediated in vivo mRNA targeting to suppress expression of specific genes in a temporospatial fashion across all tissue types. In order to prepare this in vivo platform for various reproductive applications, we first sought to establish the reproductive baseline of these mice to verify that Cas13d expression does not affect normal morphological and physiological parameters. This was validated by comparable phenotypic assessment of gross anatomy of the female reproductive tract, estrous cycling, and breeding parameters of Cas13d-expressing females relative to wild type animals. To assess the mRNA targeting ability of Cas13d within the female reproductive tract, we performed a proof-of-concept study targeting LIF regulation during blastocyst implantation. LIF is a historically well-studied protein that is secreted by the uterine endometrium and known to be indispensable for blastocyst implantation. Furthermore, by utilizing a model of blastocyst implantation, not only are we able to disrupt implantation by disrupting LIF production and secretion by the uterus, but we are able to perform an analogous reciprocal experiment by disrupting LIF regulation in the blastocyst. Leveraging this established and well validated system, we first optimized appropriate guide RNAs (gRNAs) targeting LIF using endometrial organoids. Once gRNAs were shown to disrupt LIF expression ex vivo, we injected these optimized gRNAs directly into one uterine horn of Cas13d-expressing female mice 12 hours after mating. The other uterine horn served as an internal sham control. As we observed fewer (if any) implantation sites in the injected uterine horn compared to control, our results demonstrate that

Cas13d-mediated perturbation can be successfully utilized in a reproductive context. Combined with the results of normal reproductive parameters, this mouse provides a promising platform to study hitherto inaccessible systems requiring transient gene expression, using both reproductive and non-reproductive systems. Moreover, as gRNAs targeting multiple genes may be rapidly tested using this Cas13d expressing mouse model, this in vivo platform provides additional advantages relative to standard Cas9 models. One area of active research that would greatly benefit from the use of novel technology enabling transient perturbations in gene expression in vivo, is non-hormonal contraceptives. As reversibility is at the forefront of any successful contraceptive target, we aim to leverage this unique capacity of the Cas13d mouse model to test the validity of novel non-hormonal contraceptive targets.

S27.4 - 3DMOUSEneST: A Method for Evaluation of Decidualization Efficacy in Mice Using Three-Dimensional, Label-Free Higher Harmonic Generation Microscopy

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Effective interplay between uterus and embryo is essential for pregnancy establishment, however, convenient methods to screen embryo implantation success and maternal uterine response in experimental mouse models in three-dimensions, without labeling are currently lacking. Here, we introduce a pioneering method named 3D Microscopy for Optically cleared Unlabeled implantation Site Evaluation using Second and Third harmonic generation (3DMOUSEneST) for analyzing mouse implantation sites based on 3D, label-free higher harmonic generation microscopy. Decidualization initiates extensive morphological changes around the implanted embryo, including enhanced deposition and remodeling of collagen-rich extracellular matrix. After implantation, collagen fibrils rapidly thicken and rearrange around the decidual cells. These thick fibrils have been identified as fibrillar collagen. While accumulation of collagen upon decidualization has long been acknowledged, its three-dimensional structure remains understudied. Advancements in label-free, non-linear imaging technology, namely higher harmonic generation microscopy, provide great tools to study fibrillar collagen, which has a non-centrosymmetric structure and therefore intrinsically creates strong second-harmonic generation signals. Complementary to second-harmonic generation, third-harmonic generation mainly occurs at water–lipid and water–protein interfaces in tissues, providing a broad range of structural information. Despite these technological developments, there are no reports, to the best of our knowledge, of using higher harmonic generation microscopy to study chemically cleared implantation sites in early mouse pregnancy. Briefly, to establish the 3DMOUSEneST method we collected and imaged intact, optically cleared E4.5–E6.5 mouse implantation sites with multiphoton microscopy using second-harmonic generation and third-harmonic generation. We systematically analyzed all 3D scans using Imaris software. Using second-harmonic generation, we defined a novel 3D structure, the decidual nest—the result of rapid deposition and organized arrangement of fibrillar collagen by the decidual cells around the implanted embryo. We demonstrate that volumetric analysis of the decidual nest can be used to quantify decidualization and present a standard growth pattern for the decidual nest during normal early pregnancy (E4.5–E6.5). We also demonstrate that the conceptus can be detected by third-harmonic generation signal enabling its growth measurements in immediate relation to decidual dynamics. Using

implantation sites from two mouse lines with known implantation defects and a logistic regression model, we demonstrate that decidual nest volume is a measurable readout of decidualization efficacy with 92.3% accuracy. In conclusion, 3DMOUSEneST uses second-harmonic generation to image the three-dimensional structure of decidual fibrillar collagen and third-harmonic generation to visualize the outline of the conceptus, providing simultaneous label-free insights into the embryo–maternal decidualization dynamics during early pregnancy. Our study bridges extracellular matrix biology with reproductive biology, adding new knowledge of the 3D remodeling of extracellular matrix in decidualization and applying this knowledge to establish a unique 3D analysis method to study decidual development. We envision 3DMOUSEneST to become a primary method for investigating mouse models associated with decidual defects, early embryo lethality, and fertility issues. All animal work was conducted in accordance with institutional and national policies for experimental animal care. This work was supported by grants from the Sigrid Jusélius Foundation to R.P.-H.; the University of Oulu and the Research Council of Finland Profi6 program (AF 336449) to R.P.-H. and E.K.; Health and Bioscience Doctoral Program of the University of Oulu to A.S.; Next Gen Pregnancy Award (NGP10125) from the Burroughs Wellcome Fund to D.M.; and Eunice Kennedy Shriver National Institute of Child Health and Human Development grants HD032067 to M.M.M and HD096057 and HD105800 to D.M.

S27.5 - Microfluidic Chips to Study Ovulation Reveal Novel Mechanism Regulation of Tumor Suppressors in Primary Human Fallopian Tube Tissue

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The lifetime number of ovulations is a risk factor for developing ovarian cancer. High grade serous cancers can arise from the fallopian tube epithelium. Our study was designed to investigate how ovulation may impact fallopian tube carcinogenesis. We developed a microfluidic chip that can support ovulation of murine ovaries under the influence of exogenous gonadotropin stimulation. Secreted factors from the ovulating ovary chamber are perfused to a second paired chamber that supports the growth of primary human fallopian tube tissue at an air liquid interface. The platform supports 15 paired interacting tissues. Ovaries can be stimulated to ovulate over a 28 day or abbreviated 14 day cycle and produce the steroid hormone estradiol during the follicular phase and progesterone during the luteal phase. The secreted factors produced by the ovary were collected in the microfluidic chamber effluent over the course of a menstrual cycle and subjected to proteomics to profile proteins that interact with the fallopian tube tissue over the course of ovulation. Primary human fallopian tube tissue was subjected to GeoMx DSP spatial transcriptomics to capture differential mRNA expression before and after ovulation in the ciliated and secretory fallopian tube epithelium and revealed repression of the thioredoxin-interacting protein (TXNIP) transcript that encodes a putative tumor suppressor and regulator of metabolism. TXNIP expression has been shown to be negatively regulated by IGF1. Interestingly, IGF1 was present in the proteomics from the secreted ovarian products collected on chip providing a mechanism for the repression. Fallopian tube immortalized cell lines were cultured and treated with secreted factors collected from ovaries ovulating on the microfluidic platform to confirm

whether ovulation could influence TXNIP in cell lines and to further test the significance of its downregulation. TXNIP protein was repressed by ovulatory secretion, but not by follicular phase or luteal phase secreted factors in FT194 and FT33. The FT282 cell line harbors a TP53 mutation and in this model TXNIP protein was also repressed. Mice were superovulated to monitor TXNIP expression in oviductal epithelium and protein levels were also repressed. Overall, these results indicate that ovulation may be associated with high grade serous formation from fallopian tube cells due to IGF1 repression of the tumor suppressor TXNIP.

S27.6 - Safe Magnetic Force-Based Technique to Select Pig Mature Eggs

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Egg and embryo manipulation is an important biotechnological challenge to enable positioning, entrapment, and selection of reproductive cells. Our group has previously reported a novel method for labeling mature in vitro pig oocytes with nanoparticles (NP) conjugates with OVGP1 (NPOv). To take advantage of the intrinsic superparamagnetic properties of the NP used in NPOv, we developed an assay to isolated eggs by magnetic force. We used a magnetic stand to evaluate the number of eggs attracted or non-attracted under varying times and doses of NPOv. Eggs were incubated with NPOv for 0.5, 1, and 6 h, approximately 70% to 90% of the eggs were attracted by the magnetic force. The results indicated that after 30 min of incubation with NPOv, most of the eggs were attracted by the magnetic force. However, approximately 20% of eggs were not attracted by the magnetic field. As the zona pellucida is modified during egg maturation, we speculate that incompetent eggs are less likely to bind OVGP1. To test this hypothesis, we determined the maturation stage of both attracted and non-attracted eggs. For the evaluation of oocyte maturation, cells were fixed for 15 min in 10% glutaraldehyde in PBS, stained for 15 min with 1% Hoechst 33342 in PBS, washed in PBS, and then mounted on glass slides for evaluation by epifluorescence microscopy. An oocyte was considered mature (egg) when the nucleus was in metaphase II stage and the first polar body was extruded. Most of the eggs not attracted were immature (88% vs. 12%), whereas most of those attracted were mature (74% vs. 26%, $p < 0.001$) ($n = 4$ replicates, a total of 203 oocytes, Chi-Square test, $p < 0.05$). These data document that specific binding of NPOv to the surface of the zona pellucida correlates with maturation state, with more mature eggs being attracted by magnetic forces. This method offers a rapid and noninvasive method for selecting competent eggs. Supported by the Fundación Seneca-Agencia de Ciencia y Tecnología de la Región de Murcia. (22001/PI/22) Spain, also by projects PID 2020-114109GB-I00 and PID2019-106380RB-I00 funded both by MCIN/AEI/ 10.13039/501100011033.

FOCUS SESSION 28 - GATES FOUNDATION SPONSORED SESSION ON CONTRACEPTION

S28.1 - sAC Inhibitors as Potential Nonhormonal On-Demand Contraceptives for Men

Lonny R. Levin and Jochen Buck

Nearly half of all pregnancies are unintended; thus, existing family planning options are inadequate. For men, the only choices are condoms and vasectomy, and both suffer from disadvantages which limit their

appeal and adoption. Most current efforts to develop new contraceptives for men impact sperm development, meaning that contraception is only achieved after months of continuous pretreatment, and chronic dosing must be maintained to remain infertile. Here, we provide proof-of-concept for an innovative strategy for on-demand contraception, where a man would take a birth control pill shortly before sex, only as needed. Soluble adenylyl cyclase (sAC: ADCY10) is essential for sperm motility and maturation. We show a single dose of a safe, acutely acting sAC inhibitor with long residence time renders male mice temporarily infertile. Mice exhibit normal mating behavior, and full fertility returns the next day. These studies define sAC inhibitors as leads for on-demand contraceptives for men, and they provide in vivo proof-of-concept for two previously untested paradigms in contraception; on-demand contraception after just a single dose and pharmacological contraception for men.

S28.2 - Investigating Genes Regulated by BRDT In Vitro For Non-Hormonal Male Contraception

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There is a significant paucity of available contraceptive options for men beyond largely unreliable barrier methods, such as condoms, or invasive procedures like vasectomies. Despite major advances in our understanding of male reproductive biology in the genomics era, we have seen limited advancement in the identification of suitable non-hormonal male contraceptive targets. This is surprising, given the large number of genes that are expressed exclusively in the testis throughout spermatogenesis. Recent studies from our lab have shown that meiotic prophase I represents a suitable stage for contraceptive targeting in male mice, with complete reversibility of meiotic disruption following pharmacologic inhibition. One essential regulator of meiotic prophase I that mediates transcription of over 3000 meiotic and post-meiotic genes is the testis-specific member of the bromodomain and extra terminal family of proteins, BRDT. Recent studies have shown that male mice lacking BRDT are sterile with disrupted meiotic prophase I, highlighting the potential of BRDT as a contraceptive target. However, our current study shows that *Brdt*^{-/-} mice present with global transcriptional changes that may lead to downstream consequences such as irreversible damage to reproductive cells. This would far exceed what is necessary for contraceptive control, suggesting that BRDT itself might not present as a suitable contraceptive target. ChIP-seq analysis from meiotic spermatocytes has previously identified BRDT binding sites across the genome, providing us with a long list of genes whose expression is regulated by BRDT. We therefore hypothesize that these genes whose expression is controlled by BRDT binding will serve as more suitable non-hormonal contraceptive targets. To further explore these targets in meiotic prophase I in a high-throughput manner, we developed a novel CRISPR in vitro platform to perturb cells that are progressing through meiosis. We designed gRNAs targeting our top candidate genes (*Rfx2*, *Ldhc*, *Mroh2b*, *Fbp1*, *Aqp9*, and *Ccdc60*) owing to them being expressed during meiosis and bound by BRDT. Heterogeneous single cell suspensions from the testis of Cas9-expressing male mice were cultured in media with gRNAs targeting these genes, allowing for their perturbation. Our in vitro platform utilized meiotic prophase chromosome spreads (the gold standard), and in-cell westerns as endpoints to assess progression from pre-meiotic stages, through meiotic entry and full prophase I progression. This allowed us to assess changes in meiotic progression as a result of CRISPR-mediated perturbation of our candidate genes using

desired antibodies. Any candidate gene that, when mutated, affects progression of meiosis is considered a promising contraceptive target suitable for further in vivo testing. This study is the first to assess in vitro the contraceptive potential of many genes mediated by BRDT, many of which play unknown roles during spermatogenesis. We hope that targeting such genes will provide a contraceptive effect without causing the global disruption of gene expression seen when targeting BRDT itself.

S28.3 - Knockout of PKDREJ, A Candidate Oviduct Receptor on Porcine Sperm, Reduces Fertility Assessed by Competitive Insemination

Kankanit Doungkamchan¹; Caroline Lucas²; Kevin Wells²; Leonardo Molina¹; Kristin Whitworth²; Randall Prather²; and David Miller¹

PKDREJ, a homolog of the sea urchin receptor for egg jelly, is a member of the polycystin-1 gene family and is expressed only in the spermatogenic lineage. Previous studies found that mouse sperm lacking PKDREJ exhibited normal morphology and motility. The mutant sperm also had reduced fertility that was only observed in competition with wild-type sperm due to slower capacitation and delayed entry into the cumulus-oocyte complex. We have found that PKDREJ is also a candidate receptor for oviduct glycans containing Lewis X (LeX) and multi-antennary 6-sialylated motifs. These glycans are responsible for forming a functional sperm reservoir in the porcine oviduct. In this study, we investigated the role of PKDREJ in the formation of the sperm reservoir and fertility by generating PKDREJ-knockout boars using the CRISPR/Cas9 system. We confirmed the genotype of the male founders using DNA extracted from ear notches and sperm. We identified 2 male founders for further study that were mosaics with multiple indels resulting in truncated protein and no wild-type alleles. Western blots of sperm protein from the 2 founders demonstrated a deficiency in PKDREJ. Immunofluorescence showed a reduction in PKDREJ on the head of sperm from these founder males. The motility and morphology of sperm from the PKDREJ-knockouts were indistinguishable from wild-type sperm. Preliminary experiments did not detect a reduction in mutant sperm binding to oviduct cell spheroids. A competitive insemination fertility trial inseminating an equal number of sperm from wild-type and PKDREJ-knockout boars into 7 females yielded only offspring from wild-type boars; there were no offspring from PKDREJ-knockout boars. Significantly, PKDREJ-knockout sperm exhibited complete infertility in competition with wild-type sperm. We conclude that PKDREJ is crucial for porcine male fertility in competitive insemination. This work was supported by NIH R01HD095841 to D.M. and U42OD011140 to R.P.

S28.4 - The Epididymis: Balancing The Burden & Responsibility of Fertility

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The responsibility of fertility has been disproportionately placed on women, bearing the majority of the health, emotional and financial burdens. With infertility rapidly on the rise, where 1 in 6 Australian couples seeking recourse in assisted reproductive technologies to achieve their goal of starting a family, notably >50% of cases involve a male factor. Despite these contributions, we are yet to fully comprehend the mechanisms that provide sperm with the competence to engage in fertilisation, which occur during their transit of the epididymis; a highly specialised region of the male reproductive tract. To address this knowledge gap, we have exploited high-resolution tandem mass spectrometry to characterise proteome-wide changes in the mouse sperm phosphoproteome that are associated with their transit through the epididymis and elicited in response to capacitation stimuli (maturation changes that are initiated upon contact with the female reproductive tract). Here, we report an unprecedented depth of >6,000 proteins and 14,586 site-specific phosphorylation events across these sperm populations. Contrary to the belief that epididymal maturation is driven by the uptake of additional proteins, we demonstrate that sperm shed over 56% of their proteins during this process, producing a refined fertilisation-competent cell. This reduced proteomic complexity of sperm aligned with activation of functions including sperm motility and capacitation. Despite this background of enormous complexity, we have harnessed these findings to identify key proteins and kinases which regulate sperm function. Through selective pharmacological inhibition, we have demonstrated the roles of RHOA, HIPK4, STK33 and PAK1 on mature spermatozoa to reduce sperm motility and return the rate of acrosome reaction to basal levels. Such data represent an important paradigm shift in our understanding of male fertility regulation and will contribute impactful advancements of the development of non-hormonal male contraceptives, and robust sperm selection biomarkers for infertile men

S28.5 - Absence Of Eppin and Its Closely Related Wap Four-Disulfide Core Genes Wfdc6A, Wfdc8, And Wfdc6B Leads to Defects in Spermatid Elongation and Male Sterility in Mice.

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Despite 96 million years of evolution separating humans and rodents, eleven closely-related reproductive tract-specific genes in humans—SPINT3, WFDC6, EPPIN, WFDC8, WFDC9, WFDC10A, WFDC11, WFDC10B, WFDC13, SPINT4, and WFDC3—and the thirteen reproductive tract-specific orthologous genes in mice, form highly conserved gene clusters indicative of conserved, combined critical functions in the male reproductive tract. However, despite significant progress towards a non-hormonal male contraceptive targeting the protein encoded by one of these genes, EPPIN, neither EPPIN nor any of the closely related genes within the WFDC locus have been explored functionally through genetically manipulated mouse models. In this study, we sought to clarify the involvement of the entire locus of reproductive tract specific WFDC genes in male fertility. To avert potential gene redundancy effects leading to no phenotypic effects in single gene knockouts (KOs), we strategically generated mice in parallel lacking a combined total of thirteen (S13/W3), ten (S13/W13), five (W16/W13), or four (W6a/W6b) genes within the conserved gene cluster. All KO mice were generated through our CRISPR/Cas9-electroporation of zygotes (CRISPR-EZ) gene editing approach utilizing strategically designed pairs of sgRNA sequences. Reproductive phenotype analysis of resulting KO and WT littermates was conducted in parallel. We found that the W16/W13 KO males despite normal spermatogenesis and sperm counts, were infertile due to a significant reduction in sperm motility. In contrast, the S13/W3, S13/W13, and W6a/W6b KO males showed a more severe infertility phenotype displaying decreased testes size with reduced epithelial thickness and azoospermia due to a defect in spermatid development

evident by the presence of round, but not elongated spermatids. Since W6a/W6b KO mice lack the protease inhibitors encoded by Wfdc6a, Eppin, Wfdc8, and Wfdc6b, we aimed to elucidate the specific molecular mechanisms responsible for the impaired differentiation of spermatids into elongated spermatids. Our focus was on identifying direct targets affected by the absence of these proteins, rather than generating a proteomic signature primarily influenced by the absence of elongated spermatids. To achieve this, we conducted a proteome profiling analysis of whole testes at the early postnatal age of 28 days, a stage when elongated spermatids are just initiating their development in mouse testes. Our analysis revealed altered expression of various proteins in KO testes including upregulation of serine/threonine proteases, and cell cycle, apoptosis, and meiosis regulator proteins, indicating a compensatory response to increased protease activity in the testes. Among the downregulated proteins, we found structural and epigenetic regulatory proteins and protease inhibitors, which likely result from excessive protease activity in W6a/w6b KO mice, leading to abnormal processing or degradation of key proteins required for the transition from round to elongated spermatids. Our knockout mouse studies, the first to explore an entire WFDC locus of closely related genes, not only clarified the functional requirement of WFDC locus genes in male fertility but also shed light on the involvement of EPPIN and its closely related WFDC proteins as key protease inhibitors regulating various proteolytic processes essential for spermatid development. These findings have implications for improving clinical diagnoses of male infertility and development of novel contraceptive strategies. This study has been supported by the National Institutes of Health (NIH) trainee award T32GM139534 and the A.I. & Manet Schepps Discovery Foundation fellowship (to K.K.); and NIH awards R01HD106056 and R01HD095341 and the Male Contraceptive Initiative David Sokal Innovation award 2021-302 (to T.X.G.).

FOCUS SESSION 29 - OVARY AND TIME

S29.1 - Why X-Chromosome Dosage Control Exists During Mouse Oocyte Development

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The mammalian germline is characterized by extensive epigenetic reprogramming during development into eggs and sperm. Specifically, the epigenome requires resetting before parental marks are established and transmitted to the next generation. In females, X-chromosome inactivation and reactivation are among the most prominent epigenetic reprogramming events, yet little is known about their function.

Previously, we found using an in vitro system of mouse primordial germ cell-like cell (PGCLC) differentiation that X-inactivation is followed by step-wise X-reactivation, which is mostly completed during meiotic prophase. Importantly, we found that PGCLCs, which failed in X-inactivation or reactivated too rapidly, displayed impaired meiotic and oogenic potential.

Here we asked if X-dosage control is a direct functional requirement or rather a diagnostic mark for proper germ cell and oocyte differentiation. For that we analyzed PGCLC differentiation from embryonic stem cells (ESCs) deficient for Xist, the master regulator of X-inactivation. Xist-KO ESCs underwent massive cell death and X-chromosome loss upon pluripotency exit, constituting a major bottleneck in

their development into germ cells. In this work, we investigated the underlying mechanisms why X-chromosome dosage matters and how it impacts the different steps of germ cell development towards meiosis and oogenesis.

S29.2 - Revealing the Secrets of Ovulation

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Ovulation is essential for reproduction. During ovulation, an egg is released from an ovarian follicle into the oviduct. Once released, the egg can be fertilised by the sperm and give rise to a new embryo. Ovulation takes place inside the body, impeding studies of its progression. The exact mechanisms that control ovulation have therefore remained unclear. In this work, we devised methods to study the entire process of ovulation live in isolated ovarian follicles from mice using a long-term culture and imaging system. A combined approach of confocal and two-photon microscopy allowed for the study of ovarian processes at both the cellular and whole follicle scale. We show that ovulation proceeds through three distinct phases, follicle expansion (I), contraction (II), and rupture (III), culminating in the release of the egg. Follicle expansion is driven by the secretion of hyaluronic acid, and an osmotic gradient-directed influx of fluid into the follicle. Subsequently, smooth muscle cells in the outer follicle drive follicle contraction. Follicle rupture is initiated by the formation of a stigma in the thinnest region of the follicle wall, followed by the exit of follicular fluid and cumulus cells, and finally, the rapid release of the egg. In parallel with ovulation, the oocyte progresses through the first meiotic division and thereby develops into a fertilisable egg. Ovulation was not dependent on meiotic progression however, suggesting that the ovary lacks mechanisms to prevent ovulation when oocyte meiosis fails. Taken together, these results establish a mechanistic framework for ovulation, a process of fundamental importance to reproduction.

S29.3 - Delineating Changes In The Hippo Pathway With Aging Towards Ovary Matrix Modifications

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Ovarian aging in humans begins by 30 years of age and fertility dramatically decreases over the next 10 years. The ovaries undergo a dynamic remodeling both structurally (stiffening of ovary) and functionally (decreased follicular function and failure to ovulate). The Hippo signaling pathway is one of the key cellular pathways associated with ovarian granulosa cell proliferation, differentiation and follicular development, though a gap exists in understanding the impact of aging on ovarian Hippo signaling. This pathway consists of multiple components amongst which effectors YAP1 and TAZ (also known as WWTR1) via association with various transcription factors such as TEAD, FOS/JUN, RUNX, etc. control many physiological processes. This pathway is regulated by multiple hormonal and non-hormonal factors like gonadotropins, steroids, and extracellular matrix components, which undergo alterations with age.

The hypothesis for this project is Hippo signaling components change across the ovarian lifespan and are associated with alterations in follicular function. We evaluated 4 groups of CD1 mice (n = 10 per group) representing various life stages (1) prepubertal: postnatal day 21, (2) young adult: 3-6 months, (3) menopause: 10-14 months and (4) ovarian senescence: 18-20 months. The ages correlate to various ages and reproductive physiology of women ranging from 12-13 years, 20-30 years, 38-47 years and 56-69 years, respectively. Although body weight increased significantly ($p \leq 0.05$) and remained constant over the age groups, ovarian weight increased significantly ($p \leq 0.05$) in adult mice, followed by a significant decrease ($p \leq 0.05$) in old mice, 18-20 months. Picrosirius red staining showed increased ($p \leq 0.05$) fibrosis with advanced ovarian aging. Serum LH increased ($p \leq 0.05$) with advanced age. Serum estradiol was increased ($p \leq 0.05$) in adult mice, whereas steroids such as DHEA-S, progesterone and corticosterone levels showed no age-related significant differences. qRT-PCR analysis showed an increase in fibrosis genes ($p \leq 0.05$), whereas steroidogenic genes increased ($p \leq 0.05$) until 10-14 months after which they declined. Yap1 mRNA transcripts were not different across age groups, although the ratio of phosphorylated to total YAP1 protein significantly decreased ($p \leq 0.05$) with aging. Interestingly, Taz mRNA was slightly elevated ($p \leq 0.06$) and remained constant, but the ratio of phosphorylated to total TAZ protein significantly increased ($p \leq 0.05$) during ovarian aging. We observed Hippo core proteins MST1/2 and LATS1/2 to be significantly decrease ($p \leq 0.05$) with age, however the ratio of phosphorylated to total protein remained unchanged for MST1/2 in the older mice. In contrast, the ratio of phosphorylated to total LATS1/2 protein increased ($p \leq 0.05$) over the age. The downstream YAP1/TAZ target genes Amphiregulin (Areg) and CTGF (Ccn2) increased ($p \leq 0.05$) over age. Our results indicate that Hippo pathway is disrupted and may play a role in fibrosis and steroidogenic modifications during ovarian aging. The impact of this project is novel understanding of Hippo signaling in ovarian aging and development of a pipeline of targets to better reveal the molecular alterations the occur during ovarian aging.

S29.4 - Defining the Cell and Molecular Origins of the Primate Ovarian Reserve

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Ovarian follicles are the functional unit of the ovary, with the most immature follicles (referred to as primordial follicles) making up the ovarian reserve. Primordial follicles are composed of a single arrested

meiotic oocyte surrounded by supporting ovarian somatic cells called granulosa cells. In primates, including humans, primordial follicles are established during the third trimester of pregnancy, with approximately 400,000 to 1 million primordial follicles per ovary present at birth. However, due to the challenges in accessing human fetal tissue during this gestational window, very little is known about the cell and molecular mechanisms associated with establishing the ovarian reserve. Using the nonhuman primate rhesus macaque, we mapped ovarian development from the specification of the ovary from the genital ridge in the first trimester through to follicle establishment (5 developmental timepoints, n=2-3 animals). Using spatial transcriptomics (10x Visium) in the first trimester, we determine what distinguishes the ovarian gene program from adrenal and mesonephric tissues derived from the same progenitors. Using single-cell RNA sequencing (10x Chromium) in the first through third trimesters, we characterize the nature and progression of germline and somatic cells as the ovary undergoes developmental patterning (organizing first into cords composed of germ cell nests and pre-granulosa cells that then break down into individual follicles). We find major transcriptional shifts in granulosa cell identity over time and also investigate germline apoptosis relative to follicle establishment. Throughout, immunohistochemistry analyses support our genomic findings. Our data reveals key insights into the origins of the ovarian reserve in primates, and will inform ongoing efforts to engineer ovarian follicles in vitro from stem cells to model ovarian development, disease and ovarian aging, or for contraceptive discovery. We aim to generate a reference standard against which to measure successful follicular derivation and to identify key regulatory networks to underpin future in vitro gametogenesis technologies.

S29.5 - The Age-Associated Increase in Ovarian Stiffness Impairs Follicle Development and Oocyte Quality Through Early Modulation of Follicles' Transcriptome

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Oocyte quantity and quality are severely impacted by multiple extrinsic factors, with female reproductive aging being among the most prevalent in our society. We and others reported that with aging the mouse ovary assumes a pro-fibrotic milieu, which is associated with ovarian stiffness. However, the role of stiffness on ovarian function and oocyte quality is unknown. The objective of this work is to use an alginate-encapsulated in vitro follicle culture system that mimics age-associated changes in the ovarian microenvironment to identify whether stiffness is a novel mechanism mediating the progressive decline in oocyte quality observed with advanced maternal age. To evaluate how stiffness impacts follicle development and oocyte quality, we synthesized hydrogel that replicated the soft (young: 0.5%, 1.79 ± 0.08 kPa) and stiff (old: 2%, 4.56 ± 2.03 kPa) environments. Secondary follicles from CD1 (D12-D13, N=3 replicates) were cultured in 0.5% or 2% alginate for up to 12 days. Follicles cultured in stiff environments showed significant reduction in follicle size compared to follicles in soft environments (0.5% $226.9 \pm 17.4 \mu\text{m}$, 2% $160.8 \pm 9.9 \mu\text{m}$, $p < 0.0001$). These differences were triggered by granulosa layer since no changes were detected in oocyte size (oocytes: 0.5% $66.19 \pm 5.7 \mu\text{m}$, 2% $60.6 \pm 4.5 \mu\text{m}$, $p = 0.401$; granulosa cells: 0.5% $160.04 \pm 13.4 \mu\text{m}$, 2% $103.37 \pm 16.5 \mu\text{m}$, $p < 0.0001$) suggesting that granulosa cells are not proliferating in a stiff environment. To explore this, we assessed estradiol synthesis and granulosa cell death. Estradiol levels were reduced in 2% (0.5% $11.3 \pm 15.9 \text{ ng/ml}$, 2% $0.3 \pm 0.5 \text{ ng/ml}$, $p = 0.296$) while CC3 (apoptosis-marker) intensity was increased (CC3 intensity/area, 0.5% $20.4 \pm 5.8 \text{ a.u.}$, 2% $27.1 \pm 4.3 \text{ a.u.}$, $p < 0.05$), confirming that stiff environments impact granulosa cells viability. We then evaluated whether stiff environment impact gamete quality. Oocytes from follicles at D12 were isolated and inspected

morphologically. Oocyte quality significantly declined in 2%, with $68.9 \pm 16.8\%$ of oocytes degenerated, compared to $23.6 \pm 9.2\%$ in 0.5%. We repeated all the analyses using the CB6F1 strain yielding comparable results. Since the effects of stiffness on follicle growth were already evident at day 4, we investigated early-changes in follicles' transcriptome that might mediate the observed phenotypes. 32 secondary follicles were cultured in 0.5% or 2%, and analyzed by RNAseq at 3h, 6h, 12h and 24h. We analyzed follicles' gene expression (DESeq2, R/Bioconductor) at each timepoint comparing 0.5% and 2% (significance at $\text{padj} < 0.05$; $\log_2\text{FoldChange} \geq 2$). Surprisingly, we found few differentially expressed genes (DEG) at 3h, a peak of DEG at 6h, followed by a dramatic decrease which reached a plateau at 12h and 24h. DEGs at 6h were involved in response to external stimuli and ERK1/2 cascade, pathways of proliferation and differentiation, suggesting that at 6h follicles' transcriptome is more sensitive to changes in ovarian biomechanics. To further evaluate how stiffness impacts ovarian transcriptome, we analyzed DEGs in a time-dependent manner. We identified 1033 DEGs in follicles cultured in 2% at 24h compared to 3h. Most of the upregulated genes were associated with apoptosis and matrix remodeling. In the 0.5% condition, 1295 DEGs were identified, with upregulation of genes related to metabolism and cell cycle at 24h. Overall, we demonstrated that the age-associated increase in ovarian stiffness impacts follicle development and oocyte quality by impairing granulosa cell viability. In addition, our transcriptome analysis reveals that follicles quickly respond to stiffness triggering the expression of well-known genes associated with advanced maternal age when exposed to a stiff environment. These results show that stiffness might be a novel regulator of folliculogenesis and oocyte quality. Work supported by NIH-K99/R00-HD108424 to FAR.

FOCUS SESSION 30 - GERM CELLS

S30.1 - Germ Cell Specification and Development

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Primordial germ cells are one of the earliest cell types to become specified during early development, however detailed molecular understanding of the ontogeny of these cells in bilaminar disc embryos is lacking. We employed single cell transcriptomics and wholemount immunofluorescence of a developmental time series to track the emergence of pig PGCs during peri-gastrulation. In contrast to observations in mice, pPGCs are first found scattered along the posterior epiblast at the border with the trophectoderm. Furthermore, since implantation occurs after the onset of gastrulation in pig, we determined that PGCs are specified before the amnion forms. We find that PGCs first express TFAP2C and NANOG, and soon after activate SOX17 and PRDM1. Notably, prior to their migration towards the gonad pPGCs initiate broad epigenetic reprogramming, encompassing DNA and H3K9me2 demethylation, while they transiently cease DNA replication. In this presentation I will discuss our progress developing in vitro gametogenesis using epiblast stem cells recapitulating the in vivo properties of PGC.

S30.2 - Determining The Potency of Primordial Germ Cells by Injection into Early Mouse Embryos

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Primordial germ cells (PGCs) are the earliest precursors of the gametes. During normal development PGCs only give rise to oocytes or spermatozoa, depending on the sex of the embryo. However, PGCs can acquire pluripotency in vitro by forming embryonic germ (EG) cells and in vivo during teratocarcinogenesis. Classic embryological experiments directly assessed the potency of PGCs by injection into the pre-implantation embryo. As no contribution to embryos or adult mice was observed, PGCs have been described as unipotent. Here we demonstrate that PGCs injected into 8-cell embryos can initially survive, divide and contribute to the developing inner cell mass. Apoptosis-deficient PGCs exhibit improved survival in isolated epiblasts, and can form naïve pluripotent embryonic stem cell lines. However, contribution to the post-implantation embryo is limited, with no functional incorporation observed. In contrast, in vitro-derived PGC-like cells show extensive contribution to mid-gestation chimaeras. We thus propose that PGC formation in vivo establishes a latent form of pluripotency that restricts chimaera contribution.

S30.3 - The Combined Action of Transcription Factors FOXL2 and RUNX1 Overrides the Testis Program to Enforce Granulosa Cell Differentiation During Gonad Differentiation

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Sex determination of the gonads begins with fate specification of gonadal supporting cells into either ovarian pre-granulosa cells or testicular Sertoli cells. We previously identified a new player in controlling pre-granulosa cell differentiation: the transcription factor RUNX1. From fish to human, RUNX1 is expressed in fetal ovaries during gonad differentiation. In mice, RUNX1 marks the supporting cell lineage and becomes specific to pre-granulosa cells as the gonads differentiate. While loss of Runx1 in fetal ovaries did not cause ovary-to-testis sex reversal, combined loss of Runx1 and Foxl2 resulted in masculinization of fetal ovaries. This finding implied a complementary/ redundant role of RUNX1/FOXL2 in controlling fetal granulosa cell identity. Here we aimed to determine if RUNX1, similar to FOXL2, antagonizes the emergence of the testis pathway and promote granulosa cell differentiation. We developed a mouse model with ectopic expression of RUNX1 in the somatic cells of the fetal testes. Expression of the transgene Runx1 was induced specifically in Nr5a1+ somatic cells of the embryonic gonads. Contrary to FOXL2, RUNX1 induction in the fetal testis did not result in partial testis-to-ovary sex-reversal. Instead, RUNX1 induction led to a mild downregulation of pro-testis genes and the development of smaller testes. On the other hand, induction of both FOXL2 and RUNX1 in fetal testes resulted in an almost complete testis-to-ovary sex reversal. Expression of both FOXL2 and RUNX1 in XY gonads at the time of sex determination prevented the differentiation of supporting cell precursors into

testis-specific Sertoli cells, tipping their identity toward granulosa cell differentiation. As a consequence, close to birth, these XY gonads presented an ovarian morphology that was undistinguishable from control XX gonads. In addition, XY embryos expressing both FOXL2 and RUNX1 developed a female reproductive tract instead of a male reproductive tract. While these feminized XY gonads were morphologically undistinguishable from normal fetal ovaries, immunostaining revealed that a few supporting cells in the center of the gonads maintained their testis identity, preventing a full testis-to-ovary sex reversal. Altogether, these results suggest that RUNX1 acts as a supporting player to FOXL2 in the control of supporting cell identity. Together, RUNX1 and FOXL2 are capable of overriding the testis program to drive granulosa cell differentiation in most supporting cells.

S30.4 - Confirmation of Embryonic Donor-Derived Germline Stem Cell Migration, Colonization, and Proliferation within Host Gonads

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While cloning may one day enhance the conservation of mammalian species, the large avian oocyte and inconsistent response to super-ovulation hormone treatment make cloning an unlikely tool for avian conservation. Germline stem cell (GSC) transfer is a unique technique that allows us to increase the reproductive lifespan of an individual post-mortem by transferring GSCs to a domestic host. This technique could significantly impact conservation by enabling germplasm rescue of under-represented genetically valuable individuals, increasing genetic diversity. We are currently using quail as a model system to develop this technique for endangered species management. In addition, when successful, this method will be incorporated into the passenger pigeon de-extinction project using genetically edited band-tailed pigeon germ cells. Previous studies have shown that transferred GSCs can produce functional gametes within the host gonad, leading to donor-derived offspring. Our study includes the collection of transgenic embryonic quail GSCs followed by transfer to host embryos at the embryonic stage when the endogenous GSCs are migrating to the gonadal ridge. We isolated germline stem cells from Dendra2 transgenic quail embryos, followed by transfer to stage 15-17 host chicken or wild-type quail embryos. Following transfer of embryonic Dendra2 germline stem cells, the donor-derived cell competes with endogenous germline stem cells, resulting in a chimeric gonad. The proportion of donor-derived GGCs depends on injection parameters, including number of cells, injection volume, exact embryo stage, and endogenous PGC numbers. We evaluated transgenic donor-derived germline stem cell migration, colonization, and proliferation in host gonads by fluorescent microscopic detection of the Dendra2 protein and PCR detection of the Dendra2 gene. Our fluorescent microscopy data confirmed that donor-derived cells successfully migrated to the gonadal ridge and localized to the secondary sex cords. While 57% of host gonads exhibited Dendra2-positive cells, only 43% of gonads yielded detectable Dendra2 PCR products. For germline stem cell transfer to be effective, the donor-derived cells must not only colonize the host gonad but also proliferate to levels similar to the endogenous GSCs. Therefore, microscopic cell localization and PCR are necessary to generate good data for injection parameter evaluation. We are currently developing a method to quantify the number of donor-derived GSCs that have colonized the host gonad using qPCR to determine the relative amount of Dendra2 DNA. This method will allow us to more accurately compare the effect of injection volumes, cell counts, and other variables on donor-derived cell colonization success within the host gonad.

FOCUS SESSION 31 - VIRENDRA B. MAHESH SESSION ON NEUROENDOCRINOLOGY

S31.1 - Mechanisms for Suppression of Gonadotropin Secretion During Stress in Mice

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Stress impairs reproduction in a variety of species, including humans, ruminants, and rodents. The suppression of reproductive function is mediated, at least in part, by inhibition of gonadotropin secretion. Luteinizing hormone (LH) is a critical gonadotropin that is secreted in a pulsatile pattern which supports both steroidogenesis and gametogenesis. LH pulses are organized by neurons in the arcuate nucleus of the hypothalamus that produce the neuropeptides kisspeptin, neurokinin B and dynorphin (KNDy cells). Although effects of stress on the pituitary gland have been noted in some studies, evidence for inhibition of KNDy neurons during metabolic stress will be discussed. The mechanisms by which KNDy cells become inhibited during metabolic stress is a central question. New data generated in mice exploring the role of brainstem neurons in suppression of KNDy cells, with a focus on neuropeptide Y will be discussed. Investigation of the neural pathways by which stress impairs gonadotropin secretion could influence reproductive management of humans and domestic animals.

S31.2 - Serum Concentrations of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) in KISS1 Knockout Gilts Treated with Neurokinin B, Kisspeptin, and GnRH Hormone Analogs

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Kisspeptin knockout (KISS1^{-/-}) pigs exhibit hypogonadotropic hypogonadism. Hormone analogs stimulate gonadotropin secretion and ovulation in mice and humans with induced or naturally occurring mutations in the KISS1 gene. It is unknown how such agonists affect gonadotropin secretion in KISS1^{-/-} gilts. The goal was to characterize LH and FSH secretion in gilts treated with hormone analogs activating the hypothalamic-pituitary-gonadal axis. Gilts (254 d of age) were jugular catheterized for serial blood collection before and after treatments. Concentrations of LH and FSH were quantified with RIA. Data were analyzed as repeated measures with genotype and time as fixed effects. In Experiment 1, baseline hormone secretion was established for 6 h, then gilts were treated with a neurokinin 3 receptor (NK3R) agonist (senktide; 10 mg/kg, i.v.) to activate KISS1 neurons. In Experiment 2, gilts were estrus synchronized with altrenogest (18 mg/d, 14 d) and treated with PG600 (1000 IU hCG, 2000 IU PMSG, i.m.) 12 h after altrenogest withdrawal to synchronize follicular development. At 112 h after PG600, a kisspeptin receptor agonist (C6; 0.3 nMol/kg, i.m.) was administered to activate GnRH neurons. In Experiment 3, gilts were treated with GnRH (Cystorelin; 150 ng/kg, i.v.) to stimulate gonadotroph cells. In Experiment 4, gilts received an estradiol implant and GnRH with increasing frequency every 2 wk (1500 ng/kg, 1/d; 1000 ng/kg, 2/d; 500 ng/kg, 4/d, i.v.) followed with PMSG (1000 IU) and hCG (1000 IU) 78 h apart to induce follicular development and ovulation. LH and FSH pulse amplitude, but not pulse frequency, was greater in KISS1^{-/-} and KISS1^{+/+} gilts compared with KISS1^{-/-} gilts, which had reduced serum LH and FSH concentrations ($P < 0.01$). LH but not FSH concentrations in KISS1^{-/-} and KISS1^{+/+} gilts

were increased for 60 min following senktide ($P < 0.001$), which did not affect LH or FSH in KISS1^{-/-} gilts. C6 treatment did not affect FSH but increased circulating LH in KISS1^{+/-} and KISS1^{+/+} gilts for approximately 24 h ($P < 0.05$), but C6 did not affect LH in KISS1^{-/-} gilts. The C6 treatment induced ovulation in all KISS1^{+/+} gilts but none of the KISS1^{-/-} gilts. A single acute injection of GnRH increased LH in KISS1^{+/-} and KISS1^{+/+} gilts but not in KISS1^{-/-} gilts ($P < 0.001$). LH secretion in KISS1^{-/-} gilts was increased modestly with a greater dose and frequency of GnRH ($P < 0.05$). One KISS1^{-/-} gilt ovulated 2 follicles after hCG and one had luteinized follicles, but the rest failed to ovulate. A single functional KISS1 allele is sufficient to confer normal reproductive endocrine function in pigs. This is the first report to confirm NK3R regulates LH secretion in gilts. Lack of GnRH-induced LH secretion in KISS1^{-/-} gilts may result from insufficient releasable pools of LH due to lack of sufficient GnRH priming. Optimization of treatments will be required to induce full ovulation in KISS1^{-/-} gilts. Funding FFAR 552176 and CRIS 3040-31320-001-000D. The USDA is an equal opportunity provider and employer.

S31.3 - Secretoneurin: A Paracrine Regulator of Gonadotrope Cyclicity

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Secretoneurin (SN), a proteolytic product of secretogranin II (SCG2), has been implicated as a potential regulator of gonadotropin secretion. Previous studies showed that exogenous SN can stimulate luteinizing hormone (LH) secretion from the mouse LβT2 gonadotrope cell line and goldfish pituitaries, and some have suggested that SN may provide a paracrine signal from other anterior pituitary cells to support LH production and secretion. Our group has previously confirmed the ability of SN to stimulate LH secretion from proestrous murine pituitaries. SN may also regulate gonadotropes by working in conjunction with gonadotropin-releasing hormone (GnRH). We hypothesize that (1) secretoneurin from lactotropes alters gonadotrope processes to ultimately regulate the cyclic production and secretion of gonadotropins, and (2) the production of SN is regulated cyclically. To define murine gonadotrope protein expression in response to exogenous SN and GnRH during different stages in the estrous cycle, pituitaries were collected from adult female mice expressing a Gnhrh-driven Cre and a floxed fluorescent reporter at 0900 on proestrus (n=36) and diestrus (n=36). Pituitaries were dispersed and treated with one of the following conditions for 3h: Vehicle, 1 μM SN, 1 nM GnRH, and SN + GnRH (n=9 per stage/treatment). Gonadotropes were FACS-purified, pooled, and submitted to the UAMS Proteomics Core for mass spectrometry-based proteomic analysis. Differential abundance analyses were performed to determine significant ($p < 0.05$) fold-change (FC) differences in proteins from each treatment group within and between both estrous cycle groups. Conditioned media (CM) from a novel ovine lactotrope cell line (Chappell Lab) stimulated with increasing doses of estradiol was applied to LβT2 cells to determine if the lactotrope secretome (including SN) can stimulate LH secretion. Lactotropes from the same line were also stimulated with estrous cycle levels of E2/P4, and Scg2 and proprotein convertase 2 (Pcsk2) were quantified via PCR. Proteomic analysis identified a total of 1678 and 1863 proteins in the proestrous and diestrous purified gonadotrope pools respectively. Of these, 383 (proestrous) and 387 (diestrous) proteins were significantly differentially expressed between the 4 treatment groups ($p < 0.05$).

Ingenuity Pathway Analysis (IPA) revealed multiple biological processes that were changed with each stage/treatment. For example, compared to proestrous vehicle-treated gonadotropes, proestrous SN-stimulated gonadotropes have significantly upregulated translation initiation pathways (z-score: 1.633, $p < 0.00001$) and significantly downregulated estrogen receptor signaling pathways (z-score: -1.000, $p < 0.01$). Conditioned media (CM) from lactotropes stimulated with 50pM E2 increased LH secretion 150% over baseline, nearly twice the increase seen with 50pM E2 alone, suggesting that lactotropes produce a factor/factors that can elicit LH secretion. Our data from the lactotrope cell line and mouse pituitaries indicate that E2 may regulate the expression of Scg2 and Pcsk2, and this may result in increased SN production. Together, our data indicate that SN regulates the gonadotrope proteome in a manner distinct from GnRH, and both the SN-stimulated proteome and lactotrope responses to E2 appear to be cycle/dose dependent, indicating that paracrine interactions between lactotropes and the hypothalamic-pituitary-gonadal axis play a role in the cyclic regulation of gonadotropin production and secretion.

S31.4 - Regulation of Follicle-Stimulating Hormone β Subunit Transcription by Newly Discovered Enhancers

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Follicle-stimulating hormone (FSH) is an essential regulator of gonadal function. It is a dimeric glycoprotein produced by pituitary gonadotrope cells. Expression of the FSH β subunit (Fshb) is rate-limiting in production of the mature hormone. Activins and related TGF β ligands stimulate Fshb transcription via complexes of SMADs and FOXL2, which bind to the Fshb promoter. Single nucleus ATAC-seq analysis of wild-type murine pituitary demonstrated the presence of four regions of co-accessibility upstream of the Fshb gene exclusively in gonadotropes. We refer to these as enhancers (Enh) 1-4, with Enh 1 most proximal to the gene. Gonadotrope-specific activin type II receptor knockout mice are FSH-deficient. The Fshb promoter and three of four enhancers are fully closed in these animals, with Enh 3 remaining partly accessible. Enh 2 was recently described as an enhancer that increased activin-stimulated murine Fshb promoter-reporter (luciferase) activity in gonadotrope-like L β T2 cells. In our hands, however, Enh 2 did not alter murine Fshb promoter-reporter activity in response to activin. Moreover, we observed normal FSH synthesis and secretion in Enh 2 knockout mice. Enh 3 and 4, in contrast, markedly increased basal and activin-stimulated Fshb promoter-reporter activity in L β T2 cells. In addition, Enh 3 and 4 were accessible, possessed the enhancer mark H3K27Ac, and bound SMAD3 and FOXL2 in these cells. Collectively, the data indicate that there may be four upstream, activin-sensitive enhancers that regulate murine Fshb transcription in gonadotropes. At least two of these regions bind SMADs and FOXL2, which may facilitate their association with the Fshb promoter. Whether the enhancers play redundant or complementary roles in vivo has not yet been determined. This research was supported by grants from CIHR and NIH.

S31.5 - High Androstenedione Cows Have Increased Endocrine Hormones and Inflammatory factors which may contribute to Persistent Follicles Resulting in Anovulation

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Cows with naturally occurring androgen (specifically androstenedione; A4) excess (High A4; A4 >40ng/ml in follicular fluid) have been identified in our herd. They have a 17% reduction in calving rate, theca and granulosa cell gene expression profiles indicative of altered androgen synthesis, and follicular arrest compared to controls (A4 < 20ng/ml). We hypothesized High A4 cows would have longer reproductive cycles resulting in anovulation, with endocrine, pro-inflammatory cytokine and lipid profiles analogous to women with Polycystic Ovary Syndrome (PCOS). Thus, a series of experiments were conducted to determine if High A4 cows had altered endocrine, follicular fluid and estrous cycle characteristics contributing to follicular arrest and anovulation. Serial blood samples collected during the luteal phase (day 10-11 of the estrous cycle) had elevated circulating concentrations of plasma progesterone (P4) with a tendency for (P < 0.06) increased ratio of luteinizing hormone (LH) to follicle stimulating hormone (FSH) compared to controls. Over two reproductive cycles, High A4 cows had increased follicular fluid Anti-Mullerian Hormone, and pro-inflammatory Interleukin-18 cytokines with more persistent follicles and longer reproductive cycles compared to controls. High A4 cows also tended to have increased plasma A4, and reduced estrogen plasma secretion which did not mimic rises in estrogen indicative of follicular waves of growth compared to controls. High A4 cows exhibited an altered pattern of inflammatory lipids (LysoPC), in blood plasma. Compared to control cows, their levels were lower during the Early-Luteal (EL) stage but then increased by the Mid-Luteal (ML) stage with higher levels than controls. Therefore, this lipid data indicates an increase in systemic inflammation in High A4 cows during the ML stage. Angiopoietin-1 (ANGPT1) in blood plasma increased from EL to ML in control cows; presumably, to increase vascular development and inflammation necessary for ovulation. However, in the High A4 cows this increase in ANGPT1 did not occur, potentially due to the lack of estrogen produced by the dominant follicle. Likewise, Leukemia inhibitory factor (LIF) in blood plasma was reduced in both EL and ML in controls; but in High A4 cows LIF was elevated during the EL stage. This increase in LIF during the EL phase may be due to abnormal follicular development or lack of estrogen production since LIF appears to be negatively correlated to estrogen in several different organ systems. Many PCOS-like phenotypes were present in High A4 cows including: longer reproductive cycles, increased LH/FSH ratio, elevated inflammatory markers, and persistent follicles resulting in anovulation. Therefore, the naturally occurring High A4 cow is an excellent model to understand how androgen excess may affect mechanisms regulating anovulation and female infertility.

FOCUS SESSION 32 - FERTILITY PRESERVATION

S32.1 - Fertility Preservation: Twenty-Five Years of Bold Progress

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Fertility Preservation is a success story for translational research following the progress in gamete biology and cryobiology. The advent of the clinical field was hastened by the improved understanding of cryobiology leading to better cryogenic methods followed by the report of the first successful ovarian

transplantation with cryopreserved tissue (Oktay & Karlikaya NEJM 2000). Subsequent events, including a better understanding of the molecular mechanisms of chemotherapy-induced gonadal damage, cancer-specific ovarian stimulation protocols (such as the utility of anti-estrogen agents as ovarian stimulants), flexible random start ovarian stimulation protocols based on the dynamic understanding of ovarian folliculogenesis, and others have contributed to the rapid progress within the last 25 years. The future of fertility preservation appears to be even brighter with improved ovarian transplantation techniques utilizing neovascularization-enhancing approaches, the progress with in vitro gamete production from primordial follicles in tissue, and potentially the pharmacologic approaches that enhance gamete resistance against chemotherapy-induced damage. There are also recent progress with the in vitro growth of primordial follicles when cryopreserved ovarian tissue is involved with cancer. Finally, the experience gained from cancer patients now leads to the utility of these technologies in extending the reproductive life span and delaying menopause in healthy women. After providing a historical context, this session will highlight significant new progress and pending discoveries in the Fertility Preservation and Restoration field.

S32.2 - Development of Microbead-Based Human Sperm Binding Assay

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The fertilization process relies on a well-coordinated recognition mechanism between sperm and egg. Currently, the only documented cell-surface interaction in human gametes involves the interplay between sperm IZUMO1 and egg JUNO. Sperm undergoes physiological transformations from ejaculation to fertilization, including capacitation, acrosome reaction, and crucial IZUMO1 protein relocation for successful oocyte fertilization. Analyzing sperm prepared for fertilization is challenging due to their scarcity and confinement in the perivitelline space of human eggs. To overcome this, JUNO-coated microbeads were employed to emulate the sperm-egg binding interface. The full-length human JUNO encoding gene (NM_001199206) was transfected into Drosophila S2 cells and expressing recombinant JUNO protein purified through Ni-NTA metal affinity chromatography. Microbeads (>70µm) (Cytiva, Sweden) were conjugated with recombinant JUNO protein to simulate the shape, size, and surface of the oocyte. The model was validated using human semen samples from healthy donors (BiokiBank, Spain) with prior informed consent, and the study was approved by Murcia University Human Research Ethics Committee. Ejaculates from three donors were divided into two groups, one preserved through cryopreservation and the other through vitrification. After thawing according to the supplier's recommendations, the swim-up method isolated motile sperm, which were then incubated in G-MOPS PLUS media supplemented with 2 mM CaCl₂ and 10 mg/ml progesterone for 3 h. Then, the sperm cells were co-incubated with coated microbeads (200,000 spermatozoa/30 beads) overnight in G-IVF PLUS medium (Vitrolife, Sweden). The control comprised microbeads lacking the JUNO coating. After co-incubation, the microbeads were fixed in 4% PFA (EMS, USA) and stained with Hoechst 33342 (1 µg/mL) and CD46 (2 µg/ml). Data was analysed by non-parametric Kruskal-Wallis test and the values were compared by Dunn's test when p value < 0.05. Conventional cryopreserved samples showed a mean of 5.63 ± 0.44 (n = 191) spermatozoa attached to JUNO microbeads and 2.50 ± 0.15 (n = 186) to

control microbeads, while vitrified samples resulted in 11.21 ± 0.69 ($n = 188$) and 0.98 ± 0.10 ($n = 183$), respectively. Although both cryopreservation methods exhibited a specific binding to JUNO-microbeads (JUNO-microbeads vs control-microbeads, p value ≤ 0.0001), conventional cryopreserved sperm bound less efficiently to JUNO-microbeads than vitrified samples (p value ≤ 0.0001). When assessing microbeads with at least one attached spermatozoon, no significant differences were observed in the percentage between control (86.29 ± 1.21) and JUNO (94.21 ± 2.66) in cryopreserved samples. However, significant difference was observed in vitrified samples (control 56.20 ± 1.20 , JUNO 98.94 ± 1.06 , p value ≤ 0.0001), indicating a potential non-specific binding to microbeads in the cryopreserved samples. Finally, we observed that more than 85% of spermatozoa adhered to the beads were positive for CD46 in both samples (86.39 ± 1.55 in cryopreserved and 96.66 ± 0.72 in vitrified), assuming a specific acrosome-reacted sperm bound to the microbeads. Our results demonstrate the ability of the microbead-based human sperm binding assay to select sperm capable of specific JUNO binding, considering vitrification as the preservation method that better preserves the physiological ability of the sperm to specifically recognise the oocyte. Research was supported by project PID 2020-114109GB-I00 funded by MCIN/AEI/10.13039/501100011033 (M. Jiménez-Movilla) and by the Bill & Melinda Gates Foundation (grant INV-055841 to J.E. Lee and M. Jiménez-Movilla).

S32.3 - The Growing Follicle Protection During the Chemotherapy Exposure Using Functionalized Gold Nanoparticle (AuNP)-Mediated Let-7A Delivery

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Cancer treatments such as alkylating agents are highly gonadotoxic, disrupting physiological processes such as the growth of ovarian follicles, the survival and the acquisition of oocyte competence. The current methods for fertility preservation using oocytes, embryo or ovarian tissue cryopreservation are invasive and cannot be offered to all patients. A pharmacological approach, including the microRNA therapy, is very attractive. We previously identified let-7a as a promising candidate. Its expression profile is downregulated in response to chemotherapy using a postnatal-day-3 (PND3) mouse ovaries model that allows the study of quiescent follicles. The transfected PND3 ovaries with let-7a using liposomes prior to the exposure to chemotherapy showed a reduction in apoptosis. Recently, we extended our study on chemotherapy-induced growing follicles damage. Our collaborator, the Engineering of Molecular NanoSystems Laboratory, develops a functionalized gold nanoparticle (AuNP)-based system to deliver let-7a mimics in growing follicles in vitro. To evaluate the safety and efficiency of AuNPs-mediated let-7a mimic (AuNPs-let-7a) delivery as well as the protective effect of let-7a against the chemotherapy, mechanically isolated preantral follicles from prepubertal mice were cultured in vitro for 11 days. At day 4, pre-antral follicles were cultured under different conditions; negative control (no treatment), 20 μ M of chemotherapy exposure (4-hydroperoxycyclophosphamide, 4-HC), 1 nM of AuNPs-let-7a transfection and co-treatment with 1 nM AuNPs-let-7a and 20 μ M 4-HC (AuNPs-let-7a + 4-HC). From day 4 and until the end of the culture, follicular development, follicular survival and steroidogenesis were assessed to determine the damage induced by chemotherapy and the potential toxicity of AuNPs-let-7a system. To evaluate the efficiency of let-7a mimic, AuNPs were internalized in the growing follicles and the expression profile of let-7a and its target genes were assessed. The results confirmed the impact of the

chemotherapy on the follicular development. The follicles were classified according to their morphology: healthy for normal growing pattern, delayed growth, and abnormal morphology, when the follicle is unhealthy but alive with apoptotic cells. Chemotherapy exposure induced a high level of abnormal morphology (Day 8: $p < 0.01$; Day 10: $p < 0.001$) and a decrease in the follicular survival (Day 8: $p < 0.05$; Day 10: $p < 0.001$) compared to control. The cultured follicles with AuNPs-let-7a showed no toxicity either in the analysis of survival or morphology. At day 4, the internalization of AuNPs, visualized with a fluorochrome grafted on the let-7a strand, was in perinuclear of granulosa cells area after 6h of transfection and an accumulation of AuNPs-let-7a was observed in the cumulus oocyte complex on antral follicles (day 11) after 24h and 48h of AuNPs-let-7a presence in the culture. Currently, different AuNP functionalizations by using a spacer between the AuNP and let-7a mimic are tested to assess the optimal delivery approach. In addition, hormonal assay measuring the concentration of steroid hormones secreted by the follicles and the oocyte competence are used to assess the acquisition of oocyte competence to mature and to be fertilized in the different experimental conditions.

S32.4 - Propranolol Treatment Activates Murine Primordial Follicles In Vitro and In Vivo

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The primordial follicle is the first class of follicles, consisting of the foundation of folliculogenesis called ovarian reserve. The mechanism of quiescence and activation of primordial follicles involves several factors and signaling pathways. Among which PI3K/AKT/mTOR is already very well described and plays an important role in regulating follicle dormancy and activation. Propranolol is described in the literature as an activator of mTOR (mammalian target of rapamycin) in different cell types and expressed in all follicular classes. Therefore, the objective of this study was to evaluate the effect of propranolol on follicular activation in vitro and in vivo in mouse ovaries. The experiment was divided into two phases: (1) explant culture and (2) mice treatment. In phase 1, ovaries were collected from 3-day-old animals, grown in culture medium and collected on day 1 (D1) and day 3 (D3). The ovaries were divided into 3 groups: growth medium (GM), DMSO and 10nM propranolol (dose decided by the response curve previously performed). In the second stage, in vivo tests were carried out. Four-week-old animals were treated with propranolol for 15 days intraperitoneally. The animals were divided into three groups: Control (PBS), Low (15mg/kg) and High (40mg/kg). After the end of the experimental period, all ovaries (in vitro and in vivo) were collected, fixed, stained with HE and used for follicular quantification. Follicles were classified into 6 classes: primordial, transitional, primary, secondary, antral and atretic. Only follicles containing the oocyte nucleus were quantified. All statistical analyzes were performed in GraphPad and data are presented as mean $\pm$ SEM. In explant culture, we observed a significant decrease in the number of primordial follicles on D1 (1158 ± 37 GM, 1076 ± 38 DMSO, 815 ± 10 Propranolol; $p = 0.0006$) and on D3 (933 ± 18 GM, 899 ± 22 DMSO, 729 ± 11 Propranolol; $p = 0.0004$). The number of transitional follicles increased on D1 (151 ± 5 GM, 162 ± 14 DMSO, 202 ± 7 Propranolol; $p = 0.026$) and on D3 (195 ± 9 GM, 205 ± 9 DMSO, 260 ± 9 Propranolol, $p = 0.004$). We also observed an increase in the number of primary follicles in D1 and D3 (D1: 153 ± 22 GM, 123 ± 9 DMSO, 212 ± 11 Propranolol; $p = 0.017$. D3: 211 ± 6 GM, 247 ± 11 DMSO and 318 ± 10 Propranolol ; $p = 0.0006$). In the in vivo test, we observed a significant decrease in the number of primordial follicles (223 ± 32 Control, 171 ± 23 Low, 145 ± 20 High; $P = 0.03$) and an increase in the number of primary follicles (18 ± 5 Control, 30 ± 7 Low, $57 \pm$

18 High; $P = 0.007$). We also observed an increase in the number of antral follicles (3.4 ± 0.8 Control, 5.9 ± 0.6 Low, 6 ± 0.7 High, $P = 0.03$). The rate of follicular activation in treated animals increased compared to control (19.11 ± 4 Control, 34.3 ± 2 Low, 41.4 ± 2.6 High; $P = 0.0004$). Based on our in vitro and in vivo studies, propranolol activates primordial follicles. These data suggest that propranolol may be a promising drug for a new clinical approach to rescue infertility in women with residual dormant primordial follicles. New tests are being carried out to understand the effect of this medication on late folliculogenesis and ovulation.

S32.5 - A Wild Semen Bank for African Elephants

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Elephants play vital roles in their ecosystems and are among the most intelligent and charismatic land mammals. Yet, both the Asian (*Elephas maximus*) and the African savanna elephant (*Loxodonta africana*) are listed as Endangered, and the African forest elephant (*Loxodonta cyclotis*) as Critically Endangered on the IUCN Red List of Threatened Species. In the current sixth mass extinction with increasingly fragmented and confined populations, the role of zoos as an ark to safeguard backup populations and genetic diversity will be of increasing importance to the future of entire species. Breeding success of elephants in zoological institutions is compromised by a lack of suitable breeding bulls, decreasing genetic diversity and pathologies associated with prolonged non-reproductive periods in females, resulting in infertility. The development of assisted reproductive technologies (ART), especially artificial insemination (AI) with fresh and cryopreserved semen has substantially contributed to maintain genetically healthy and self-sustainable captive elephant populations. Between 2000 and 2022, 12.4 and 5.0 % of captive-born African and Asian elephants, respectively, were produced via AI. By collecting semen from the wild, international captive breeding programs can be linked with wild populations avoiding costly and stressful transportation of animals. To this end, in 2023, we collected semen of wild African elephants in 2023 in Selati Game Reserve, South Africa. We immobilised 22 elephant bulls using etorphine (5.6-6.4 mg/kg), thiafentanyl (8.4-9.6 mg/kg), hyaluronidase (5000 IU) and azaperone (30 mg) via dart from a helicopter. Before electroejaculation (Seager, USA) with a customized rectal probe, a brief ultrasound examination of testes and accessory glands was performed. In absence of an epididymis, the ampullae of the ductus deferens are the site of temporary sperm storage in elephants. Ampulla size and appearance (echogenicity of the content) in addition to testis size proved to be most informative to predict high-quality semen suitable for cryopreservation. Ejaculates of 14 bulls were obtained and diluted 1:1 with BotuCrio (Nidacon, Sweden) extender immediately upon collection and assessed using a field CASA system (Androscope, Minitube, Germany). If suitable for cryopreservation (i.e. progressive sperm motility $\geq 60\%$; average $76 \pm 13\%$; $n=9$), semen was transported to the lab by helicopter where it was diluted to a final concentration of $175 \pm 65 \times 10^6$ sperm/ml. After filling semen into hollow glass

tubes (8 ml), the tubes were placed in a water bath at 5-7°C for equilibration and then cryopreserved by directional freezing (MTG-550, IMT Ltd., Ness Ziona, Israel). Post-thaw motility averaged 36±8% (range: 30-45%). Previously, wild African elephant semen was collected twice: In 2008, which due to low semen quality did not generate any pregnancy following AI, and in 2009, from which ~2l of cryopreserved semen with post-thaw progressive motilities of ≤30 % (100-150 x 10⁶ sperm/ml) were used to sire 11 calves using AI. The improved quality and higher concentration of the newly cryopreserved semen are promising for further enhancing AI success rate and zoo elephant genetic diversity; it is attributable (1) to the discovery that only a minority - i.e. solitary bulls, especially ones following a herd – provide large volumes of high quality semen, unlike bulls in bachelor groups. (2) ultrasound-informed reproductive assessment and (3) on-site assessment of ejaculates help to further focus resources and time on the most suitable candidates and samples. Finally, (4) an enhanced cryopreservation protocol and abstaining from centrifugation to remove seminal plasma resulted in overall higher post-thaw motilities.

TRAINEE PLATFORM COMPETITION

T.1 - Neurotensin Mediates Follicular Vascular Endothelial Cell Function Via Both NTSR1 and SORT1 During Ovulation

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Neurotensin (NTS) is a small peptide which has recently been described as a paracrine mediator of ovulation. NTS mRNA is highly expressed by granulosa cells of the mammalian ovary in response to the luteinizing hormone (LH) surge. The ovarian microvascular endothelial cells (OMECs) are responsive to NTS signaling. Ablation of NTS signaling in vivo interferes with typical follicular angiogenic events occurring in response to the LH surge, inducing an apparent increase in vascular permeability, with red blood cells pooling outside the vasculature and in the follicle antrum. In vitro, NTS both increases OMEC migration and decreases OMEC monolayer permeability. Ovarian microvascular endothelial cells express the NTS receptors NTS receptor 1 (NTSR1) and sortilin (SORT1, also known as NTS receptor 3). We hypothesize that both NTSR1 and SORT1 are critical mediators of NTS action on OMEC angiogenic activity within the developing vasculature of the ovulatory follicle. To identify the role of NTSR1 and SORT1 in ovulation and ovulatory angiogenesis in vivo, dominant follicles of the cynomolgus macaque (*Macaca fascicularis*) were injected prior to ovulation with either: 1) sterile water as a vehicle control, 2) the general NTS receptor antagonist SR142948, 3) the NTSR1-selective antagonist SR48692, or 4) the SORT1-selective antagonist AF38469. Immediately after the follicle injection surgery, hCG was administered systemically to initiate ovulatory events. 48 hours after hCG, the injected ovary was surgically removed and histologically assessed for follicle rupture and antral red blood cell pooling. While evidence of a rupture site was observed on each dominant follicle in the water-, SR142948-, and AF38469-injected groups, red blood cell extravasation and pooling in the antrum and granulosa cell layer was increased in the SR142948 and AF38469 follicles when compared to water-injected controls. Injection of the NTSR1 antagonist SR48692 blocked ovulation and also induced red blood cell extravasation and antral pooling. These results show that NTS signaling via NTSR1 and SORT1 may be critical for ovulatory angiogenic changes. The importance of NTSR1 and SORT1 in NTS-stimulated vascular changes was investigated in

vitro using primary macaque OMECs (mOMECS). We quantified changes in both migration and permeability in response to NTS, with or without ablation of NTS/NTSR1 activity via either: 1) NTSR1 antagonist SR48692 or 2) siRNA knockdown of NTSR1. NTS significantly increased mOMEC migration over untreated control in a trans-well migration assay. In the absence of NTS, neither the antagonist nor the NTSR1 knockdown changed mOMEC migration. However, after administering the NTSR1 antagonist or NTSR1 knockdown, NTS no longer effected mOMEC migration. Similarly, while mOMEC permeability was decreased by NTS treatment in a trans-well permeability assay, knockdown of NTSR1 ablated the NTS effect on permeability. Similar experiments were also performed with the SORT1 antagonist AF38469 and siRNA knockdown of SORT1. These studies also demonstrated ablation of NTS-mediated changes in migration and permeability after NTS/SORT1 signaling was impaired. Together, these data demonstrate for the first time that both NTSR1 and SORT1 act as critical mediators of NTS-stimulated changes in the ovarian microvasculature. These novel insights into the signaling of this ovulation-critical molecule improve our understanding of the regulation of the vascular changes which are integral to ovulation. This work was supported by NIH HD097675 to DMD and TEC.

T.2 - The Heterogeneity of Ovary-Derived Hydrogels Elucidates the Matrisome Properties That May Influence Follicle Growth and Survival In Vitro

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Premature ovarian insufficiency (POI) is characterized by a decline in ovarian function resulting in infertility and reduced ovarian hormone production. With the global prevalence of POI at approximately 3.5%, there are over 138 million women experiencing subfertility or complete infertility with disruption of sex hormones. However, there are many limitations to the current fertility preservation and restoration options that result in suboptimal outcomes. Additionally, oncofertility patients with metastatic disease within their ovary are ineligible for transplantation of tissue in its native form. This leaves a large population of individuals without desired biological children or necessary hormones. As such, future methods for fertility preservation and restoration will include isolating ovarian follicles to grow in vitro or transplant into a bioprosthetic ovary. These methods necessitate a deeper understanding of the ovarian microenvironment in order to define an engineered environment that best supports follicle growth and maturation. The human ovary is compartmentalized into two regions: the cortex and the medulla. Our lab has shown that there are differentially expressed matrisome proteins across these regions. Here, we developed compartmental decellularized extracellular matrix (dECM) hydrogels by processing 10 bovine ovaries into cortical tissue (CTX) and medullary tissue (MED) then decellularizing the tissue slices to generate hydrogels. Using atomic force microscopy, we determined that CTX-dECM and MED-dECM hydrogels were similarly rigid (537Pa, 468Pa). Thus, we hypothesized that differences observed in downstream applications of these gels are due to differences in biochemical cues or protein composition and relative abundance. ECM proteins' chemical and structural properties influence cellular behavior. So, we performed second harmonic generation scanning microscopy and determined that MED-dECM hydrogels had significantly wider and longer fibers than CTX-dECM and a lower fiber angle alignment. To evaluate the gel's ability to serve as a bioscaffold, we selected two biologically unique CTX and MED-dECM hydrogels, encapsulated primary murine follicles (~110µm), and assessed growth and survival. Interestingly, we observed increased follicle growth in CTX-dECM 1 and MED-dECM 2 when

compared to our control alginate. Day 8 follicle diameter for CTX-dECM 1 was significantly larger compared to follicles grown in CTX-dECM 2 (241.75, 189.95µm). Similarly, follicles grown in MED-dECM 2 were significantly larger at day 8 compared to follicles grown in MED-dECM 1 (212.12, 173.02µm). These data indicate biological differences in the dECM hydrogels. To investigate these differences, we performed proteomics on these four biologically unique hydrogels. A total of 604 proteins were detected. Further analysis comparing the uniquely present or absent proteins between the two selected biological replicates for each compartmental hydrogel resulted in 150 proteins unique to CTX-dECM 1, 51 in CTX-dECM 2, 70 in MED-dECM 1 and 82 unique in MED-dECM 2. Gene ontology (GO) analysis of biological processes was performed for each hydrogel based on the differentially present proteins. Interestingly, the two compartmental hydrogels that resulted in follicles with larger diameters than the control (CTX-dECM 1 and MED-dECM 2) had comparable GO biological terms with an overrepresentation of proteins involved in metabolic processes. However, further work must be undertaken to identify how this heterogeneity in the hydrogels influenced follicle growth. With further interrogation of these differences, we can engineer an environment that supports follicle growth and increases the production of functional MII oocytes. Importantly, this work aims to lay the groundwork for designing enhanced biomaterials that improve fertility restoration options for a variety of individuals.

T.3 - Development and Characterization of Novel Follicle-Stimulating Hormone Receptor (FSHR) Inhibitors

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Follicle-stimulating hormone (FSH), a heterodimeric glycoprotein hormone, plays a crucial role in regulating female reproduction. Its actions are mediated by binding to its receptor (FSHR), a Class A G protein-coupled receptor (GPCR), which was previously thought to primarily couple to the Gas/adenylyl cyclase (AC)/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway. However, it is now clear that FSH/FSHR can activate additional G protein dependent and independent pathways, to regulate ovarian follicle growth, granulosa cell proliferation, aromatase expression, and oestrogen production. In addition to its reproductive functions, emerging evidence suggests extragonadal roles of FSH/FSHR. Specifically, that elevated FSH levels experienced during perimenopause/menopause may directly act on bone, adipose and brain, contributing to increased susceptibility to the development of associated co-morbidities of osteoporosis, alterations in adipose tissue distribution and Alzheimer's disease, respectively. Inhibiting the actions of FSH/FSHR therefore presents an appealing therapeutic strategy, offering potential use as a non-steroidal contraceptive and/or inhibiting the development of menopause-related co-morbidities. The aim of this study therefore was to develop, screen, and characterize a panel of novel AI identified FSHR small molecule inhibitors. Accordingly, 84 small molecule compounds (SMCs) were identified by an AI screen as potential FSHR inhibitors. SMCs were screened for their ability to inhibit FSH-dependent cre-luciferase activity in HEK293 cells expressing FSHR. Three potential inhibitors (SMC48, -74, and -80) demonstrated over 90% inhibition of FSH-dependent cre-luciferase activity and were taken forward for further investigation. Using the live kinetic Glo sensor cAMP assay to monitor their temporal and kinetic effects, all three inhibitors exhibited concentration-dependent inhibition of FSH-dependent cAMP accumulation, achieving >90% inhibition at 100µM,

displaying similar IC50s of ~20 μ M. Additionally, assessment of selectivity to FSHR revealed minimal cross-reactivity with the related glycoprotein hormone receptor, luteinizing hormone receptor (LHR). All inhibitors were shown to enhance the binding affinity of FSH to FSHR in radioligand binding assays, suggesting non-competitive behaviour possibly at an allosteric site. Interestingly, the inhibitors displayed differential potencies in modulating FSH-dependent ERK1/2 phosphorylation, indicating potential signal bias in their actions. To determine the effects of the SMCs on ovarian function, mouse ovarian fragments were cultured +/- FSH and SMCs. All three SMCs inhibited FSH-dependent induction of aromatase (Cyp19a1) expression, as determined via qPCR. This study has identified three novel FSHR inhibitors, as promising therapeutic candidates for targeting menopause-related co-morbidities and/or developing non-steroidal contraceptives.

T.4 - Differential Transcript Usage in Bovine Blastocysts Exposed to Varying Concentrations of Energy Substrates

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According to the Developmental Origins of Health and Disease (DOHaD), energetic imbalance during critical developmental periods may impact long-term the offspring health, predisposing to the development of cardiovascular and metabolic diseases in adulthood. While epigenetic processes have been proposed as bona fide mediators of DOHaD, the mechanisms establishing its early onset remain poorly understood. Recently, the expression of different transcript isoforms of given genes, defined as differential transcript usage (DTU), has been observed during development and implicated in the onset of various diseases, making DTU a possible player in the landscape of developmental plasticity. Notably, transcript isoforms can arise by different mechanisms, among which change of the transcription start/end sites, alternative splicing, or activation/repression of different promoters. Consequently, functionally different transcript isoforms are produced, carrying changes in the coding sequence or the regulatory, untranslated regions. Therefore, even in the absence of noticeable changes in gene expression, the production of two or more transcript isoforms of a gene can exert biological effects. DTU, however, goes mainly undetected when routinely analyzing RNAseq datasets if specific pipelines are not applied. With this in mind, we aimed to analyze the expression of transcript isoforms in bovine blastocysts developing in varying concentrations of energy substrates. The preimplantation embryo development has been chosen as the window of exposure for the intense genome reprogramming and epigenetic remodeling to capture the earliest events of DOHaD. Bovine ovaries were obtained from a slaughterhouse and cumulus-oocyte complexes were collected from follicles of 2-8 mm in diameter. After in vitro maturation and in vitro fertilization with X-sorted semen, zygotes were cultured in a serum-free system with varying concentrations of energy substrates, i.e. increasing or decreasing concentrations of glucose, pyruvate, lactate, citrate, and amino acids. Expanded blastocysts (pools of 5 blastocysts/group, 3 biological replicates) were harvested for RNA extraction giving a minimum yield of 289 pg/ μ L and RIN >8.6 and sequenced on an Illumina platform. Transcript levels were quantified and genes having multiple transcript isoforms were filtered with DRIMSeq. Subsequently, DEXSeq was employed to distinguish which of these isoforms were differentially expressed (adjusted $p < 0.1$). Almost 6000 genes with multiple isoforms were identified, of which approximately 90 had differential isoform

expression in response to varying concentrations of energetic levels, for a total of 250-280 isoforms in the decreased and increased energy substrates, respectively. Enrichment analysis conducted to understand which molecular functions were mainly affected by energy supplementation revealed a general implication of 'palmitoyl-CoA' and 'acyl-CoA hydrolase activity' that seem concordant with the experimental model. Notably, other molecular functions involved were 'histone deacetylase binding' in the condition of increased supplementation and 'transcription factor binding' when energy levels were decreased, pointing at possible interferences with epigenetic and nuclear reprogramming mechanisms. To validate these observations, the acetylation of histone residues was monitored by immunofluorescence and confocal microscopy on expanded blastocysts (7-8 blastocysts/group, 2 biological replicates), revealing a 25-50% increase in the relative intensity of the acetylated signal when embryos were cultured with higher energetic substrates ($p < 0.01$, unpaired T-test, two-tailed). Overall, these observations contribute to a deeper understanding of how energetic excess impacts early embryonic development at the molecular level, showing for the first time the implication of DTU in the early onset of epigenetic modifications that might lead to DOHaD. Financial support: MIUR PRIN2017, No. 20172N2WL3_002 (DeSTINE); MUR PRIN2020, No. 20209L8BN4 (InfinitEGG)

T.5 - Single-Nuclei Sequencing Reveals Compositional Alterations in Ovarian Cell Types of AMH Deficient Mice

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Anti-Müllerian hormone (AMH), produced by granulosa cells (GC) of growing follicles, plays a crucial role in regulating folliculogenesis. AMH acts as a gatekeeper of follicle growth by inhibiting primordial follicle recruitment and follicular atresia, as well as inhibiting FSH sensitivity of small antral follicles. However, the precise cell type-specific mechanisms through which AMH regulates follicular development remain largely unknown. Due to the heterogeneous composition of ovary tissue, our previous bulk RNA sequencing analyses were unsuccessful to identify AMH regulated genes. Therefore, we conducted single-nuclei RNA sequencing to identify transcriptional changes in a cell type- and follicle-stage specific manner. Ovaries from 4-month-old AMH deficient (AMHKO) mice (n=5 per group) and wild type (WT) littermates (n=6 per group) were isolated at diestrous. Chromium Single Cell Multiome ATAC + Gene Expression (10x Genomics) was performed. Following ambient RNA removal and filtering, we performed normalization and doublet removal, then analyzed the combined data using Seurat. Transcriptional cellular clusters and GC subclusters were profiled using relevant literature-based markers. Subsequently, we evaluated whether differences in ovarian cellularity reflected variations in ovarian composition between WT and AMHKO mice by analyzing the cell percentage of cell clusters per sample. After quality control analyses, we included 63,236 cells from AMHKO mice and 73,313 cells from WT mice for characterization. Unbiased clustering and uniform manifold approximation and projection (UMAP)

analysis revealed eight distinct clusters. The two largest clusters comprised GC (n=49,459 cells) and mesenchymal cells of the ovarian stroma (n=46,703 cells). Furthermore, two clusters of corpora lutea (CL) were identified: one active CL cluster (n=21,867 cells) and one regressing CL cluster (n=9,101 cells). In addition, four smaller clusters of other cell types were identified, including endothelial cells, ovarian surface epithelial cells and two clusters of immune cells. The GC cluster segregated into five distinct subclusters, including preantral (n=5,596 cells), antral-mural (n=16,175 cells), mitotic (n=13,073 cells), atretic (n=14,172 cells), and an unknown subcluster (n=443 cells). This unknown subcluster was characterized by high expression of Ki67 and Cenpf, involved in cell proliferation, along with other granulosa cell markers (Fst, Slc18a2, Nr5a2, Inha). Apart from previously established markers, further transcriptomic profile analysis revealed potential novel gene signatures within each identified GC subcluster, necessitating further verification. Analysis of the cellular composition revealed a ~50% ($P < 0.01$) and ~30% ($P < 0.01$) increase in AMHKO-derived preantral and mitotic GC compared to WT mice, which may be consistent with the increased number of healthy small antral follicles in AMHKO mice. Also, the percentage of AMHKO-derived atretic GC was significantly increased compared to WT mice. In contrast, the percentage of AMHKO-derived active CL cells was decreased by ~35% ($P < 0.01$) compared to WT mice, suggestive of a suboptimal CL function. Investigations into single-cell transcriptional differences within each GCs cluster between WT and AMHKO mice are ongoing, as well as analysis of intercellular signaling networks using CellChat. In conclusion, our study shows that loss of AMH signaling alters the ovarian cellular distribution, which will aid in identifying differentially expressed gene in a cell-cluster specific manner.

T.6 - Assessment of Premature Reproductive Aging in IVF Female Offspring Using a Mouse Model

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The female reproductive system experiences early functional decline, affecting multiple physiological processes. Environmental factors may expedite reproductive aging, triggering primary ovarian insufficiency (POI). In vitro fertilization (IVF) has been linked to adverse placental and fetal outcomes. Considering critical reprogramming of primordial germ cells during development, IVF may influence their physiology. Our hypothesis is that IVF may alter ovarian development, impacting follicle formation and function. Utilizing an IVF mouse model, we aim to assess changes in IVF-offspring ovaries compared to a Naturally conceived group (Naturals) at embryonic day (E) E18.5 (12 Naturals/12 IVF, 5 litters), 12-weeks (12 Naturals/16 IVF, 5 litters), and 39-weeks (12 Naturals/15 IVF, 5 litters). At 12- and 39-weeks, IVF-offspring exhibit decreased ovary weight to body weight ratio, lower estradiol levels, and fewer small follicles. Additionally, at E18.5, 12-weeks, and 39-weeks ovaries display a reduction in global DNA methylation. Finally, at all three timepoints RNA sequencing revealed distinct gene clustering of IVF versus Naturals, with enriched affected pathways linked to follicle development, cell division, inflammation, autophagy, and chromosomal integrity. Finally, IVF-offspring exhibited smaller litter sizes and longer inter-pregnancy intervals compared to Naturals during four consecutive pregnancies. In conclusion, IVF-offspring, compared to Naturals, exhibited decreased ovary weight to body weight ratio, estradiol levels, primordial follicles, and global DNA methylation. Plus, changes in gene expression of pathways associated with follicle development, and changes in litter size (subfertility). Interestingly, most of these markers have been previously identified on POI. Future directions will identify cell-specific changes and developmental timepoint that are driven POI.

Poster Abstracts

D-1 - The oxidative metabolism dictates the histone lactylation levels during in vitro maturation of bovine oocytes

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The lysine histone lactylation (Kla) has emerged as a newly identified epigenetic marker, playing a crucial role in maintaining the transcriptional machinery of cells, with its reliance on lactate metabolism. Our latest findings reveal the presence of Kla and its regulation dependent on oxygen metabolism during the bovine morula and blastocyst stages. However, the existence of Kla and its relationship with oxygen regulation during oocyte maturation remains unknown. The objectives of this study were: 1) to confirm the existence of Kla in bovine oocytes and 2) to assess the global nuclear levels of Kla during the maturation of bovine oocytes cultured under two different oxygen tensions (low [5% O₂] or high oxygen tension [20% O₂]). For this purpose, during in vitro maturation (IVM), the oocytes were randomly assigned to two groups [5% O₂ or 20% O₂] and subsequently collected at 8 or 24 hours of IVM. The oocyte Mitochondrial Membrane Potential (MMP) was assessed using Mitotracker™ Red CMXRos dye (ThermoFisher), Reactive Oxygen Species (ROS) were measured using CellRox Green® (ThermoFisher), and the nuclear levels of Kla were determined through immunostaining with the polyclonal antibody for pan histone lactylation (PTM-1401). Image acquisition was performed with a fluorescence microscope, and subsequent analysis was conducted using ImageJ software. Data analysis was carried out using Student's t-test, with significance set at $p < 0.05$. We observed an elevated level of MMP in oocytes cultured in 5%O₂ compared to 20%O₂ at 8h of IVM ($p=0.0400$), but no significant differences were found at 24h of IVM ($p=0.4642$). ROS analysis did not reveal any alterations in either period of IVM (8h - 5%O₂ vs 20%O₂ - $p=0.4628$ / 24h - 5%O₂ vs 20%O₂ - $p=0.1777$). However, at 8h of IVM, both oxygen treatments led to increased MMP and ROS levels compared to 24h of IVM (MMP: 5%O₂ - 8h vs 24h - $p < 0.0001$; 20%O₂ - 8h vs 24h - $p=0.0097$ / ROS: 5%O₂ - 8h vs 24h - $p < 0.0001$; 20%O₂ - 8h vs 24h - $p < 0.0001$). Furthermore, we identified a positive correlation between MMP and ROS levels at 8h (5%O₂ - $p=0.0012$ - $r=0.4419$; 20%O₂ - $p=0.0139$ - $r=0.362$), indicating heightened oxidative metabolism activity at the beginning of IVM. The differences in oxygen tension did not influence oocyte Kla levels at 8h (5%O₂ vs 20%O₂ - $p=0.3882$) or at 24h (5%O₂ vs 20%O₂ - $p=0.5121$) of IVM. Upon closer examination, we observed an increase in KLA levels at 8h when compared to 24h of IVM in oocytes exposed to 20%O₂ (5%O₂ - 8h vs 24h - $p=0.0970$; 20%O₂ - 8h vs 24h - $p < 0.0001$), suggesting that the oxidative metabolism coordinates the global Kla levels during IVM. In summary, to the best of our knowledge, this study presents the first description of Kla in a bovine oocyte model. It proposes that variations in O₂ availability during IVM elicit alterations in oxidative metabolism, thereby influencing the lactylation levels, reinforcing the association between the metabolic status and the epigenetic mechanisms responsible for regulating embryonic transcriptional machinery during IVM. ACKNOWLEDGEMENTS: Federal University of ABC (UFABC), São Paulo Research Foundation (FAPESP) (2017/18384-0 and 2023/10575-2) and CAPES.

D-2 - Analytical measurement and microscopy of the Human Sperm Properties

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Details of the bio molecular and atomic construction of human sperm have not been profiled at this point. The current examination is meant to give a profound knowledge into the normal construction of human sperm. For this purpose, analytical and microscopic methods were applied. High-performance Liquid Chromatography (HPLC) and flowcytometry were used to quantify the DNA nucleotide components. As well fluorescent, confocal and advanced light microscopy were distinguished to identify the stained sperm DNA by chromomycinA3 (CMA3) and 5-methylcytosine antibody (5-mc). The mean upsides of nucleotide base rates in the construction of the sperm DNA went through HPLC sequenced by 27.6%, 8.92%, 27.05%, and 35.36%. Likewise, quantitative flowcytometry of global 5-methylcytosine showed an unpredictable vacillation in people with ordinary sperm while there is a lasting expansion in unique 50% percoll inclinations. The measure of CMA3-positivity levels adversely corresponded with sperm collect by percoll gradients ($p < 0.0001$) and positively associated ($P < 0.05$) with global methylation as dictated by flowcytometry. Curiously, in this content microscopy of stained cells showed an alternate perspective on the sperm head. These investigations propose some new substance levels of nucleotides and cytochemistry of sperm head structure. This investigation incites further examination of actuated new suppositions in atomic harmony and nuclear equilibrium in the axiom of the DNA ladder in human sperm.

D-3 - Non-Caloric Sweeteners are Associated with Decreased Sperm Viability and Morphology and Altered Testicular Gene Expression in Male Mice

Michelle Kearns, Clare Reynolds, Sabine Koelle, Caoimhe Neville

Infertility is global issue affecting approximately 15% of couples worldwide, with male infertility contributing up to 50% of cases. There is growing evidence that obesity can alter sperm parameters and negatively affect sperm function and induce epigenetic changes to spermatozoa. Non-caloric sweeteners (NCS) are widely consumed owing to their intense sweetness while providing minimal or no energy, however their detectability in different body tissues, including the reproductive system is unclear. Limited rodent studies have shown high NCS intakes can reduce sperm function, however most studies to date have looked at excessive NCS consumption. The present study was carried out to evaluate the impact of modest NCS intakes, in combination with High fat diet (HFD) on sperm parameters and glucose tolerance in male mice. 32 adult male mice were randomly assigned to a control diet (CD) (10% kcal fat) or HFD (45% kcal fat) and assigned to 8 groups of 4 mice each and received either water (control), fructose (20% solution in water) or NCS (Ace-k at a concentration of 12.5mM solution in water or Reb A at a concentration of 1mM solution in water). Mice were weighed weekly, and food and solution intakes were monitored. Following 8 weeks, mice were humanely killed. Testes were weighed and sperm isolated from the cauda epididymis and parameters, including sperm viability and morphology were evaluated. Sperm abnormal morphology and live viability were evaluated for at least 200 spermatozoa of each animal. The percentage of morphologically abnormal spermatozoa and viable sperm were assessed by Eosin and Eosin-Nigrosin staining respectively and imaged using light microscopy. RNA was extracted from testes. The expression of 84 genes relevant to male infertility was analysed using the Mouse Male Infertility RT2 Profiler PCR Array (SABioscience). Following 8 weeks on the diet, HFD mice gained significantly higher body weight than CD mice ($p < 0.001$) with a mean body weight of $30.1 \text{ g} \pm 1.0$ for CD and 33.7 ± 1.4 for HFD mice. Following NCS consumption, mice had increased abnormal

sperm morphology (Ace-k 68.0 ± 9.2 , Reb-a 78.3 ± 10.3) compared to CD fed mice (CD control 59.6 ± 6.8 , HF control 64.2 ± 9.0). Sperm viability decreased in CD NCS groups (Ace-k 27.9 ± 1.1 , Reb-a 39.9 ± 11.2) compared to the CD Con group (43.2 ± 8.5), however sperm viability increased in both HFD NCS mice (Ace-k 38.1 ± 0.6 , Reb-a 46.6 ± 4.0) compared to CD NCS fed mice. Among the CDRebA groups there was downregulated expression of Boll gene (0.24 ± 0.01 , $p < 0.05$) and HFCon downregulated expression of Akap14 gene (0.5 ± 0.04 , $p < 0.05$). This study has shown that NCS consumption reduced sperm parameters and testicular gene expression in male mice. Furthermore, consumption of a HFD had a protective effect on sperm viability in mice consuming artificial sweeteners compared with control and fructose groups. HFCon mice had downregulated expression of Akap14, thought to play a role in sperm motility. Our results indicate that modest consumption of artificial sweeteners may induce negative effects on sperm parameters in mice, however these negative effects on sperm viability may be reduced with the addition of a high fat diet. Funding is acknowledged from the Ad Astra Fellowship Programme, UCD; Funding is acknowledged from the UCD Wellcome Institutional Strategic Support Fund, which was financed jointly by University College Dublin and the SFI-HRB-Wellcome Biomedical Research Partnership (ref 204844/Z/16/Z)

D-4 - Innovations in 3D Culture Systems for the Study of Sperm-Oviduct Interactions

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The oviducts play a crucial role in reproduction by connecting the ovary to the uterus, facilitating the fertilization of the oocyte, and initiating embryonic development. Understanding the cellular processes that occur during the transport of gametes through the oviduct is essential for improving the reproductive efficiency of these animals. In this context, the present study proposes a magnetic 3D culture system to understand the interaction between the isthmus and bovine sperm, aiming to enhance the reproductive efficiency of these animals. For this purpose, epithelial and stromal cells from the bovine oviduct were separately cultured in a monolayer system and magnetized by centrifugation using nanoshuttle-PL and the magnetic 3D system [Greiner Bio-One CELLSTAR, Germany] for spheroid formation. In experiment 1, our goal was to determine the spheroids responsiveness to steroid hormones mimmetizing the estrous cycle. For that, the spheroids were divided into two experimental groups with different hormonal treatments [Control (no hormone) Progesterone-100 ng/mL 14 days or Progesterone-100 ng/mL 11 days followed by Estradiol-300 pg/mL for 3 days] and subjected to cell viability analysis using Hoescht and Propidium Iodide staining (Sigma-Aldrich). In experiment 2, our goal was to verify if the method of semen processing would affect the ability of sperm cells to adhere to the spheroids. For that, frozen semen from one bull were submitted to two different seminal processing methods (Swim-up in TALP medium, supplemented with Hyaluronic Acid (10 μ g/mL HA), or Percoll discontinuous gradient 90/45%) and stained with Mitotracker Red CMXRos (ThermoFisher) to stain the middle piece of the sperm cell and allow its identification after attachment to the spheroid. Sperm cells (3×10^6) were co-cultured with one spheroid for 12 hours and the number of red dots were assessed by fluoresce microscopy. For both experiments, images were analyzed in ImageJ software and groups were compared by ANOVA followed by LSD's test (experiment 1) and Student's t test (experiment 2). In experiment 1, both hormonal treatments showed a significant response of the spheroids, and the treatment using

Progesterone and Estradiol together demonstrated a lower proportion of necrotic cells compared to Progesterone alone [$P < 0.0008$]. In experiment 2, both sperm processing resulted in the same adherence capability to the spheroid [$p=0.14$]. This finding suggests that spheroids generated by our system present responsiveness to hormonal treatments and allow sperm adherence in vitro. The development of this study model may bring significant advances in the reproductive efficiency of cattle. Financial support: FAPESP 2019/25982-7, 2021/11747-6, 2024/04153-0.

D-5 - Arginine Methylation Plays a Critical Role in Bovine Preimplantation Development

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Protein arginine methyltransferase 1 (PRMT1) is a key enzyme responsible for catalyzing the asymmetric dimethylation of arginine residues on histone and non-histone proteins. The methylation of substrates occurs within glycine and arginine-rich domains, relying on the association of PRMT1 with co-regulators, which leads to crosstalk with other post-translational modifications on lysine (K) in histones. PRMT1 and asymmetric arginine dimethylation (me2a) are highly expressed in bovine oocytes and preimplantation embryos. To assess the role of PRMT1 in early development, we disrupted PRMT1-mediated arginine methylation with pharmacological methods in bovine embryos. When the inhibitors successfully reduce PRMT1-mediated H4R3me2a, we observed a significant reduction in the recruitment of the histone marks H3K27 trimethylation and a significant enhancement increase in the levels of H3K9 acetylation, suggesting an impact on chromatin dynamics through crosstalk. Of note, inhibition of PRMT1 also resulted in reduced expression of SOX2, a vital transcription factor for embryonic development. Despite blastocyst rates in bovine embryos being unaffected by PRMT1 inhibition, a notable decrease in the cell counts of total cells, inner cell mass (ICM) cells, and trophectoderm (TE) cells was observed. Furthermore, PRMT1 disruption also reduced depletion of glucose and pyruvate during the blastocyst stage, indicating altered metabolic activity in the presence of PRMT1 inhibitors. In summary, our findings reveal an important role of PRMT1 in orchestrating the intricate regulatory network governing the epigenetic landscape and gene expression profile of pre-implantation bovine embryos. The inhibition of PRMT1 activity not only disrupts histone modifications but also impacts transcription factor expression and metabolic processes, ultimately influencing embryonic cell fate determination and metabolic competence.

D-6 - Maternal Obesity During Pregnancy: Impacts on pancreatic proteome of offspring

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Maternal obesity, resulting from an excess- of nutrition during pregnancy, can impair development of the pancreas in the offspring and result in an increased incidence of diabetes.. Proteomic analysis of the cellular processes, signaling pathways, and molecular mechanisms underlying these changes could offer insight about these conditions. We hypothesize that maternal obesity during pregnancy leads to reduced expression of proteins associated with β -cell function and increased expression of proteins associated with cellular apoptosis. C57BL/6J (N&#3f 6) female mice were fed either a high fat diet (HFD; n= 3; TD.07011 Pellet Envigo, Somerset, NJ) or a control

diet (CON; n=3) for fourteen weeks prior to breeding and for the duration of pregnancy and lactation. All pups from both treatment groups were weaned at 4 wks of age and maintained on a CON diet regardless of maternal treatment. The pups were euthanized at 8 wks of age. Proteomics analysis of the pancreatic tissue from the pups was performed via ultra-performance liquid chromatography-tandem mass spectrometry Q Exactive HF mass spectrometer; ThermoFisher Scientific, Waltham, MA; University of Connecticut Proteomics and Metabolomics Facility, Storrs, CT). The number and identity of differentially expressed proteins were determined using the average precursor intensity in Scaffold v5.3.2 ($P \leq 0.05$; Proteome Software, Inc; Portland OR). A total of 2329 proteins were identified in each of the treatment comparisons for CON vs HFD male and CON vs HFD female offspring. A subset of 165 differentially expressed (DE) proteins were parsed from these groups (CON vs HFD male: 145; CON vs HFD female: 20; $P \leq 0.05$) for further evaluation. In the male offspring, 14 DE proteins were up-regulated, whereas 131 were down-regulated in HFD and CON males, respectively. In contrast, of the 20 DE proteins identified in female offspring, 13 were up-regulated and 7 were down-regulated in HFD and CON females, respectively. Following further assessment of the above-described DE proteins, four were identified as common in both the male and female offspring (Mgat2: Alpha-1,6-mannosyl-glycoprotein 2-beta-N acetylglucosaminyltransferase; Lama2: Laminin subunit alpha-2; Isyna1: Inositol-3-phosphate synthase 1, and Got2: Glutamic-oxaloacetic transaminase 2). Among these, expression of Mgat2, Isyna1, and Got2 decreased in HFD males but increased in HFD females compared to their respective CON offspring. Conversely, expression of Lama2 increased in both males and females. Additionally, in male HFD offspring, there was reduced expression of proteins involved in the regulation of cellular apoptosis. Several of these proteins are needed to inhibit apoptosis (Ppib: Peptidyl-prolyl cis-trans isomerase B; Mcts1: Malignant T-cell-amplified sequence 1) while others induce apoptosis (Hrg: Histidine-rich glycoprotein; Hspa1: Heat shock 70 kDa protein 1; and Mecr: Enoyl-[acyl-carrier-protein] reductase, mitochondria). Proteins implicated in mitochondrial function and insulin secretion, such as mitochondrial fission 1 protein (F1S1), vesicle-fusing ATPase (NSF), and malate dehydrogenase, mitochondrial (Mdh2), exhibited reduced expression patterns in male HFD offspring. Pathway analyses indicated that the greatest proportions of DE proteins were associated with metabolic pathways. Notably, a total of 6 DE proteins in HFD vs CON males involved multiple neurodegenerative pathways. The results support our proposed hypothesis, and suggest that excess nutrition during pregnancy impacts pancreatic regulatory mechanisms in the offspring.

D-7 - PCOS Associated Gene TOX3 Regulates Androgenic Cell Differentiation in Chicken Gonads

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Polycystic ovary syndrome (PCOS) stands out as a prevalent cause of female infertility, impacting approximately 10% of women during their reproductive years. Despite its high incidence, the precise etiology of PCOS remains incompletely understood, involving a complex interplay of genetic and environmental factors. While rodent models have historically been utilized for PCOS

research, they do not naturally develop PCOS-like phenotypes. Conversely, hens develop spontaneous Polycystic Ovarian Condition (PCOC), rendering them a promising model for studying this condition. PCOC in layer hens is linked with ovarian inflammation, which affects egg laying/ovulation rates and raises concerns for animal welfare and economic implications within the egg industry. Unlike mammals, chicken gonads are steroidogenically active during early embryonic stages. Embryonic chicken ovaries not only harbor estrogenic cells but also contain a higher proportion of androgenic cells compared to embryonic testes. We have identified the PCOS-associated gene TOX3 as a novel transcription factor expressed in embryonic Sertoli cells of the chicken embryo testis. Our study focuses on characterizing the expression pattern of TOX3 in developing chicken gonads, elucidating its regulatory mechanisms, and exploring its potential role in gonadal supporting and steroidogenic cell differentiation. TOX3 knockdown resulted in an increase in CYP17A1-positive androgenic cells. Conversely, TOX3 overexpression in both male and female gonads led to a significant reduction in CYP17A1-positive androgenic cells. Furthermore, TOX3 expression was found to be negatively regulated by estrogens *in vivo*, while it was not induced during estrogen inhibition-induced masculinization. These findings suggest a model where TOX3 modulates the androgenic cell population, and its dysregulation may contribute to increased steroidogenic capacity, leading to PCOC/PCOS. In addition, the chicken embryo may serve as a tractable model for studying the etiology and progression of PCOC to identify molecular players and treatment avenues.

D-8 - Differential Transcript Usage in Bovine Blastocysts Exposed to Varying Concentrations of Energy Substrates

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According to the Developmental Origins of Health and Disease (DOHaD), energetic imbalance during critical developmental periods may impact long-term the offspring health, predisposing to the development of cardiovascular and metabolic diseases in adulthood. While epigenetic processes have been proposed as bona fide mediators of DOHaD, the mechanisms establishing its early onset remain poorly understood. Recently, the expression of different transcript isoforms of given genes, defined as differential transcript usage (DTU), has been observed during development and implicated in the onset of various diseases, making DTU a possible player in the landscape of developmental plasticity. Notably, transcript isoforms can arise by different mechanisms, among which change of the transcription start/end sites, alternative splicing, or activation/repression of different promoters. Consequently, functionally different transcript isoforms are produced, carrying changes in the coding sequence or the regulatory, untranslated regions. Therefore, even in the absence of noticeable changes in gene expression, the production of two or more transcript isoforms of a gene can exert biological effects. DTU, however, goes mainly undetected when routinely analyzing RNAseq datasets if specific pipelines are not applied. With this in mind, we aimed to analyze the expression of transcript isoforms in bovine blastocysts developing in varying concentrations of energy substrates. The preimplantation embryo development has been chosen as the window of exposure for the intense genome reprogramming and epigenetic remodeling to

capture the earliest events of DOHaD. Bovine ovaries were obtained from a slaughterhouse and cumulus-oocyte complexes were collected from follicles of 2-8 mm in diameter. After in vitro maturation and in vitro fertilization with X-sorted semen, zygotes were cultured in a serum-free system with varying concentrations of energy substrates, i.e. increasing or decreasing concentrations of glucose, pyruvate, lactate, citrate, and amino acids. Expanded blastocysts (pools of 5 blastocysts/group, 3 biological replicates) were harvested for RNA extraction giving a minimum yield of 289 pg/μL and RIN >8.6 and sequenced on an Illumina platform. Transcript levels were quantified and genes having multiple transcript isoforms were filtered with DRIMSeq. Subsequently, DEXSeq was employed to distinguish which of these isoforms were differentially expressed (adjusted $p < 0.1$). Almost 6000 genes with multiple isoforms were identified, of which approximately 90 had differential isoform expression in response to varying concentrations of energetic levels, for a total of 250-280 isoforms in the decreased and increased energy substrates, respectively. Enrichment analysis conducted to understand which molecular functions were mainly affected by energy supplementation revealed a general implication of 'palmitoyl-CoA' and 'acyl-CoA hydrolase activity' that seem concordant with the experimental model. Notably, other molecular functions involved were 'histone deacetylase binding' in the condition of increased supplementation and 'transcription factor binding' when energy levels were decreased, pointing at possible interferences with epigenetic and nuclear reprogramming mechanisms. To validate these observations, the acetylation of histone residues was monitored by immunofluorescence and confocal microscopy on expanded blastocysts (7-8 blastocysts/group, 2 biological replicates), revealing a 25-50% increase in the relative intensity of the acetylated signal when embryos were cultured with higher energetic substrates ($p < 0.01$, unpaired T-test, two-tailed). Overall, these observations contribute to a deeper understanding of how energetic excess impacts early embryonic development at the molecular level, showing for the first time the implication of DTU in the early onset of epigenetic modifications that might lead to DOHaD. Financial support: MIUR PRIN2017, No. 20172N2WL3_002 (DeSTINE); MUR PRIN2020, No. 20209L8BN4 (InfinitEGG)

D-9 - Developmental Patterns and Gene Expression of Bovine Blastocysts Exposed to Imbalanced Energetic Levels

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The Developmental Origins of Health and Disease (DOHaD) states that an imbalanced maternal diet has long-term effects on the health of the offspring. In humans and animal models, an inadequate nutrient supply during pregnancy may impact post-natal life, predisposing to cardiovascular and kidney diseases, metabolic disorders, and poor female fertility in the absence of overt developmental defects. Given the extensive genome and epigenome reprogramming during the preimplantation embryo development, in this study we asked if exposure to nutritional imbalance during this window triggers changes in developmental patterns and gene expression in the blastocyst. We modelled this stage using in vitro bovine embryo production to strictly control the environmental conditions and reduce the number of animals used for experimentation. Furthermore, the amount of in vitro produced bovine embryos has increased exponentially in the

last decades, surpassing the number of embryos produced in vitro since 2016, posing the need to carefully evaluate the long-term safety of embryo culture. Bovine blastocysts were produced in vitro from abattoir-derived oocytes using X-sorted semen. The zygotes were cultured for 8 days in a modified, serum-free culture medium. The composition of the medium was changed to contain either 0.5 (energetic restriction) or 1.5-fold (energetic excess) of the standard content of energetic substrates. RNA was extracted from a pool of 5 expanded blastocysts per treatment and amount and quality were assessed by BioAnalyzer. Three biological replicates per experimental group with a minimum RNA amount of 289 pg/μl and RNA integrity number 8.6-10 were used in downstream procedures. Libraries were prepared and sequenced on NovaSeq 6000 generating 150 bp paired-end reads. Sequenced reads were mapped and quantified using Salmon or HISAT2/HTSEQ. Differential gene expression analysis was performed in R using DESeq2 package. Genes with adjusted $p < 0.1$ were considered differentially expressed. Markers of cell lineage specification, SOX2, CDX2, and NANOG, were traced by immunofluorescence. The obtained blastocysts were also characterized morphologically by recording the overall blastocyst rate and morphology, and nuclei counting. Principal component analysis evidenced that increased levels of energetic substrates induced a clear clustering compared to the control, in contrast to energetic restriction. Furthermore, only a few genes were differentially expressed, without substantial differences between the two bioinformatic pipelines, when conducting paired comparisons. When considering gene expression patterns across the 3 treatments, 2 patterns were generated: increasing and decreasing expression along with increased energy substrates. Consistent with these findings, the blastocyst rate, comprising the expansion and hatching rates, the number of cells, and the expression of the first cell lineage markers were comparable. A lack of major disruption of the expression profile and normal development patterns seem in line with previous observations made in vivo that a mild nutrient restriction in the mother does not prevent the development to term and the birth of healthy-looking offspring, which will only be affected later in life. We are therefore tempted to speculate that at this stage of development embryo plasticity allows to adjust in response to energetic imbalance. However, such adjustments might come at the cost of heritable alterations of the epigenetic profiles that will impact cells and organ functions later in life. For instance, overexpression of MUC13, as consistently observed in the energetic excess treatment, has been linked with changes in the methylation levels of its promoter and with higher glucose uptake.

D-10 - Maternal Obesity During Pregnancy Impacts Pancreatic DNA Methylation and Protein Expression in the Offspring

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It has been postulated that DNA methylation is one of the mechanisms by which developmental programming causes long term, multi-generational impacts on the developing offspring. However, the role this molecular mechanism has in mediating the effect of maternal obesity on the offspring's pancreas is limited. We hypothesize that maternal obesity during pregnancy will result in changes to offspring pancreas DNA methylation that would be reflected as corresponding changes in protein expression. C57BL/6J (N&#3f 6) female mice were fed a high fat diet (HFD; n = 3; TD.07011 Pellet Envigo, Somerset, NJ) to induce obesity or a control diet (CON; n = 3) for fourteen

weeks prior to breeding. These animals also remained on their treatment diets for the duration of pregnancy and lactation. Pups were euthanized at 8 wks of age and pancreas tissue was collected. Pancreas tissue DNA was isolated and whole genome bisulfite sequencing was performed at Genewiz (Illumina Hi-Seq; Azenta, Cambridge, MA). Data were analyzed in conjunction with CD genomics (New York, NY). Read alignment to the *Mus musculus* reference genome (mm39) were performed using Bismark (v0.24.2; Babraham Bioinformatics). Differentially methylated regions (DMR) and the associated locations were determined using methylKit v3.18 (Bioconductor; $q \leq 0.05$). Proteomics analyses were performed via ultra-performance liquid chromatography-tandem mass spectrometry (ThermoFisher Scientific Q Exactive HF mass spectrometer; University of Connecticut Proteomics and Metabolomics Facility, Storrs, CT). The number and identity of differentially expressed proteins were determined using the average precursor intensity in Scaffold v5.3.2 (Proteome Software, Inc; Portland OR). From our analyses we were able to identify 41,515 and 44,958 DMR for CON vs HFD males and females respectively ($q \leq 0.05$). 30.23% of the top 25% of DMR were hypomethylated whereas 69.76% were hypermethylated in CON vs HFD females. Likewise, 39.61% of the DMR identified were hypomethylated and 60.39% of DMR were hypermethylated in CON vs HFD males. We were able to identify a subset of proteins in our treatment comparisons whose change in expression match the change in methylation status for that DMR. 3 targets (heparan sulfate proteoglycan 2, collagen type VI alpha 2 chain, and collagen type IV alpha 2) exhibited a reduction in DNA methylation that corresponded with an increase in protein expression in CON vs HFD males. 31 targets in the CON vs HFD males exhibited a reduction in protein expression that corresponded with increased DNA methylation of the DMR. For CON vs HFD females, Cathepsin A expression was reduced while there was increased DNA methylation at an associated DMR. Three proteins (immunoglobulin heavy constant gamma 1, isopentenyl-diphosphate delta isomerase 1, and glutamic-oxaloacetic transaminase 2) exhibited increased expression while having reduced DNA methylation at an associated DMR. In conclusion, we have demonstrated that there is a relationship between the DMR identified and changes in the pancreatic protein expression of the offspring from HFD-fed dams. This multi-omic approach has provided valuable insight into the interplay between epigenetics, maternal diet, and the pancreas tissue of the offspring.

D-11 - Epigenomic Crosstalk in H3K27ac Regulation and RNAseq: An Integrative Approach Assessing Metabolically Modulated Bovine Embryos

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In the intricate embryonic development dynamics, the chromatin landscape and the expression patterns are tightly coordinated responding to metabolic cues that shapes the fate of the implantation embryo. This study delves into the molecular choreography of bovine embryos, elucidating the interplay between the H3K27ac epigenetic marker and gene expression profiles under the influence of metabolic modulators - DCA (dichloroacetate, a pyruvate analog that increases the pyruvate to acetyl-CoA conversion) and IA (iodoacetate, which inhibits the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), leading to glycolysis inhibition), from day 5 of culture. Both modulations were reported previously as affecting targeted metabolic pathways and acetylation levels for H3K27. The goal of this study was to integrate results from RNASeq and H3K27ac Cut&Tag (Cleavage Under Targets and Tagmentation) of inner cell masses from

metabolically modulated bovine embryos and find crucial relationships between the datasets. For this, in vitro produced bovine embryos were cultured from day 5 in 3 experimental groups [synthetic oviductal fluid with amino acids (SOFaa) + 4% bovine serum albumin]: Control (CO - no additional supplementation), DCA (addition of 2mM of DCA) or IA (addition of 2µl of IA). Blastocysts were collected on Day 7 and their inner cell masses were submitted to RNASeq and Cut&Tag analysis. 3 ICM per group per replicate (3 replicates) were used for RNA extraction or for the Cut&Tag procedure. Samples were sequenced by the Illumina Genome Analyzer II (NextSeq PE40 high output). Results from both datasets were submitted to quality control with FastQC, genome alignment (Bos_taurus_UMD_3.1.1, NCBI) was done by Bowtie2, normalization and differential expression measurement of RNASeq data was performed by Deseq2. Peak-calling was done with MACS2 and the genomic annotation by ChipSeeker. Regarding the RNASeq dataset, comparing to CO, 855 transcripts were found differentially expressed in DCA, while 659 were found in IA. Assuming that H3K27ac is described as a permissive mark, the subsequent step was to consider unique peaks from each group confronted with DEGs, to retrieve the regulatory behavior among group comparisons. Therefore, for CO vs. DCA, 126 matches were found between CO peaks and CO upregulated DEGs, mainly associated with translation activity, mRNA processing and protein folding, while 184 matches were present between DCA peaks and DCA upregulated DEGs, linking to glucose homeostasis, chromatin organization, cell differentiation and positive regulation of transcription from RNA polymerase, making up to 36% of complementary IDs across datasets. Concerning the contrast between CO vs. IA, 118 IDs were found upregulated in IA together with their mirroring peaks, aligned with molecular functions such as DNA topological change, mitochondrial ATP synthesis, positive regulation of DNA replication and translation. Along with 119 matches between upregulated DEGs in CO and their counterpart peaks pointing to in utero embryonic development, positive regulation of embryonic development and regulation of transcription from RNA polymerase II promoter, equally accounting for 36% of compatible IDs. This integrative approach revealed the important role H3K27ac in controlling gene expression and reinforces the metabolism as a key regulator of epigenomic remodeling during the early embryogenesis. Acknowledgements: Federal University of ABC (UFABC), Sao Paulo Research Foundation (FAPESP, grant numbers: 2019/25982-7 and 2022/14472-0)

O-1 - Investigating Ribosome Dynamics in the Aging Mammalian Oocyte

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The female reproductive system is the first system to experience age-dependent functional decline in mammals. In humans, advanced maternal age severely compromises the likelihood of successful conception and pregnancy, due to decreased fertility and increased risks of pre-eclampsia, miscarriage, birth defects, and pre-term birth. This age-dependent decline has become a significant concern to society, as more women than ever are delaying childbearing until their advanced maternal years. Oocyte quality is the greatest barrier to fertility at advanced maternal age. Though the impact of oocyte quality is appreciated, our understanding of its molecular culprits is limited. One aspect of oocyte quality that remains understudied is translation, the production of proteins by macromolecular machines called ribosomes. Oocytes are unique in that they rely entirely upon ribosomes to modulate gene-regulation during meiotic maturation and the oocyte-to-embryo transition. Furthermore, the oocyte ribosomes provide for the zygote until zygotic ribosome

biogenesis becomes active. Ribosomes are therefore an important - but poorly characterized - factor of oocyte quality. If ribosome production or activity is perturbed with age, oocyte quality would be significantly compromised. To address this gap in knowledge, we conducted single-cell RNA sequencing from oocytes isolated from reproductively young and old female mice. The pathways identified to be the most enriched with age were those associated with ribosome biogenesis and translation. We then measured the levels of ribosomal RNA (rRNA), the rate limiting factor of ribosome biogenesis, within oocytes. As expected, we found that rRNA also significantly increases in oocytes with age. This observation compliments the finding that cytoplasmic ribosome populations in oocytes significantly increase with age. To determine the functional output of these ribosomes, global translation assays were conducted in germinal vesicle oocytes from reproductively young and old mice. The results revealed significantly lower translation levels in old oocytes. Taken together, we surmise that ribosome numbers increase while translation decreases. These paradoxical findings point to translation perturbations within the aging oocyte that warrant further investigation. We hypothesize that as oocytes age, the regulation of ribosome production and translation are compromised. Continued investigation of age-associated changes in ribosome production and translation activity in oocytes will provide new insight to oocyte quality and how it changes with age.

O-2 - Mechano-Hydraulic Control of Mammalian Ovarian Follicle Development

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The formation of functional eggs in the ovarian follicles is a critical process of early mammalian development. While genetic studies have revealed key genes for oocyte functions, the underlying mechanisms driving follicle growth remain enigmatic. Recent studies showed that follicle growth is sensitive to mechanical environment, calling for a need to understand mechanical signalling within follicles. Here, we investigate the mechanical functions of theca cells (TCs), which sit on a basement membrane encapsulating the follicles. Using a combination of biophysical, bioengineering, molecular and ex vivo approaches, we demonstrate that the TCs are highly contractile and exert active compressive stress to tune follicle pressure and mechanics, thereby regulating somatic cell signalling and follicle growth. We further found that the TC proliferation, migration and hormonal activities are mechanosensitive to stiffness, stretch and curvature, suggesting that changes in follicle geometry and basement membrane mechanics may in turn regulate TC functions in vivo. We hypothesise that such mechanical feedback is a key design principle for regulating follicle growth and oocyte functions through mechanotransduction pathways. Another long-standing question in the field is the mechanism driving the formation of fluid-filled cavity (lumen) in the antral follicle stage, failure of which is known to lead to infertility. Using label-free biophotonics and machine learning, we were able to, for the first time, characterise the spatiotemporal dynamics of lumen growth and cell motion during this phase. Our work will have profound implications for understanding infertility to help improve follicle health and IVF/IVM outcomes. References: 1. Biswas et al. Squeezing the eggs to grow: The mechanobiology of mammalian folliculogenesis. *Front. Cell Dev. Biol.* (2022) 2. Chan CJ, Bevilacqua C, Prevedel R. Mechanical mapping of mammalian follicle development using Brillouin microscopy. *Comm.*

Biology (2022)3. Chan CJ, Hirashima T. Tissue Hydraulics in Reproduction. Semin. Cell Dev. Biol. (2022) Keywords: Folliculogenesis, ovarian follicles, mechanical stress, tissue mechanics, theca cells Supported by: Ministry of Education, Singapore; National University of Singapore; National Research Foundation, Singapore; Bia-Echo Asia Centre for Female Reproductive Longevity & Equality at the National University of Singapore.

O-3 - Long-Lived Rodents as Emerging Model of Long-Term Female Fertility in Mammals

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Mammalian oocytes are believed to be established during prenatal development as a finite pool. They are essential for propagation of species yet are among the oldest cells in the body – they can be several decades old in long-lived species. Moreover, number of oocytes decreases with age, until ovulation ceases permanently. The suboptimal quality and quantity of oocytes, interconnected with the declining properties of their ovarian niche, are among main factors causing female age-associated fertility decline. This is mostly studied in laboratory mouse, however, growing number of studies reveal remarkable differences when other species are explored. African mole-rats and blind mole rats are two distinct groups of rodents that are subterranean, long-lived and with long female reproductive span. While blind mole rats are solitary, African mole-rats live in a social system where only one female breeds and ovulates, while the remaining females are non-ovulating non-breeders. This provides a unique opportunity to study how these characteristics impact oocyte properties, age-associated quality decline over long fertility span, the effects of ovulations, and how they evolved within rodent lineages. We identified unique characteristics of mole-rat oocytes and their ovarian niche. These comprise differences in the oocyte transcriptome such as the absence of epigenetic factors considered essential for mammalian oocytes, different transposon expression, high genome integrity during aging, human-like meiotic spindles, and resistance to inflammaging, age-associated chronic pro-inflammatory response. Moreover, we revealed that repeated ovulations play an important role in oocyte quality decline, and that their ovaries comprise large ovarian reserve of early oocytes.

O-4 - The Impact of Leukocyte-Derived Cytokines on Intracellular Signaling Cascades in Human Periovulatory Granulosa Cells

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Mid-cycle gonadotropin-induced ovulation, a pivotal event in female reproductive physiology, shares many features with an acute inflammatory response, notably marked by a massive influx of leukocytes into the dominant follicle. Despite this, the precise impact of leukocytes on the ovulatory process remains largely elusive. Our previous finding, derived from single-cell RNA-sequencing (scRNA-seq) analysis using follicular aspirates obtained from in vitro fertilization (IVF) patients has revealed the presence of ten distinct leukocyte subpopulations. Based on the single-cell transcriptomic datasets, we found that inflammatory cytokines – e.g., IL1B, IL10, IL16, IFNG,

TNF, LTA, and LTB – were specifically expressed in leukocytes, while the expression of their corresponding receptors – IL1R1, IL1RAP, IL10RA, IL10RB, CD9, IFNGR1, IFNGR2, TNFRSF1A, and LTBR – was abundantly detected in follicular (granulosa and theca) cells. To demonstrate the action of the leukocyte-derived cytokines and their cognate receptors in granulosa cells, primary human granulosa-lutein cells (hGLCs) were treated with $\pm$ human chorionic gonadotropin (hCG, 1 IU/ml) $\pm$ recombinant human (rh) protein of leukocyte-derived cytokines for 36 hours. Western blot analysis was performed using the cultured hGLCs to assess whether leukocyte-derived cytokines activate intracellular signaling pathways in periovulatory granulosa cells. Treatment of rhIL1B (1, 5, 10 ng/ml), rhIFNG (1, 5, 10 ng/ml), rhTNF (1, 5, 10 ng/ml), and rh1LTA:2LTB (a heterotrimer of LTA and LTB; 10, 20, 50 ng/ml) increased the levels of phosphorylated IRAK4 (pIRAK4), pSTAT1, pIKK α/β , and pRelA, respectively, which are key mediators for MYD88, STAT1, and canonical Nk κ B signaling pathways. These in vivo and in vitro findings showed that specific cytokines and their corresponding receptors are expressed in leukocytes and follicular cells, respectively. Furthermore, these cytokines activated specific intracellular signaling pathways in granulosa cells expressing their receptors. Collectively, this study suggests that leukocyte-derived cytokines regulate granulosa cell functions by activating specific signaling pathways, thereby potentially facilitating the ovulatory process in the human ovary. (Supported by P01HD71875, R03HD095098, and R01HD096077)

O-5 - The Nuclear Envelope in Regulation of Oocyte Developmental Competence and Mechanotransduction

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Nuclear Envelope Membrane Protein 1 (Nemp1) is a highly conserved transmembrane nuclear envelope protein required for fertility in *Drosophila*, *C. elegans*, zebrafish, and mice (Tsatskis et. al, 2020). Human genome-wide association studies show an association between Nemp1 and early menopause. The mammalian ovary is composed of a mechanically stiff, highly cross-linked cortex filled with primordial follicles that constitute the ovarian reserve. Nemp1KO female mice have a drastically reduced ovarian reserve. Loss of ovarian reserve can lead to ovarian failure that results in premature menopause. Atomic Force Microscopy (AFM) and micropipette aspiration technique revealed that NEMP protein supports the mechanical stiffness of the nucleus in cell lines and Nemp1KO oocytes respectively (Tsatskis et. al, 2020). These data led us to hypothesize that Nemp1 has a role in mechano-transduction. Here I present data indicating that loss of NEMP1 leads to a germline checkpoint, mediated by ATM and CHK2, and that loss of NEMP1 leads to DNA damage and premature aging

O-6 - Virilizing Testosterone Decreases Pre-Antral Follicle Size and Oocyte Mitochondrial Volume in a Transgender Male Swine Model

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There are roughly 1.6 million transgender Americans, 80% of whom are on gender-affirming hormone therapy. Although transgender men (female $\rightarrow$ male) are counseled to undergo fertility preservation prior to initiation of testosterone hormone therapy, a large percentage of transgender youth do not undergo fertility preservation prior to beginning treatment. There are conflicting data in the human literature on the impact of virilizing testosterone on ovarian antral follicle size and number, and no information exists on the effect of virilizing testosterone on pre-antral follicle

classes or oocyte mitochondria. The objective of this pilot study was to assess the impact of virilizing testosterone on ovarian follicle size and number and on oocyte mitochondrial volume and number in a transgender male swine model. Three sexually mature female pigs were housed individually, were fed 1.5 kg of feed daily, and were heat checked daily. Testosterone enanthate (TE) was administered via silastic implants as follows: placebo (0 g TE, n=1), transgender male (2.24 g TE, n=2). Fasting blood samples for measurement of androgens and estrogens by LC-MS/MS were collected on days 1-3 and then every three days post-implantation for 42 days. Ovaries were collected and fixed in 4% paraformaldehyde, and H&E histology was performed for follicle counts, whereas transmission electron microscopy was performed for oocyte mitochondrial assessment. An individual blinded to treatment assessed follicle and oocyte mitochondrial counts and size in Qupath software. Follicles were classified as primordial, primary, secondary, tertiary, or tertiary-atretic. Each pig had three separate images analyzed for follicles and oocyte mitochondria, respectively. Serum hormone measurements were analyzed with repeated measures in time ANOVA. The non-parametric Mann-Whitney test was used to analyze ovarian and oocyte morphology and count measurements. The level of significance was set at 0.05 for all statistical tests. Serum testosterone levels consistent with those of transgender men were achieved with the 2.24 g TE dose (transgender male pigs). Transgender male pigs remained in anestrus during the study. Serum estrogens, androstenedione, and dihydrotestosterone were not different between placebo female and transgender male pigs. Serum dehydroepiandrosterone (DHEA; a weak androgen) was lower in transgender male pigs compared to the placebo female pig. Transgender male pigs had greater numbers of primordial, antral, and atretic antral follicles than the placebo female pig. However, with respect to follicle size, transgender male pigs had smaller primary and secondary follicles than the placebo female pig. Ongoing histology image analysis is focused on granulosa and theca cell size in primary and secondary follicles and the number of granulosa cell layers in secondary follicles. Although the number of oocyte mitochondria did not differ between treatment groups, the oocyte mitochondrial volume was decreased for transgender male pigs as compared to the placebo female pig. These results suggest that virilizing testosterone adversely affects aspects of ovarian folliculogenesis and markers of oocyte health that may negatively impact the fertility of transgender men wishing to conceive. Future research will focus on replicating these findings in a larger cohort of pigs and exploring whether testosterone withdrawal reverses these effects.

O-7 The Effects of RGD and Hedgehog Ligands on Theca Cell Differentiation in Three-Dimensional Culture System

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Bacterial-derived dextran is a bioinert, nontoxic, and biocompatible polysaccharide. Therefore, dextran-based hydrogels have been widely used in the biomedical and pharmaceutical fields. We recently established a three-dimensional (3-D) ovarian tissue culture system supported by dextran hydrogel. To mimic the natural environment, we modified dextran hydrogel with Arg-Gly-Asp (RGD) peptide, a cell adhesion motif derived from extracellular matrix (ECM) proteins. The ovarian tissues

at 14 days old cultured in the RGD-modified dextran hydrogel significantly promoted antral follicle development and oocyte quality compared with those in dextran hydrogel alone. We also determined that RGD ligand directly stimulated the ovarian interstitial cell population to induce cell migration and steroidogenic gene expression, suggesting RGD peptide contributes to theca cell differentiation in our 3-D culture system. In the ovary, two Hedgehog (Hh) pathway ligands, Indian hedgehog (Ihh) and Desert hedgehog (Dhh), are produced by granulosa cells to regulate theca cell specification and differentiation. To examine the effects of RGD and Ihh ligands on theca cell differentiation, ovarian interstitial cells were isolated from immature murine ovaries and cultured in dextran hydrogels under four different conditions: RGD-/IHH- (R-/I-), (R+/I-), (R-/I+), and (R+/I+). After 7 days of culture, cell migration was observed only in the aggregates cultured with RGD peptide: (R+/I-) and (R+/I+). In particular, cell aggregates in the (R+/I+) condition had expanded areas compared to the (R+/I-) condition. In the presence of IHH, mRNA levels of steroidogenic related genes were dramatically increased in the cell aggregates compared with those cultured without IHH. Furthermore, the combination of RGD and IHH effectively upregulated the gene expression levels in the cell aggregates. Our results demonstrated synergistic effects of RGD and Ihh ligands on theca cell differentiation using the 3-D culture supported by dextran hydrogel.

O-8 - Proteomic Analysis of Follicular Fluid in Polycystic Ovary Syndrome

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Objectives: Polycystic ovary syndrome (PCOS) is a complex and common endocrine disorder which afflicts women of reproductive age. Ovulatory dysfunction is recognized as a primary factor contributing to infertility of PCOS. However, patients with PCOS have a reduced potential to produce high-quality oocytes and embryos. In this study, we used a quantitative proteomics analysis based on mass spectrometry to investigate the differences in proteomics profiles for follicular fluid obtained from patients with or without PCOS to identify the potential markers associated with oocyte quality. **Material and methods:** Follicular fluid samples were collected from infertile patients with (n=10) or without (n=10) PCOS during IVF cycle. Total protein was extracted and analyzed with a label-free quantitative proteomics using liquid chromatography-mass spectrometry (LC-MS). An in-depth understanding of the differentially expressed proteins (DEPs) and their networks was achieved by using a bioinformatics analysis including the protein annotation, unsupervised hierarchical clustering, functional classification, functional enrichment and clustering, and protein-protein interaction analysis. Selected DEPs were confirmed by ELISA, and correlation analysis was performed between these DEPs and the clinical characteristics. **Results:** In this study, we have identified 612 proteins, including 29 DEPs (11 upregulated proteins, and 18 downregulated proteins in follicular fluid from PCOS patients compared with women without PCOS). GO enrichment analyses revealed that aminoglycan catabolic process, cytokine secretion, and extracellular matrix organization were the top three biological processes between women with PCOS and controls. The localization of DEPs showed high enrichment in catenin complex indicated by cellular component terms between PCOS patients and controls. Based on ELISA results, PLTP and HYOU1 were differentially expressed between patients with and without PCOS. Follicular PLTP showed a positive correlation with embryo quality. **Conclusions:** Our study identified 29 DEPs in the follicular fluid of patients with PCOS compared with controls. PLTP and HYOU1 were deregulated in PCOS, which may play an essential role in the pathogenesis of infertility of PCOS.

O-9 - Reframing Women's Health and Aging through the Lens of Ovarian Function

Jennifer L. Garrison **Abstract Text:** Reframing Women's Health and Aging through the Lens of Ovarian Function Jennifer L. Garrison^{1,2}

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Ovaries are the architects of health and the pacemaker for aging in females. Beyond reproduction, ovaries signal to nearly every tissue in the body to promote general health. Aging in the ovary dramatically affects aging in somatic tissues, yet we know little about the mechanisms regulating healthy aging in reproductive organs, nor how this crosstalk is achieved. Understanding how and why ovaries age prematurely may provide important clues about aging in the rest of the body; ovaries also represent an elusive human model to test longevity interventions on a relatively short timescale. This presentation will discuss a global movement to advance scientific research focused on ovarian aging through funding, infrastructure, resource-building, and advocacy to accelerate the pace of discovery toward useful therapies. It will cover the state of research, the gaps, and where it's headed as we forge a collaborative dialogue about ovaries, health and aging.

O-10 - Local Effect of Allopregnanolone in Rat Ovarian Steroidogenesis, Follicular and Corpora Lutea Development

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Allopregnanolone (ALLO) is a known neurosteroid and a progesterone metabolite synthesized in the ovary, central nervous system, peripheral nervous system, adrenals and placenta. Its role in the neuroendocrine control of ovarian physiology has been studied, but its in situ ovarian effects are still largely unknown. The aims of this work were to characterize the effects of ALLO intrabursal administration on different ovarian parameters, and the probable mechanism of action. ALLO administration increased serum progesterone concentration ($p=0.0010$, $n=5$) and ovarian 3β -HSD ($p=0.0451$, $n=4$) while decreasing 20α -HSD mRNA expression ($p=0.0137$, $n=4$). ALLO increased the number of atretic follicles ($p=0.0077$, $n=4$), and the number of positive TUNEL granulosa and theca cells (theca: $p=0.0019$ and granulosa: $p<0.0001$, 16 sections/ovary, 5 ovaries/group), while decreasing positive PCNA immunostaining (theca: $p=0.0312$ and granulosa: $p<0.0003$). Meanwhile, the diameter of corpora lutea ($p<0.0001$, $n=300$ CL) and the PCNA immunostaining increased ($p<0.0001$, 16 sections/ovary, 5 ovaries/group), while decreasing TUNEL positive cells ($p<0.0001$, 16 sections/ovary, 5 ovaries/group). Ovarian angiogenesis (α -actin $p=0.0005$; Von Willebrand $p=0.0058$) and the GABAA receptor immunohistochemical expression also increased after ALLO treatment. To evaluate if the ovarian GABAA receptor was involved in these effects, we conducted a functional experiment with a specific antagonist, bicuculline. The administration of bicuculline returned the number of atretic follicles ($p=0.0002$, $n=5$ animals/group) and the diameter of corpora lutea ($p<0.0001$, $n=5$ animals/group, 51 CL/group) to normal values. These results show ALLO actions on the ovarian physiology of the female rat during the follicular phase, some of them

through the GABAAR. Intrabursal ALLO administration alters several processes of the ovarian morpho-physiology of the female rat, related to fertility and oocyte quality.

O-11 - Does Diet-Induced Obesity Alter Metabolic and Reproductive Traits of Female Mice Lacking AMH Signaling?

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Obesity is closely associated with reproductive disorders, including polycystic ovary syndrome (PCOS) and infertility. Diet-induced obesity in female mice has been suggested to accelerate primordial follicle recruitment leading to premature ovarian aging. Early depletion of primordial follicle pool is also observed in mice lacking anti-Müllerian hormone (AMH) signalling, such as AMH deficient (AMHKO) mice and AMH type 2 receptor deficient (MRKI) mice. On the other hand, diet-induced obesity also results in disrupted estrous cyclicity and ovulatory failure, a phenotype resembling PCOS. To address these opposing aspects of infertility, ovarian aging and PCOS, female AMHKO and MRKI mice were exposed to a high fat high cholesterol diet (HFHCD). Female AMH signalling deficient mice and their corresponding wild-type (WT) littermates at 30 days of age were fed a HFHCD or control diet (CD) for 14 weeks. Metabolic and reproductive parameters were assessed at the end of the treatment period. HFHCD led to significantly increased body weight and total fat mass in WT, AMHKO and MRKI mice ($P < 0.01$). Consistently, increased total serum leptin levels were observed in the HFHCD-fed mice. Serum adiponectin levels were decreased in HFHCD-fed WT mice compared to CD-fed WT mice ($P < 0.05$). Interestingly, CD-fed AMHKO mice had significantly lower adiponectin levels than CD-fed WT mice ($P < 0.05$), which was not further affected upon HFHCD exposure. HFHCD treatment induced glucose intolerance, as assessed by IPGTT ($P < 0.01$). However, AMH signaling deficient mice did not differ from WT mice. HFHCD-fed mice had a ~15% lower respiratory exchange ratio compared to CD-fed mice ($P < 0.01$). Energy expenditure (EE) was significantly increased in HFHCD-fed WT mice compared to CD-fed WT mice. Interestingly, CD-fed AMHKO mice tended to have a higher EE compared to CD-fed WT mice ($P = 0.06$) - which was not further increased upon HFHCD treatment. HFHCD treatment did not affect physical activity in all groups. Estrous cyclicity was irregular in HFHCD-fed mice compared to CD-fed mice, with HFHCD-fed mice showing a ~65% reduction in number of complete estrous cycles ($P < 0.01$). No genotype difference was observed. Our preliminary ovarian composition data confirmed the increased number of total growing follicles in AMHKO mice compared to WT mice. However, no dietary effect was observed among groups. In line with the disrupted estrous cycles, both HFHCD-fed WT and AMHKO mice tended to have a decreased number of corporal lutea compared to CD-fed mice. Interestingly, HFHCD-fed WT mice displayed ~50% higher serum AMH levels compared to CD-fed mice ($P < 0.05$), suggestive of dysregulated granulosa cell function. Analysis of the primordial follicle pool is ongoing. In summary, diet-induced obesity disrupts estrous cyclicity and alters granulosa cell function. AMH signalling deficiency had mild effects on studied metabolic phenotypes.

O-12 - SUMOylation is Required for Nuclear Organization in Mouse Oocytes

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In developed countries, women are choosing to have children later in life. During a woman's reproductive years, the quality and quantity of oocytes decrease due to several factors, including chromatin organization, mitochondrial dysfunction, and loss of protein homeostasis. SUMOylation is a post-translational modification involved in protein homeostasis, subcellular localization, and the formation of membraneless organelles by phase separation. SUMOylation becomes dysregulated during aging, leading to dysregulation of its target proteins. In the ovary, loss of the SUMOylation conjugation enzyme, Ube2i (Ubc9), from postnatal oocytes causes female infertility, with oocytes that arrest in metaphase I of meiosis I, but the mechanism by which loss of SUMOylation disrupts oocyte development is not fully characterized. Normally, as the oocyte reaches its terminal growth state, transcription is repressed and the oocyte uses stored RNA transcripts for later use in meiosis and embryonic development. Ube2i-Zp3cre oocytes show defects in these processes as transcription continues and some maternal RNAs decrease. We hypothesize the lack of SUMOylation disrupts the ability of Ube2i-Zp3cre oocytes to form phase-separated condensates essential for transcriptional regulation and mRNA processing in the nucleus, thereby preventing proper development. Confocal imaging of fully grown, germinal vesicle (GV) stage Ube2i-Zp3cre oocytes show mislocalization of SUMO1 and SUMO2-3, including loss of SUMO1 and SUMO2-3 condensates in the nucleus. We further analyzed the number of SC-35 positive speckles and their volume, as these speckles are closely tied to active transcription, mRNA processing, and have stable associations with nearby chromatin. We found that SC-35 positive nuclear speckles are greatly decreased in number and size in the fully grown GV Ube2i-Zp3cre oocytes as compared to similar stage wildtype oocytes. We hypothesize this may be related to speckle collapse due to a lack of SUMOylation and proper association with chromatin. Because nuclear speckles are sites of RNA processing and splicing, we analyzed splicing events in RNA-sequencing data comparing Ube2i-Zp3cre oocytes to controls. Informatic analysis showed almost 600 alternative splicing patterns, including genes associated with actin binding and the nuclear membrane essential for proper nuclear structure. This work shows that in the absence of SUMOylation nucleus becomes disordered leading to a loss of controlled transcription in GV oocytes. These studies were supported by NIH grant R01HD085994 and NSF GRFP.

O-13 - Digital Analysis of Ovarian Tissue: Generating a Standardized Method of Follicle Classification

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Ovarian follicles develop through a series of growth stages that correspond with morphological changes in the oocyte as well as the surrounding granulosa and theca cells. Classical follicular stages assessed in histological sections of ovarian tissue include primordial, primary, secondary, and antral stages. However, follicles transitioning between primordial and primary stages display key molecular changes that define activation and determine their subsequent development, and these transitional stages are not currently classified. This is due, in part, to the lack of standardized methods for classifying, counting and morphologically analyzing follicles. QuPath is a user-friendly open-source software extensively used in digital pathology. In addition to its multiple functions for image analysis, it supports collaboration by enabling sharing of exported annotated data and scripts between users. We hypothesize that establishing an automated pipeline of analysis to classify follicles based on key histomorphometric parameters using QuPath will support a more robust, rigorous and reproducible follicle classification method, ultimately defining early healthy follicles stages as they are activated within human ovary sections. Hence, this project aimed to develop a pipeline that requires minimum input from the user and allows automatic detection of principal elements of the ovarian follicle in digitized H&E-stained whole slide images. We used samples from healthy human ovarian biopsies. By using a supervised approach, follicles were selected and classified by expert ovarian morphologists. Then, segmentation of the whole follicle area, individual granulosa cell nuclei and oocyte was done using manual and automatic QuPath annotation tools. Feature extraction regarding size and shape and distribution analyses were performed to determine cut-off values that represented each follicle stage. Then, a tunable script was generated using Groovy programming language to automatically classify follicles. Finally, validation of follicle classification was performed. We were able to establish the morphometric parameters to classify follicles into primordial, transitional, primary, secondary and antral stages. Granulosa cell eccentricity was shown to be an accurate parameter to distinguish flat versus cuboidal granulosa cells in primordial and transitional stages, and granulosa cell number and follicle area were sufficient to distinguish all follicle stages. Future studies will focus on color deconvolution normalization of H&E staining to minimize inter-stain differences and define intensity parameters of the oocyte and granulosa cells as potential markers of oocyte degeneration and granulosa cell death. Altogether, in-depth digital analyses of ovarian follicle features may be useful for the assessment of morphometrical follicle normality, with the end goal of reaching a more transparent, unbiased and standardized method to assess different diseases or exogenous conditions that may affect the ovary against healthy folliculogenesis. Funding sources: Gesualdo Foundation Research Scholar Fund (MML), NIH U01HD110336 (MML, HM, ET), NIH F30HD107966 (ET), NIH T32HD094699 (HM).

O-14 - How Small Extravesicles (Sevs) from PCOS Follicular Fluid Modified Granulosa Cells Activity. Use of Proteomic and Mirna Approach

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The development of the ovarian follicle and the maturation of the oocyte both require the presence of several factors which come from the blood and follicular cells. Among these factors, extracellular vesicles (EVs) represent an original communication pathway inside the ovarian follicle and the follicular fluid (FF). Recently, EVs have been shown to present potential roles in follicular development and reproduction-related disorders including the Polycystic ovary syndrome (PCOS), the most common hormonal disease among women of childbearing age. To our knowledge, only one study has focused on protein content of FF exosomes and has detected one protein associated to PCOS (Li et al. 2020). The objective of our project was to better decipher the role of EVs derived from FF. We have performed a functional study with an incubation of small EV from PCOS FF and control FF on the human granulosa cell line (KGN). In order to obtain a "global" view of the proteins content, 6 sEV from PCOS (BMI < 25) and 6 sEV from control (BMI < 25) were analysed by proteomic and miRNA approaches. First results, sEVs purified from FF expressed exosomal markers such as CD9, CD63 and hsp70. The proteomic analysis of sEVs derived from FF in comparison to sEVs derived from blood has shown that FF sEVs possess proteins expressed by steroidogenic cells (StAR, Cyp11a1, 3 β HSD) such as granulosa cells. The characterization by using nanoparticle tracking has shown that sEVs from control and PCOS patients presented same morphological parameters. However, we have identified difference in the functionality of EVs in cell culture when they were purified from control some PCOS patients. Both sEVs (PCOS FF and control FF, $p < 0.05$) stimulated granulosa cell proliferation. But proliferation was more increased (+192%) with control sEVs in contrast to PCOS sEVs (+131%, $p < 0.05$). In addition, sEVs from FF (PCOS FF and control FF) increased cell migration of granulosa cells which was enhanced when sEVs were derived from PCOS patients. The elevated migration rate was associated with a lower β -catenin protein cell content. Moreover, the progesterone and estradiol secretion were more produced by granulosa cells after a 48h of exposition with control sEVs. The increase in steroids secretion was associated with an elevated expression of proteins involved in steroidogenesis (StAR, 3 β HSD). However, the stimulating activity of the steroid production was lost when the sEVs derived from PCOS patients were used. In order to identify difference in sEVs content (PCOS FF and control FF), the proteomic approach was performed and has identified 706 proteins in sEVs including 68 proteins that were significantly enriched in PCOS sEVs. These 68 proteins are involved in immunity functions, cell-cell and matrix adhesion and regulation of actin cytoskeleton. Analysis of the miRNA content has revealed 239 miRNA specific of the FF sEVs targeting genes involved in steroid biosynthesis, cell cycle, Oocyte meiosis, tight junctions and Insulin signaling pathway. Specific miRNAs were expressed specifically in PCOS sEVs, showing a specific signature. In conclusion, this study demonstrated that EVs are able to modify the granulosa activity suggesting a role in the follicle development. The results show that proteins and miRNA content in sEVs are significantly different in PCOS patients leading to perturbation of the granulosa cells functions. The miRNA and proteins profile in sEVs could be used as biomarkers of the follicle health. Project supported by AgenceBiomedecine

O-15 - Preovulatory Follicles of GnRH-II Receptor Knockdown Gilts Possess Fewer Hypertrophic Theca Cells, but Similar Numbers of Hypotrophic Granulosa Cells Compared with Littermate Controls

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The second form of gonadotropin-releasing hormone (GnRH-II) and its associated receptor (GnRHR-II) are only produced in one mammalian livestock species, the pig. Unlike the classical GnRH system, this ligand-receptor complex is more abundant in peripheral tissues. Regarding female reproduction, GnRH-II and its receptor are present in multiple ovarian-derived cell lines (human, primate, rodent) and GnRH-II has been discovered in human granulosa (GC) and theca (TC) cells. Recently, our laboratory measured GnRH-II within follicular fluid and detected GnRHR-II protein within both GC and TC of porcine preovulatory follicles. To examine how GnRH-II and its receptor mediate biological processes within preovulatory follicles, we utilized a transgenic swine line with ubiquitous knockdown (KD) of GnRHR-II (GnRHR-II KD). Preliminary studies revealed that GnRHR-II KD females: 1) secrete 20% less serum 17 β -estradiol than littermate controls during peak concentrations [-44 to -8 h prior to initiation of behavioral estrus (0 h)] within the follicular phase of the estrous cycle, despite similar circulating levels of gonadotropins; 2) produce 40% less GnRHR-II protein in TC of preovulatory follicles compared with control TC or GC from either line; 3) ovulate 16% fewer follicles than control littermates, although they possess similar numbers of large antral follicles; and 4) possess preovulatory antral follicles that are 10% larger in diameter than corresponding follicles from control females. Therefore, the objective of the current study was to characterize the morphology of GC and TC within preovulatory follicles collected from GnRHR-II KD (n = 4) and littermate control (n = 4) gilts. Mature females were euthanized during proestrus (Day 18-20 of the estrous cycle). Follicle diameter was measured using vernier calipers and large antral follicles (≥ 6.5 mm) were excised from ovarian stroma, fixed in 4% paraformaldehyde, and paraffin embedded. Sequential sections (approximately 6-7 μ m thick) were collected, mounted, and stained with hematoxylin and eosin. Sections with the largest measured follicular area were imaged (100X) at 8 random locations within the theca interna and mural GC layers. Cell numbers were quantified within a pre-set rectangular area; cell areas were determined using the closed polygon tool of the cellSens imaging software (Olympus Life Science, Waltham, MA) by tracing the plasma membrane of 40 GC and 40 TC exhibiting a defined plasma membrane and visible nuclei. Statistical analyses performed using the MIXED procedure of SAS included models for both cell area and cell number, with line as a fixed effect, litter as a random effect, and follicular area as a covariate. Log transformations were performed on all variables to meet normality assumptions. The number of GC within preovulatory follicles did not differ ($P > 0.05$) between lines; however, GC in GnRHR-II KD tended ($P < 0.06$) to be smaller ($31.6 \pm 0.9 \mu\text{m}^2$) than those within littermate control ($33.1 \pm 0.9 \mu\text{m}^2$) follicles. Interestingly, GnRHR-II KD females tended ($P < 0.08$) to possess fewer TC (38.4 ± 1.8 cells) than littermate controls (42.2 ± 1.8 cells). Moreover, TC from GnRHR-II KD follicles were significantly ($P < 0.001$) larger ($94.0 \pm 3.2 \mu\text{m}^2$) than TC in control ($81.4 \pm 3.2 \mu\text{m}^2$) follicles. Combined with our previous results, these data suggest GnRH-II and its receptor are critical to porcine TC function within preovulatory follicles. Supported by USDA/NIFA AFRI EWD predoctoral

fellowship (2022-11301) and Hatch Multistate (NEB-26-244) funds. USDA is an equal opportunity provider, employer and lender.

O-16 - Entering the Twilight Zona: Exploring the Role of UCHL1 in Regulating Oocyte Proteostasis

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Depletion of the ovarian reserve directly affects female procreative potential and leads to an altered endocrine environment that negatively impacts ovarian and extragonadal health. As the female reproductive system ages faster than any other organ systems in the human body, it is of the utmost importance to understand the specific mechanisms which underlie ovarian aging and promote efforts to preserve the ovarian reserve which is critical for female fertility, longevity and quality of life. Towards this goal, our laboratory has identified Ubiquitin C-Terminal Hydrolase L1 (UCHL1) as highly enriched in mouse and human oocytes throughout the reproductive lifespan and have found that female mice lacking UCHL1 exhibit severe subfertility, morphologically abnormal oocytes, altered folliculogenesis, and systemic endocrine disruption. We have observed that loss of UCHL1 results in increased rates of granulosa cell (GC) apoptosis and a thickened appearance of the oocyte's zona pellucida (ZP), which has been associated with poor oocyte quality, poor IVF outcomes in humans, and observed in women of advanced maternal age. The formation of the ZP plays an important role in the bidirectional communication between the oocytes and their surrounding somatic GCs, particularly via the connexin proteins of the gap junctions, with disruption resulting in impaired follicle development and female infertility in the mouse. Critically, this communication is also required for ovarian hormone production, specifically estradiol and progesterone, whose serum concentrations directly affect function of the hypothalamic-pituitary-gonadal axis. Our lab has observed increased levels of Zona Pellucida Protein 3 (ZP3) expression in UCHL1 KO oocytes, although differential expression of ZP3 is not observed at the mRNA level, suggesting that this phenotype is a result of altered protein turnover. We have also detected a change in the organizational pattern of Connexin 37, the predominant connexin in the oocyte, suggesting that in the absence of UCHL1, gap junctions may not properly form. To better understand molecular mechanisms of UCHL1 function in the mammalian oocyte, we performed a pilot proteomic analysis on oocytes isolated from UCHL1 KO and WT mice, finding significant dysregulation of over 1000 peptides, including ZP3 and Zona Pellucida Protein 2, indicating altered oocyte proteostasis in these animals. Interestingly, UCHL1 is a Ubiquitin Proteasome Pathway (UPP) regulator which is known to maintain the cellular balance of monoubiquitin and thus regulate

protein turnover. Currently, no current studies are investigating the molecular underpinnings of the UPP in the context of ovarian aging. While our exciting findings point to an essential role of UCHL1 and the ovarian UPP in the long-term functioning of the mammalian ovary, our previous work has been limited by the neurological aging phenotypes of full UCHL1 KO mice, which are not able to live past six months of age due to rapid motor neuron decline. Furthermore, interpretation of these results has been confounded by the intersection of functions of the neuroendocrine tissues in which UCHL1 is also expressed. To circumvent these obstacles, and to understand the oocyte-specific roles of the UPP in reproductive longevity, we have generated an oocyte-specific UCHL1 KO mouse line which will help elucidate the effect of oocyte-specific loss of UCHL1 on long-term fertility, fecundity, ovarian aging and proteostasis. This research will illuminate the molecular mechanisms which underlie oocyte quality and ovarian aging with the potential to inform our understanding of idiopathic female infertility, reproductive outcomes, and overall women's health.

O-17 - Long In Vitro Culture of Oocytes from Early Antral Follicles in Prepubertal Lambs

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Long in vitro culture (LIVC) of oocytes derived from early antral follicles (EAFs) has emerged as a potential reproductive technology for producing large numbers of competent oocytes. In a previous study, we demonstrated that LIVC of cumulus-oocyte complexes (COCs) from EAFs supports oocyte survival, growth, and acquisition of meiotic competence in adult sheep. This promising approach offers opportunities for preserving valuable and endangered animals, shortening generation intervals, and increasing genetic gain, especially when ovaries are collected from prepubertal animals as a source of EAFs. With this in mind, our objective was to investigate the efficacy of LIVC of COCs collected from EAFs in lambs. To achieve this, lamb ovaries were collected, and COCs were retrieved from EAFs (350-450 µm) by rupturing the follicle wall using a 21-gauge needle. Subsequently, they were cultured in a 96-well plate (one COC per well) containing TCM199 supplemented with 0.15 µg/mL zinc sulfate, 10-4 IU/mL FSH, 10 ng/mL estradiol, 50 ng/mL testosterone, 50 ng/mL progesterone, and 5 µM Cilostamide. After 5 days of culture, the following parameters were evaluated: COC morphology, oocyte diameter, chromatin configuration, gap junction communications, meiotic competence, levels of reactive oxygen species (ROS), mitochondrial activity, and distribution. Following the LIVC process, the results indicate a significant increase in oocyte diameter (108.76 vs. 113.44 µm, $p < 0.000$) and in mitochondrial activity ($p < 0.005$), with a change in mitochondrial distribution pattern from 'fine' to 'granular' following the LIVC and IVM process ($p < 0.01$). Furthermore, a notable transition in chromatin configuration was observed, characterized by a shift from a non-surrounded nucleolus (NSN) to a surrounding nuclear envelope (SNE) stage ($p < 0.000$), coupled with spontaneous meiosis resumption after LIVC (MI/MII, 26.7%), and an increase in meiosis resumption after IVM ($p < 0.000$). However, these results were accompanied by a significant increase in low quality COCs (based on

morphological evaluation) ($p < 0.05$) and an increase in ROS levels ($p < 0.000$). Most COCs also exhibited closed gap junction communications before and after LVC ($p < 0.01$), indicating a defective cell coupling between oocytes and cumulus cells.

O-18 - RGD-Modified Dextran Hydrogel Promotes Follicle Growth and Theca Cell Migration Through Integrins $\alpha\beta 3/\alpha\beta 5$ in Three-Dimensional Culture

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Due to ovarian damage that might result from chemotherapy and/or radiation treatment, cancer patients run the risk of losing their fertility. In vitro follicle culture is a promising technology to preserve the fertility of cancer patients. We recently established a three-dimensional (3-D) ovarian tissue culture system supported by a biochemically defined dextran hydrogel. To functionalize the dextran hydrogel, extracellular matrix-derived triple Arg-Gly-Asp (RGD) peptide was supplemented in the gel. Murine ovarian tissues at 14 days old cultured in the RGD-modified dextran hydrogel significantly promoted antral follicle development and oocyte quality compared with those in dextran hydrogel alone. Next, we determined the RGD-binding target integrins expressed in the murine ovary. In mammals, the family of integrins is composed of 24 $\alpha\beta$ subtypes, and 8 integrin subtypes recognize and bind to the RGD motif. Using qPCR and immunohistochemical analyses, we found that integrins $\alpha\beta 3$ and $\alpha\beta 5$ are the main targets of RGD in the immature ovary. To examine the effect of RGD-integrin interaction on follicle development, we used Cilengitide (Ci), an inhibitor for integrins $\alpha\beta 3/\alpha\beta 5$. Ovarian tissues were cultured in dextran hydrogels under three different conditions: RGD⁻, RGD⁺, and RGD+Ci. In the RGD⁺ condition, follicle development and theca cell differentiation were significantly enhanced in the ovarian tissues. However, in the presence of Ci (RGD+Ci), RGD-induced follicle differentiation was dramatically suppressed, similar to the RGD⁻ condition. Next, we examined the direct effect of RGD on ovarian interstitial cells. The aggregates of ovarian interstitial cells were 3-D cultured under the three conditions. The RGD⁺ condition significantly enhanced cell migration and the expression levels of theca cell marker genes in the aggregates. The RGD⁻ induced cell activity was totally suppressed in the RGD+Ci condition, similar to those in the RGD⁻ condition. To examine theca layer formation, interstitial cell aggregates were isolated from transgenic Gli1ERcre/td tomato (tdT) mice. Since Gli1 is a known marker for theca cells, tamoxifen was used to inject Gli1ERcre/tdT pups. This caused cre-mediated recombination and the expression of tdT fluorescence in cells that expressed Gli1 and all their offspring. The transgenic-derived cell aggregates were co-cultured with the wild-type (WT) secondary follicles under the three conditions. In the RGD⁺ aggregates, tdT-positive cells migrated to form the cell layers around follicles. In contrast, cell migration and cell layer formation were absent in both RGD+Ci and RGD⁻ aggregates co-cultured with follicles. In conclusion, these results suggest that RGD peptide promotes follicle growth and theca cell differentiation via binding integrins $\alpha\beta 3/\alpha\beta 5$ expressed in the ovarian interstitial compartment in our 3-D culture system.

O-19 - NEDDylation: An Essential Regulatory Pathway for Oocyte Quantity and Quality

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Reproductive decline and infertility in women are largely caused by diminished oocyte quality and quantity. The mechanisms underlying this decline are not yet fully understood but are attributed to various factors, including mitochondrial dysfunction, improper oocyte-granulosa cell communication, and changes to proteostasis. Neddylation, a ubiquitin-like post-translational modification that affects protein homeostasis and quality control, is implicated in various health conditions, but its role in oocyte biology remains unknown. Using CRISPR/Cas9 gene editing, we generated a novel conditional allele of Uba3, an essential enzyme in the NEDDylation pathway, and crossed it with the oocyte-specific cre driver line, Gdf9-icre. Continuous mating for 6-months of Uba3 conditional knockout (“cKO”) females failed to produce any pups, indicating that NEDDylation is required for female fertility. By histological and morphometric analysis, 12 week old Uba3 cKO ovaries are nearly devoid of follicles - severe premature aging phenotype. Prior to ovulation, GV oocytes of Uba3 cKO mice have significantly higher levels of reactive oxygen species (ROS). Transmission electron microscopy showed that mitochondrial morphology was significantly altered in Uba3 cKO oocytes. Additionally, Uba3 cKO oocytes lack well-developed transzonal projections (TZPs), structures that span the zona pellucida to allow for bidirectional communication with the oocyte and surrounding somatic cells. By transcriptomic analysis of fully grown GV oocytes, we discovered changes in genes related to cell adhesion as well as cell projection. Thus, NEDDylation appears to be essential for maintaining oocyte organelle integrity and overall female fertility. Further studies will seek to elucidate NEDDylation targets as mediators of reproductive disease and oocyte aging. These studies were supported by NIH grants T32HD098068 and R21HD109807.

O-20 - The Heterogeneity of Ovary-derived Hydrogels Elucidates the Matrisome Properties that May Influence Follicle growth and Survival In Vitro

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Premature ovarian insufficiency (POI) is characterized by a decline in ovarian function resulting in infertility and reduced ovarian hormone production. With the global prevalence of POI at approximately 3.5%, there are over 138 million women experiencing subfertility or complete infertility with disruption of sex hormones. However, there are many limitations to the current fertility preservation and restoration options that result in suboptimal outcomes. Additionally,

oncofertility patients with metastatic disease within their ovary are ineligible for transplantation of tissue in its native form. This leaves a large population of individuals without desired biological children or necessary hormones. As such, future methods for fertility preservation and restoration will include isolating ovarian follicles to grow in vitro or transplant into a bioprosthetic ovary. These methods necessitate a deeper understanding of the ovarian microenvironment in order to define an engineered environment that best supports follicle growth and maturation. The human ovary is compartmentalized into two regions: the cortex and the medulla. Our lab has shown that there are differentially expressed matrisome proteins across these regions. Here, we developed compartmental decellularized extracellular matrix (dECM) hydrogels by processing 10 bovine ovaries into cortical tissue (CTX) and medullary tissue (MED) then decellularizing the tissue slices to generate hydrogels. Using atomic force microscopy, we determined that CTX-dECM and MED-dECM hydrogels were similarly rigid (537Pa, 468Pa). Thus, we hypothesized that differences observed in downstream applications of these gels are due to differences in biochemical cues or protein composition and relative abundance. ECM proteins' chemical and structural properties influence cellular behavior. So, we performed second harmonic generation scanning microscopy and determined that MED-dECM hydrogels had significantly wider and longer fibers than CTX-dECM and a lower fiber angle alignment. To evaluate the gel's ability to serve as a bioscaffold, we selected two biologically unique CTX and MED-dECM hydrogels, encapsulated primary murine follicles (~110µm), and assessed growth and survival. Interestingly, we observed increased follicle growth in CTX-dECM 1 and MED-dECM 2 when compared to our control alginate. Day 8 follicle diameter for CTX-dECM 1 was significantly larger compared to follicles grown in CTX-dECM 2 (241.75, 189.95µm). Similarly, follicles grown in MED-dECM 2 were significantly larger at day 8 compared to follicles grown in MED-dECM 1 (212.12, 173.02µm). These data indicate biological differences in the dECM hydrogels. To investigate these differences, we performed proteomics on these four biologically unique hydrogels. A total of 604 proteins were detected. Further analysis comparing the uniquely present or absent proteins between the two selected biological replicates for each compartmental hydrogel resulted in 150 proteins unique to CTX-dECM 1, 51 in CTX-dECM 2, 70 in MED-dECM 1 and 82 unique in MED-dECM 2. Gene ontology (GO) analysis of biological processes was performed for each hydrogel based on the differentially present proteins. Interestingly, the two compartmental hydrogels that resulted in follicles with larger diameters than the control (CTX-dECM 1 and MED-dECM 2) had comparable GO biological terms with an overrepresentation of proteins involved in metabolic processes. However, further work must be undertaken to identify how this heterogeneity in the hydrogels influenced follicle growth. With further interrogation of these differences, we can engineer an environment that supports follicle growth and increases the production of functional MII oocytes. Importantly, this work aims to lay the groundwork for designing enhanced biomaterials that improve fertility restoration options for a variety of individuals.

O-21 - Single-nuclei Sequencing Reveals Compositional Alterations in Ovarian Cell Types of AMH Deficient Mice

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Anti-Müllerian hormone (AMH), produced by granulosa cells (GC) of growing follicles, plays a crucial role in regulating folliculogenesis. AMH acts as a gatekeeper of follicle growth by inhibiting primordial follicle recruitment and follicular atresia, as well as inhibiting FSH sensitivity of small antral follicles. However, the precise cell type-specific mechanisms through which AMH regulates follicular development remain largely unknown. Due to the heterogeneous composition of ovary tissue, our previous bulk RNA sequencing analyses were unsuccessful to identify AMH regulated genes. Therefore, we conducted single-nuclei RNA sequencing to identify transcriptional changes in a cell type- and follicle-stage specific manner. Ovaries from 4-month-old AMH deficient (AMHKO) mice (n=5 per group) and wild type (WT) littermates (n=6 per group) were isolated at diestrus. Chromium Single Cell Multiome ATAC + Gene Expression (10x Genomics) was performed. Following ambient RNA removal and filtering, we performed normalization and doublet removal, then analyzed the combined data using Seurat. Transcriptional cellular clusters and GC subclusters were profiled using relevant literature-based markers. Subsequently, we evaluated whether differences in ovarian cellularity reflected variations in ovarian composition between WT and AMHKO mice by analyzing the cell percentage of cell clusters per sample. After quality control analyses, we included 63,236 cells from AMHKO mice and 73,313 cells from WT mice for characterization. Unbiased clustering and uniform manifold approximation and projection (UMAP) analysis revealed eight distinct clusters. The two largest clusters comprised GC (n=49,459 cells) and mesenchymal cells of the ovarian stroma (n=46,703 cells). Furthermore, two clusters of corpora lutea (CL) were identified: one active CL cluster (n=21,867 cells) and one regressing CL cluster (n=9,101 cells). In addition, four smaller clusters of other cell types were identified, including endothelial cells, ovarian surface epithelial cells and two clusters of immune cells. The GC cluster segregated into five distinct subclusters, including preantral (n=5,596 cells), antral-mural (n=16,175 cells), mitotic (n=13,073 cells), atretic (n=14,172 cells), and an unknown subcluster (n=443 cells). This unknown subcluster was characterized by high expression of Ki67 and Cenpf, involved in cell proliferation, along with other granulosa cell markers (Fst, Slc18a2, Nr5a2, Inha). Apart from previously established markers, further transcriptomic profile analysis revealed potential novel gene signatures within each identified GC subcluster, necessitating further verification. Analysis of the cellular composition revealed a ~50% ($P < 0.01$) and ~30% ($P < 0.01$) increase in AMHKO-derived preantral and mitotic GC compared to WT mice, which may be consistent with the increased number of healthy small antral follicles in AMHKO mice. Also, the percentage of AMHKO-derived atretic GC was significantly increased compared to WT mice. In contrast, the percentage of AMHKO-derived active CL cells was decreased by ~35% ($P < 0.01$) compared to WT mice, suggestive of a suboptimal CL function. Investigations into single-cell transcriptional differences within each GCs cluster between WT and AMHKO mice are ongoing, as well as analysis of intercellular signaling networks using CellChat. In conclusion, our study shows that loss of AMH signaling alters the ovarian cellular distribution, which will aid in identifying differentially expressed gene in a cell-cluster specific manner.

P-1 - The effect of resveratrol on the developmental competence of feline oocytes after vitrification

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Recent studies have shown that an increase in the level of ROS in thawed oocytes causes a temporary, dynamic loss of mitochondrial potential, which leads to a decrease in ATP production, which may ultimately impair embryo development. Resveratrol is an antioxidant with quite a wide range of uses, and among its most prominent effects are its anti-aging capabilities. The objective of this research was to assess the viability and developmental potential of feline oocytes following in vitro maturation (IVM), vitrification, and post-warming incubation with resveratrol. The oocytes after vitrification and thawing procedure, were incubated with 0.2 μ M, 2 μ M, or 20 μ M resveratrol for 2 hours. Then viability test was performed. Oocytes treated with 0.2 μ M resveratrol had the highest viability (68.89%), as assessed by fluorescein diacetate and ethidium bromide staining, while higher concentrations were associated with diminished oocyte viability. In the second stages of experiment, the warmed oocytes were inseminated (IVF) following the 2-hour incubation with the three concentrations of resveratrol. The presumptive zygotes were then maintained in culture and their development evaluated. The highest cleavage rate was observed when the oocytes had been incubated with 0.2 μ M resveratrol (88.34%), which was higher than for the control group (without resveratrol (75%)). Moreover, this concentration of resveratrol also augmented the blastocyst formation rate. While the vitrification of oocytes often results in diminished developmental potential in the ensuing embryos, attributed to cryopreservation-induced injury, the utilization of low concentrations of resveratrol enhances the procedure's efficacy.

Funding: This research was funded by the National Science Centre, grant nr 2022/06/X/NZ9/01562

P-2 - Tests Préimplantatoires Non Invasifs Pour L'étude des Aneuploïdies Embryonnaires

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Preimplantation Genetic Diagnosis (PGD) is an invasive method that allows for the detection of serious hereditary diseases or non-viable chromosomal anomalies in embryos resulting from in vitro fertilization (IVF) before implantation into the uterus. A biopsy of one or more embryonic cells is necessary to obtain genetic material for analysis. Recently, new non-invasive methods for studying embryonic aneuploidies have emerged, relying on the analysis of biomarkers contained in embryonic culture media or on the study of morphokinetic parameters of embryonic development provided by time-lapse technology (TLT). In this work, we propose to present the new non-invasive methods for detecting embryonic aneuploidies. An updated international literature review was conducted using the following keywords: "PGD," "aneuploidy," "biomarkers," and "non-invasive genetic tests." Non-invasive Preimplantation Genetic Testing (niPGT) has been proposed as an alternative to invasive embryonic biopsy, avoiding potential alterations to the implantation and future development potential of the embryo, and reducing the risk of miscarriages. niPGT involves analyzing acellular DNA contained in the culture medium after amplification by next-generation

sequencing (NGS) or comparative genomic hybridization (aCGH) array. These tests, based on embryonic acellular DNA, show high concordance with biopsies of the trophectoderm, inner cell mass, and whole blastocyst. niPGT can now be performed for the definitive diagnosis of trisomy 21, certain recessive and X-linked disorders. However, the main limitation is embryonic mosaicism. On the other hand, a new non-invasive approach involves measuring certain biomarkers in spent embryonic culture media, such as specific miRNAs or proteins. Overexpression of miR-191 has been observed in the culture media of aneuploid embryos, suggesting their use as biomarkers for embryonic aneuploidies. Analysis of glutamine levels in culture media by gas chromatography and mass spectrometry has shown a significant increase in glutamine consumption in aneuploid embryos. Glutamine has been proposed as a molecular indicator for evaluating embryonic ploidy. Protein secretome analysis of blastocysts in spent culture media has shown different profiles based on embryonic ploidy. Increased and differentially significant expression of lipocalin-1 in the secretome of aneuploid blastocysts compared to euploid blastocysts suggests the potential use of this protein as a biomarker for embryonic aneuploidy. In addition to the secretome, other non-invasive methods focus on morphological and morphokinetic parameters of embryos derived from Time-Lapse incubator systems. Euploid embryos exhibit faster kinetics of segmentation and blastocyst expansion. The combination of these data with artificial intelligence has allowed the development of algorithms to predict embryonic ploidy with high efficiency.

Bien que les méthodes non invasives d'étude des aneuploïdies embryonnaires soient moins perturbatrices pour l'embryon et plus efficaces en termes de temps et de coût que le DPI invasif, d'autres études sont nécessaires pour valider ces méthodes et déterminer dans quelle mesure elles reflètent le véritable statut génétique de l'embryon.

P-3 - Semen Collection from Rhinoceros During Standing Sedation

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Assisted reproductive technologies can facilitate genetic management of ex situ wildlife populations but rely on access to sperm samples. Sperm collection via electroejaculation (EEJ) and urethral catheterization (UC) is established for black (*Diceros bicornis*), greater one-horned (GOH; *Rhinoceros unicornis*), and southern white rhinoceros (*Ceratotherium simum*), but rely on anesthesia which can be risky and costly. Our main objective is to develop and optimize a protocol for sperm collection from rhinos that does not require general anesthesia, via UC during standing sedation. A procedure, which to our knowledge, has not been reported in any species. The success of anesthesia-based UC sperm collection appears to be promoted by alpha-2 agonists, which may stimulate epididymal contractions aiding the movement of sperm into the urethra. Alpha-2 agonists, such as medetomidine, are a commonly used sedative for rhinos. As a secondary objective, we also aimed to assess the use of intramuscular administration of oxytocin, a peptide hormone demonstrated to promote sperm collection in some species. Thus far, two trials of standing sedation UC were conducted with one white and one GOH rhino. For both procedures, oxytocin (20 IU) was administered alongside the sedatives, and once sedation was achieved, a rectal ultrasound was conducted to visualize sex accessory glands. The males were trained for rectal palpation leading up to the procedure and did not display any signs of discomfort during the

exam. For both, the penis was fully extended following ultrasound exam and a sterile catheter was slowly inserted into the urethra. A highly concentrated (1.25×10^9 sperm/mL) and highly motile (~90%) sample was collected from the white rhino. The GOH rhino did not provide a spermic sample. There are several possible reasons to explain why the outcomes differed, ranging from physiological variations between individuals or species to procedural differences. To address the second objective, oxytocin was administered (20 IU) to four GOH males opportunistically during veterinary procedures and serum samples were collected at 0, 15, 30, 45, and 60 min post injection. Serum samples were used to validate an enzyme immunoassay for measuring oxytocin in rhinoceros' serum (Oxytocin Kit; Arbor Assays). Parallelism was confirmed between serially diluted pooled sample and the standard curve. Inter- and intra- assay coefficients of variation were maintained at < 15%, except for time 0 samples which fell below the detection limit of the assay. Results revealed oxytocin concentrations ~2X – 5X above basal were detectable at 15 min and remained high for the full 60 min. This study is ongoing; additional collections and efforts to optimize techniques will aid in the development of a protocol that may reduce risk and cost of sperm collection procedures and allow for collection of genetic material from a wider range of individuals.

P-4 - Oestrus Synchronization: A Tool for Sustainable Breeding and Conservation Program in Smallholder Sheep Farming

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Indigenous sheep breeds play a vital role in rural communities of South Africa's livestock agriculture. Breeds such as Namaqua Afrikaner, Bapedi sheep, Zulu and Damara are recognized as landrace breeds according to the South African Animal Improvement Act (Act 62 of 1998). They possess traits of low maintenance and adaptation, crucial for withstanding common challenges encountered by smallholder farmers such as diseases, heat stress and limited feed resources. These breeds also demonstrate high fertility rates, optimizing reproductive success in their native environments. Despite their significance, indigenous breeds encounter various threats including genetic dilution and low productivity in smallholder farms. Amongst key factors contributing to their population decline and genetic erosion are inbreeding, uncontrolled mating, resource limitations and limited farm information. Of all assisted reproductive biotechnologies, oestrus synchronization has been studied as a tool for conservation programs and assist in preventing genetic erosion. However, most oestrus synchronization research has been conducted in controlled research environments (in situ/ in-vivo). Thus, integrating oestrus synchronization into community conservation programs may facilitate knowledge exchange and capacity building among farmers, leading to tangible socio-economic benefits. This includes controlled mating for increased lambing rates and uniform lamb crops which elevates incomes and food security. Moreover, synchronized breeding enables farmers to strategically time lamb sales, therefore maximizing economic returns from their livestock enterprises. Ultimately, this aids in preserving genetic integrity and mitigating the risk of genetic erosion in rural communities. Oestrus synchronization protocols are relatively simple to implement and require minimal infrastructure and financial investment, making them accessible to resource-constrained farmers. By integrating synchronization techniques into conservation strategies, local communities can take ownership of their genetic resources,

enhancing resilience and sustainability for both farming communities and indigenous sheep populations.

P-5 - Improved Detection Method of Estrus Behaviors in Individually Housed Kiko Goats Using YOLOv8

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Observing reproductive behavioral patterns is a critical tool in assessing animal health status among a variety of agricultural applications. Numerous reports indicate time and labor were the two limiting factors in performing observational studies among various farm animal species. Apart from these factors, using human observation-based data is very subjective and may yield variation in results. This approach can increase inaccurate information and potential missteps in the decision-making process. Additionally, collecting data using human observation is very limiting when working on a large sample size. These collective challenges warrant the need to adopt artificial intelligence. In recent years, numerous studies used machine learning models to detect reproductive behavioral patterns in cattle, pigs, and companion animals. Despite the progress, adopting machine learning tools to detect reproductive behavior, particularly estrus, in goats remained underserved. To improve traditional detection methods, our study proposes a novel approach for goat estrus detection by using the You Only Look Once Version 8 (YOLOv8) machine learning model. Prior to model development, an ethogram was established to ascertain distinct estrus behaviors in individually housed Kiko goats. The ethogram was later used to determine tail wagging as a behavioral pattern of interest in our trained YOLOv8 model. Based on the performance metrics, results from our model indicate promising results as the precision, recall, F1 value and AP50 was above 95%. Our research findings can be instrumental in improving livestock production by increasing labor efficiency and promoting early detection of anomalous estrus behaviors.

P-7 - JNJ-7706621 Treatment during the Post-activation Period Enhances the Developmental Competence of Mouse Somatic Cell Nuclear Transfer Embryos

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Somatic cell nuclear transfer has undergone extensive investigation for animal cloning, but embryos produced through this method display reduced developmental potential compared to naturally reproduced counterparts. During the post-activation period, cytochalasin B is conventionally used in somatic cell nuclear transfer to maintain the successful activation and formation of diploid nuclei in somatic cell nuclear transfer embryos. Despite its benefits, cytochalasin B has associated toxic effects, prompting exploration into alternative compounds. This study aimed to enhance animal cloning efficiency by optimizing post-activation through the

substitution of cytochalasin B with JNJ-7706621, a specific inhibitor of cyclin-dependent kinase 1. Parthenogenetic embryos were cultured with cytochalasin B (5 µg/mL) and various concentrations of JNJ-7706621 (1, 10, and 50 µM). The 10 µM JNJ-7706621 and cytochalasin B groups exhibited significantly higher rates of parthenogenetic embryo development than the 1 and 50 µM JNJ-7706621 groups. The 10 µM JNJ-7706621 treatment increased trophectoderm and total cell numbers while decreasing apoptotic cells in parthenogenetic blastocysts compared with the cytochalasin B group. Similarly, in somatic cell nuclear transfer embryonic development, the 10 µM JNJ-7706621 treatment improved blastocyst formation, trophectoderm, inner cell mass, and total cell numbers while decreasing apoptotic cells in blastocysts compared with cytochalasin B treatment. Moreover, the 10 µM JNJ-7706621 treatment resulted in a significantly higher intensity of F-actin in somatic cell nuclear transfer embryos compared to cytochalasin B treatment. It also reduced the incidence of abnormally distributed cortical actin, uneven division, and blastomere fragmentation compared to cytochalasin B treatment. Furthermore, the 10 µM JNJ-7706621 treatment led to a significantly higher intensity of α-tubulin than cytochalasin B treatment at the one-cell and two-cell stages of somatic cell nuclear transfer embryos. The 10 µM JNJ-7706621 treatment reduced the incidence of chromosome misalignment and abnormal spindle organization during metaphase in somatic cell nuclear transfer embryos compared to cytochalasin B treatment. Additionally, it was confirmed that JNJ-7706621 treatment significantly increased efficiency in cloned mouse production compared to cytochalasin B. Moreover, JNJ-7706621 treatment significantly decreased placenta-fetal weight ratio in cloned mice compared to cytochalasin B. These results suggest that post-activation treatment with JNJ-7706621 enhances somatic cell nuclear transfer embryo full-term development, highlighting potential progress in cloning technology through improved distribution and organization of the cytoskeleton. This research was supported by the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Research Initiative Program (KGM4252432, KGM1012412), Republic of Korea.

P-8 - Beyond Mommies and Daddies: Mental Health Needs of Gender Expansive Patients

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The authors of Chapter 17 - Transgender Assisted Reproductive Technology, in Cambridge University Press' Fertility Counseling: Clinical Guide (published November of 2022) explore the pivotal role of assisted reproductive technology (ART) in supporting the reproductive justice and autonomy of transgender individuals. The chapter emphasizes the importance of considering fertility preservation and family-building options, such as the use of personal gametes or gestational carriers, prior to medical transitions involving hormones or surgeries. The chapter also underscores that these considerations are integral to the informed consent process in gender transition. With the advancement of progressive thinking and modern medicine, there has been a notable increase in transgender individuals utilizing ART for family creation. This demographic shift necessitates a comprehensive approach in fertility counseling, demanding not only diverse medical knowledge but also cultural sensitivity and inclusivity. The chapter delves into the nuances of providing gender-affirmative care in reproductive endocrinology and fertility settings, highlighting

the importance of understanding specific terminologies, employing a trauma-informed care approach, acknowledging nonbinary gender systems, and avoiding heteronormative and cisnormative biases. Furthermore, the chapter reviews guidelines and considerations set forth by leading organizations such as the American Society for Reproductive Medicine (ASRM), The Endocrine Society, World Professional Association for Transgender Health (WPATH), and American College of Obstetrics and Gynecology (ACOG) for fertility and preservation in transgender populations. The findings and discussions presented offer a comprehensive overview of the challenges and opportunities in enhancing ART services for transgender individuals, ultimately contributing to a more equitable and inclusive reproductive healthcare landscape.

P-9 - Identification of Bioactive Small Molecules altering Endometrial Receptivity and Embryo Implantation In Vitro and In Vivo

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Background and Aims: Implantation remains a major impediment to success in in vitro fertilization (IVF) treatments. The endometrial receptivity window in humans is limited to the mid-secretory phase of the cycle. Modulating endometrial receptivity using pharmacological agents represents a potential strategy to improve implantation rate and IVF outcomes. **Method:** A high-throughput spheroid attachment assay using BeWo cells and receptive Ishikawa endometrial epithelial cells was utilized to screen the Library of Pharmacologically Active Compounds (LOPAC), comprising 1,280 bioactive small molecules. Lead compounds enhancing attachment were validated in non-receptive endometrial lines (HEC-1B, AN3CA). Their effective concentrations and cytotoxicity were determined. Their effects on embryo implantation were evaluated by transcervical injection into mouse uterus on 1.5 days post-coitum (dpc) and examining implantation sites on 5.5 dpc and 18.5dpc. **Results:** Screening identified 255 up-regulators and 173 down-regulators of spheroid attachment. Three lead molecules (L, O, and P) significantly enhanced attachment in non-receptive HEC-1B and AN3-CA cells. Conversely, Molecule J reduced attachment in receptive Ishikawa and RL95-2 cells but increased attachment in non-receptive HEC-1B and AN3CA cells. These four molecules all significantly elevated implantation sites in the mouse model compared to the control in 5.5dpc and 18.5dpc (n=10-15). **Conclusion:** Three molecules were found to increase spheroid attachment and embryo implantation in vivo and in vitro. Another molecule J reduced the spheroid attachment in receptive cell lines while increasing attachment in the non-receptive cells and in mouse model. Four identified pharmacological agents that can modulate endometrial receptivity and augment embryo implantation in mice, representing potential adjuvants to improve implantation and IVF success rates. [This study is partially supported by the CRCG and GRF grants 15162211 and 17120720 to KFL]

P-10 - Effect of lactate, pH and microbiomes in regulating human endometrial receptivity and pregnancy outcomes

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Study Question: What is the role of Lactates and pH and Lactobacillus on human endometrial receptivity and pregnancy outcome? Summary answer: D- and L-lactates at 10 mM, and both acidic (pH 6.4) and alkaline (pH 8.1) pH did not significantly affect trophoblastic spheroid attachment onto endometrial cells. High concentrations of D- and L-lactates suppress spheroid attachment, invasion of trophoblast cells, angiogenesis of HUVEC cells and cytokines production of endometrial cells. What is known already: Lactobacillus is the most abundant in the vagina and a Lactobacillus dominant uterine microenvironment is associated with better pregnancy outcomes. Study design, size, duration: In vitro laboratory study and in vivo pregnancy outcome association from 98 patients. Participants/materials, setting, methods: In-vitro cell culture and analysis, and patient uterine and vaginal fluid collection. Main results and the role of chance: High D- and L-lactate concentrations (>10 mM) inhibited the cell viability and proliferation of the human endometrial epithelial Ishikawa and trophoblastic BeWo cells. Culture media at pH 6.1 (acidic), 7.2 (neutral), and 8.1 (alkaline) did not affect the BeWo spheroid attachment on the treated Ishikawa cells. Moreover, high lactate concentrations (>10 mM) inhibited the invasion of trophoblastic BeWo by the treated Ishikawa cells, as well as tube formation (angiogenesis) in the treated human endothelial HUVEC cells. D- and L-lactate (≥ 10 mM) induce the production of cytokines in Ishikawa cells including TIMPs, MMPs, IL6, IL6R, and certain Th1/Th2 cytokines. Lastly, Lactobacillus and Bifidobacterium are the most abundant bacteria inside the uterine and vaginal fluid, but their abundance did not correlate pregnancy outcome in IVF patients. Limitations, reasons for caution: This is in vitro study using cancer cell lines. The sample size of uterine and vaginal fluid is small and limited to our IVF patients. Wider implications of the findings: Our findings suggested lactate and pH levels did not affect spheroid attachment. The effect of lactate-derived cytokines on trophoblast development, and the whether Lactobacillus, and Bifidobacterium could be promising indicators for pregnancy outcomes prediction warrant further investigation. Study funding/competing interest(s): This project is partly funded by an HMRF grant (06173976) to KFL.

P-11 - Using a Time-lapse System to Examine the Association Between Morphokinetic Patterns and Bovine Embryo Gender

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Determining the gender of a preimplantation embryo is often a challenging procedure. Although pregnancy diagnostics are used for human embryos, these procedures have not been utilized in domestic animal reproductive management. In cattle, shifting the offspring gender ratio (50:50) might have high economic merit because it reduces the proportion of unwanted genders. Sex-sorted semen can be used to increase the desired embryo gender; however, it was found to reduce the pregnancy rate by 5-8%. Therefore, other approaches are required. The morphokinetics of the

developing embryo have been used to assess its quality. Here, we examined whether the morphokinetic parameters throughout the early developmental stages are associated with the gender of the embryo. Two sets of experiments were conducted using an in-vitro model of the production of bovine embryos. In the first set, cumulus oocyte complexes (COCs; n=416) were aspirated from ovaries collected at a local abattoir, and in-vitro matured for 22 h. COCs were fertilized by using Y- or X-sorted semen (Semex, Canada). Then, embryos were individually cultured in an incubator equipped with a time-lapse system (TLS) for ~190 h and the embryo morphokinetic parameters were monitored continuously. Blastocysts (n=25) were collected for gender validation; DNA was extracted (High Pure PCR Template Preparation Kit, Roche, Austria) from each single embryo, followed by polymerase chain reaction (PCR) using a Y-chromosome gene (TSPY and BOV97M). The proportion of oocytes that cleaved (74.1 ± 7.3 vs. $70.8 \pm 7.1\%$; $P=0.7$) and those that developed to blastocysts did not differ between the Y- vs. X-sorted groups, respectively. Of the kinetic parameters, the time from fertilization to the 8-cell stage and to the morula stage was shorter for the Y- vs. the X-sorted group (87.5 ± 4.9 vs. 107.5 ± 4.5 hpf; $P=0.02$ and 119.3 ± 1.9 vs. 129.6 ± 5.1 hpf; $P=0.03$, respectively). Moreover, the time from fertilization to the early blastocyst stage tended ($P=0.06$) to be shorter in the Y-sorted group. No difference was found in the morphology of the embryos that were derived from the Y- vs. the X-sorted semen, throughout all the embryonic developmental stages. Note that only the gender-validated blastocysts were analyzed, suggesting that male embryos differ from female embryos in their kinetics rather than in their morphological parameters. This understanding was taken into account in the second part. In the second set, in-vitro matured COCs (n=466) were fertilized with non-sorted semen and cultured in an incubator equipped with TLS for ~190h. The morphokinetic parameters were evaluated continuously and blastocysts (n=112) were collected for gender validation based on DNA extraction followed by PCR. Multiple discriminant analysis (JMP 17 software) was conducted by combining kinetic parameters as covariates in accordance with PCR validation. By using the kinetic parameters, i.e., those parameters found to differ between genders in the first experiment, it was possible to predict that 59.7% of the embryos would be male and 49% would be female. Using a single kinetic parameter as a covariate, the time from fertilization to the 8-cell stage revealed a probability of 64.5% to predict a male embryo, whereas the time from fertilization to the early blastocyst stage revealed a probability of 60% to predict a female embryo. We concluded that kinetic parameters can be used to select a preferred gender of in-vitro-derived embryos before transferring them. However, this selection might shift to some extent the offspring gender ratio without affecting the pregnancy rate.

P-12 - Investigations on Antimicrobial Properties of Stallion Seminal Plasma

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When collecting semen from stallions, bacterial contamination cannot be completely avoided. To prolong the storage life of sperm, semen extenders therefore include antibiotics to inhibit bacterial growth. This approach, however, meets the definition of excessive or wrong antimicrobial use, considered a major cause for the development of antimicrobial resistance. We were therefore interested in characterizing antimicrobial properties of stallion seminal plasma (SP) with the final

aim to further exploit such properties in cooled-stored stallion semen. Semen was collected by artificial vagina from 5 healthy normospermic stallions (6-29 years) of different breeds. For collection of SP, raw semen was centrifuged (10 min, 1800xg, 4 °C), the supernatant checked for absence of sperm, centrifuged again (10 min, 15000 x g, 4 °C) for removal of remaining bacteria and the supernatant then frozen at -80 °C. For extraction of antimicrobial peptides (AMP) from SP, ultrafiltration (10,000xg for 85min) was performed (omega membrane 10kDa; Pall Life Sciences, Portsmouth, UK), followed by electrophoresis (Tricine-SDS-PAGE) and Orbitrap LC-MS/MS analysis. The AMPs psoriasin and equine lysozyme were detected in very low abundance. For determination of antimicrobial effects of SP, bacterial isolates of *Streptococcus* (Sc.) *equi* ssp. *zooepidemicus* and *Escherichia* (E.) *coli* were used in a liquid growth inhibition assay (Schulze et al. 2019; *Theriogenology* 157:335-340) with minor modifications. The SP (from 2 ejaculates of each of 5 stallions) was diluted 1:1 (v:v) with tryptic soy broth (TSB), then 100 µl mixed with 800 µl bacterial suspension in TSB in two trials (starting concentrations of bacteria: 1x10⁵ and 1x10³ CFU, respectively). As controls, TSB with SP and bacterial suspension in TSB were used. Optical density (OD) was measured (spectrophotometer, 595 nm) at 0h and after 24h incubation (37°C). At 24h, serial dilutions were incubated at 37 °C on Columbia agar for 24h for determination of CFU. Statistical analysis of OD values was performed with two-way repeated measures ANOVA (IBM-SPSS statistics 29.0.1.0). For analysis of differences between CFU values, non-parametric tests were used. Based on changes in OD, there was a bactericidal effect of SP on growth of Sc. *zooepidemicus*, but only a minor effect on E. *coli* (time p< 0.001, bacterial species p< 0.001, time x bacterial species p< 0.001) in both trials. When analysed by CFU, bacterial growth was inhibited in comparison to controls irrespective of the bacterial species (p< 0.01). We conclude that stallion SP has antimicrobial properties effective against both, gram-positive and gram-negative bacteria. These are most likely not caused by the low concentrations of equine AMPs determined in this study. Stallion SP is therefore suggested to contain non-protein substances with antimicrobial activity. Further characterization of these substances may help to develop additives with antimicrobial properties to replace antibiotics in semen extenders.

P-14 - Sperm Capacitation Status Correlation with Porcine IVF Cleavage Rates

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Sperm capacitation is a key biochemical and physiological change spermatozoa must undergo before fertilization. Differing percentages of sperm can undergo this process, which requires minutes to hours to complete depending on species and sire. Distinct localization patterns of zinc ions (Zn²⁺) have been identified across various species, denoted as zinc signatures, which correlate with capacitation status. In the boar, four zinc signatures have been discovered. Zinc signature 1 is indicative of a non-capacitated sperm cell with Zn²⁺ localized across the head and whole tail. Signature 2 reflects early stages of capacitation with Zn²⁺ uniformly found in the head and midpiece of tail. Signature 3 is associated with intermediate to late capacitation stages with Zn²⁺ localized exclusively in the tail's midpiece. Lastly, Signature 4 reflects a fully capacitated or dead sperm cell, distinguished by the absence of Zn²⁺. This study aims to determine if there is a

correlation between sperm capacitation status and prediction of in vitro embryo cleavage rate. Fresh sow ovaries were collected from a local harvest facility. Follicles ranging from 3-5mm in size were aspirated. Oocytes containing at least two layers of cumulus cells were selected and placed in TCM-199 based maturation medium containing 10 ng/mL of EGF & FSH and 0.5 µg/mL of LH and incubated at 38.5° C in 5% CO₂. After 40-44 hours, cumulus cells were removed by pipetting with 0.1% (wt/vol) hyaluronidase for 1 minute. Oocytes with a visible polar body were selected and placed in 50 µL droplets of modified Tris-buffered medium (mTBM) and co-incubated with sperm for 4 hours from 9 boars. After fertilization, potential zygotes were placed in MU4 culture medium at 38.5° C in 5% O₂ and CO₂. Cleavage percentages were checked on day 4 after fertilization. Boar samples used for in vitro fertilization (IVF) were analyzed by image-based flow cytometry to observe the zinc signature state before (0 hour) and after incubation in mTBM (1 and 4 hours) to observe what proportion of sperm cells capacitated. Sperm cells were labeled with zinc indicator FluoZin-3 AM; acrosome remodeling indicator lectin PNA-AF647; and live/dead cell, plasma membrane-integrity reflecting DNA stain propidium iodide to observe precise capacitation states. Sperm motility was recorded using a computer-assisted sperm analysis system. Correlation coefficients were calculated by using Python. Features with strong positive correlations include sperm progressive motility after 1 hour, signature 3 at 4 hours, and difference in signature 2 population from 4 hour to 1 hour, and difference in plasma membrane integrity from 4 hour to 0 hour. Features with a strong negative correlation include total motility changes from 4 hour to 1 hour, and signature 3 at 4 hours. Further statistical analysis will be performed with ongoing IVF trials. Work done here can help provide livestock producers with tools to predict a sire's potential fertility and help aid human assisted reproduction technologies. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2022-67015-36298 from the USDA National Institute of Food and Agriculture and National Pork Board Award no. PR005493.

P-15 - Studies on The Impact of Hyaluronidases and Low Molecular Weight Hyaluronan on Preimplantation Embryo Development, Morphokinetics, Pregnancy and Live birth rates in Sheep

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There is increasing interest in understanding the role of the hyaluronan (HA) system in mammalian embryo development and implantation. We have previously shown size-specific effect of HA on embryo development and the beneficial effects of hyaluronidase-2 (hyal-2) on improving blastocyst rate and quality. The effect of HA is mediated through binding to its receptors CD44 and RHAMM present in bovine embryos. Here we compared the effect of hyal-2 and sperm hyaluronidase (Spam-1; PH20) and the corresponding low molecular size HA fragments produced by these enzymes [LMWHA (15-40kDa) and very LMWHA (< 10kDa)] respectively, on sheep embryo development in vitro. Survival rate after vitrification, pregnancy and live birth rate after blastocyst transfer to estrus synchronized recipient donors were compared. Pregnancy was assessed by ultrasound scanning on day 35, and number and normality of lambs were recorded. In addition, impact of hyal2 on morphokinetics of cell division in the bovine embryos was analyzed. Cleaved 2-cell embryos were cultured in the absence or presence of 300IU/ml hyal-2 in an Embryoscope time-lapse imaging incubator. For each experiment, sheep embryos were produced in four independent repeats after in vitro maturation and in vitro fertilization of abattoir-derived oocytes. Hyal2

supplementation increased blastocyst rate (58% v 28% in control) ($p < 0.001$). This effect was abrogated in the presence anti- CD44 and RHAMM and no blastocyst could be produced in the presence of anti-RHAMM even in the presence of hyal-2. No difference was observed in blastocyst rate between control and Hyal-1 treatment groups. VLMWHA at 2 $\mu\text{g/ml}$ significantly increased blastocyst and hatching rates ($p < 0.001$). Higher percentage of the blastocysts produced in VLMWHA survived after vitrification as observed by re-expansion and hatching after re-culture (80% v. 52.2%, $P < 0.05$). Higher number of pregnancies and live birth was observed in ewes received hyal-2 [83% (10/12)] and VLMWHA [75% (9/12)] treated blastocysts versus control (6/11; 55%). No abnormality was observed in the lambs' weight, behavior and survival. Presence of hyal-2 accelerated cell division to different stages of the embryos with and overall average of 16h faster development of blastocyst as compared to control group. The findings of these studies provide significant results leading to the potential use of VLMWHA or hyal-2 as part of the preimplantation culture media. This project was funded in part by Origio Ltd. (currently Coopersurgical Company).

P-16 - Pre-Stimulation Level of Serum Anti-Müllerian Hormone is Associated with Subsequent Quality of Ovarian Response to eCG/pLH in the Domestic Cat (*Felis catus*)

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Serum anti-Müllerian hormone (AMH) concentration is a standard diagnostic marker used in human fertility clinics to predict the quality of the ovarian response to gonadotropin stimulation in advance of assisted reproductive technologies (ARTs). While similar diagnostic utility has been demonstrated in livestock species, it is unclear whether serum AMH is equally informative in felids. Here, we present preliminary data from domestic cats ($n = 13$) that underwent ovarian stimulation with equine chorionic gonadotropin (eCG) and porcine luteinizing hormone (pLH). Blood samples were collected during interestrus (progesterone $< 0.5 \text{ ng/mL}$) 2-25 days before eCG injection to determine pre-stimulation AMH concentrations. Cats were spayed 15 days after pLH injection, and pre-stimulation AMH was compared to post-stimulation outcomes measured at the time of spay, including serum progesterone and number of ovulation sites. Linear regression revealed that pre-stimulation serum AMH concentration significantly predicted post-stimulation serum progesterone ($R^2 = .34$, $p = .046$), with greater pre-stimulation AMH levels associated with greater subsequent progesterone output. Additionally, cats with greater levels of AMH prior to eCG/pLH tended to have greater numbers of ovulation sites following stimulation. We then performed receiver operating characteristic (ROC) curve analysis to determine an AMH threshold that distinguished cats with high or low ovulatory responses to eCG/pLH. We defined a high response as ≥ 6 ovulation sites and a low response as ≤ 5 , because 5-6 ovulations are average for a domestic cat after natural coitus. As indicated by the ROC curve, a pre-stimulation AMH threshold of 4.46 ng/mL classified our cats as high vs low ovulatory responders with 100% sensitivity and 75% specificity. For comparison, control cats ($n = 7$) were induced to ovulate during behavioral estrus by simulated coitus via vaginal swab. In contrast to eCG/pLH-treated cats, control cats exhibited no clear relationships between pre-stimulation AMH and post-stimulation progesterone or number of ovulation sites. These preliminary results suggest that pre-stimulation serum AMH may have diagnostic value to predict

the quality of the response to exogenous ovarian stimulation, but not to simulated coital stimulation, in domestic cats.

P-17 - Normal Rates of Pregnancy and Foaling Achieved Following Transfer of Large Expanded Equine Blastocysts Vitrified After Blastocoele Collapse

Jenin Cortez, Kylie Hardwicke, Danton Cervi, Christopher Grupen

The vitrification of the embryos collected from donor mares is integral to advanced horse breeding programs.

P-18 - Novel Therapies to Protect Sperm Quality In ART

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The manipulation of sperm for assisted reproductive technology (ART) results in a significant decrease in sperm quality, especially higher levels of DNA damage. Elevated levels of DNA damage in sperm have been linked to poor-quality embryos and increased pregnancy loss after ART. Utilizing the novel therapeutic BGP-15, we have previously shown that treating the whole ejaculate prior to and during cryopreservation increases sperm quality compared with frozen/thawed untreated samples. In this study, we investigated the potential of this BGP-15 therapy to preserve sperm quality during semen washing prior to IVF/ICSI. Donated human semen specimens excess to ART (N=341), were treated with 10 μ M BGP-15, and sperm motility and DNA damage (DNA fragmentation and oxidation) were first examined to understand the effect of incubation time in whole semen. Samples were then washed using different clinical sperm washing methods: simple wash, swim-up and density gradient centrifugation (DGC); and purified sperm underwent assessment for motility, vitality, ROS levels, mitochondrial membrane integrity, mitochondrial membrane potential, and DNA damage. Statistical analysis was two-tailed paired Student's T test when comparing between two groups, or repeated measures Two-Way ANOVA when comparing between three groups. Post-hoc pairwise comparisons were two-tailed paired Student's t-tests to analyze matching data pairs for each patient. Semen samples incubated with BGP-15 demonstrated 15% improved sperm motility ($p=0.002$) and 57% reduced oxidative DNA damage levels ($p=0.03$). Comparison between the untreated groups for each washing method, showed that simple washed samples had the highest sperm recovery rate, which was 55% and 21% higher than DGC ($p<0.005$) and swim-up ($p<0.0005$), respectively. Additionally, sperm recovered by swim-up had 18% and 27% increased vitality compared to DGC ($p<0.001$) and simple wash ($p<0.0001$), respectively. Swim-up sperm also presented DNA oxidation levels that were reduced by 40% and 76% when compared to simple wash ($p=0.01$) and DGC ($p<0.0001$), respectively. However, swim-up sperm also had the lowest mitochondrial membrane potential, that was 28% lower than simple wash and DGC ($p<0.03$). Comparison between untreated and BGP-15 treated groups for each sperm washing method showed that DGC sperm had 11% increased mitochondrial membrane potential after BGP-15 treatment ($p=0.0006$). Moreover, BGP-15 treatment only reduced sperm DNA fragmentation in washed samples (22% reduction, $p=0.03$), but it lowered DNA oxidation by 48% in

washed ($p=0.002$), by 42% in swim-up ($p=0.04$) and by 29% in DGC ($p<0.0001$) sperm. We further tested the safety of sperm treatment with BGP-15 in a mouse model and analyzed its effect on IVF, pre-implantation embryo development, embryo cryopreservation and thawing. After surgical embryo transfer, surrogate female mice were assessed for pregnancy success, gestation length and litter size. Litters were also assessed for growth trajectory, righting reflex and survival to weaning at 21 days old. There were no differences between untreated and BGP-15 groups in any measurement analyzed, indicating there was no detectable effect of in vitro BGP-15 treatment of sperm on embryo, pregnancy or offspring outcomes ($n=6$ males/pools of embryos/surrogate females/litters per treatment group; $p>0.1$). Our findings indicate that the utilization of BGP-15 during semen preparation can protect and improve on its quality. Our preclinical models further highlight that the addition of BGP-15 could be used clinically to improve the outcomes of patients undergoing ART.

P-19 - Preimplantation development of bovine IVF embryos obtained from the blastomere separation at 2-cell stage and gene expression homology in pair demi-blastocysts.

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In vitro fertilization (IVF) is valuable technology of producing bovine embryos for embryo transfer (ET). To solve the problems of low conception rates and high frequency of large offspring syndrome (LOS) in bovine IVF embryos, it is necessary to evaluate of gene expression status in IVF embryos using ET. In order to evaluate gene expression in IVF embryos actually used for ET, biopsy should be performed on trophoblast cells at the blastocyst stage. However, mRNA contained in the biopsied sample (10 - 20 cells) dissected by microblade is extremely small. Thus, the amount of cDNA after RT-PCR process is insufficient to analyze various genes. In order to obtain a sufficient sample for gene expression analysis and be less damaging to the embryo using ET, we attempted to make pair demi-blastocysts, which are one for ET and the other for gene expression analysis, by the blastomere separation at 2-cell stage. A total of 64 IVF embryos at 2-cell stage were separated and 128 blastomeres. These blastomeres were cultured using the Well of the Well culture system. The blastocyst formation rate from separated blastomeres (56.3%) was not significantly different compared with control IVF embryos with and without Zona Pellucida (63.6% and 71.8%, respectively). The rate of embryos that formed demi-blastocyst in pairs was 50.0% (32 pairs of 64 sets). In turn, we attempted to determine the gene expression homology in pair demi-blastocysts. Gene expression status in individual 40 demi-blastocysts from 20 pairs were analyzed by RT-real time PCR. The relationships of OCT-4, CDX2 and IGFBP-3 transcript levels in the pair demi-blastocysts were evaluated using scatter plot analysis. The homology of gene expression levels in the pair embryos was high. The developmental ability and epigenetic homology of the bovine demi-blastocysts obtained by the separation at 2-cell stage were demonstrated in this study. This technology enables not only gene expression evaluation of the transferred embryos, but also genomic selection based on single nucleotide polymorphism (SNP) genotypes at the early embryo stage which will contribute to the efficient production of high-performance cattle using IVF technology.

P-20 - Exploring the Influence of Extracellular Vesicle Supplementation on the Equine Oocyte Fertilization Rate after ICSI

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The efficiency of in vitro embryo production in horses is limited compared to other animal species. This is due to the lack of an effective superovulation method, low availability of oocyte-cumulus complexes (COC), along with the limited rates of in vitro oocyte maturation (IVM) and poor fertilization and blastocyst rate. Extracellular vesicles (EVs) are nanoparticles secreted by all body cells, playing a significant role in intercellular communication within the ovarian microenvironment, due to their ability to transport bioactive components. They are used in various studies as potential biomarkers and supplementary ingredients that allow to imitate the physiological conditions during IVM and, therefore, enhance the oocyte developmental potential. The hypothesis of the conducted research was that EVs obtained from small ovarian follicles (< 20 mm) could improve the fertilization rate in mares. Follicular fluid was aspirated postmortem from ovaries lacking a corpus luteum, and small EVs were extracted using ultracentrifugation and characterized. The IVM process was performed in the commercial EQ-IVM medium (IVF-BioScience) with the addition of EVs (200 µg of EV protein/ml) or without supplementation (control). Additionally, EVs internalization during IVM was examined using fluorescent particle labeling and confocal microscopy. In vitro fertilization was performed by intracytoplasmic sperm injection (ICSI) of oocytes and presumptive zygotes were cultured in vitro in TCM 199 (Earl's salts, with the addition of FBS and gentamicin) for 5-7 days in the time-lapse system. Confocal microscopy confirmed the internalization of EVs by both cumulus cells and the oocyte. Nanoparticle tracking analysis showed that the average size of EVs obtained from follicular fluid was 110.7 nm, and flow cytometry confirmed the presence of the surface markers, tetraspanin CD81 and CD63 in 56.5% and 22.4% of the particles, respectively. Using transmission electron microscopy, the disk shape characteristic of EVs was observed in the isolates. Total of 532 COCs designated for IVM were obtained from 75 slaughtered mares. After IVM, 196 oocytes (36.84%) exhibited the presence of a first polar body, with the experimental and control groups showing IVM rates of 39.76% and 34.28% respectively. A higher fertilization rate (34.7% vs. 20.2%) ($p < 0.05$), a slightly lower degeneration and improvement in the cleavage efficiency ($p < 0.1$) were observed in the experimental group compared to the control. Although in both groups the embryonic development arrested at the early stages, the results indicate the potential of follicular fluid-derived EVs as a factor supporting IVF in horses. This research was funded in part by [National Science Centre, Poland] [2022/45/N/NZ9/01795]. We also acknowledge financial support from the University of Agriculture in Krakow [grant number: SUB020013-D015].

P-21 - Reprogramming Conditions for Induced Pluripotent Stem Cells using Immortalized Mink Cells

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Despite the tremendous potential of induced pluripotent stem cells (iPSCs) across diverse applications, the optimal establishment conditions for species other than humans and mice remain insufficiently explored. One obstacle hindering the optimization of reprogramming conditions in such species is the lack of uniformity in the cell lines used in each report, even within the same species, which poses obstacles to reproducibility. Identifying immortalized cells, particularly from public cell banks, capable of reprogramming could significantly enhance experimental reproducibility. This study investigated the reprogramming potential of mink-derived immortalized cell line, Mv.1. Lu (NBL-7) cells. Mv.1. Lu cells were obtained from the JCRB Cell Bank in Japan. The constructs, including Tet-on-inducible reprogramming factors, were induced into the cells with the piggyBac transposase system. Post gene introduction, the cells were cultured in DMEM with 10% FBS for 48 hours. Subsequently, the cells were seeded onto mitomycin C-treated mouse embryonic fibroblasts and cultured in NSM medium (N2B27 medium supplemented with 5% KSR, human LIF, CHIR99021, PD0325901, A83-01, and Forskolin) with Doxycycline added to induce reprogramming. The medium was refreshed every two days. Initial attempts using Yamanaka four factors (OCT4, KLF4, SOX2, MYC) showed no colony formation within the three weeks. However, the introduction of Yamanaka factors with three factors (Nanog, Glis1, Lin28a) and three more factors (Kdm4d, p53DD, Klf2) resulted in alkaline phosphatase-positive colony formations. The induction of the ten factors led to the appearance of naïve-like colonies within one week of culture in the NSM medium. However, these colonies did not persist upon passaging, with or without Dox, and differentiated into cyst-like structures. Our findings demonstrate that Mv.1. Lu cells can be induced to form iPSC-like colonies through the introduction of reprogramming factors. This suggests the potential utility of this cell line as an assay system for investigating reprogramming induction conditions within the mink genetic background.

P-22 - Glycaemic Response in Pigs Derived from Assisted Reproductive Technologies: A Long Term Follow-Up Study

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Emerging evidence suggests that assisted reproductive technologies (ART) may predispose individuals to an increased risk of metabolic disorders including diabetes. Previous studies have reported a different glycaemic response in growing pigs derived from ART, but it is unknown whether these differences persist into adulthood and old age. The aim of the present work was to gain insight into the evolution of glycaemic response of ART-derived pigs across their life. Pigs born through artificial insemination (AI group) and transfer of in vitro-produced embryos (IVP group) underwent a glucose challenge at young age (45 days), adulthood (365 days), and old age (1250 days). The number of animals, respectively for AI and IVP groups, were 16 and 29 (young), 13 and 21 (adult), and 9 and 13 (old). Oral glucose tolerance test (OGTT; 1.75g/kg body weight) was performed after overnight fasting and 1h water withdrawn. Blood samples were obtained from auricular vein

prior (T=0 min) and 15, 30, 45, 60, 90, 120, 150 min following glucose intake. Glycemia was immediately determined by glucometer test strips (GlucoMenLX Plus). The area under the curve analysis was used to identify differences ($p < 0.05$) between age and experimental groups. Minimum and maximum glycemia recorded during the three OGTT tests were 22.0 and 319.0 mg/dl respectively. Both the origin of the animals (AI vs. IVP) and the age (young-adult-old) significantly affected the glycaemic response ($p < 0.05$). At a similar age, there were no differences in fasting glucose between AI and IVP groups (young: 81.70 ± 4.67 and 90.25 ± 8.27 mg/dl; adult: 91.13 ± 7.41 and 87.70 ± 7.14 mg/dl; old: 65.12 ± 3.50 and 59.93 ± 4.12 mg/dl) being all data within the physiological range for pigs (60.00-100.00 mg/dl). Mean glycaemia during OGTT was 25-30 mg/dl higher in young and adult animals compared to old pigs ($p < 0.05$). Evolution of glycemia curves showed a monophasic pattern during youth in both groups, as it is commonly observed in young pigs and we have previously described. Interestingly, the response pattern shifted into a biphasic curve in adult-AI pigs, returning to a monophasic response during old age, whereas in IVP animals the biphasic response appeared only during old age. The lack of studies in older pigs makes difficult to interpret the biphasic response which in other species is associated with a certain resistance to insulin. Moreover, differences between origin of animals were observed at young and old ages ($p < 0.05$). Therefore, young-IVP animals had higher AUC during the initial response to glucose challenge (15, 30 and 45 min) than young-AI pigs. However, old-IVP had lower AUC than old-AI pigs at the intermediate time of 60 min due to the biphasic glucose peak around this time. No differences between groups were observed during adulthood. In conclusion, naturally and artificially conceived pigs show different responses to glucose challenge across life. The clinical relevance and implications of such differences should be further studied. This study is part of project PID2020-113366RB-I00 funded by MCIN/AEI/10.13039/501100011033/ and “FEDER Una manera de hacer Europa”.

P-23 - Predicting the success of Laparoscopic Artificial Insemination in Sheep

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Artificial insemination (AI) plays a critical role in facilitating rapid genetic and production gains for the sheep industry, yet variable success rates and diminished confidence have reduced the use of AI and high-merit semen. While several factors are thought to influence sheep fertility, their relevance during laparoscopic AI and the interplay between factors is unknown. As such, this study examined how various female factors collected at the time of AI and male semen factors assessed in vitro influence the success of intrauterine laparoscopic AI in sheep. Over 3 breeding seasons, data was collected during 30 different AI programs in Australia using Merino rams (N=387) and ewes (N=30,254). Ewes were synchronised and laparoscopically inseminated as per industry standards. During AI, the ID tag, age, uterine tone score (1; pale/flaccid-5; turgid/pink), intra-abdominal fat score (1; little to no fat present-5; high fat), time of insemination post CIDR removal and sire used, was recorded for each ewe. A subset of the semen used for insemination underwent advanced in vitro semen assessment. Thawed samples were diluted (1:0.5) in PBS + 0.3% BSA and immediately assessed for volume (mL), subjective motility (phase contrast microscopy), sperm concentration ($\times 10^6$ spm/mL; NucleoCounter SP-100), and morphology (% abnormal; $\times 400$

objective). Samples were then incubated and aliquots were taken at 0, 3 and 6h for motility and kinematics assessment (Computer-assisted semen analysis; HT CASA IVOS II) as well as sperm viability and acrosome integrity (FITC-PNA/PI), membrane fluidity (M540/Yo-Pro), mitochondrial superoxide production (Mitox Red/Sytox Green), lipid peroxidation (Bodipy C11) levels of intracellular Reactive Oxygen Species (H2DCFDA) and DNA fragmentation (Acridine Orange) via flow cytometry (CytoFLEX and Aroura 3L). Transcutaneous ultrasound was performed approx. 55 days post AI. Statistical analyses (RStudio) included correlations, CASA Principal Component Analysis (PCA) and a binomial multivariable regression analysis. The concentration at which sperm were frozen ($p < 0.001$), CASA PCA at 0h (PCA; $p = 0.03$), the percentage of viable, acrosome intact sperm at 6h ($p = 0.02$), percentage of abnormal sperm ($p < 0.001$), as well as uterine tone ($P < 0.001$) and intra-abdominal fat ($p = 0.03$) of ewes significantly influenced the likelihood of pregnancy following AI. A 1% increase in the percentage of acrosome intact, viable sperm, a 1% increase in morphology abnormalities and an additional 100×10^6 sperm/mL frozen corresponded to a 1.01% increase (OR = 1.10, 95% CL: 0.34, 2.56), 1.07% decrease (OR = 0.99, 95% CL: 0.98, 1.00) and 5.09% increase (OR = 1.05, 95% CL: 1.05, 1.05) in the probability of a ewe falling pregnant, respectively. Furthermore, each standard deviation away from the CASA PCA average led to a 0.37% decrease in the probability of pregnancy (OR = 1.00, 95% CL: 1.00, 0.99). The odds of pregnancy increased by 7.21% (OR = 1.07, 95% CL: 0.44, 2.60) and 6.05% (OR = 1.07, 95% CL: 0.78, 1.47) when semen was inseminated into an ewe with a uterine tone and intra-abdominal fat score of 4+5 compared to ewes which scored 1+2 ($p < 0.001$ and $p = 0.047$, respectively). The results demonstrate the benefit of analysing multiple male and female factors recorded during AI on individual ewes. The identified factors could now be used to standardise and further enhance AI protocols. Screening semen before AI can help minimise variability in success rates, improve the adoption of reproductive technologies, and ultimately advance the rate of genetic gain for the sheep industry.

P-24 - Microfluidic Sperm Selection Improves Number and Quality of In Vitro Produced Bovine Embryos

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For successful in vitro embryo production, male factors such as sperm motility and DNA integrity play an important role. Sperm selection by density-gradient centrifugation (DGC) before in vitro fertilization (IVF) is widely used to select progressive motile sperm but potentially damages sperm DNA. Therefore, a microfluidic sperm selection (MFSS) method has been developed to reduce DNA damage by avoiding centrifugation. While MFSS is expected to improve the production efficiency of IVF embryos, the impact of sperm DNA integrity before selection on the efficiency of MFSS remains unknown. Thus, the present study aimed to determine the impact of MFSS on in vitro production of bovine embryos using sperm with high or low DNA integrity. Cryopreserved semen samples from 11 Simmental bulls were selected depending on their DNA fragmentation index (%DFI), where six bulls showed low %DFI values (%DFI < 3.5%) and five bulls had high %DFI values (%DFI > 7.0%). Progressive motility, viability, and %DFI of sperm were analyzed before (Pre-selection (PS)-sperm)

and after selection with DGC using Bovipure™ (DGC sperm) or microfluidic chips (Fertile Plus, KOEK Biotechnology: MFSS sperm). Abattoir-derived cumulus-oocyte complexes of dairy cows were matured in vitro and fertilized with DGC- or MFSS- sperm. At 18 h post-insemination, the number of pronuclei was determined to evaluate the fertilization rate. Another cohort of zygotes was examined by time-lapse microscopy for 48 hours to determine the timing of the first cleavage and its pattern, then cultured for seven days. At day 7, the bioenergetic profile of the embryos was analyzed by measuring the oxygen consumption rate (OCR) of pools of five expanded blastocysts using an extracellular FLUX-analyzer (Seahorse XFp, Agilent). Progressive motility and viability increased, and %DFI decreased in MFSS-sperm compared to PS-sperm and DGC-sperm in all bulls, regardless of pre-selection %DFI values ($P < 0.01$). In bulls with high %DFI pre-selection, %DFI increased in DGC-sperm ($P < 0.0001$) compared to PS-sperm ($P < 0.01$). Compared to the DGC-IVF zygotes, MFSS-IVF showed a lower proportion of abnormal fertilization (more than two pronuclei, 34.5% vs. 22.7% in low DFI bulls; 36.1% vs. 7.3% in high DFI bulls, $P < 0.05$). The proportion of embryos showing direct cleavage, the cleavage from one cell to three or more blastomeres, was lower in MFSS-IVF (12.5% and 2.5% in low and high DFI bulls, respectively, $P < 0.05$) than DGC-IVF (32.4% and 22.4% in low and high DFI bulls, respectively). The developmental rate to the blastocyst was higher in MFSS-IVF (35.0% and 29.5% in low and high %DFI bulls, respectively, $P < 0.05$) compared to DGC-IVF (28.5% and 21.8% in low and high %DFI bulls, respectively). In high %DFI bulls, the OCR of blastocysts was higher in MFSS-IVF embryos than in the DGC-IVF embryos ($P < 0.05$), whereas DGC-IVF and MFSS-IVF embryos showed comparable OCR values in low %DFI bulls ($P > 0.05$). In summary, MFSS substantially improved bovine sperm quality and embryonic development compared to DGC. These improvements occurred both in ejaculates with low and high DNA damage before the selection. The blastocysts produced with MFSS-sperm also showed a better bioenergetic profile, but only in ejaculates with high DNA damage. These results indicate an increase in the number of bovine embryos after IVF with sperm with low and high DNA damage and an improvement of embryonic quality after IVF with sperm with high DNA damage by using microfluidics compared to DGC for sperm selection.

P-25 - Gene Editing affects Embryo Quality in Rhesus Macaques

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Because of their close evolutionary relationship to humans, rhesus macaques (*Macaca mulatta*) are an important animal system to create nonhuman models of disease allowing for studying various diseases and developing treatments. Advances in gene editing and Assisted Reproductive Technologies have made it possible to routinely generate embryos with specific mutations in nonhuman primates. Edited blastocysts can be used for embryo transfers with the goal to create infants with specific mutations of interest. We are invested in learning how gene editing alters embryo development, and we hypothesize that it may have adverse effects on embryo quality. To assess embryo quality, we analyzed 367 blastocysts that were generated in the ART Core at the ONPRC from 2021-2024 including projects that involve gene editing using CRISPR or piggyBac technology ($n=219$) and non-editing projects that commonly involve IVF between heterozygous carriers of specific gene mutations ($n=148$). Blastocyst quality was evaluated considering their individual age and morphology. In rhesus, blastocyst development usually occurs from day six to

ten, with the older embryos being of lower quality. To query whether gene editing influences the day of blastocyst development, we performed a t-test which revealed a significant difference between the mean days (age) of edited and unedited blastocysts (7.959 ± 0.06 days vs 7.655 ± 0.06 days, $p < 0.001$). By applying the guideline for embryo grading from the Society for Assisted Reproductive Technology, we defined blastocysts into good, fair and poor based on their morphology of inner cell mass and trophectoderm. A chi-square test of independence revealed that the relation between embryo projects (edited/unedited) and embryo grade (good, fair, poor) was significant, $\chi^2(6, n=384) = 402.172$, $p < .001$). Unedited embryos are more likely to be of good quality, while edited embryos are more likely to be of fair or poor quality. Furthermore, to assess if there is a similar potential for blastocysts from edited and non-edited projects to lead to implantation and pregnancy following an embryo transfer, we analyzed 72 embryo transfers and found no statistically significant difference performing a t-test (edited: $n=51$, 12 pregnancies, unedited: $n=21$, 6 pregnancies, $p = 0.6588$). Our analysis provides strong evidence that gene editing affects embryo development and blastocyst morphology. We observed a deceleration in their general development rate as well as an adverse effect on their morphology. Gene editing technology is known to have the potential to cause cytotoxicity and also double stranded DNA breaks, which could be the reason. By providing more detailed insight into the quality of blastocysts and working cooperatively, gene editing technology can focus on improving those adverse effects.

P-26 - Efficiency of the CryoEyelet® Device in Simultaneously Vitrifying Large Numbers of Mouse Oocytes

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Current vitrification devices, designed for handling a limited number of oocytes, lead to significant time consumption and vary in effectiveness based on user experience. This necessitates an easy-to-use device that guarantees reproducibility and efficacy when attempting to store a large number of oocytes. Our study compared the CryoEyelet® device's efficiency to a commercial device, consisting of a transparent film strip attached to a plastic handle, in vitrifying up to 25 mouse oocytes by device, focusing on their fertilization and development post-thawing and Intracytoplasmic spermatozoa injection (ICSI) using piezo pulses targeting. MII oocytes were vitrified following Kitazato Co.'s instructions, then stored in liquid nitrogen. After thawing, oocytes were incubated in KSOMaa medium at 37°C in 5% CO₂ in humidified air. Embryo development was assessed at 1 (two-cell stage) and 3.5 days (blastocyst stage) post-ICSI. Statistical analysis showed no significant difference in two-cell stage outcomes between fresh oocytes, CryoEyelet®, and commercial devices (100% (165/165), 97.4% (149/153), 98.6% (70/71), respectively). Blastocyst development rates were 81.2%^a (134/165) for fresh oocytes, 69.3%^b (106/160) for CryoEyelet®, and 62.0%^b (44/70) for commercial device ($p < 0.05$). The CryoEyelet® device offers high storage

capacity, saving time, and ensuring high viability of mouse oocytes. Funded by MCIN/AEI/10.13039/501100011033 and the EU “NextGenerationEU”/PRTR (PDC2021-120767-I00).

P-27 - Assessing Cryotolerance in Rabbit Embryos Across Three Consecutive Generations: The Impact of Vitrification on Offspring

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Cryopreservation is a method for long-term embryo storage, potentially preserving genetic diversity. However, its effectiveness in maintaining viability over extended periods and the cryotolerance of embryos across successive generations are not well understood. This knowledge is crucial for applications such as regenerating live animals and restoring lost breeds. This study focused on assessing the cryotolerance of embryos over three consecutive generations in rabbits, aiming for successful offspring regeneration. Natural conceived females were super-stimulated (3 µg of Corifollitropin alfa) and artificial inseminated with monospermic sperm dose (20x10⁶ millions) and late morulae and early blastocysts were recovered and vitrified using a French ministraw and a two-step vitrification protocol (EQ: 10% DMSO and 10% EG for 2 minutes; V: 20% DMSO and 20% EG for less than 1 minute). After warming, embryos (14-16 embryos by female) were transferred to natural conceived foster mothers to establish F1 population. Once the animals from the F1 population reached adulthood, females and males were bred, deliberately avoiding mating between animals with common grandparents to reduce inbreeding. One-two female from each sire family was super-stimulated to produce embryos to establish the F2 generation. This process was repeated to generate the F3 generation. A total of 1,104 vitrified embryos were transferred; 100 for F1, 310 for F2, and 694 for F3 to 7, 18 and 48 naturally conceived females respectively, and the offspring rate was recorded by generation. The offspring rate significantly decreased across generations (65%^a, 34%^b, and 17%^c for F1, F2, and F3, respectively, $p < 0.05$). These data suggest that embryos may lose their cryotolerance capability over vitrification generations. The study was funded by the Conselleria de Innovación, Universidades, Ciencia y Sociedad Digital (CIAICO/2022/072).

P-28 - Morphokinetics as a Tool to Identify Embryos with Superior Ability to Survive Cryopreservation

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Currently, in vitro-produced embryos represent over 2/3 of the global production of bovine embryos. The number of transfers of cryopreserved embryos has been increasing. However, pregnancy per transfer is lower for cryopreserved embryos than for fresh embryos. The objective was to use morphokinetic endpoints of development to identify embryos with superior ability for

survival to cryopreservation. Survival was determined post-thawing during 72 h of culture. Embryos were produced in vitro and individually cultured in a time-lapse incubator (Miri®TL6, Esco Medical) from ~ 17 h after fertilization until day 7.5. Images were captured every 5 min in different focal planes and a final video was obtained for each embryo. Several morphokinetic features were evaluated, including timing of cell divisions, size of blastomeres at the 2-cell stage (equal vs unequal), type of first cleavage (1-cell to 2-cell, 1-cell to 3-cell, and reverse cleavage), appearance at various stages (including paleness of cytoplasm and occurrence of extruded cells), blastocyst grade and stage as well as duration of the lag-phase in temporary cessation in development occurring between the 4 to 16-cell stage (77% of lag phases were initiated between the 5 and 8-cell stage). On day 7.5, blastocysts (n=132) were vitrified using a Cryolock device. Subsequently, each blastocyst was thawed and cultured individually in the Miri®TL6 and survival characteristics (re-expansion of the blastocoele and hatching from the zona pellucida) were evaluated at 24, 48 and 72 h of culture. Data were analyzed using chi-square test and logistic regression procedures of SAS. The overall percent of blastocysts that re-expanded at 24, 48 and 72 h was 59, 59 and 54%, respectively and the percent that hatched was 14, 33 and 38%, respectively. Morphokinetic indicators were largely similar for time points after thawing. Here data at 48 h post-thawing are reported. Among the determinants of whether an embryo survived cryopreservation, whether measured as the percent that re-expanded or that hatched, was timing of first cleavage, timing of formation of the compact morula, duration of the lag-phase and blastocyst stage and grade at the time of cryopreservation. For example, the time of first cleavage was 28.3 ± 0.5 h for embryos that re-expanded vs 30.4 ± 0.6 h for embryos that did not re-expand ($P=0.004$). The time of compact morula formation was 134.5 ± 1.3 h for embryos that hatched vs 140.3 ± 1.5 h for embryos that did not hatch ($P=0.004$). The lag-phase duration was 34.3 ± 1.2 h for embryos that re-expanded vs 39.0 ± 1.2 h for embryos that did not re-expand ($P=0.016$). Embryos that were expanded blastocysts at time of vitrification were more likely to re-expand (79%) than blastocysts (51%) or early blastocysts (26%) ($P < 0.0001$). Blastocysts that were classified as grade 1 prior to vitrification were more likely to hatch as compared to embryos of other grades (53% vs 26%; $P=0.012$). Size of blastomeres at the 2-cell stage, type of first cleavage, embryo paleness and degree of blastomere extrusion were not related to cryosurvival. Results demonstrate that timing of development and blastocyst characteristics at vitrification are important variables associated with survival post-thawing. Machine learning approaches and development of an in vivo model to characterize survival may further improve the accuracy of morphokinetic endpoints for identifying embryos with superior cryosurvival. (Support: BARD IS-5474-22).

P-29 - Changes in Liver Metabolism in Mouse Offspring Born after In Vitro Embryo Culture

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During in vitro culture (IVC) the embryo can be exposed to oxidative stress. We recently show that IVC induces oxidative changes in lipid droplets in preimplantation embryo, and that lipid changes

are maintained during fetal development. Because the liver plays a central role in lipid metabolism, here we hypothesized that IVC can cause changes in hepatic lipids in offspring, which can predispose to steatosis and liver disease. C57BL6 embryos collected one day following mating of 8 pairs of 3-4 months old mice were collected and cultured in vitro in KSOM medium using standard procedures. Total of 95 blastocysts were then transferred to pseudopregnant recipients for further development (8-12 embryos/female). Four months old male offspring obtained following IVC and control offspring obtained following natural mating were randomly selected for liver analysis (n=8/group). Collected liver was homogenized and subjected to evaluation of lipid peroxidation, antioxidant mechanism status and proteome. Statistical significance ($p < 0.05$) was assessed by Mann-Whitney test, data are described as Mean $\pm$ SEM. High level of lipid peroxidation product, malondialdehyde (MDA) (27.63 ± 9.03 vs. 18.99 ± 1.93 nmol/mg of protein, $p = 0.03$) measured by Thiobarbituric acid reactive substance (TBARs) assay was demonstrated in livers of IVC offspring. Furthermore, pro-oxidative status of liver from IVC offspring was shown by an increased levels of oxidative stress markers: GSH (1.11 ± 0.2 vs. 0.2 ± 0.06 nmol/mg of protein, $p = 0.002$); GSSG (0.37 ± 0.05 vs. 0.16 ± 0.04 nmol/mg of protein, $p = 0.0093$) and high superoxide dismutase activity (SOD) (4.0 ± 0.7 vs. 2.2 ± 0.2 U/mg of protein, $p = 0.03$) evaluated by colorimetry assay. Tandem Mass Tag (TMT) proteomic analysis revealed 88 differentially expressed proteins (DEPs) in IVC group (34 upregulated and 54 downregulated DEPs). Enrichment analysis indicated that most of up-regulated proteins are involved in lipid metabolism pathways, such as, fatty acid oxidation, PPAR signalling pathway, beta-oxidation, alcoholic liver disease, lipid accumulation, and hepatic steatosis. Most of 54 down regulated DEPs were involved in oxidative stress and redox pathway, glutathione metabolism, fatty acid beta-oxidation, synthesis of polyunsaturated fatty acids and other metabolic pathways. Altogether, liver of males born after embryo culture of mouse embryos is characterized by oxidative damage of lipids and altered expression of proteins regulating lipid metabolism. During early stages of development the liver is sensitive to changes in the environment. Our study indicates that in vitro environment can induce embryonic lipid damage and subsequent development of the liver. Consequent to embryonic lipid damage deregulation of hepatic lipid homeostasis may predispose to steatosis and liver disease later in life. Funding: Study supported by National Science Centre, Poland GA 2022/44/C/NZ4/00076; 2021/41/B/NZ3/03507; 2019/35/B/NZ4/03547..05

P-30 - Long-term Phenotypical Implications of Assisted Reproductive Technologies in Cattle

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Assisted reproductive technologies (ART) are regularly used in cattle breeding to produce animals of high genetic value. In 2022, over 1 million in vitro-produced embryos were transferred worldwide. However, recent studies suggest that in vitro-produced animals may differ in health, milk production, fertility, and gestational length compared to those derived in vivo. Currently, the long-term effects of ART on growth have not been studied. We aimed to investigate whether there are differences in IGF-1, T4 and cortisol levels, and their interactions with growth parameters (body weight, withers height, thoracic circumference and body length), between cattle of in vivo and in

vitro origin. The 19 animals included in the study were either derived from artificial insemination (AI; 5 males and 2 females) or from embryos produced in vitro with either reproductive fluids as source of protein (RF-IVP; 4 males and 1 female) or BSA (C-IVP; 4 males and 3 females). Growth parameters were assessed at 0, 3, 7, 15, every 15 days until day 360, 550, 750, 900, 1100, 1300 and 1500 days of age. Blood samples were collected at 75, 150, 360, 550, 900, 1100 and 1500 days of age and centrifuged. Plasma hormone concentrations were determined using a solid-phase, enzyme-labeled competitive chemiluminescent enzyme immunoassay. Data were analyzed using a linear mixed-effects model. Benjamini-Hochberg was used for p-value correction. Data were considered significant when $p < 0.05$. IGF-1 levels were different between both in vitro groups, being 1.6 folds higher in RF-IVP than in C-IVP, but no differences were found between the in vitro groups and the AI group. There was no correlation between IGF-1 and weight. Still, there was a positive correlation between IGF-1 and the rest of the growth parameters studied. Higher IGF-1 concentrations were correlated with higher height at withers (0.078 ± 0.029 cm/ng/mL), and higher body length (0.16 ± 0.04 cm/ng/mL). Additionally, in the RF-IVP group, higher IGF was associated with higher thoracic circumference (0.28 ± 0.12 cm/ng/mL). T4 levels were reduced with age, and this reduction was smaller in males than in females. Additionally, in the AI group, T4 was 1.6 folds higher in females than in males at 75 days of age. With regard to the growth parameters, T4 showed a negative correlation with weight (-13.26 ± 6.49 Kg/ μ g/dL), a positive correlation with thoracic circumference in the RF-IVP group (5.06 ± 2.67 cm/ μ g/dL), and no correlation with either withers height or body length. In males, a small but significant increase in cortisol level was observed with age. Additionally, a positive correlation was found between cortisol and weight in the AI group (34.11 ± 25.78 kg / μ g/dL). On the contrary, there was a negative correlation between cortisol and body length in males, and bigger height at withers tended to be associated with lower cortisol concentration ($p=0.06$). There was no correlation between cortisol and thoracic circumference. In conclusion, we determined for the first time the relationship between growth parameters and IGF-1, T4 and cortisol, from birth to 4 years of age in cattle from in vivo and in vitro origins. It was found that in vitro embryo production supports the development of healthy animals, in terms of growth parameters and hormonal regulation, with no clinically significant differences compared to AI animals. This work was funded by AGL2015-66341-R; PID2020-113366RB-I00; IJC2019-039404-I; Maria Zambrano CO-MZ-14.

**P-31 - Non-invasive Targeted Gene Knockdown of GDF9 and BMP15 Genes with LNA™
GapmeRs: Implications for Bovine embryo development**

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In recent decades, substantial progress has been achieved by applying genetic engineering techniques to manipulate genes. RNA interference (RNAi) has been identified as a natural

mechanism regulating gene expression in various higher organisms. RNAi technologies like small interfering RNA (siRNA) are widely used to down-regulate any gene expression without disrupting the expression of other genes. For delivery of these siRNAs into mammalian cells various methods have been used like microinjection, transfection reagents, and electroporation. However, there are some pitfalls in using these assisted delivery methods, such as cellular toxicity, variable transfection efficiency, and off-target effects. Hence it is necessary to deliver RNA Silencing molecules without any assisted delivery methods. In the current preliminary study, we tested the efficiency of commercially available Antisense LNA® (Locked nucleic acids) GapmeRs (Qiagen, 339511) for the knockdown of specific target genes in bovine embryos without any assisted delivery (like transfection or microinjection). For this, we chose GDF9 (Growth differentiation factor 9) and BMP15 (Bone morphogenetic protein 15), two genes involved in folliculogenesis, oocyte quality and embryo development for knockdown by using GapmeRs. To do so, we targeted three exon regions of each target gene (GDF 9 and BMP15), along with negative control. We used routine in vitro maturation and fertilization methods but in vitro culture was supplemented with GapmeRs (100nM) targeting GDF9 (Group A; n = 98, 4 replicates), BMP15 (Group B; n = 99, 4 replicates), a combination (Group C n = 102, 4 replicates), a negative control (Group D; n = 102, 4 replicates), whereas culture without any supplementation served as control (Group E). The presumed zygotes (n = 501 in 4 replicates) were cultured until 8 days post-insemination (dpi), at which time blastocysts were collected and snap-frozen to further validate gene knockdown with RT-qPCR. Our results demonstrated a compromised embryo development following the gene knockdown, which was most pronounced for BMP-15. A drop in cleavage rate was observed in group B ($74\% \pm 7.06$), which was significant when compared to group A ($87\% \pm 8.64$, $p=0.045$). Moreover, a significantly lower blastocyst rate was observed in Group B ($26\% \pm 9.35$) when compared to Group A ($40\% \pm 15.83$) and Group E ($40\% \pm 12.64$) ($p=0.039$, $p=0.023$ respectively), RT-qPCR analysis provided additional proof that GDF9 and BMP15 had a knockdown impact on blastocysts that were collected on 8 dpi. Interestingly, the knockdown of GDF9 increased the gene expression of BMP15 significantly (Fold change (FC): 4.5; $p < 0.05$) compared to the control group (FC: 1.0), similarly knockdown of BMP15 induced a lower gene expression of GDF9 compared to control (FC: 0.4; 1.2, $P < 0.05$ respectively). No significant gene expression changes were observed in other groups. Overall, using this unassisted Antisense oligonucleotides GapmeRs method could help in determining the functional impact of target genes (GDF9; BMP15) during bovine embryo development without using any delivery methods.

P-33 - Toward Decoding Sub-Fertility in Boars.

Bradley A. Didion, Michael Kleve-Feld

It is well understood within human and livestock species on the importance of semen assessment prior to the production of semen doses for use in artificial insemination (AI). When preparing semen doses for AI there are several methods and assays used to accept or decline a semen collection that will proffer enough functional sperm to achieve fertilization. Nevertheless, even though a semen collection can be accepted based on established semen quality thresholds (i.e. concentration, motility, morphology) the assays are unable to identify the sub-fertile male. Towards a better understanding of the sub-fertile boar, there is, assumedly, a transition period during which the animal moves from a healthy or “wellness” state to an unhealthy state wherein the production of functioning sperm is compromised and yet passes all assays suggesting normality and

functional. The notion of identifying a sub-fertile male at the time of sexual maturation via semen evaluation or breeding soundness exam is already too late assuming sub-fertility to be the 'tip of the iceberg'. Albeit a hurdle will be to quantify and characterize an individual male's wellness state. And yet perhaps leveraging the application of omics could provide molecular signatures and biomarker reference values over the course of a male's life as associated with wellness allowing abnormal states detectable months and/or weeks ahead of breeding age. As a boar grows from a piglet to a weaned pig, to a grower, to adult, and to older adult he progresses through phenomes at various times so the male phenome is continuously changing over time. Phenomics measure three elements (genome, behavior, and environment) that come together to form a phenome at a given point in time. These three fundamental elements could be characterized in boars by looking at proteins, metabolites, lipids, and blood biomarkers per a longitudinal approach from newborn to sexual maturation and beyond. Studying the phenome would provide a reference as to what is normal/healthy/functional versus what is unhealthy/abnormal/low functional. The notion to collate omics towards characterizing a boar's wellness over an 18-month period is proposed. Establishing reference values for various metabolites, lipids, hematology is a step in the direction of a future approach that would identify boars possessing biomarkers that are counter-productive to being a fully functional breeding male having acceptable fertility.

P-34 - The Correlation Between Anti-Müllerian Hormone and Antral Follicle Count in Mares Undergoing Estrous Cycle Manipulation

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Anti-Müllerian hormone (AMH) concentration is correlated with antral follicle count (AFC) in the mare. Because of this relationship, potential lies in the ability of AMH to act as a prognostic tool for assessing a mare's overall fertility or, more specifically, predicting a mare's potential as an oocyte donor for the production of in vitro-derived embryos. It is unknown if AMH concentrations remain correlated when mares are undergoing hormone therapy protocols aimed at increasing AFC to promote oocyte aspiration efficiency. The aim of this project is to determine if AFC is correlated to AMH values in mares undergoing cycle manipulation with only follicle ablation (controls) or undergoing hormone therapy targeted to increase AFC with either progesterone and estradiol (P&E) or with a proprietary drug that supports follicular growth. We hypothesize that AMH concentration will be positively correlated with AFC in mares undergoing cycle manipulation. For this study, data was analyzed from two previous experiments. In the first experiment, 10 mares underwent a crossover design in which mares did (P&E group) or did not (Control group) receive a dose of a slow-release formulation of progesterone and estradiol 17 β (P&E, a total dose of 1.5 g and 50 mg, respectively) on Day 0. On Day 0, all mares underwent transvaginal follicle ablation. On Days 0, 3, 6, and 10, AFC was determined via transrectal ultrasound and serum was stored for AMH enzyme-linked immunosorbent assay (ELISA). In the second experiment, a group of 6 mares (Proprietary Drug group) underwent transvaginal follicle ablation (Day -10) followed by prostaglandin (5 mg dinoprost tromethamine IM, Day -4) to lyse existing luteal tissue and act as a means of cycle synchronization. The proprietary drug was administered on Days 0 through 4. On Days 0 through 4 and Day 7, AFC was determined via transrectal ultrasound and serum samples were stored for AMH

ELISA. Data was analyzed for normality via Shapiro-Wilk and correlation was determined by Kendall rank correlation coefficient. Day 0 AMH and AFC data was removed prior to analysis, as this was the first day in which cycle manipulation (i.e., drug administration or ablation) occurred in most mares. Significance was set at $P < 0.05$ for all analyses and performed in R version 4.3.1. There was no significant correlation ($R = 0.17$; $p = 0.19$) between AMH and AFC in the Control group ($n=10$), a moderately positive correlation ($R=0.51$; $p = 0.00011$) in the P&E group ($n=10$), and a weak positive correlation ($R = 0.36$; $p = 0.0062$) in the Proprietary Drug group ($n=6$). This study demonstrates that there is a statistically significant positive correlation between AMH concentration and AFC in mares undergoing two treatments that support follicular growth. Future studies are needed to determine if AMH concentration can be a tool used to help assess oocyte aspiration potential and fertility in the mare.

P-35 - Development of Non-Invasive Cervical Insemination in Rhesus Macaques

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With the high demand of rhesus macaques for biomedical research, breeding programs are under pressure to increase reproductive efficiency while maintaining genetic diversity through intentional breeding strategies. One such strategy is artificial insemination (AI). Transcervical AI in the Rhesus macaque is challenging due to the tortuous path of the cervical canal. However, successful pregnancies have been previously reported by both direct intrauterine insemination (IUI) using animals pre-screened with less cervical impedance (57%) and by ultrasound-guided percutaneous insemination through the abdominal wall and directly into the uterine lumen (33%). Both methods are invasive and require veterinary support. As a refinement, we hypothesized that we could achieve similar pregnancy rates by depositing semen into the external os of the cervix. In this pilot study, regularly cycling adult rhesus females ($n=10$; average age $13.7 \pm \text{SEM } 0.46$ years) underwent a total of 17 insemination cycles. Animals were either inseminated once ($n=5$), or twice ($n=12$) during the periovulatory window. In dual inseminating cycles, semen from different males were used for each insemination to allow for genetic testing of the offspring to identify which insemination resulted in conception. Starting 7 days from the onset of frank menses, we collected blood daily to monitor estradiol (E2) over the periovulatory phase. Inseminations occurred between one day prior and 3 days post-E2 peak (average E2 peak = $360 \pm \text{SEM } 19.1$ pg/ml). Sedated animals were placed in dorsal recumbency with pelvis elevated and monitored by pulse oximetry. Perineum was cleaned of gross contaminants, and a lightly lubricated nasal speculum was inserted in the vaginal canal. Utilizing a surgical headlamp, the external cervical os was located and a Smith's Insemination Catheter was inserted with placement confirmed by ultrasonography. Following fresh semen collection, at least 50 million motile spermatozoa suspended in 0.5ml TALP-Hepes with BSA were infused into the external cervical os. The catheter was then removed and the pelvis remained elevated for 5 minutes while the animal recovered. Pregnancy was diagnosed approximately 30 days later by ultrasound assessment of the uterus. Data were interrogated by 2-way ANOVA with significance considered $p < 0.05$. Of the 5 cycles where animals underwent a single insemination,

no females conceived. From the 12 cycles involving dual insemination, 25% conceived and went on to produce healthy offspring. Genetic testing of the offspring confirmed conception occurred from inseminations performed on days 1-2 after the E2 peak. These data show that dual inseminations resulted in higher pregnancy rates than single inseminating cycles ($p < 0.05$). Further, age had a significant impact on conception rates even though all animals were considered reproductively fecund. Those who conceived were on average younger than females who did not conceive ($11.3 \pm \text{SEM } 1.28$ vs. $14.2 \pm \text{SEM } 0.39$ years, respectively; $p < 0.05$). Overall, non-surgical cervical insemination resulted in a 25% pregnancy rate when using dual inseminations during the periovulatory window. Despite the lower pregnancy rate, when the invasiveness of both cervical cannulation for IUI and percutaneous intrauterine insemination are considered, cervical insemination offers an excellent alternative. This ongoing project will continue to test optimal insemination timepoints around E2 peak, methods to increase conception, and if utilizing cryopreserved sperm yields a similar outcome.

P-36 - Impacts of Immunomodulatory Feed Supplementation on Ovum Pick-Up Collections in Beef Cattle

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Assisted reproductive technologies are implemented to improve the efficiency of genetic selection with ovum pick-up (OPU) becoming a popular strategy for obtaining oocytes from genetically elite cattle. However, in vitro production (IVP) of embryos from these oocytes remains suboptimal. The focus of this work is to explore schemes for improving oocyte quality of donor females. OmniGen-AF® (OG; Phibro Animal Health Corporation, Teaneck, NJ, USA) is an immunomodulatory supplement formulated to improve stress responses in dairy and beef cattle. Several benefits of OG to fertility have been identified, but limited research has explored how OG may specifically influence OPU efficiency and oocyte quality. We propose that OG supplementation to beef cows will improve oocyte and/or cumulus cell quality and these benefits will improve OPU efficiency. Commercial Angus and Charolais cows ($n=7/\text{treatment}$) were blocked by breed and body condition and sorted into one of two groups: control animals fed a base supplement daily (1 kg cracked corn, 30 g molasses) and OG animals were supplemented daily with the base supplement and 56 g OG. All animals were on pasture with ad libitum access to tall fescue grass, hay, and water. The diets began 28 days prior to the beginning of OPU. Ovulation was synchronized and induced during this period so cows would be at 7-day post-estrus on the first OPU event. Ovum pick-up sessions were completed 2x/week at 3-4 day intervals for 5 weeks. Total follicle numbers and diameters of follicles ≥ 5 mm were recorded at each OPU event. Retrieval rate was evaluated for all punctured follicles (≥ 5 mm). Follicular fluid was collected once weekly from the largest follicle. Cumulus oocyte complexes (COC) were scored (IETS scoring system) and ooplasm quality was scored based on color and granularity. Both datasets were analyzed using the GLM procedure in SAS. The MIXED repeated measures in SAS with type AR (1) covariance structure was used to examine total follicle numbers, greatest follicle size at each collection, average follicle size ≥ 5 mm, number of follicles aspirated, and COC recovery rate. Neither OG treatment, time, nor their interaction influenced the

number of non-aspirated ($< 5\text{mm}$) and aspirated ($\geq 5\text{mm}$) follicles and COC retrieval rates. There was a reduction ($P=0.05$) in the mean diameter of the largest follicle when comparing the OG group with controls ($8.37\pm.35$ vs $9.45\pm.35$ mm, respectively). Additionally, mean aspirated follicle size tended to be reduced ($P=0.07$) with OG supplementation. No effects of OG treatment, time, or their interaction were detected for any of the COC quality measurements. In summary, OG supplementation did not have a positive influence on morphometric assessments of follicle development and oocyte quality. Work is underway to explore whether OG supplementation benefits follicular fluid composition and the ability of COCs to generate IVP embryos. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2021-67015-34485 and 2023-67011-40399 from the USDA National Institute of Food and Agriculture, from the Virginia Agricultural Council Project no. 825, and Pratt Research Fellowship. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity employer.

P-37 - Preliminary Study of Sex Hormone Levels Throughout the Estrous Cycle in the Saliva of 4.5-year-old Sows Born by ART

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Estradiol (17-beta-estradiol, E2) and progesterone (P4) play a key role in the regulation of the estrous cycle. Their levels fluctuate throughout the cycle, triggering processes that are essential for the expression of mating behaviour. The rising levels of E2 during the follicular phase triggers the initiation of behavioural and physiological changes associated with heat in sows, whilst P4 is essential for early embryo development and uterine preparation for implantation. The evaluation of sex hormones is typically performed on plasma or serum. However, daily blood collection puts stress on the animals and requires specialized staff, and the extraction may alter the results of some metabolites. Since the small size of these molecules allows them to penetrate the membranes, such as those of the salivary glands, using saliva to measure sex hormones could offer a non-invasive approach to facilitate the collection of sex hormone data. Yet, studies on sex hormones in pig saliva are very scarce, limiting its application in the field. Here, the correlation between the levels of E2 and P4 were evaluated in plasma and saliva, at three separate timepoints, in 22 Large White and Landrace crossbreed sows. These animals were born through different assisted reproductive technologies from a previous study (Paris-Oller et al., 2021), with their ages around 4.5 years old. Their exclusion from production lines allows them to have natural estrous cycles, be kept in an open enclosure, and fed under identical conditions. Furthermore, 12 sows of the same herd [6 born via artificial insemination (AI group) and 6 born after surgical transfer of in vitro-produced embryos (IVP group)] were used to measure the fluctuation of both E2 and P4 in saliva during the estrous cycle. Plasma was obtained through the centrifugation (1000 G, 10 min) of ophthalmic venous sinus blood collected in lithium heparin tubes. Saliva samples were collected using Salivette® tubes containing a polystyrene sponge previously chewed by the sows for 10 secs. These tubes were then centrifuged (1000 G, 5 min) and the samples stored (-80°C) until hormone analysis. Both plasma and saliva samples were later analysed by quimioluminescence (Immulite).

The correlation between the levels of E2 in saliva and plasma, analysed using a Pearson correlation coefficient, showed a statistically significant correlation ($p < 0.05$) with a moderate negative relationship (-0.311). The average level of plasma E2 was 25.78 pg/mL whilst the saliva E2 was 49.05 pg/mL. However, the correlation between P4 in saliva and plasma was not significant, with an average concentration of 15.23 ng/mL and 0.54 ng/mL in plasma and saliva, respectively. Analyses of the hormone levels in saliva showed no significant differences for both hormone levels between the AI group and IVP group, nor for P4 levels among the days of the estrous cycle. Nevertheless, significant differences were found in the levels of E2 per day of the estrous cycle ($p < 0.001$). Moreover, in addition to the peak expected towards the end of the follicular phase, another peak was observed around days 12-14 of the cycle. This peak is essential for the maternal recognition of pregnancy in pigs and has been identified in blood but has never been reported in saliva of non-pregnant animals. This study is part of project PID2020-113366RB-I00 funded by MCIN/AEI/10.13039/501100011033/ and “FEDER Una manera de hacer Europa”.

P-38 - Comparative Application of a Natural, Sustainable Plant Extract with Antimicrobial Properties in Fish, Rooster, Bull, and Boar Spermatozoa

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Antimicrobial resistance (AMR) is one of the challenges for humankind, mainly caused by the overuse of antibiotics. Applying natural plant extracts, rich in polyphenols and with antimicrobial properties, could positively contribute to solving this problem. Semen extenders (used in the millions of liters yearly) usually contain wide-spectra antibiotics, being a target for new formulations using antimicrobial extracts. However, spermatozoa are sensitive to bioactive molecules, and it is necessary to establish compatible concentrations. This communication presents preliminary results on sperm motility from applying grape marc extracts, obtained by an environmentally friendly method, on spermatozoa from species of commercial interest: sturgeon, carp, trout, chicken, pig, and cattle. This information was obtained in the NeoGiANT H2020 project (<https://neogiant.es>) for replacing antibiotics in animal production with plant extracts obtained from a sustainable, environmentally friendly, and patented method. Some details are temporarily limited due to intellectual property protection issues. Sperm doses were collected and diluted using species-specific methods and extenders and evaluated after refrigerated storage. In total, samples from 30 sturgeons, carps, and trouts, 20 roosters, 10 boars, and 10 bulls were used. In all cases, we tested extracts obtained using a glycol and an alcohol (both in the chemical GRAS category), following a progressive experimental design: a first trial was focused on concentrations

around 1%, and successive ones focused on higher or lower ranges, depending on results (including control with no extracts). Sperm motility (CASA, computer-assisted sperm analysis) was assessed at day 0 and after 3, 6, and 9 days at 5 °C for fish, 1 and 2 days at 5 °C for chicken, 3 and 7 days at 17 °C, and 24 h at 5 °C for bull. Data were analyzed by linear mixed-effects models. Results indicated that fish and boar spermatozoa were sensitive to these polyphenol-rich extracts, but low concentrations were compatible and potentially beneficial for sperm storage. On the contrary, bull spermatozoa tolerated the polyphenol-rich extracts well, with an excellent potential for application in sperm preservation. Rooster spermatozoa showed moderate compatibility, but the tolerated concentration was higher than that of fish and pig. Establishing these ranges will enable the development of suitable extenders with no or reduced antibiotic contents. Further studies should focus on sperm physiology to determine how the extracts modulate it during storage and assess their effects on sperm cryopreservation, which is particularly interesting for the cattle industry.

Supported by NeoGiANT 101036768 (H2020-LC-GD-2020-4) and PID2022-137640OB-I00 (MCIN/AEI/10.13039/501100011033, by "ERDF A way of making Europe"). Thanks to AIM/Topigs-Norsvin, V. Pascual, B. Sánchez, and Á. López.

P-39 - Incidence and Timing of Pregnancy Loss Following Timed Artificial Insemination or Timed Embryo Transfer with a Fresh or Frozen In Vitro Produced Embryo

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The aim of this study was to characterize the incidence and timing of pregnancy loss from service event (timed artificial insemination, AI or timed embryo transfer, ET) to parturition. Lactating Holstein Friesian cows received either AI (n = 243) or ET (n = 863) with a fresh or frozen in vitro produced (IVP) blastocyst produced using oocytes collected from the ovaries of elite dairy donors (n = 14 Holstein Friesian and n = 8 Jersey; ET-DAIRY) and elite beef donors (n = 21 Angus; ET-BEEF) using transvaginal ovum pick-up. Blood samples were collected on d 7 from all cows to determine progesterone concentration and from subsets of cows on d 18 (n = 524) and d 25 (n = 378) to determine mRNA abundance of interferon-stimulated gene-15 (ISG15) and pregnancy-specific protein B concentration (PSPB), respectively, to provide an early pregnancy diagnosis. Transrectal ultrasonography was conducted to determine pregnancy status on d 32, d 62 and d 125 after synchronized ovulation. Parturition date was recorded for all cows that reached a term delivery. Probability of pregnancy (%) varied at each time point depending on treatment (AI: 70.9, 67.3, 57.3, 48.8, 47.0, 44.8, 44.3; Fresh ET: 100.0, 73.2, 60.1, 56.1, 48.4, 46.0, 44.6; Frozen ET: 100.0, 67.6, 52.9, 41.6, 32.9, 31.3, 29.7). Irrespective of treatment, the largest proportion of pregnancy loss occurred in the period from service event (AI on d 0 or ET on d 7) to d 18, with minimal loss occurring between d 62 and parturition (AI: 1.8%, ET fresh: 1.9%, ET frozen: 3.5%). Differences in P/S were detected between fresh vs frozen ET on d 32 (P = 0.0001) and both AI and Fresh ET vs Frozen on d 62, 125 and at parturition (P < 0.05). There was greater probability of pregnancy loss (P = 0.002) between d 32 and 62 following ET (Fresh: 11.3%, Frozen: 18.0%) than AI (4.0%). The percentage of cows that calved following transfer of a fresh embryo was similar to AI. In conclusion, AI and fresh ET led to a greater probability of a cow becoming pregnant and maintaining the pregnancy to term than frozen ET. Cows that were still pregnant on d 62 had a very high likelihood of maintaining the pregnancy to parturition. Further work is required to improve probability of

pregnancy and reduce embryonic and fetal mortality following transfer of a cryopreserved IVP embryo.

P-40 - Sperm Injection at The Para-Polar Body Site in Piezo-ICSI Improves the Survival Rate of Treated Metaphase II Oocytes.

Yoshikazu Nagao; Shiori Ashibe; Yoku Kato; Rui Miyamoto; Borjigin Sarentonglaga

Conventional and piezo-intracytoplasmic sperm injection (ICSI) are generally performed at the 3 o'clock position of a metaphase II (MII) oocyte when the first polar body (PB) is positioned at either 6 or 12 o'clock in order to avoid damage to the meiotic spindle. The choice of this injection site is based on the assumption that the meiotic spindle is located near the PB. However, it is possible that the ooplasmic cell membrane might be damaged in piezo-ICSI during drilling of the zona pellucida with piezo pulses; such damage might result in a reduction in the rate of successful development to the blastocyst stage. Here, we designed a new piezo-ICSI method in which the PB is set at either the 2 or 4 o'clock position. In this new method, zona drilling and sperm injection is performed through the para-PB site, which is the widest position in the perivitelline space. We examined how piezo pulses during the drilling of the zona pellucida affected the ooplasmic cell membrane and subsequent embryonic development. We performed three experiments. In the first experiment, we investigated the influence of the distance between the cell membrane and edge of the micropipette when drilling the zona pellucida: this was performed by varying the position of the micropipette. After drilling the zona pellucida, we added piezo pulses for 0.5 sec with an intensity 1 or 12. In the second experiment, we examined the effect of the position of the injection site on the integrity of the meiotic spindle in MII oocytes by performing a sham injection of a conventional piezo group and para-PB piezo group. In a third experiment, we evaluated the effect of the injection site position in bovine ICSI on the survival of the oocytes; we also investigated competence to develop to the blastocyst stage and chromosomal integrity in blastocysts. We performed ICSI with the PB of the MII oocyte in either the 6 or 12 o'clock position (conventional piezo group), or 2 or 4 o'clock position (para-PB piezo group). In experiment 1, the survival rate of the oocytes decreased as the distance between the micropipette and ooplasmic cell membrane became smaller. An analysis of cytoplasmic levels of free calcium ions (Ca^{2+}) showed that these increased in oocytes as the distance between the micropipette and ooplasmic cell membrane was reduced. In experiment 2, there were no significant differences in meiotic spindle integrity. Even if the spindle was on the side, it was not damaged when displaced by the stretching cell membrane under pressure of the micropipette. In experiment 3, the survival rate in the para-PB piezo group (90.1%) was significantly higher than that in the conventional piezo group (75.0%; $P < 0.05$). There were no significant differences in the rate of developing to the blastocyst stage of the surviving oocytes or the proportion of blastocysts showing chromosome damage. In conclusion, the present study showed that our new piezo-ICSI method (para-PB site piezo-ICSI) is an improvement on current ICSI technology for animal and human reproduction.

P-41 - Quercetin Affects Boar Sperm Motility During Refrigerated Storage

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Polyphenols are a group of bioactive molecules produced by the secondary metabolism of plants and other organisms. They have many properties, including being antimicrobials. This is a very exciting application since antimicrobial resistance (AMR) is a critical challenge for humankind. Polyphenols (with no risk for promoting AMR on substances of medical importance) could substitute antibiotics in many applications. Animal production, being an area with extensive use of antibiotics, could benefit, including animal reproduction and the production of sperm media. Specifically, the pig industry annually uses a considerable amount of semen extenders containing wide-spectra antibiotics. Whereas the production of pure polyphenols is often expensive and therefore impractical for the requirements of the pig industry, new developments have enabled the production of plant extracts through cost-effective and environmentally friendly procedures. These extracts might allow the reduction or substitution of antibiotics in boar semen doses, improving the industry's sustainability. However, the effects of polyphenols on sperm physiology and fertility are still little researched, making it necessary to perform preliminary studies to increase our knowledge and refine the application of complex extracts. In this regard, quercetin is a particularly interesting polyphenol from the flavonol class, which is found in appreciable quantities in plant extracts obtained by many methods. It offers considerable promise as a potent antioxidant, anti-inflammatory drug, and modulator of detoxification pathways. Considering the effects on spermatozoa, quercetin could modulate motility by blocking $(Ca^{2+}+Mg^{2+})$ ATPases, and it has been reported in ruminant spermatozoa (e.g., doi:10.1016/0005-2736(83)90063-9). Still, more information on boar spermatozoa must be available, especially during refrigerated storage. Therefore, we designed a panel to test quercetin in a wide range of concentrations (semi-logs from 32 nM to 100 μ M), using no supplementation and vehicle (0.1% DMSO) as controls, and the polyphenol gallic acid (GA, usually used as a reference for polyphenol quantification) for comparison. Semen doses were obtained from a stud center, pooled, and split among the treatments. Spermatozoa were stored at 17 °C and assessed by CASA (Computer Assisted Sperm Analysis) at 37 °C on days 0, 2, and 6 (3 and 7 days of storage). The experiment was triplicated, and data were analyzed by linear mixed-effect models and heatmaps (effects relative to the control). Results indicated that the vehicle did not affect motility. Quercetin did not affect total or progressive motility or improve it compared to the control on days 0 and 2 in the nM-low μ M range, decreasing them at 10 μ M-100 μ M. On day 6, results were neutral at 32 and 316 nM, with lower motility for the other concentrations. Comparatively, GA only showed a negative effect on day 6 by 10 μ M-100 μ M, being positive in the low range of concentrations. Nevertheless, on day 2, quercetin was superior to GA by 32–316 nM. Considering the kinematic variables, quercetin showed a trend of lowering sperm velocity dose-dependently on day 0. It was less clearly associated with concentration on days 2 and 6, whereas linearity was promoted overall. In conclusion, plant extracts for pig sperm diluents with a low quercetin content should be selected. However, future studies must consider the combined effects on plant extracts and the possible benefits of other sperm features. Supported by NeoGiANT 101036768 (H2020-LC-GD-2020-4) and PID2022-137640OB-I00 (MCIN/AEI/10.13039/501100011033, by "ERDF A way of making Europe"). Thanks to AIM/Topigs-Norsvin and Á. López.

P-42 - In Vitro Embryo Production Alters the Liver Transcriptome Preferentially in Female Compared to Male Calves

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According to data collected by the International Embryo Technology Society, the number of in vitro produced (IVP) bovine embryos transferred annually exceeds 1.5 million, far surpassing the number derived from traditional superovulation techniques. Most studies using IVP embryos report pregnancies/embryo transfer but few follow up on the birth and postnatal development of IVP calves. Culture of the embryo in an suboptimal in vitro environment until transfer potentially results in epigenomic modifications of the embryo genome, including the transcriptome, leading to postnatal alterations in organ function. Recent studies have reported that dairy cows conceived by IVF have a higher incidence of ovarian cysts, which could be associated with de-regulation of energy metabolism ((Lafontaine et al., 2023). Furthermore, 3-month-old male dairy calves derived from an IVP or in-vivo embryo exhibited divergence in hepatic and muscular energy regulation (Rabaglino et al., 2021); however, the molecular changes in these organs in females are unknown. Taking advantage of a large-scale field study examining the feasibility of using IVP embryos in a seasonal pasture-based system of production, the objectives of this study were to identify differences in the liver transcriptome between male and female dairy calves conceived by IVF or AI. Biopsy tissue samples were collected using a percutaneous punch technique from the livers of 4-month-old male and female Holstein-Friesian calves derived from AI or IVP (n = 4 per sex per group) and were processed for RNA sequencing. Raw RNAseq data was aligned to the ARS-UCD1.3 genome, and the processed data were analyzed with the R software. Principal component analysis revealed a distinct separation between the liver transcriptomes of AI and IVP calves, irrespective of sex; however, the distinction was much more pronounced for females than for males. Consistent with that observation, differential expression analysis revealed 7377 differentially expressed genes (DEG) in IVP vs AI female calves compared to just 273 in their male counterparts (false discovery rate (FDR) < 0.05). Of these, 178 genes were commonly differentially expressed between AI and IVP calves, irrespective of sex. Of the total number of DEG between the livers of female calves derived from IVP vs AI, downregulated DEG (n=3566) enriched pathways involving RNA processing, ATP binding, metabolism of RNA, and catalytic activity as well as processes associated with cell cycle and DNA repair. Upregulated DEG (n=3811) strongly enriched pathways involved in the immune system including the innate immune system, protein localization and catabolic processes. There were considerably fewer DEG in the liver of male calves derived from IVP vs AI (121 downregulated and 152 upregulated). While the 121 downregulated genes did not enrich any pathways, the 152 genes upregulated enriched pathways such as metabolism, the endoplasmic reticulum, and cellular response to organic substance. Of the 178 common DEG between IVP vs AI calves irrespective of sex, 103 were upregulated and 62 were downregulated. Only the commonly upregulated genes enriched gene pathways, and these were particularly associated with angiogenesis. In conclusion, these results provide evidence that the IVP process preferentially

alters the liver transcriptome in the female postnatal calf. The consequences for the lifetime production characteristics remain to be elucidated.

P-43 - Assessing Developmental Competence through miRNA Markers in Aneuploid Human Blastoids

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The Centers for Disease Control and Prevention's 2020 report on Assisted Reproductive Technology (ART) revealed that only 45% of embryo transfer procedures result in live birth deliveries. The current criteria for embryo transfer rely on simple evaluations like grading of morphology and timing of compaction. However, a more thorough evaluation of an embryo's status using preimplantation genetic testing for aneuploidy (PGT-A) requires the biopsy of a trophectoderm cell(s), and this method overlooks the possibility of genetic mosaicism in an embryo. Aneuploidy is a frequent cause of embryo developmental failure during the pre-implantation stages. Studies have found unique miRNA markers in different trisomy cell lines. According to recent research, these miRNA markers show potential as predictors of successful implantation. MicroRNAs (miRNAs) released by mammalian embryos into the culture media could be important, unbiased, and non-invasive markers for evaluating embryo quality. Unfortunately, using human embryos as a research model is challenging due to sample limitations and ethical concerns. Our laboratory has established a blastoid protocol that models human blastocyst development using pluripotent naïve-like H9 human embryonic stem cells (hESCs). By inhibiting the Hippo, TGF- β , and ERK pathways in aggregated naïve-like hESCs, we efficiently generated blastoids that resemble human blastocysts. Blastoids present a fluid-filled cavity and the self-organization of three primary cell lineages. We evaluated the presence of all three primary lineages and their proportions using RT-qPCR and immunofluorescence microscopy. $n > 3$. To induce aneuploidy before blastoid generation, we treated hESCs with 0.5 μ M reversine, a spindle assembly checkpoint inhibitor, for 24 hours. miRNA markers with links to developmental competence (e.g. miR-191, miR-320a, and miR-372) were evaluated in blastoids and their spent culture media fractions using RT-qPCR. Unlike non-treated controls, we observed a much lower percentage of cavitation in reversine-treated blastoids. We also identified a higher frequency of cell death in blastoids exposed to reversine treatment. Elevated levels of miR-320a and miR-372 were found in the spent culture media fraction of reversine-treated blastoids compared to non-treated blastoids. Overall, our study presents blastoids as a plausible and reflective human embryo model. These results provide insights into identifying candidate miRNA markers of developmental competency via blastoids generated from hESCs of known aneuploid status.

P-44 - Functional Capacitation of Stallion Sperm Promotes Interspecies Embryo Chimerism and Equine In Vitro Blastocyst Development from Cryopreserved Sperm

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In vitro fertilization (IVF) is highly optimized in many species but has historically failed in horses. Equine IVF failures are often attributed to incomplete functional capacitation of stallion sperm, despite the efficacy of similar techniques in other species. Lack of data describing conditions that promote capacitation while maintaining sperm function remains a significant obstacle to the success of equine IVF. Herein, we report the first equine embryo produced by IVF from cryopreserved stallion sperm, achieved through a thorough characterization of conditions that promote sperm capacitation conducive to interspecies and equine fertilization. The capacitation status of stallion sperm was characterized using fresh, cooled, and frozen sperm (2-4 ejaculates; 5 stallions). Sperm isolation was achieved by Percoll centrifugation, Swim-up, or microfluidics (VetMotl), depending on experiment. Four incubation conditions were tested: 1) fresh sperm maintained at 38°C and 6% CO₂, 2) fresh sperm incubated at room temperature (RT), 3) cooled sperm incubated at RT (5°C for 24 and 48 hr), and 4) frozen-thawed sperm incubated at RT. Sperm were diluted to 20 x 10⁶ total sperm/mL in capacitating media and evaluated at 0, 4, 8, 12 and 22 hr (fresh and cooled sperm) or at 0, 1 and 2 hr (frozen-thawed sperm) for kinematics (CASA system), acrosome integrity (FITC-PSA) and capacitation status (tyrosine phosphorylation). No significant differences across time in all sperm parameters were identified for Percoll vs. Swim-up. For fresh sperm, total motility (TM) dropped quickly over time (0-22 hr) when samples were incubated at 38°C (87%, 43%, 24%, 21%, 15%). Therefore, experiments were repeated with fresh sperm maintained at RT, where TM remained constant for the first eight hours (0 hr = 80%, 4 hr = 69%, and 8hr = 65%). When sperm were cooled and then held at RT for 22 hr, no interaction was detected between cooling time (24 and 48 hr) and incubation time for all variables. The motility of previously cooled sperm remained above 50% for 8 hr (70%, 55%, 51%, 47%, 39%). For all sperm holding conditions except cryopreserved sperm, maximum capacitation status was reached at 4 hr of incubation (45-73%), while acrosome integrity remained above 75% for fresh and 57% for cooled samples. Frozen-thawed sperm isolated by microfluidics exhibited maximum motility (73%) and capacitation status (80%) immediately after thawing. To challenge sperm fertilizing ability and discern reasons for failed equine IVF success that may falsely be attributed to stallion sperm factors confounded by suboptimal culture systems, IVF was conducted in a highly optimized bovine embryo culture system to generate interspecies embryo chimeras. Fresh stallion sperm maintained at RT for 4 hr prior to gamete co-incubation resulted in 41% cleavage (171/380) and 7% blastocyst development (22/380; n = 4 reps). Equine IVF was then performed using pooled frozen-thawed sperm from two stallions selected by microfluidics. Sperm were diluted in capacitating media and added immediately to fertilization drops containing 6 oocytes aspirated from a 19-year-old mare and matured for ~28 hr. Two embryos developed to the expanded blastocyst stage. In summary, the temporal characterization of capacitation suggests that stallion sperm do not require prolonged pre-incubation for successful fertilization. Optimization of sperm conditions for in vitro production of blastocysts by interspecies and equine IVF indicates a requirement for motility maintenance while promoting capacitation status and preserving acrosome integrity.

P-45 - Effects of the Supplementation of in Vitro Maturation Media with Fatty Acid Binding Protein on the in Vitro Developmental Rates of Bovine Embryos

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Despite the growing popularity of in vitro embryo production in the cattle industry, there is significant potential for further improvement of the in vitro process to enhance blastocyst development, which is crucial for cattle producers to increase the number of offspring produced, ultimately making the production system more efficient. Maturation of recovered oocytes is the first, crucial stage of in vitro embryo production and requires meiotic and cytoplasmic changes to be completed at the proper time, sequence, and rate. Previous research indicates that in vitro matured oocytes typically contain a higher lipid content. Fatty acid binding protein (FABP) may play a role in effective maturation and development by facilitating lipid movement within the cells and across the plasma membrane which is supported by results indicating that FABP-3 was higher in embryos that reached the blastocyst stage compared to those that arrested at the 8-16 cell stage, from the same culture. The aim of this study was to investigate whether there was a difference in the cleavage and blastocyst developmental rates of embryos from oocytes matured with varying concentrations of FABP-3. Follicles were aspirated from slaughterhouse ovaries. Selected oocytes were randomly placed in either control drops or drops supplemented with FABP-3 at concentrations of 1, 10, or 20 µg/ml (10 oocytes per 50 µl drop). Matured oocytes were fertilized (50 oocytes per 500 µl well), vortexed to remove cumulus, and placed into culture (10 presumptive zygotes per 50 µl drop). After maturation, ova in one randomly selected drop per group were stripped of cumulus cells and evaluated for the presence of a polar body. In vitro maturation (18-20 hours), fertilization (18-20 hours), and culture were performed under an oil overlay at 38.5°C in a fully humidified 5% CO₂, 5% O₂, 90% N environment. On the 7th day of culture, the cleavage (≥ 2 cells) and blastocyst rates were evaluated and analysed via Chi Square. Results collected over 3 replicates indicated that cleavage was significantly higher ($P < 0.05$) in the group of 20 µg/ml ($74.1\% \pm 6.0$) compared to control and 10 µg/ml ($55.9\% \pm 5.1$, $51.7\% \pm 4.6$; respectively). There was no significant difference between 20 and 1 µg/ml ($63.7\% \pm 4.8$), 10 and 1 µg/ml, Control and 1 µg/ml, or 10 µg/ml and Control. There was no difference in blastocyst development among the groups (1; $16.7\% \pm 3.7$, 10; $17.5\% \pm 3.5$, 20; $22.2\% \pm 5.7$, Control; $21.5\% \pm 4.3$). Polar body extrusion was similar among the groups (1; $63.0\% \pm 9.3$, 10; $78.6\% \pm 7.8$, 20; $61.5\% \pm 9.5$, Control; $69.2\% \pm 9.1$). Supplementation with 20 µg/ml of FABP enhanced cleavage rates but did not have a significant impact on blastocyst development. Perhaps, lipids play a role in the cleavage process; however, research has demonstrated that cleavage is not a reliable predictor of blastocyst development. Further research is needed to expand the range of FABP concentrations beyond 20 µg/ml in the maturation media and to evaluate the lipidomic profile and the relationship with FABP to gain further insights into lipid management mechanisms and their effect on in vitro embryo development.

P-46 - Healthcare Provider Perspective on In-Vitro-Fertilization and Inclusivity in Reproductive Healthcare

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This paper asks how healthcare providers in the field of in-vitro-fertilization make IVF healthcare inclusive to the diverse group of patients they serve. In this study, eight healthcare providers with different jobs were interviewed from the same IVF healthcare facility. These interviews provide

evidence for the arguments made in this paper. Provider word choice matters due to the political implications of human embryo science. Providers attempt to neutralize their word choices as much as possible in order to minimize their influence on patient decisions, as well as include as many patient identities as possible. In addition to word choice, language is also important for inclusive care. Specifically, language interpretation, translation, and time constraints are ways in which providers work to ensure inclusive care, even if they fall short due to limitations by the US medical system. Providers also use images, plain language, and information prioritization strategy to be able to communicate with patients effectively. Finally, providers work to elevate cultural competency to cultural humility.

P-47 - From Lab to Clinic: Mastering Reproductive Health through the MS in Reproductive Science and Medicine Program

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Reproductive science spans endocrinology, embryogenesis, and reproductive tract biology, informing fertility, contraception, pregnancy, and more. Despite the crucial role of reproductive health to human well-being, a shortage of trained professionals exists, emphasizing the need for continuous educational opportunities. To support the pipeline of training in our field, the Northwestern Center for Reproductive Science (CRS) established the Master of Science in Reproductive Science and Medicine (MS-RSM) program in 2016. This program, which is based in the Feinberg School of Medicine, focuses on human reproduction and merges basic and clinical research. Now in its eighth year, the MS-RSM program has ample data to analyze the educational framework utilized in training the next generation of leaders in reproductive science and medicine. The MS-RSM program curriculum is comprehensive and includes didactic coursework coupled with hands-on laboratory research opportunities. To provide a more personalized education opportunity that can meet various career goals, the MS-RSM program provides two tracks: thesis and non-thesis. Both tracks require the same coursework but differ in research project requirements (thesis, 1.5-year independent research; nonthesis, 6-month collaborative research project). Foundational courses cover key aspects of reproductive science, complemented by advanced topics in emerging research in reproductive science and medicine, medical management of fertility, fertility preservation and oncofertility, and human reproductive health and disease. Professional development, responsible conduct of research, and scientific communication are also requirements of the program, ensuring graduates are equipped with essential skills in research ethics and science communication. Students benefit from a supportive community within the CRS, engaging in regular events, lectures, and extracurricular activities. Additional opportunities for leadership and skill development are provided through participation in the CRS subcommittees, which are focused on events and programming, diversity, and community engagement. Since the program's inception in 2016, 82 students have graduated and 12 are currently in the program. The student body is predominantly female (85%), with diverse domestic and international

representation (88% and 12% respectively). Notably, 19% of students are URM. MS-RSM students contribute significantly to the field through publications and conference presentations. Between September 2016-July 2023, MS-RSM trainees have published 49 papers during their time in the program, including 20 first-authors publications. These papers have been cited 430 times and the average citation per document is 8.78. By the time of program completion, many MS-RSM graduates have secured their next positions and overall, career outcomes differ based on the chosen track. Those who complete the thesis program (45 students to date) generally pursue advanced training (44%), research (36%), clinical jobs (15%), and industry jobs (5%). For those who graduate from the nonthesis program (37 students to date), most pursue clinical jobs (53%) with an equal distribution of those who pursue industry jobs (18%), research jobs (15%), and advanced training (15%). To ensure trainees have a network of peers to mentor them through the program and as they transition into their careers, we strive to maintain connections to alumni. Many of our alumni remain actively involved via the Mentorship and Ambassador programs. Overall, the MS-RSM program is impacting the future of the reproductive sciences field by cultivating the next generation of medical doctors, research scientists, embryologists, and clinical scholars.

P-48 - Revolutionizing Female Fertility: Pioneering In-Vitro Culture of Multiple Ovarian Follicles on a Reproductive Tissue Engineering Scaffold for the Advancement of Transplantable Artificial Ovaries

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Implementing tissue engineering for safe in-vivo transplantation of an artificial ovary poses challenges in reproductive medicine. Taking advantage of the recently developed ovarian biomimetic scaffolds, the present research has been designed to demonstrate the role in supporting ovine folliculogenesis as a key proof-of-concept to translate its use in clinic herald new strategy for fertility restoration. The Reproductive Tissue Engineering (REPROTEN) approach has been used to demonstrate the bio-functionality of poly(epsilon-caprolactone) (PCL) electrospun patterned scaffolds in orchestrating sequential in-vitro folliculogenesis/ivF from preantral follicles/PA up to oocyte maturation. This study proposes an innovative multiple-follicles long-term culture (18-days) to reproduce a fragment of ovary (Artificial Ovary/AO). The goal is to replicate the natural microenvironment guiding follicles transition from PA to early-antral/EA stages, completing the phase of oocyte-growth and acquiring meiotic competence. Upon reaching the antral-stage, the follicle-enclosed-oocyte is exposed to human Chorionic Gonadotropin/hCG surge to trigger meiotic maturation. PA (250±4), mechanically isolated from slaughterhouse lamb ovaries, were cultured on scaffold individually (Control/Ctrl) or in groups (10 PA/well; AO) on PCL-Patterned scaffolds. To preserve the ratio scaffold-surface and volume/follicle, Ctrl and AO were placed on 96 and 48-well plates, respectively. The cultural media were serum-free, supplemented with 4IU/mL equine Chorionic Gonadotropin (growth-phase) or 25IU/mL hCG (maturation-phase). Follicle development was assessed by considering follicle-growth, antrum differentiation and CYP19A1-expression. The culture influence on germinal compartment is recorded by considering oocyte increase, chromatin configuration, Metaphase-II and parthenogenetic activation-rate. AO

synchronously supported follicle and oocyte growth. The AO displayed slower follicular growth with a delay in antrum differentiation on Day 12 (44.8%) vs. Ctrl (84.6%; $p<0.001$). Therefore, the long-term ivF was better faced by AO with a consistent lower rate of degeneration (5% vs. 28% Ctrl; $p<0.001$). Furthermore, almost the totality of follicles in AO differentiated the antrum cavity (95% vs. 72% Ctrl). Additionally, the transition from PA to EA in AO group was accompanied from a great upregulation of CYP19A1 (2.5-fold change PA vs. EA; $p<0.0001$), significantly higher than that recorded in Ctrl-EA (1.5-fold; $p<0.01$). The synchronous in-vitro development of somatic and germinal compartments is confirmed by the meiotic competence acquisition (2.6-fold change PA vs. EA in AO; $p<0.001$). The long-term culture in AO improved also the quality of the collected oocyte in term of chromatin configuration (53.3% Surrounding-Nucleolus; 26.7% Surrounding-Nuclear-Envelope vs. 100% Non-Surrounding-Nucleolus in Ctrl; $p<0.001$), Metaphase-II (62% vs. 48% Ctrl; $p<0.05$) and parthenogenetic activation rate (66.7% vs. 15.4% in Ctrl; $p<0.001$). The ability to bypass the pronuclear block in AO-system may be attributed to a more mature chromatin organization in AO-derived oocytes, with a nuclear remodeling distribution rate exceeding 50% in Surrounding-Nucleolus and Surrounding-Nuclear-Envelope (compared to 100% Non-Surrounding-Nucleolus in Ctrl; $p<0.0001$). The study delves into the prospective application of the REPROTEN solution to face female infertility. PCL-scaffolds showcase the ability to replicate functionally the native ovarian matrix by supporting multiple and coordinate follicle development. This opens two potential applications: optimizing current ivF protocols with biomimetic scaffolds, potentially mimicking gonadotropin-insensitive/low-sensitive phases; using PCL to construct ovarian fragments from frozen ovaries, onto which early-stage developing follicles can be transferred for transplantation, reducing cancerous cell transfer risk. The study emphasizes the crucial role of assisted-reproductive-techniques in enhancing female fertility by recovering early-stage follicles, highlighting potential future applications in ovarian transplantation. This research was funded by the European Union—Next Generation EU. Project-Code:ECS00000041; Project-CUP:C43C22000380007; Project-Title: Innovation, digitalization, and sustainability for the diffused economy in Central Italy—VITALITY.

P-49 - Pyruvate Carboxylase Mediated Macrophage Polarization Regulate Male Reproductive Function Under High-fat Diet

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Objective: To explore the effects of HFD on male reproductive tissues, and the mechanism of pyruvate carboxylase(PC)-mediated macrophage polarization regulation of male reproductive function under HFD. **To provide a new metabolism-immune regulation mechanism for obesity-induced male infertility.** **Methods:** By establishing an obese mice model induced by HFD, the effects of HFD on metabolism and systemic organs in mice were studied, and their reproductive function was tested. Flow cytometry was performed to detect the number and polarization of macrophages in the male reproductive tissues of HFD and normal diet (ND) mice. RNA-Seq sequencing was performed on male reproductive tissues to analyze differential genes and validated through fluorescence quantitative PCR. A co-culture system was established for macrophages and adipocytes, which were treated adipocytes with different concentrations of PC inhibitors, namely Erianin (Eri), the number and polarization of macrophages were measured. **Results:** ① Compared with the ND group mice, the HFD group mice showed a significant increase in body weight and blood sugar, disrupted lipid metabolism, and altered organ morphology. The morphological

changes in the testes and epididymis of mice fed with HFD diet resulted in a decrease in sperm quality. ②Flow cytometry analysis showed that HFD induced an increase in the number of macrophages in epididymal adipose tissue and epididymal tissue, and differentiated towards M1 type. This was accompanied by an increase in local inflammatory factors in mouse epididymal fat tissue and epididymal caput tissue. The RNA seq sequencing results showed that the expression of PC gene increased most significantly in epididymal fat. ③When mature adipocytes and macrophages are co-cultured in vitro, they can significantly increase the number of macrophages and polarize towards the M1 type. Eri can inhibit macrophage proliferation and polarization towards the M1 type, and the inhibitory effect becomes more pronounced as the concentration of Eri increases. Conclusions: The sperm motility and sperm viability of obese mice induced by HFD were significantly reduced. HFD-induced obesity resulted in a significant increase in the expression of adipose tissue PC in the epididymis, inducing polarization of epididymal macrophages towards M1 type, thus leading to local chronic inflammation of the epididymis and damaging sperm quality and male reproductive function.

P-50 - Developing Nanotechnology Based Lateral Flow Bioassay Capturing Pregnancy Circulatory Microrna for Early Pregnancy Diagnosis In Buffaloes

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In India, over 73 million artificial inseminations are performed in bovine annually (National Dairy Development Board, Year). Conventional pregnancy detection assays for livestock have a detection limit of 40-45 days post artificial insemination with poor sensitivity causing significant economic loss to Indian dairy farmers. Therefore, an early pregnancy detection kit with an ability of detecting pregnancy as early as day 25 post artificial insemination can be a game changer in India's dairy industry with increasing the product revenue. In the pursuit of solutions to early pregnancy detection in bovine, this study focuses on developing the circulatory microRNA based lateral flow bioassay which is an invasive-paper based detection kit that can be used directly by the farmer on the field. The designed bioassay uses gold nanoparticles that embeds the capture and detection probe. The tagged nanoparticles detects the pregnancy specific microRNAs from the blood plasma of animals and gives a signal in form of a red color line which can be seen through naked eyes. The assay is simple, easy and robust in detecting the early pregnancy in buffaloes as early as day 25 post artificial insemination. The test is capable of estimating as low as 0.01nm concentration of pregnancy associated circulatory miRNA thus making it more sensitive as compared to the existing technology. The ultrasonography results also support the outputs obtained using the developed lateral flow bioassay. Since, the results are seen as a change in color with naked eye, without using an expensive laboratory equipment and expertise of handling the equipment, it is suitable for detection of early pregnancy diagnosis in bovine at farmers site. However, the newly developed bioassay needs further validation to gain more confidence for cow side test. The novel microRNA based lateral flow bioassay is expected to have a promising positive impact on animal sciences especially in bovine dairy farming.

P-51 - LEUTX Regulates Non-Rodent Mammalian Embryonic Genome Activation and Somatic Cell Reprogramming

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Emerging evidence highlights the regulatory role of PRD-like homeobox transcription factors (TFs) in embryonic genome activation (EGA). However, the majority of PRD-like genes are lost in rodents, thus urging an investigation for PRD-like TFs in non-rodent mammals. Here we showed that PRD-like TFs, such as ARGFX, DPRX, DUXB, LEUTX, TPRX, were transiently expressed during EGA in non-rodent fertilized embryos, including human, monkey, and pig, yet all these TFs exhibited inadequate expression in their cloned embryos. In this study, applying pig as the research model, we identified LEUTX as a key PRD-like activator of porcine EGA through reporter selection assay, combined RNA-seq data analysis with LEUTX genomic profiling via CUT&Tag. Furthermore, we found that LEUTX overexpression restored EGA failure (reactivated 76% EGA genes), improved blastocyst formation (elevated from 13.9% to 26.2%) and cloning efficiency (elevated from 0.75% to 4.11%) in porcine cloned embryos. Mechanistically, by conducting co-IP and proximity ligation assay, we revealed that LEUTX opened EGA-related genomic regions and established histone acetylation via recruiting acetyltransferases p300 and KAT2A. Utilizing PRD-like TFs' DNA-binding motif, we also developed a reporter system for real-time EGA monitoring to select early embryos with good quality. These findings highlight LEUTX as a conserved PRD-like TF governing non-rodent mammalian EGA, which also benefits somatic cell reprogramming in these mammals.

P-52 - Processes and Pathways Associated with In Vivo Derived and In Vitro Produced Male and Female Bovine Conceptus Proteins

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Early pregnancy in cattle involves a protracted period of development whereby the conceptus remains unattached to the endometrium for nearly three weeks. During this time, the conceptus and endometrium secrete factors that reciprocally modulate their activities such as conceptus elongation, immune tolerance, and subsequent attachment. Studies suggest that bovine

conceptus sex [male (MC) or female conceptus (FC)] and origin [in vitro produced (IVP) or in vivo derived (IVD)] impact the endometrial transcriptome via conceptus secreted proteins (CSP). Previously, we identified IVD and IVP-CSP that may impact the endometrial transcriptome. Here, we aimed to identify CSP and associated processes or pathways specific to conceptus origin within sex. To achieve this, Angus-Holstein heifers underwent estrus synchronization and were divided into one of two groups: 1) bred by artificial insemination on Day 0 to produce IVD conceptuses or 2) underwent IVP embryo transfer on Day 7 to produce temporally and sire-matched IVP conceptuses. On Day 16, IVD and IVP conceptuses were flushed from heifer uteri. The conceptuses were then used to develop the following treatments in 1 mL of RPMI medium: 1) culture medium only (base medium; n=7), 2) IVD conceptus only, or 3) IVP conceptus only. After 12 h, conceptuses were sexed using PCR and media from the treatments were collected and analyzed for protein content by LC-MS/MS. Proteomic data were analyzed to identify origin specific proteins within sex [IVP-MC (n=3) vs IVD-MC (n=4) and IVP-FC (n=2) vs IVD-FC (n=3)]. Compared to base medium, culture medium from IVD-MC had fewer DAP than the IVP-MC (564 and 838, respectively, $P < 0.05$). Of the DAP, 262 and 535 were specific to IVD-MC and IVP-MC, respectively. Processes or pathways associated with the upregulated IVD-MC specific DAP (191) were related to complement activation whereas the upregulated IVP-MC specific DAP (490) were related to protein translation ($P < 0.0001$; FDR < 0.05). Compared to the base medium, culture medium from IVD-FC had more DAP than the IVP-FC (912 and 759, respectively, $P < 0.05$). Of the DAP, 531 and 378 were specific to IVD-FC and IVP-FC, respectively. Processes or pathways associated with upregulated IVD-FC specific DAP (435) were related to glycolysis whereas upregulated IVP-FC specific DAP (317) were related to microtubule-based processes ($P < 0.0001$; FDR < 0.001). In conclusion, compared to IVD conceptuses of the same sex, IVP-MC may have increased translation and protein secretion whereas IVP-FC may have reduced glycolytic activity. This may partially explain why IVP male and female conceptuses are more susceptible to reproductive failure compared to those that are IVD. This study was supported by the USDA National Institute of Food and Agriculture (NIFA) award number 2020-67015-31615.

P-53 - Exploring Sex Differences in Prenatal Human Fetal Mammary Bud Development

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The early stages of mammary development are independent of sex steroid hormones but at 15 weeks of fetal development, breast tissue is transiently sensitive to testosterone. Milk ducts are formed by weeks 20–32 unless significant testosterone exposure prevents alveolar ductal system development. Mammary ducts and lobules are present in both sexes meaning that like women, men can develop breast cancer although accounting for less than 1% of all diagnosed cases. Despite tissue similarities, female mammary gland development is far more dynamic, starting during fetal life, pausing after birth, then resuming at puberty in response to hormonal cues and culminating at the end of pregnancy with functional lactogenesis. Little is known about sex-dimorphism in the human prenatal mammary bud; indeed, the nipple is not visible in

1st and early-mid 2nd trimesters. With this study, we have begun analysis of region-specific transcriptomes to provide novel data on the regulation of sex-specific development of the prenatal mammary bud. Collection of human fetuses from normally progressing pregnancies was approved by the National Health Service (NHS) Grampian Research Ethics Committees (REC 15/NS/0123). Dissected tissues were fixed overnight in 10% Neutral Buffered Formalin prior to dehydration and paraffin embedding processes. Fetal sex was further confirmed by PCR. Three male and 3 female mammary bud (15-16 gestational weeks) sections were mounted as recommended by Nanostring®. Adipose tissue was identified using FABP4 immunolabelling while vimentin and PanCK immunolabelling highlighted stroma and epithelium, respectively. We compared differential gene expression (DEG) in similar mammary bud regions (regions of interest) between sexes using an unpaired t-test ($p\text{-value} \leq 0.05$ and $|FC| \geq 2$). As a follow-up, proliferating and dying epithelial cells within male and female mammary buds were immunolabelled using KI67 and cleaved-Caspase3 as respective markers. Counting is currently ongoing. Visualisation of the dissimilarities in gene expression between regions of interest, by PCA biplot, showed clear separation of samples based on region of interest type but no separation based on fetal sex. Indeed, PC1 and PC2 accounted for 32% and 22% of expression variability, respectively. The first dimension separated stroma from other region of interest types while the second dimension separated epithelial regions (central and basal) from the nipple area. More gene expression changes were sex-related in the central epithelium compared to other regions of interest (301 versus 192 in stroma and 135 in basal epithelial layer). Gene set enrichment analysis based on sex related DEGs in stroma, central epithelium and basal epithelial layer showed significant enrichment of the “DNA replication” pathway ($p\text{-value}=0.0002$, $p\text{-value}=0.005$ and, $p\text{-value}=0.0007$, respectively). Sex related DEGs in the basal epithelial layer were significantly enriched in pathways such as “DNA methylation” ($p\text{-value}=0.0002$) and “estrogen-dependent gene expression” ($p\text{-value}=0.0009$). Genes encoding for proteins involved in lipid metabolic process such as MVK (mevalonate kinase, $p\text{-value}=0.00734$) and CH25H (cholesterol 25-hydroxylase, $p\text{-value}=0.00312$) were upregulated in female central epithelium compared to male. Surprisingly, prolactin transcript was almost 3-fold more highly expressed in male compared to female central epithelium ($p\text{-value}=0.00919$). No sex differences in prolactin receptor transcript level were observed in basal or central epithelium. These data represent early but important steps towards better understanding of sex-dependent human prenatal development of the mammary bud and may contribute to better understanding of sex differences in breast cancer risks. This work was supported by the NHS Grampian (EA5009) and European Union’s Horizon 2020 FREIA project (EU Grant Agreement no. 825100).

P-55 - Chemerin in Egg White, Oviduct and During the Embryo Development in Pekin Duck

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Breeding companies goal's is the production of viable and robust one-day old duckling. But, a major issue is the uncontrolled embryo mortality during egg incubation. Thus, the intensive genetic selection has resulted in the production of animals with reproductive disorders. New fertility biomarkers are necessary to improve reproductive performances in genetic program. The aims were to (1) quantify chemerin concentrations in egg white during the cycle of laying for two successive generations of pekin ducks (2) determine a link between chemerin concentrations in egg white and egg and reproductive performances (egg weights, laying, fertility and hatchability) (3) evaluate a heritability on two generations and (4) investigate the presence of chemerin in reproductive tract and during the embryo development. Eggs from 50 mother and respective offspring pekin ducks were collected during five days at three period: before and after the laying peak and at the end of laying. For each egg, an albumen sample was collected to measure chemerin concentrations by ELISA assay. The reproductive tract was also collected to determine the chemerin expression in magnum. Moreover, pekin eggs were incubated to determine the expression of chemerin in embryonic annexes (allantoic and amniotic sac) during the embryo development. A third experiment consisted to inject pekin eggs with different treatments: control (PBS), IgG and chemerin antibodies (0.01, 0.1 and 1 µg/mL) at embryonic day 9 of incubation to evaluate the embryo mortality. During the cycle of laying, chemerin concentrations in egg white significantly decreased. Chemerin amounts in egg white were negatively correlated with albumen weight ($r = -0.33$, $p = 0.036$) and not associated with any reproductive parameters. Between generations, we obtained an estimation of the heritability of 0.29 ± 0.17 . By RT-qPCR, chemerin expression was higher in magnum compared to the other region of oviduct. During the incubation, chemerin levels in allantoic fluid were unchanged during the incubation whereas significantly increased from ED16 in amniotic fluid. Eggs injected with the high quantity of anti-chemerin antibody (1 µg/mL) showed a significant increase of embryo mortality (82.45 ± 1.44 %) compared to eggs injected with control treatment (28.65 ± 6.15 %). The concentration of chemerin in egg white was variable during the cycle of laying for duck breed. Chemerin is a hormone locally produced by the magnum region of the oviduct tract, leading to its accumulation in the egg white during the egg formation. During egg incubation, chemerin in egg white was transferred into the amniotic sac to support the embryo growth. Our findings suggest that chemerin could exert a beneficial role in pekin duck development. Our biomarker seems to be heritable to the offspring and it could be used as new tool in genetic selection. Keywords: chemerin, egg white, embryo, pekin duck, genetic selection

P-56 - Metabolic Enhancement of Mammalian Developmental Pausing

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Mammalian developmental timing is adjustable in vivo by preserving pre-implantation embryos in a dormant state called diapause. Over 130 mammalian species use this reproductive strategy to ensure that their offspring is born under most favorable conditions, thereby safeguarding survival. Inhibition of the growth regulator mTOR (mTORi) pauses mouse development in vitro. However, how embryonic dormancy is maintained is not known. Proteomics analyses of mTORi paused embryonic and trophoblast stem cells revealed that embryonic, but not trophoblast stem cells switch to fatty acid degradation as primary energy source. Moreover, diapausing embryos accumulate fatty acids into large lipid droplets in the trophectoderm, but not in the epiblast. The transfer of fatty acids from the cytosol into mitochondria requires their conjugation to carnitine by carnitine palmitoyltransferase 1 (CPT1). We reasoned that supplementing the embryos with free L-carnitine may enhance fatty acid degradation in the epiblast and promote it in the trophectoderm, thus alleviating the current constraints and maintaining the paused embryos longer in culture. Notably, L-carnitine balances lipid consumption, puts the embryos in deeper dormancy and boosts embryo survival from 15 to 34 days. Our results lift a constraint on in vitro embryo survival and suggest that lipid metabolism may be a critical metabolic transition relevant for longevity and stem cell function across tissues.

P-57 - Effects of Superovulation with Follicle-Stimulating Hormone on Embryo Production and Cryopreservation

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The OPU technology plays a significant role in oocyte collection globally and is the most efficient means of enhancing genetic advancement in cattle through maternal lines. However, studies on cryopreservation and pregnancy rates of embryos produced from oocytes obtained through OPU IVP and superstimulation are unclear. In this study, we examined the quality and yield of oocytes between the FSH group and the control group, evaluated chromatin configuration, assessed embryo productivity, checked the quality of embryos after freezing and thawing, and ultimately compared pregnancy rates after embryo transfer. Oocytes were collected from 20 control groups and 15 female Hanwoo treated with follicle stimulating hormone (FSH). Reduced doses of FSH (36, 36, 24, 24 mg 12-hour intervals), regulated internal drug release, estrogen and prostaglandin were used for synchronization and weak stimulation. In vitro embryos were produced through in vitro maturation, fertilization and culture. This study demonstrated that FSH improves the efficiency of

the production, freezing, and preservation of embryos during the OPU-in vitro embryo development.

P-59 - Assessment of Responsiveness and Sensitivity of the Spindle Assembly Checkpoint during the First Cell Division of the Human Embryo

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Human pre-implantation embryos frequently exhibit chromosomal abnormalities with the majority arising during the first mitotic divisions. In somatic cells, the spindle assembly checkpoint (SAC) ensures proper chromosome segregation during mitosis. The chromosomal passenger complex (CPC) ensures correction of non-bipolar kinetochore-microtubule attachments and signals the SAC to initiate anaphase onset, thereby preventing chromosome missegregation together. However, whether the SAC is fully operational in the human zygote is uncertain. Human zygotes have a pronounced epigenetic asymmetry for trimethylation of histone H3 at Lysine 9 (H3K9me3) on paternal (sperm) and maternal (oocyte) chromatin. Maternal chromatin shows global H3K9me3-enrichment whereas on paternal chromatin it is only retained at the pericentromeric regions. CPC localization to the inner centromeres is important for its function. A previous study from our group observed H3K9me3-rich maternal chromatin to have an advantage in CPC recruitment. This raises the question if paternal chromosomes may be more prone to misalignment. We aim to assess SAC responsiveness and sensitivity during the first cell division of the human embryo and whether differences exist in susceptibility to misalignment between maternal and paternal chromosomes. This study utilized human surplus mono- and triprounuclear zygotes with approval from the Dutch Central Committee on Research Involving Human Subjects (CCMO, NL38053.000.11/NL82597.000.22). Zygotes were cultured in a time-lapse incubator (EmbryoScope, Vitrolife) in culture medium with or without low-dose (5nM) or high-dose (25 or 50nM) GSK923295, or 62.5nM Cpd-5. Zygotes were imaged throughout the first cell cycle or fixed in 4% formaldehyde (MeOH-free) one hour post nuclear envelope breakdown for immunofluorescent staining for the centromeres, centrosomes, H3K9me3 and microtubuli. Spindles were imaged using the Leica SP8 STED Stellaris confocal microscope (Leica Microsystems) and 3D-analysed using Huygens software (Scientific Volume Imaging, The Netherlands). First, the kinetochore-binding microtubule motor protein CENP-E was inhibited using GSK923295 to disrupt chromosome congression. Analysis of fixed spindles showed that the average percentage of misaligned chromosomes increased from 6.2% in the controls (n=6) to 8.5% in low (n=8) and 36.6% in high-dose (n=7), respectively. No difference in misalignment frequency was found when differentiating between maternal and paternal chromosomes. Time-lapse imaging of the first cell cycle revealed no difference in cell cycle progression between the control (n=21) and low-dose (n=19) condition, but a severe delay or complete arrest at M-phase was observed at the high-dose conditions (n=21). Second, a low concentration Cpd-5, an inhibitor of mitotic kinase Mps1 responsible for recruiting components for SAC assembly, compromises SAC establishment and attachment error correction in somatic cells. In zygotes, low dose Cpd-5 resulted in early anaphase onset in 30% of the analysed fixed zygotes

and induced misaligned chromosomes in 50% (n=12). Preliminary findings reveal a higher proportion of misaligned paternal chromosomes. However, more spindles should be analysed to confirm the susceptibility of paternal chromosomes to misalign. To conclude, this study demonstrates that the SAC is responsive in the first cell division of the human embryo. However, the sensitivity of the SAC seems low as only high proportions of misaligned chromosomes cause a delay or arrest at M-phase. Besides, our preliminary data suggest Mps1 inhibition increases the susceptibility of paternal chromosomes to misalign, but not CENP-E inhibition. This is in line with our previous findings that paternal chromosomes have a disadvantage in recruiting CPC proteins and are thereby more prone to misalignment which seems independent of congression by CENP-E.

P-60 - Investigating the Presence of Acetylated Histone Post-Translational Modifications in Bovine Preimplantation Embryos

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Following fertilization, the preimplantation embryo must undergo a series of cellular and molecular changes that are essential for successful development. A key event in preimplantation development is the activation of embryonic transcripts, or zygotic genome activation (ZGA). This process of switching from maternally provided transcripts to activating the embryonic genome is essential for survival of the embryo and the development of embryonic tissue and placental membranes. Throughout preimplantation embryo development, gene expression is regulated by several different epigenetic mechanisms, including DNA methylation, chromatin conformation, small non-coding RNAs, and histone post-translational modifications (PTMs). Changes to the epigenetic landscape at different stages of preimplantation embryo development is a critical component in the precise transcriptional regulation of genes that are necessary for embryo survival and lineage differentiation. One essential mechanism of epigenetic regulation is the attachment of PTMs to histone tails, thus altering chromatin accessibility and inducing or silencing gene transcription. The addition of acetyl groups to histone lysine residues activates gene transcription by opening the chromatin, thus allowing binding of DNA polymerase and other transcription factors. While the specific timing and location of histone PTMs during preimplantation embryo development has been well characterized in the mouse and human, little is known about the presence and timing of histone PTMs during bovine preimplantation embryo development. The aim of this study was to investigate the presence of activating histone PTMs and associated chromatin modifying enzymes in bovine preimplantation embryos. We first analyzed previously published RNAseq data from bovine blastocysts and found robust expression of several histone acetyltransferases, including: Kat5, Kat7, Kat8, Hat1, CBP, and p300. We next utilized immunofluorescence to determine the abundance and location of two activating epigenetic marks. Acetylation of lysine 27 on histone 3 (H3K27ac) is associated with active enhancers and promoters. Acetylation of lysine 9 on histone 3 is associated with active promoters (H3K9ac). Both have demonstrated regulatory roles in gene expression and may indicate important roles during and after ZGA. Immunofluorescence was also performed for the histone acetyltransferase CBP, which acetylates H3K9ac and H3K27ac. Frozen, IVF generated embryos collected at day 7.1 of development were used for this initial study. Immunofluorescence was utilized to determine the

presence of H3K27ac, H3K9ac, and CBP at the blastocyst stage of development. Both H3K27ac and H3K9ac are localized to the nuclei of the inner cell mass and trophectoderm of the blastocysts. Interestingly, CBP appears to be expressed in the cytoplasm of blastocysts, thus suggesting other histone acetyltransferases are involved with H3K9/27ac in the bovine embryo. The study sought to answer 1) Is the quantity and location of H3K27ac and H3K9ac consistent with previous findings in other mammals, 2) What are the specific epigenetic mechanisms regulating the early bovine embryo, and 3) Can the localization of activating histone modifications indicate quality of the blastocyst. The findings indicate the location of these modifications throughout bovine preimplantation development could be potential biomarkers for assessing quality and viability and that these epigenetic marks are conserved across species.

P-61 - TEAD3 and TEAD4 Exhibit Redundant Roles in Morula-To-Blastocyst Transition During Bovine Early Embryonic Development

Kun Zhang and Haotian Yu

Members of the TEA domain family are specifically expressed in the trophectoderm of mammals at the blastocyst stage. The role of TEAD4 in promoting trophectoderm lineage specification has been clarified during the transition from morula to blastocyst in mice. However, TEAD4 knockout (KO) does not impair embryo development in cattle, suggesting a species-specific function of TEA domain family members. Given that TEAD3 is uniquely expressed in the trophectoderm of cattle but not in mice, this study aims to identify the functional roles of TEAD3 and TEAD4 in bovine early embryos and the mechanisms involved. TEAD3 knockdown (KD) and TEAD4 KO embryos are constructed using RNA interference and a cytosine base editor, respectively. While TEAD3 KD does not affect embryo development, embryos with concurrent TEAD3 KD and TEAD4 KO fail to develop past the morula stage, implying an overlapping function of TEAD3 and TEAD4 during morula-to-blastocyst transition in cattle. RNA-seq analysis reveals dysregulation in 215 genes, with 53 being upregulated and 162 downregulated in bovine embryos deficient of TEAD3 and TEAD4. This analysis shows a decrease in trophectoderm genes KRT8, KRT18, and EZR in the TEAD3 KD and TEAD4 KO embryos, along with a reduction in the HIPPO signaling pathway gene WWC3. These changes suggest that developmental failure stems from impaired trophectoderm epithelium function and altered Hippo signaling. Immunofluorescence analysis corroborates the minimal presence of KRT8 in the treated group. Notably, the combined KD of TEAD3 and KO of TEAD4 does not affect the expression of trophectoderm lineage-specific factors such as TFAP2C and GATA3 or inner cell mass lineage-specific factors like OCT4, which contrasts with TEAD4's role in mice. In summary, our research demonstrates that TEAD3 and TEAD4 have redundant functions during the morula to blastocyst transition in cattle, highlighting species-specific roles in early embryonic development.

P-62 - Systematic Evaluation of Retroviral LTRs as cis-regulatory Elements in Mouse Embryos

Jian Yang, Lauryn Cook, Zhiyuan (ZY) Chen

In mammals, many retrotransposons are de-repressed during zygotic genome activation (ZGA). However, their functions in early development remain elusive largely due to the challenge to simultaneously manipulate thousands of retrotransposon insertions in embryos. Here, we applied CRISPR interference (CRISPRi) to perturb the long terminal repeats (LTR) MT2_Mm, a well-known ZGA and totipotency marker that exists in ~2667 insertions throughout the mouse genome. CRISPRi

robustly perturbed 2485 (~93%) MT2_Mm insertions and 1090 (~55%) insertions of the closely related MT2C_Mm in 2-cell embryos. Remarkably, such perturbation caused down-regulation of hundreds of ZGA genes and embryonic arrest mostly at the morula stage. Mechanistically, MT2 are globally enriched for open chromatin and H3K27ac and function as promoters/enhancers downstream of OBOX/DUX proteins. Thus, we not only provide direct evidence to support the functional importance of MT2 activation in development, but also systematically define cis-function of MT2 in embryos by integrating functional perturbation and multi-omic analyses.

P-63 - Identification of Amnion Progenitor-like Cells at the Amnion-Epiblast Boundary in the Primate Peri-gastrula

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Amniogenesis is triggered in a collection of pluripotent epiblast cells as the human embryo implants. To gain insights into the critical, but poorly understood, transcriptional machinery governing amnion fate determination, we examined the evolving transcriptome of a human pluripotent stem cell (hPSC)-derived amnion model at the single cell level. This analysis enabled the identification of several markers specific to the different stages of amnion lineage progression, and revealed a previously unrecognized CLDN10+ amnion progenitor cell type, as well as formation of some primordial germ cell-like cell (PGCLC) in this model. Strikingly, in peri-gastrula cynomolgus macaque embryos the expression of CLDN10 is restricted to the amnion-epiblast boundary region. This bolsters the notion that, at this stage, the amnion-epiblast boundary is a site of active amniogenesis. Bioinformatic analysis of published primate peri-gastrula single cell sequencing data further confirmed that CLDN10 is expressed in cells progressing to amnion. Notably, in the absence of CLDN10 in the hPSC-amnion model, more PGCLC are formed at the expense of amnion formation. Overall, this study identifies a previously unrecognized CLDN10+ amnion progenitor-like cell population and validates the presence of these fate transitioning cells at the boundary of the developing amnion.

P-64 - Pronuclear Migration in Zygotes With Sperm Centrosome is Regulated by Microtubules Oriented from Centrosomes

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After sperm penetration, parental pronuclei come close to each other and form mitotic spindle. In mouse zygotes without centrosomes, the pronuclear migration requires both of actin and microtubule cytoskeletons. However, the cytoskeletons responsible for pronuclear migration and the roles of centrosomes in non-rodent mammalian zygotes with centrosomes are not fully understood. To address this, we investigated pronuclear migration in bovine zygotes by live-cell imaging and immunofluorescence. Depolymerization of actin cytoskeleton by cytochalasin B treatment had no effects on the pronuclear migration, whereas depolymerization of microtubule cytoskeletons by nocodazole treatment significantly inhibited the pronuclear migration. Immunofluorescence analyses revealed that the centrosomes derived from a sperm accumulated gamma-tubulin, protein that is required for microtubule nucleation, and organized cytoplasmic microtubule network in zygote. In addition, the microtubule motor, cytoplasmic dynein accumulated to the both membranes of parental pronuclei and localized to the sides where the pronuclei face each other. Depolymerization of microtubules caused diffusion of dynein on the pronuclear membranes. On the other hand, removal of nocodazole caused re-elongation of microtubules from centrosomes toward pronuclei and re-localization of dynein on the pronuclear interface. When centrosomes were not located between the parental pronuclei in zygotes, the two pronuclei did not appose. Our results indicate that the pronuclear migration in mammalian zygotes with centrosomes mainly depends on the microtubules nucleated from centrosomes. The localization of dynein to the side of pronuclear interface on the pronuclear membrane was regulated by microtubules. Thus, it is predicted that the retrograde movement of dynein on the pronuclear membrane toward centrosomes along microtubules mediates pronuclear migration. The centrosome positioning is believed to be important for regulating the direction of pronuclear migration.

P-65 - Investigating Centromere Dynamics in Bovine Early Development

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Centromeres are chromosomal loci essential for correct chromosome segregation during cell division. Defects in their function can lead to aneuploidy and cancer. Centromeres are epigenetically defined by the presence of a histone variant called Centromere Protein A (CENP-A), which directly replaces histone H3 in the octamer core of centromeric nucleosomes. Previous studies have highlighted the importance of the transgenerational inheritance of paternal CENP-A: in the fruit fly (*Drosophila melanogaster*), fertilisation of eggs with sperm depleted of CENP-A results in the missegregation of the paternal chromosomes and abnormal embryogenesis. In mice, studies indicate that paternal CENP-A is inherited and maintained in the male pronucleus until at least the 2-cell stage. However, the impact of centromere proteins and function on the fidelity of the first cell cycle in mammals has not yet been investigated. Bovine in vitro fertilisation (IVF) studies have shown that a longer cell cycle impacts successful blastocyst formation, with early-cleaving embryos being more likely to develop to the blastocyst stage. Recently, the clustering of the genomes of parental pronuclei has been identified as a critical step in the prevention of aneuploidy during the first cell division in bovine and human embryos, occurring approximately 1.5 hours

before nuclear envelope breakdown and chromatin condensation. However, the position and spatial organisation of the centromeres during the first cell cycle remains unknown. Preliminary results from our laboratory using bovine zygotes derived from IVF have shown that at the time of parental genome clustering, centromeres are positioned away from the pronuclear interphase where the centrosomes are located. This unexpected localisation poses questions about how centromeres become re-arranged during chromatin condensation in this short period of time to allow faithful chromosome segregation during the first mitotic division. We aim to uncover the topology of centromeres in the developing bovine zygote during key phases of the first cell division cycle. Our long-term goal is to study whether perturbing centromere function, or cell cycle progression (in zygotes generated using high or low fertility sperm) affects the fidelity of chromosome segregation in the first mitosis.

P-66 - Zinc Supplementation Influences 8-cell Stage Global Transcriptional Activity And Improves Preimplantation Embryo Development In Cattle.

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One of the unsolved questions in mammalian early embryogenesis relates to the morpho-functional and molecular processes that drive transcriptional reactivation of the embryonic genome after fertilization. This gap of knowledge limits the ability to recapitulate this transition in vitro and further improve the efficiency of in vitro embryo production. In this study, we tested the hypothesis that zinc, emerging as a key component in several conserved processes regulating female germ cell growth, fertility, and pregnancy, is a critical determinant of transcriptional regulation in bovine early embryogenesis. To this end, immature oocytes collected from abattoir-derived ovaries were subjected to standard in vitro maturation and fertilization, and then cultivated in control conditions, which do not include the supplementation of trace elements (control) or in the presence of two concentrations of zinc (0.15 or 1.5 µg/ml) in the form of zinc sulfate. Zinc concentrations were chosen based on the content in the follicular fluid and the female genital tract. In the first set of experiments, the global transcriptional activity was directly assessed by uridine incorporation with a commercial Imaging Kit in embryos cultured in the absence (control) or in the presence zinc after 64 hours of culture when bovine embryos typically reach the 8-cell stage. Whole mount samples were imaged using an EclipseE600 fluorescence microscope equipped with a Sight DS-U3 camera (Nikon) at 40X magnification. For each embryo, multiple images were acquired at different focal planes, using the nuclear staining with DAPI as a reference. Only embryos whose nuclei corresponded to the number of blastomeres observed under bright field conditions were considered, while embryos showing multinucleated or anucleated blastomeres were discarded. A total of 478 blastomeres (of 66 embryos at the 5-8 cell stage) and 312 blastomeres (of 28 embryos at the > 8 cell stage) were included in the analysis. The blastomeres' fluorescence intensity was calculated with ImageJ after background subtraction. Data analysis showed that transcriptional activity levels of the blastomeres did not significantly differ between the control and the treatment with a higher zinc concentration. On the contrary, there was a significant increase in levels of transcriptional activity when low concentrations of zinc were used (Two-way ANOVA followed by

Tukey's multiple comparison test, $p < 0.05$). Subsequently, we tested the effect of supplementation of the two doses of zinc on the ability to reach the blastocyst stage of development after 8 days of culture. A total of 584 oocytes in 5 biological replicates were included in the study. Data analysis showed that the low concentration promotes a higher blastocyst rate when compared to the higher one (Kruskal-Wallis followed by Dunn's multiple comparison test, $p < 0.05$), while the control group showed intermediate blastocyst rates. In conclusion, our studies indicate that zinc supplementation has a dose-dependent effect on the ability of the embryo to develop in vitro. The increase in developmental competence obtained in the presence of low concentrations of zinc appears to be correlated to a stimulation of the global transcription process of the embryo. Further studies are necessary to identify which molecular mechanisms govern this process. Funded by RL PSR2014-2020 No.202102146691 (R-INNOVA) and PSR2022 Piano di Sostegno alla Ricerca: Linea 2 – Azione A - Molecular and structural responses to stressors in different cells and tissue models.

P-67 - Oviduct Epithelial Spheroids during in vitro Culture Enhance Bovine Embryo Development, Mitigate the Negative Impact of Oxidative Stress and Induce Changes in Embryonic Transcriptome

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The mammalian oviduct provides an optimal environment for early embryo development. On the contrary, in vitro embryo production exposes the embryos to oxidative stress with deleterious effects on blastocyst development and quality. Considering the antioxidant capacity of the oviduct epithelium, we developed an in vitro model co-culturing bovine oviduct epithelial spheroids (OES) and embryos. We hypothesized that co-culture of OES with in vitro produced embryos up to the time of embryonic genome activation (5 days) or blastocyst stage (7-8 days) could mitigate the effects of oxidative stress conditions. Oocytes were collected from bovine ovaries from a slaughterhouse and in vitro matured for 22 h. Then, oocytes were fertilized using frozen-thawed Percoll-washed bull semen at 38.8°C. After 24h (day 0), groups of 25 presumptive zygotes were in vitro cultured in 25 µL droplets of SOF medium with 5% fetal calf serum, at 38.8°C, 5% CO₂, and under 5% or 20% O₂, with or without OES and during two co-culture times (5 or 8 days), as follows: 1) controls-5%; 2) controls-20%; 3) 5dOES-5%; 4) 8dOES-5%; 5) 5dOES-20%; and 6) 8dOES-20%). Cleavage rates were evaluated on day 2 and blastocyst rates on days 7 and 8. Expanded blastocysts on days 7-8 were either evaluated for total cell numbers (Hoechst staining) or for gene expression analysis by Illumina RNA-sequencing. Embryo development rates were compared by ANOVA, Tukey's post-tests. RNA-seq data was processed to remove adapter sequences and low-

quality bases. Differentially expressed genes (DEGs) were identified with EdgeR (FDR≤0.05). Functional analysis of DEGs was performed using Metascape. No differences were found on cleavage rates among the different culture conditions (73-81%, 8 replicates). Under 5% O₂, the presence of OES for 5 or 8 days did not affect blastocyst rates compared to controls without OES (27-32% vs. 30% on day 8) but increased the number of cells per blastocyst when OES were co-cultured for 8 days (137.6 ± 10.8 vs. 102.6 ± 8.4 cells; P< 0.05). Under 20% O₂, blastocyst rates on days 7 and 8 were significantly higher in the presence of OES compared to controls without OES (30.7% and 31.8% in 5dOES-20% and 8dOES-20% vs. 19.8% in controls-20%; P< 0.05). Furthermore, cell numbers per blastocyst were significantly increased compared to controls with no difference between 5 and 8 days of co-culture (112.7 ± 7.8. and 138.1 ± 10.5 cells, respectively, vs. 82.1 ± 4.5 cells in controls; P< 0.0001). Under 5% O₂, 545 and 992 DEGs were identified in blastocysts co-culture with OES for 5 and 8 days, respectively, compared to controls. Under 20% O₂, 627 and 1580 DEGs were identified in blastocysts co-cultured with OES for 5 and 8 days, respectively, compared to controls. The most enriched cellular components of DEGs were mitochondrial membrane, endosome membrane and intercellular bridge. The most enriched pathways involved chromatin organization, cell-cell adhesion, cell proliferation, membrane trafficking, and lipid metabolism. In conclusion, OES co-culture improved embryo quality in all groups and mitigated the negative impact of high oxidative stress conditions on in vitro embryo development. OES co-culture up to the time of embryo genome activation (5 days) was enough to induce this beneficial effect. Finally, the OES altered the embryonic transcriptome with highest impact under oxidative stress conditions, evidencing for the first time a modulation of the embryo-OES dialog according to the environment.

P-68 - Non-Invasive Metabolic Imaging of Early Embryos Is Associated With Altered Metabolic Signatures In Aging

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Selecting optimal embryos is pivotal for successful assisted reproduction. Hence, there is a crucial demand for reliable, non-invasive methods to assess embryo quality, enhancing reproductive outcomes. Non-invasive imaging of embryos, evaluating metabolic activity, emerges as a promising avenue. Alterations in metabolism could be the cause of aneuploidy, inducing cell stress, abnormal development, reduced implantation potential and or poor reproductive outcomes. Predicting these issues through non-invasive metabolic measurements is conceivable. However, the influence of aging on embryo metabolic activity, morphology, and reproductive outcomes remains unexplored, presenting an essential area for further investigations. Thus, this study aimed to assess the metabolic activity of blastocyst embryos from both young and old mice, aiming to establish its relationship with embryo morphology and reproductive outcomes. Super-ovulated female mice; young: 5-8 weeks old, n=14; old: 45-52 weeks old, n=35; were subjected to in-vitro fertilisation (IVF).

Inseminated oocytes (Young:304; Old:351) were assessed for fertilisation and embryo development to the blastocyst-stage. Embryos from study groups were assessed by non-invasive metabolic imaging to investigate autofluorescence of nicotinamide adenine dinucleotide [NAD(P)H], flavin adenine dinucleotide (FAD) and the optical redox ratio (ORR) levels during embryo development. Subsequently, embryo transfers were performed. F1 (CBA/C57Bl6) mouse strain was used. NAD(P)H and FAD levels were measured during embryo development using confocal microscopy (Olympus FV1200) and a custom-made light-sheet on-a-chip microsystem. A confocal Z-stacking function was used to record 15 focal planes using a 20x/0.95NA air objective of entire embryos, opening the confocal pinhole system completely. Then, images were collected and analysed using FIJI software (v2.0.0-rc-69/1.52n), using arbitrary units (AU). Embryo culture experiments revealed significant variations in blastocyst formation rates between study groups (Young: 73.6%; Old: 22.9%; $p < 0.05$). Morphology analysis of blastocysts produced showed no significant difference for good quality embryos between young and old females ($p > 0.05$). Embryos that failed to reach the blastocyst stage exhibited distinct NAD(P)H activity profiles during development compared to those that did ($p < 0.05$). Additionally, abnormal embryos also presented significantly decreased NAD(P)H activity levels at the 2-cell stage to the morulae stage ($p < 0.05$). Furthermore, metabolic images of 2-cell embryos predicted blastocysts formation with an AUC of 0.974. Developed blastocyst showed a significant decrease in NAD(P)H levels in the inner cell mass (ICM) of blastocyst from old females (Young: 424.6 ± 26.0 AU; Old: 280.7 ± 43.6 AU; $p < 0.05$). Furthermore, an increased metabolic activity in the ICM of blastocyst from old females was also observed (ORR Young: 901.5 ± 6.2 AU; Old: 937.0 ± 7.3 AU; $p < 0.05$). On another hand, trophoectoderm cells from old females showed no differences in terms of metabolic activity (ORR; $p > 0.05$), however, increased NAD(P) levels (Young: 3431 ± 19.13 AU; Old: 4609 ± 346.1 AU; $p < 0.05$) and decreased FAD levels were observed respectively (Young: 4275 ± 43.56 AU; Old: 3615 ± 159.7 AU; $p < 0.05$). This study unveils altered metabolic activity levels in embryos that do not form blastocyst and decreased levels of NAD(P)H in the ICM of blastocyst from old females. Increased metabolic activity was similarly observed in the ICM with altered levels of NAD(P)H and FAD in trophoectoderm cells, which it could be indicative of abnormal metabolic activity. Non-invasive measurements of metabolic imaging could be applied to determine the link of embryo metabolic activity with poor reproductive outcomes in aging. Financial support: Monash University, University of New South Wales & Fertility Society of Australia and New Zealand, Ferring grant.

P-69 - Knock-in on the Y chromosome

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The Rosa26 locus is commonly used for autosomal knock-in, as Rosa26-targeted knock-in is easy to be implemented with stable gene expressions and has little effect on other endogenous genes. For sex chromosomal knock-in, the housekeeping genes Hprt and Pgk1 are generally used for the X chromosome as targeting loci. On the other hand, no commonly used targeting loci have been established for the Y chromosome unlike above cases. However, in a previous study, knock-in mice with tdTomato in the Ddx3y (DEAD-Box Helicase 3 Y-Linked, Dby) locus on the Y chromosome were generated because Ddx3y knock-out mice are non-lethal and normally fertile (Hirata et al., 2022).

Therefore, in this study, we followed up the Y chromosomal knock-in using the Dby locus with the GFP gene and a gene cassette longer than the GFP gene. In addition, we aimed to increase the knock-in efficiency. In order to perform knock-in only on the Y chromosome, it was favorable to microinject genome editing tools into the male pronuclei before pronuclear fusion. To increase knock-in efficiency, we employed the co-injection of a plasmid with homologous arms at both ends of the gene cassette and Cas9 ribonucleoprotein (Cas9 RNP), which is Cas9 protein and gRNA complex, into the S-phase pronucleus in early embryos (Abe et al., 2020). After knocking in the GFP gene into the Dby locus, we verified the knock-in efficiency and the presence of nonspecific insertions. We performed in vitro fertilization in ICR mice and microinjected the GFP-gene containing plasmid and Cas9 RNP into the male pronuclei 6~8 hours after insemination. After culturing the embryos to blastocysts, the presence or absence of GFP fluorescence was examined under a fluorescence microscope and then embryos were genotyped. Next, a gene cassette of 2887 bp, which is even longer than the GFP gene, was knocked-in into the Dby locus. In vitro fertilization was performed to obtain zygotes from hybrid mice (B6D2F1), and after microinjection into the male pronuclei as described above, embryos were cultured to the 2-cell or blastocyst stage, and then transferred to pseudopregnant ICR mice by oviductal transfer or Non-Surgical Embryo Transfer (NSET) using the mNSET Device for Mice 60010 (ParaTechs), respectively, and the resulting pups were genotyped. For knock-in of the GFP gene, we observed a 4.5% (1 out of 22 injected embryos) efficiency of knock-in in the appropriate locus. For knock-in of the 2887 bp gene cassette, the efficiency of obtaining knock-in mice was 21.4% (3 out of 14 pups). In a previous study, knock-in mice were obtained at a 2.4% efficiency, in which the GFP gene was inserted into the intergenic region sequence of the Ddx3y and Uty genes by microinjection in the cytoplasm (Zhao et al., 2019). Compared with the report, the efficiency of acquiring knock-in mice in the present study is relatively high. The knock-in tool injection in the S-phase pronucleus (Abe et al., 2020), which was effective for knock-in into the Rosa26 locus, was also effective for that on the Y chromosome. The knock-in technique used in this study should be applicable for knocking in other genes on sex chromosomes in the future.

P-70 - NEK2 Plays an Essential Role in Mitotic Division and DNA Damage Response by the Wnt/ β -Catenin Signaling Pathway in Porcine Embryonic Development

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NIMA-related kinase 2 (NEK2) is a serine/threonine protein kinase involved in cell cycle regulation, differentiation, and the DNA damage response system during a variety of cellular events. Inhibition of NEK2 is known to cause abnormal nuclear morphology in a variety of cell types and disrupt cell cycle progression, including the G2/M phase. However, the role of NEK2 in porcine embryonic development is unknown. In this study, we investigated the role of NEK2 in embryonic development and its underlying regulatory mechanisms using the NEK2-specific inhibitor, JH295. Inhibition of NEK2 after parthenogenetic activation significantly reduced cleavage and blastocyst formation

rates, trophectoderm and total cell numbers, and cell survival compared to control. NEK2 inhibition delayed cell cycle progression at all stages from interphase to cytokinesis during the first mitosis. Additionally, NEK2 inhibition caused abnormal nuclear morphology in 2-cell and 4-cell stage embryos. Moreover, NEK2 inhibition significantly increased DNA damage and apoptosis, and it altered the expression levels of DNA damage repair- and apoptosis-related genes. Intriguingly, NEK2 inhibition downregulated the expression of β -catenin and Wnt signaling-related genes. To determine the relationship between Wnt/ β -catenin signaling pathway and NEK2 in porcine embryonic development, we cultured porcine embryos in JH295-treated medium with or without Wnt activator CHIR99021. CHIR99021 treatment strongly restored developmental parameters reduced by JH295 treatment to control levels. In conclusion, these results demonstrate that NEK2 plays an essential role during porcine embryonic development by regulating mitosis and DNA damage repair systems by the Wnt/ β -catenin signaling pathway. This research was supported by the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Research Initiative Program (KGM4252432, KGM5382423), Republic of Korea.

P-71 - Exploration of Transposon-Derived Chimeric Transcripts Expressed in Mammalian Embryos

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Transposons are mobile DNA elements and constitute ~40% of mammalian genome. During zygotic genome activation (ZGA), some of transposons are activated and form chimeric transcripts with downstream genes in mammalian embryos though their activation is suppressed generally. Although some of these chimeric mRNAs have been found to have different functions from the canonical transcripts and contribute to cell proliferation and differentiation in mouse embryos, high-quality chimeric mRNA libraries have not yet been constructed and many of them may remain undiscovered. This could be due to the technical limitations of short-read sequencing. Although short-read sequencing is mainstream sequencing method due to its high accuracy, mapping transposon-derived sequencing is difficult because they exist in numerous numbers in the genome. Here, we applied long-read RNA-sequencing (RNA-seq) on mouse and bovine ZGA stage embryos to comprehensively search for chimeric transcripts involved in embryonic development. In addition, we performed comparative analysis of short- and long-read sequencing data to verify differences in detection and quantification of transcripts. Two hundred mouse or bovine embryos at the ZGA stage were collected and mRNA was extracted. The mRNA was reverse transcribed to cDNA and sequenced based on the cDNA-PCR Sequencing Kit from Oxford Nanopore Technology Inc. After base calling and quality checking, reads were mapped to the mm10 or bosTau9 reference genome. The short-read RNA-seq data of mouse and bovine embryos were downloaded from the GEO dataset (GSM1845301 and GSM4275382) for comparative analysis. After mapping to RefSeq transcript datasets and quantification, Pearson correlation analysis was performed. Expression levels were normalized by RPT10K (Reads Per Transcripts per 10 K mapped reads). To compare the number of detected transcripts between short- and long-read sequencing, we extracted those with RPT10K more than 0 from the quantified data. In both mouse and bovine, several chimeric transcripts listed in previous studies were detected. Especially, Prmt6 and Cdk2ap1 were identified as chimeric transcripts common to mouse and bovine. We recaptured the phenomenon that the type of transposon used as promotor for chimeric transcripts differed between species; for

example, Cdk2ap1 was transcribed from the LTR elements of endogenous retrovirus in mouse, while in bovine it was transcribed from the fusion region of the DNA transposon and LINE. Comparative analysis between short- and long-read sequencing data did not show high correlation coefficients. These results could be due to incorrect mapping of transposon-derived reads in short-read sequencing, or due to overestimation of quantitative values caused by PCR or normalization bias in long-read sequencing. In addition, there was no significant difference in the number of transcripts detected between short- and long-read sequencing in both mouse and bovine, suggesting that the ability to detect transcripts in long-read sequencing is comparable to that of short-read sequencing. These results are expected to help discover novel chimeric transcripts during mammalian embryonic development.

P-72 - How Important is Fast Re-expansion of FETs After Thawing on Implantation Rate?

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Objective: to evaluate the importance of fast blastocyst re-expansion on frozen-thawed embryos at the time of transfer on implantation rates. Design: Retrospective single-centre observational cohort study. Materials and method: 303 FETs were reviewed from July to December 2023. All FETs with full detailed information about the embryo re-expansion after thaw were included. These cycles included patients using autologous, donor oocytes, and donor embryos with patients ranging from ages 23 to 49 years with infertility diagnoses. Embryos were evaluated by at least one senior embryologist or a senior and a junior embryologist to register embryo re-expansion and categorized them into 3 features (fully re-expanded, re-expanding, or collapsed) 2 hours after thawing or one hour before the transfer. When DET took place, if any embryo was re-expanded or expanding then that category was considered. After a general analysis, we decided to also divide patients by group age (< 35 years old, 35-38 years old, 38-40 years old, >40 years old), PGT or not, and egg donor program (including all programs offered at the clinic). Results: From all FETs analyzed, 87% of them (264) were considered fully re-expanded or re-expanding before transfer. 40% resulted in positive beta results and 60 % resulted in negative beta. From the positive beta group, only 3 (2.5%) were collapsed at transfer time, the rest of them were either fully-expanded or re-expanding. The negative beta group included 80% fully re-expanded or re-expanding embryos and 20% collapsed. We applied the Chi-square test to evaluate the correlation between the variables and found the features collapsed or re-expanded/ing as an important aspect for the embryo to implant (p-value< 0.05). When analyzing PGT or not, this feature did not show a correlation with implantation results (p-value >0.05). The feature donor or own egg also did not show a correlation (p-value > 0.05). Finally the group age showed a correlation for the implantation process positioning the younger group as the most advantageous (p-value< 0.05). Lastly, we calculated the difference between the observed cases and discovered that an embryo re-expanded/ing has 37% more chances of implantation when compared to a collapsed one. There seems to be a correlation between fast expansion after thaw and positive beta. This may mean that collapsed embryos have more challenges to implant. Limits and improvements: We had a limited number of cycles with full information to evaluate. We only evaluated implantation rate, as a way of predicting the embryo's potential to lead to a possible pregnancy. Implantation does not only depend on the embryo's fast expansion ability. We did not include % of survival rate after thawing as a feature.

P-73 - Germ Cell Nuclear Factor Regulates Craniofacial, Axial and Reproductive Development

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Germ cell nuclear factor (GCNF/NR6A1/RTR) is an orphan nuclear receptor essential for reproduction and embryonic development. Loss of GCNF in mouse results in neural tube closure defects, axial truncation, and failure of chorioallantoic fusion, culminating in embryonic lethality by E10.5. Expressed in the germ cells, placenta and developing nervous system, GCNF functions as a transcriptional regulator. During embryogenesis, GCNF is known to repress the pluripotency-associated factors Oct4 and Nanog. However, little is understood about the network of genes regulated by GCNF beyond these factors that make the orphan nuclear receptor so crucial to development. Here, we describe an additional role for GCNF in embryonic development as a regulator of neural crest cell formation, epithelial-mesenchymal transition and survival. Neural crest cells are a transient migratory cell population that generate the craniofacial skeleton and peripheral nervous system among other tissues during embryogenesis. Specified along the dorsal axis of the embryo in the neural plate, neural crest cells undergo an epithelial-mesenchymal transition, delaminate and migrate to target destinations throughout the embryo for differentiation. In situ hybridization and transcriptomic analysis showed GCNF is expressed at the right time and location to regulate neural crest cell development during embryogenesis. Downregulation of neural crest cell-specifier and epithelial-mesenchymal genes revealed these processes were perturbed in GCNF null embryos. Lineage tracing confirmed GCNF null embryos have a deficiency in migratory neural crest cells, and immunostaining determined this was due to a disruption in the specification of neural crest cells within the neural plate. Neuroepithelial cells of the neural plate were maintained in a proliferative, undifferentiated state, aligning with GCNF's critical activity as a repressor of Oct4 and Nanog. Competition binding, EMSA and targeted ChIP assays demonstrated GCNF directly binds to putative DR0 binding sites located in the promoter regions of neural crest cell-specifier and epithelial-mesenchymal genes. These results suggest GCNF acts as a regulator of neural crest cell formation and epithelial-mesenchymal transition through the repression of pluripotency-associated genes and activation of neural crest cell-specifier and epithelial-mesenchymal transition genes. Altogether, we have identified an important role for GCNF during embryogenesis as a regulator of neural crest cell development. Overall, our findings have expanded our understanding of the transcriptional network governed by GCNF and thereby have the potential

to inform other processes affected by GCNF regulation such as axial elongation, germ cell development and reproduction.

P-74 - Radiographic Candling: A New Method to Evaluate Embryo Survival in Speckled or Thick-Shelled Eggs

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Egg candling is essential to managing bird egg incubation, as it allows us to determine embryo growth, survival, and position within the egg. However, embryos are difficult to distinguish in birds with dark, mottled, or thick shells using a traditional light candler. Radiographs are often used at the end of incubation to ascertain the position during hatch of valuable chicks. We therefore decided to evaluate the practicality of using radiographs earlier in incubation to “candle” thick-shelled eggs. Depending on when the bones become detectable by radiographs during development, this technique could be a useful new tool to monitor embryo development and mortality in speckled or thick-shelled eggs. To determine at which stage ossification of the cartilaginous skeleton is sufficient to be consistently detectable, embryos were euthanized at days 12 (stage 38), 12.5, 13 (stage 39), 13.5, 14 (stage 40), 14.5, 15 (stage 41), and 18 (stage 44) of incubation. In addition, embryos were fixed, cleared, and stained with Alizarin red (stain calcified bone) and Alcian blue (stain glycoproteins in cartilage) to visualize the amount of ossification at each stage of embryo development. Our results showed that bone development was first detectable by radiographic analysis by days 12.5 to 13 (stages 38-39). This timeframe is within the early part of the rapid bone mineralization process that occurs from day 10 through 17 (stages 36-43), as previously shown by Thompson et al. (1989). Our study shows that incubation managers can monitor the growth of avian embryos in thick-shelled eggs using radiographic candling from stages 38-39, which could significantly impact conservation efforts. Due to the potential harm, we envision radiographs being used at stage 39 to confirm the presence of an embryo, followed by a second radiograph several stages later to confirm continual growth and/or hatch position. This will minimize the number of radiographs and potential harm to the embryo’s health and germline. Actual detection will depend on equipment resolution and operator experience, as the early bones were difficult to distinguish from shell artifacts at stage 38. In summary, we found that embryos in speckled or thick-shelled eggs were readily detectable using radiographic candling by stage 39.

P-75 - Correlation Between MicroRNAs in Amniotic Fluid and mRNAs in Maternal Blood at 42 days of Gestation in Cattle

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Estimating normal pregnancy progression and foetal pathologies through maternal blood molecular profile in animals is scarcely explored. In a previous study (1), we employed an asynchronous transfer model in heifers to produce pregnancies of 10 male foetuses whose weight ranged from 1.25 to 1.69 gr at 42 days of gestation, and we used co-expression cluster analyses to identify a maternal blood expression signature that accurately predicted foetal weight ($R^2=0.93$). However, it is still unknown how the bovine foetus could influence the maternal blood transcriptome, given that the superficial placentation in ruminants limits the circulation of cell-free foetal RNA, as observed in humans. On the other hand, microRNAs (miRNAs), which are short, non-coding RNAs that regulate gene expression, may be involved in direct communication between foetus and maternal blood. Indeed, we have previously reported sexually dimorphic differences in the expression of miRNAs in amniotic fluid (AF) and maternal blood plasma in association with the process of sex determination and gonad differentiation in cattle (2). Therefore, we hypothesized that specific circulating miRNAs in the AF of foetuses at the end of organogenesis are correlated with mRNAs in maternal blood, which in turn, are influenced by the foetal weight. To test this hypothesis, AF samples were collected from the 10 foetuses obtained at slaughter on Day 42 of gestation (1). Samples were directly aspirated from the amnion and centrifuged. The supernatant was subjected to miRNA extraction, library preparation with the SMARTer Small RNA kit, and sequencing with a HiSeqXten platform, generating ~15 million raw reads per sample in FASQ format. Raw files were processed with miRDeep2, obtaining the reference sequences for the bovine from the miRBase database (<https://www.mirbase.org/>). The rest of the analyses were done with R packages, including in the analysis the transcriptomic information from the maternal blood, which was obtained as described previously (1). Correlations between the abundance of the 1030 mature bovine miRNAs in AF and the expression of 17501 mRNAs in maternal blood were evaluated using the non-parametric Kendall rank correlation coefficient. There were 130 miRNAs in AF whose abundance was correlated with the expression of 2768 mRNA in maternal blood ($p < 0.01$, $R > 0.75$), corresponding to 3554 miRNA-mRNA pairs. Next, we determined if the genes significantly correlated with AF miRNAs were influenced by foetal weight using the DESeq2 package. Of the 222 genes changing with foetal weight ($FDR < 0.1$), 63 significantly correlated with AF miRNAs. Thus, there was a significant association between genes affected by foetal weight and those correlated with AF miRNAs, as determined by a chi-square test ($p < 0.0001$). Functional analysis with the DAVID software (<https://david.ncifcrf.gov/>) of the 63 blood mRNAs revealed significant enrichment ($FDR < 0.1$) of defence response to virus, given the presence of interferon-induced protein 44 and interferon regulatory factor 1, and antigen processing and presentation, because of MHC class II antigen (BLA-DQB) and proteasome activator subunit 1 and 2, among others. In conclusion, these results support the notion that miRNAs of foetal origin can influence the expression of certain genes in the maternal blood according to foetal phenotype characteristics, such as weight. Further studies will be aimed to determine if these AF miRNAs are also circulating in maternal blood. Supported by a H2020-MSCA-Individual Fellowship (Proposal 101021311) to MBR.(1)Rabaglino et al., Biol Reprod. 109:749-758. 2023.(2)Sánchez et al., Biol. Reprod. 105:345–358. 2021.

P-76 - Suppressing CXCL12-CXCR4 Chemokine Axis During Implantation in Sheep Results in Shorter Fetal Limbs and Altered Expression of Glucose and Amino Acid Transporters in Fetal Tissues at Midgestation.

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Placental insufficiency is a root cause of preeclampsia, fetal growth restriction, and fetal morbidity and mortality. The chemokine, CXCL12 and its receptor CXCR4 are implicated in pregnancy complications including miscarriage and preeclampsia but precise roles of this axis during placentation are unclear, especially at the fetal-maternal interface. Using a sheep model, we previously demonstrated suppressing CXCL12-CXCR4 signaling with a pharmacological inhibitor (AMD3100, 1X dose) at the fetal-maternal interface during implantation reduces trophoblast invasion, disrupts uterine remodeling, and diminishes placental vascularization by d20 of gestation. At midgestation, these dams also exhibit placental insufficiency. In the current study a more in depth investigation into fetal growth and development was conducted concurrent with investigating impacts to fetus with increasing AMD3100 concentrations delivered. We hypothesized greater amounts of AMD3100 (1.5X and 3X doses) during implantation would cause dose-response negative impacts to fetal growth and development at midgestation. On d12 post-breeding, osmotic pumps were surgically installed in ewes to deliver two different amounts (1.5X or 3X) of AMD3100 or saline (control) into uterine lumen ipsilateral to corpus luteum for 14 days. On d90 fetuses were collected, weighed and growth measurements for fetal body, brain, liver, and heart weight, along with crown-rump length, thoracic circumference, and fore- and rear-limb lengths recorded. Fetal pancreas, small intestine, liver, and muscle were harvested, snap frozen in liquid nitrogen and stored at -80°C. Expression of mRNA for select glucose and amino acid transporters were analyzed using qPCR. The objectives were to determine if suppressing CXCL12-CXCR4 actions with varying doses of inhibitor during implantation impacts fetal growth/development and/or expression of glucose and amino acid transporters in select fetal tissues. Fetal body, brain, and liver weights did not differ between control and AMD3100 treatments. Crown-rump length and thoracic circumference was also similar. However, both fore and rear limb lengths were shorter ($P < 0.05$) in fetuses from all AMD3100-treated ewes compared to control regardless of sex. In fetal liver, females had greater GLUT1 and SNAT4 expression with 1.5X AMD3100 compared to male and female controls. Conversely, males exposed to 1.5X AMD3100 displayed greater SNAT1 and CAT1 expression than control males or females. In muscle, GLUT1 expression was suppressed by AMD3100 treatments in male and female fetuses. Control males expressed more SNAT1, SNAT2, and SNAT4 than females but when exposed to AMD3100 treatments, males displayed decreased transcripts. Control females had greater LAT1 expression than males however both sexes expressed less LAT1 with 3X AMD3100. Regardless of sex, LAT2 expression decreased with AMD3100 treatments while less CAT1 was observed with 3X AMD3100 compared to control. In fetal pancreas regardless of sex, SNAT2 expression decreased with 3X AMD3100 compared to control. In small intestine, GLUT3 expression was less with 1.5X AMD3100 and less SNAT1, SNAT2, and SNAT4 with 1.5X and 3X AMD3100 compared to control regardless of sex. Conversely, greater LAT2 and CAT1 expression occurred with 3X AMD3100 compared to control. Shorter limb lengths and altered expression of glucose and amino acid transporters in fetal tissues may indicate glucose and amino acid sparing mechanisms to ensure fetal survival and growth of brain and liver. Our model may prove useful to study pathogenesis of pregnancy disorders like preeclampsia and the corresponding impacts to the developing fetus. Research supported by National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health (NIH). Grant number 1SC1GM139712-01.

P-78 - Effects of Dietary Supplementation of Creatine-Monohydrate on Fetal-Placental Development in Mid- and Late-Gestating Gilts

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The creatine-creatine kinase-phosphocreatine (Cr-CK-PCr) system maintains intracellular ratios of ATP/ADP for support of metabolism and multiple cellular functions. Postnatally, Cr synthesis begins in the kidneys with arginine and glycine converted into guanidinoacetate (GA) by arginine-glycine amidinotransferase. GA is released from the kidneys into vasculature and transported to the liver for methylation by guanidinoacetate-n-methyltransferase to form Cr which is released into vasculature for uptake by target cells and tissues. Within target cells and tissues, CK converts Cr into PCr, which rapidly regenerates ATP. The Cr-CK-PCr system has been characterized at the placental-uterine interface of rodents, primates, and sheep, and is hypothesized to support fetal-placental development. The ATP buffering capacity of the Cr-CK-PCr system is important for muscle cell function and may be involved in fetal muscle development. This study aimed to determine effects of dietary supplementation of creatine to gestating gilts on fetal-placental growth and development and on number and ratio of primary and secondary muscle fibers, respectively in fetal pigs at Days 60 and 90 of gestation. Reproductively mature gilts were synchronized to estrus using Matrix and observed for estrus (Day 0) in the presence of a fertile boar and were artificially inseminated 12h and 24h later. Gilts were individually housed from Day 9 and fed 2lbs of a 14% crude protein diet twice daily that meets the nutrient requirements for pregnant gilts. Gilts were assigned to either control (CON) (n=9 gilts/Day of gestation) or creatine (Cr) (n=10 gilts/Day of gestation) treatment groups. Creatine (15g twice daily) was provided to gilts assigned to Cr treatment group from Day 10 to either Day 60 or Day 90 of gestation, whereas CON gilts were unsupplemented. Gilts were euthanized and hysterectomized on Day 60 or 90 of gestation. These protocols were completed in replicates, in which gilts were synchronized in spring then euthanized in summer or synchronized in fall then euthanized in winter. Litter size, fetal crown-rump length, fetal sex, and fetal body weight was recorded. Three female and male fetuses closest to mean body weight within each litter was selected and mean fetal brain, kidney, liver, spleen, and biceps-femoris muscle weight was recorded. Data were analyzed to determine effects of treatment, Day of pregnancy, season, and fetal sex on litter size, fetal measurements, and incidence of intrauterine growth restriction. There was significant treatment effect of greater mean kidney weight for fetuses from Cr-treated than CON-treated gilts in both Days 60 ($P < 0.0001$) and 90 ($P = 0.0410$) of gestation. Fetuses from CON gilts tended ($P = 0.0609$) to have heavier brains than fetuses from Cr gilts on Day 60 of gestation. Mean weights of fetal body weight, kidney, liver, and biceps-femoris from winter euthanized replicates on Day 60 of gestation were significantly greater ($P < 0.0001$) than those of summer euthanized replicates. Mean fetal spleen and liver weights were significantly greater ($P < 0.001$) in summer euthanized replicates than winter euthanized replicates from Day 90 of gestation. Results of this study suggest that dietary supplementation of creatine in gestating gilts had limited effects on fetal organ development. Studies on treatment effects on placental and fetal muscle

fiber development are ongoing and further mechanistic analyses should be performed to investigate the observed effects on organ development. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2022-67015-36376 from the USDA National Institute of Food and Agriculture.

P-79 - The Role of IVF Culture Media mRNAs in Embryo-Mother Communication in Cattle

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Embryo mortality and suboptimal reproductive performance are major causes of economic loss in dairy production systems. Though there are many factors leading to pregnancy loss, insufficient embryo-mother communication is assumed to be one of the major factors. Our objective was to identify extracellular vesicle (EV) mRNAs expressed in the culture media of IVF derived bovine embryos and investigate their roles in embryo-mother communication. Following isolation of EVs from six IVF media samples, RNA extraction and amplification, and RNA sequencing, three genes were identified to have reads across all exons in all samples: SLBP2, ACCSL, and DPPA3. We selected DPPA3 for further investigation, and in-vitro transcription was utilized to produce mRNA for this gene. This mRNA was then transfected into bovine endometrial epithelial cells (BEnEpCs). Transfected and control cells were subsequently analyzed for RNA sequencing to examine the effects of DPPA3 transfection on gene expression in these cells. A total of 25 genes were found to be upregulated and 1 gene was downregulated (FDR < 0.01) following DPPA3 transfection. Out of the 18 annotated genes, 17 have been discovered to possess known functions in pregnancy recognition in ruminants or have been previously identified to be upregulated in pregnant animals. In addition to RNA sequencing, transfected and control cells were also subjected to proteomic analysis. In total, 34 proteins were found to be differentially expressed (FDR < 0.01, $p < 0.05$, fold change > 1.5) with 19 being upregulated and 15 being downregulated. Two proteins, ISG15 and MX1, were found to overlap with the differentially expressed mRNAs. Overall, our results demonstrate that DPPA3 mRNA secreted from IVF embryos may have a role in early embryo-mother communication and maternal recognition of pregnancy prior to major interferon tau secretion.

P-80 - RAB35 small GTPase Activity Coordinates Endosome Dynamics to Facilitate Apicosome Formation in a Human Model of Epiblast Formation

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Formation of a central lumen in the developing human epiblast is a critical step that occurs during implantation. However, there is a significant knowledge gap in the mechanistic understanding by which epiblast cells initiate polarization and form a central lumen. Using a human model of epiblast formation, our previous studies have shown that apical polarization in such model is initiated by the formation of an apicosome, an intracellular membrane-bound structure with

apically polarized features including microvilli and primary cilium. During apicosome formation, apically polarized vesicles accumulate and fuse at the peri-nuclear region, and gives rise to a small apicosome, which grows in size and matures overtime. Here, we comprehensively characterized endosomal trafficking dynamics during apicosome formation, and identified RAB35 as an apicosomal RAB that plays a critical role in apicosome formation by controlling early, recycling, and late endosome dynamics. Unlike previously characterized RABs that encase, but not directly associate with, apicosomal membrane, RAB35 co-localizes with apicosome membrane and membrane associated proteins. Strikingly, in the absence of RAB35, several apicosomes that are smaller in size are formed, and defective early and recycling endosome dynamics are seen. Mechanistically, RAB35 GTPase activity is critical, as, while the expression of wild-type or a constitutively active form of RAB35 leads restoration of single apicosome formation, the expression of a dominant negative form of RAB35 leads to the multi-apicosome phenotype. Together, these results show that RAB35 organizes membrane trafficking that aids in vesicle fusion to promote single apicosome formation.

P-81 - Activin A in Mouse Preimplantation Development and Reproduction: Deciphering the Role of Protein of Zygotic and Maternal Origin

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The successful development of a mammalian embryo relies on growth factors secreted by the mother's reproductive system and the embryo itself. Activin A is an example of such a multiple-source protein, which is widely expressed among mammals. Although dysregulation of the activin A signaling pathway in humans is correlated with the occurrence of miscarriages and ectopic pregnancies, due to the various origins of this protein, its exact role in mammalian embryonic development remains ambiguous. To this end, we investigated the consequences of the deletion of zygotic protein in knockout mouse embryos, along with the results of the decreased level of activin A provided by the maternal reproductive system in a hypomorphic mouse model. By combining a time-lapse imaging system and lineage-specific markers detection, we prove that zygotic activin A is dispensable for the preimplantation development of mouse embryos, however, this protein may be involved in the embryo implantation process, as indicated in the blastocyst outgrowth assay. Furthermore, our findings highlight the importance of maternally provided activin A in mouse reproduction, as females with a hypomorphic mutation of the activin A gene display disrupted estrous cycles and significantly reduced fertility. This study provides insight into the role of activin A in the earliest stages of mouse embryogenesis and reproduction and is the first one to distinguish the function of the zygotic and the maternally derived protein in these processes. The project is financed by the grant OPUS 17 2019/33/B/NZ3/02906 from the National Science Centre (Poland).

P-82 - Bovine Trophectoderm has the Metabolic Capacity to Convert Glucose into Glycogen

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Mouse and bovine blastocysts have surprising flexibility in their use of different energy substrates. While studies have shown that bovine blastocysts metabolize glucose primarily to lactate and pyruvate, not all metabolized glucose is accounted for. In this study we asked whether bovine blastocyst could convert glucose into glycogen as a means of energy storage for later use. Two approaches were taken. The first involved staining Day 7-8 blastocysts with the Periodic Acid-Schiff (PAS) method to determine if they stained positive for glycogen. The second used gas chromatography-mass spectrometry (GC-MS) to determine if stable isotope labeled [$^{13}\text{C}_6$]-glucose could be incorporated into glycogen. In vitro produced bovine embryos on day 6 post fertilization were obtained from ART, Madison WI and cultured in SOFBE1 at 38.5°C in 6% CO_2 , 6% O_2 and balance N_2 until they became expanded blastocysts on Day 7 or 8. Blastocysts were fixed with 4% formaldehyde and stained according to the PAS kit directions (Abcam). Strong positive staining was observed in both trophectoderm and inner cell mass. The diastase treated controls, which should eliminate staining due to glycogen, retained strong staining suggesting that the stained substances were predominantly mucins (i.e., neutral and acid simple non-sulfated and acid complex sulfated types). To resolve the question as to if any of the glucose was being stored as glycogen, the metabolic processing of glucose in bovine trophectoderm cell line (CT-1) was assessed using stable isotope-based GC-MS analysis. Feeder-free CT-1 cells were grown in six-well Corning Cellbind™ plates and cultured in DMEM with 4.5 g per L glucose and 4 mM glutamine, 2 mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 U per ml penicillin/streptomycin and 10 % FBS at 37.5°C in 5% CO_2 . Prior to treatment, cells were washed with SOFBE1 lacking pyruvate, lactate, and hexose. Cells were incubated in 2 ml of SOFBE1 supplemented with 0-, 1-, 5-, and 25-mM glucose containing 50% [$^{13}\text{C}_6$]-glucose. Cells were incubated for six hours, collected, washed twice with PBS, and stored at -20°C before being processed for GC-MS. Briefly, the glycogen pellet was extracted with ice-cold ethanol (95%), washed twice with ice-cold ethanol to remove any residual-free glucose, and dried at room temperature. The glucose units in glycogen were liberated by incubation (1 h at 55°C) with 0.5 mg of amyloglucosidase (31.2 units/mg of solid) followed by di-O-isopropylidene acetate derivatization of glucose units and GC-MS analysis of the ^{13}C -isotopomers of glucose units from glycogen. Increasing amounts of enrichment in M+3 and M+6 isotopomers of glucose units from glycogen were observed in a concentration dependent manner with 25 mM > 5 mM > 1 mM glucose in the media (percent mole excess for M+3: 1.36 ± 0.47 ; 1.03 ± 0.29 ; 0.51 ± 0.08 [$R^2 = 0.36$, $F(3, 24) = 4.555$, $p = 0.0116$] and for M+6: 2.89 ± 0.97 ; 2.28 ± 0.61 ; 0.57 ± 0.11 [$R^2 = 0.41$, $F(3, 24) = 5.503$, $p = 0.0051$], respectively). No labelled M+3 or M+6 isotopomers were detected in the control (no glucose supplementation). These results suggest that bovine trophectoderm cells have the metabolic capacity to incorporate and store available glucose into glycogen. This work was funded by Agriculture and Food Research Initiative Competitive Grant No. 2019-67015-29412 from the National Institute of Food and Agriculture of the U.S. Department of Agriculture.

P-83 - Generation of Porcine Fetuses with Monoallelic HNF1A Mutation by CRISPR/Cas9 Electroporation in Oocytes

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Genetically modified pigs are a promising model for advancing biomedical applications and for studying human diseases. Frequently, haploinsufficiency in hepatocyte nuclear factor-1 alpha (HNF1A) is widely recognized as a significant contributor to the human monogenic diabetes form, maturity onset diabetes of the young (HNF1A-MODY). Creating HNF1A heterozygous knockout pig models would serve as a valuable tool for studying human HNF1A-MODY. Although CRISPR/Cas9 microinjection into porcine zygotes has proven successful in generation of gene knockout (KO) piglets, the process requires individual manipulation of each embryo. Hence, the microinjection process is quite time consuming, resulting in low throughput. Delivery of CRISPR/Cas9 by electroporation has been developed to circumvent the drawbacks of microinjection. Past experiments with electroporation of porcine embryos [30V, 1ms pulse, 100ms pulse interval, and 5 pulses (bipolar)] were successful for efficiently generating biallelic edits with CRISPR/Cas9 introduction into zygotes. However, creation of monoallelic edits requires adjustments of the procedure. To generate potential monoallelic mutation in the HNF1A gene, matured porcine oocytes were electroporated with CRISPR/Cas9 (1:2 ratio) using 2 pulses [25V, 1ms pulse, 100ms pulse interval] immediately before insemination. Cleaved embryos were transferred to a recipient approximately 44 hours later. A total of 7 conceptuses were recovered on day 19. DNAs from these conceptuses were isolated and sequenced, and the sequence results were analyzed by ICE and TIDE analysis tools. Three of the seven conceptuses had monoallelic edits, retaining wild type allele (proportions ranging from 44% to 60%).

P-85 - Regulation of Transposable Elements by TRIM28 Protects the Sex-Determination program in PGCs.

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Recent reports have shown that dynamic TE expression is a hallmark of gonadal somatic cells as they undergo sexualization. Here, we ask whether similar dynamics exist in Primordial Germ Cells (PGCs), the founding cells of the germline. As uncontrolled TE activity represents a threat to the integrity of the germline genome, we hypothesized that any balance between TE function and genomic threat is balanced by TRIM28, an epigenetic scaffolding protein which organizes the deposition of repressive heterochromatin at targeted loci. As TRIM28 is a well-characterized regulator of TEs in pluripotent stem cells (PSCs), we reasoned it may reprise a similar role in mammalian PGCs. To test this hypothesis, we used an in vivo PGC-specific conditional TRIM28 knockout (TCKO) model, focusing on PGCs between E10.5 and E14.5, during which they downregulate the early PGC program, exit the indifferent state, and undergo sex determination. We detected TRIM28 in both male and female PGCs and observed that the abundance of TRIM28 was

both sex- and stage-dependent. Likewise, we found that in control PGCs the expression of TEs was highly dynamic between E11.5 and E13.5 in both sexes. Upon knockout of TRIM28, we examined expression of TEs in TCKO PGCs at E11.5, E12.5 and E13.5 and found that TEs, especially those of the Long Terminal Repeat (LTR) class, were derepressed. At the chromatin level, ATAC-seq revealed that TRIM28-targeted loci are sex-specific, in line with TE regulation by TRIM28 being required for proper sexualization of PGCs. To assess whether TCKO PGCs could acquire competency for germline differentiation we examined expression of Dazl, an RNA-binding protein thought to facilitate the downregulation of the early PGC program. We found that TCKO PGCs were heterogeneous in their expression of Dazl. Interestingly, we found that male, but not female, TCKO PGCs fail to fully downregulate the early PGC program despite observed deficiencies in Dazl expression. In line with these findings, male TCKO PGCs fail to differentiate into prospermatogonia, while female TCKO PGCs inefficiently progress to meiotic germ cells. Collectively, our results suggest that regulation of TE expression dynamics in the germline is both necessary for proper transition between indifferent and sex-determined PGCs, and that TE regulation during this window is sex-specific. Furthermore, these results suggest that TE reprogramming during sex-determination of PGCs likely licenses the PGC epigenome for gametogenesis. This work is funded by R01HD058047 to AC, F31 HD113346 and a UCLA Whitcome Fellowship to JD.

P-86 - Effect of MAT2A on Porcine Embryonic Development

Xiao-Han Li and Xiang-Shun Cui

Methionine adenosyl-transferase 2A (MAT2A) is an essential enzyme that creates S-adenosylmethionine (SAM) by reacting methionine and ATP. MAT2A provides methylation donors (SAM) for histone and DNA methylation processes. Histone methylation plays a key role during zygotic genome activation (ZGA). However, the effect of MAT2A in porcine ZGA remains unclear. To investigate the function of MAT2A and the underlying mechanism in porcine ZGA, MAT2A was knocked down via dsRNA injection in the one-cell stage. MAT2A was highly expressed at every stage in porcine embryos. The percentage of four-cell stage embryos and blastocysts was lower in the MAT2A-knockdown group than in the control group. Notably, depletion of MAT2A decreased the levels of H3K4me2, H3K9me2/3 and H3K27me3 in the four-cell stage and MAT2A knockdown reduced transcriptional activity of ZGA gene. Furthermore, MAT2A knockdown led to a decrease in the expression of EED and EZH2. Additionally, MAT2A knockdown caused a significant increase in DNA damage and apoptosis. In conclusion, MAT2A is involved in the regulation of transcriptional activity and is essential for regulating histone methylation during porcine ZGA.

P-87 - Extension of Development (ED) of Mouse Blastocyst leads to Activation of Steroidogenesis

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It is believed that preimplantation mouse embryo is not capable of steroidogenesis, unlike species with an extended period of preimplantation development (ruminants, pigs and horses). We previously demonstrated in mice that when implantation is prevented by ovariectomy, the blastocyst switches from carbohydrate to lipid metabolism at 8 days post coitum (dpc). This leads us to hypothesise that mouse blastocyst is able to synthesize steroids once its preimplantation development is extended. Naturally mated C57BL/6 female mice were ovariectomised at 3 dpc to remove the source of oestradiol and prevent embryo implantation. Then, embryos were collected at 4, 6, 8, 10 and 12 dpc (control: E4; experimental groups: E6-8-10-12). Embryo collection media, considered as a representation of embryonic content (bearing in mind nonactive uterus of ovariectomised mice) were processed for exosome isolation. In collected ($n > 10/\text{group}$) blastocysts and exosomes, lipid profile was analysed using Fourier Transform Infrared (FTIR) and Raman spectroscopies. Moreover, gene expression and cellular structures such as lipid droplets, autophagosomes and mitochondria (where the first step of steroidogenesis takes place) were analysed in blastocysts by mRNA sequencing and transmission electron microscopy (TEM). Finally, blastocyst growth was evaluated by fluorescence immunolabeling and cross-sectional measurement of semi-thin sections. FTIR analysis of blastocysts demonstrated temporal increase of cholesterol, oxidised cholesterol and polyunsaturated fatty acids (PUFAs) absorbance that are recognised modulators of steroidogenesis (0.227, 0.381, 0.231 for E8 vs. 0.216, 0.338, 0.218, for E4, respectively, $p < 0.05$) during ED. Exosomes analysis show that total lipid level increases at E10 (FTIR: 0.164 vs. 0.125, $p < 0.05$; Raman: 0.067 vs. 0.006, $p < 0.05$); lipid peroxidation level decreases during ED (15.213, 4.142, 5.963, 10.304, 4.736 for E4, E6, E8, E10 and E12, respectively, $p < 0.05$) and PUFAs level increases at E10 (0.021 vs 0.016, $p < 0.05$); one-way ANOVA with Tukey's post hoc test. Moreover, the expression of genes regulating cholesterol metabolism (Apol6, Scap, Npc2 and Cyb5a) and steroidogenesis (Dhrs11, Hsd17b, Hsd3b, Cyp4, Cyp26a and Cyp27a) was activated during ED. Interestingly, Cyp27a expression indicates the generation of 27-hydroxycholesterol that is considered to be an ancestral oestrogen. Sequencing data are expressed as log2 fold change, differentially expressed genes were considered when false discovery rate < 0.05 . TEM analysis revealed indications of steroidogenesis changes in cell composition during ED, such as autophagy, demonstrated by the presence of lysosomal bodies and autophagosomes, and degradation of lipid droplets and changes in the profile of mitochondria (i.e. the organelle where the biosynthesis of steroid hormones occurs). Mean number of blastocyst cells at E8 and E4 was 60 ± 2.5 vs. 51.2 ± 2.5 cells $\pm$ SEM, $p = 0.04$, for trophoctoderm, and 19 ± 0.5 vs. 21 ± 1.8 cells $\pm$ SEM for inner cell mass. There was a progressive increase of the cross-sectional area of blastocysts ($2785.298 < 2915.040 < 3760.608 < 5041.595 \mu\text{m}^2$) at E4, E6, E8 and E12, respectively. We provide the evidence of steroidogenic activity in mouse blastocysts during ED. The observation of naturally occurring and experimentally induced implantation delay in mice demonstrates that ED blastocysts are capable of implantation and development to term. Thus, activation of steroidogenesis may be an evolutionary-conserved mechanism that facilitates implantation under specific conditions such as ED, in all mammals.

P-88 - Deregulation of Oxidative Phosphorylation Pathways in Embryos Derived In Vitro From Prepubertal and Pubertal Heifers Based on Whole-Transcriptome Sequencing

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Mitochondria are essential for the oocyte maturation and embryonic development by ATP production through oxidative phosphorylation. The transcriptomic profile of embryos could be used as a predictor for embryo's individual developmental competence. The aim of the study was to characterise and compare transcriptomic profile of blastocysts derived from prepubertal and pubertal heifers. Bovine cumulus-oocyte complexes were obtained by ovum pick-up method from prepubertal and pubertal heifers. After 24 hours, the mature cumulus-oocyte complexes (COCs) were fertilized in vitro using commercial bull semen. At 24 hours after fertilization, the embryos were transferred to culture medium and cultured for another 7 days to the blastocyst stage. Total RNA was isolated from two groups of blastocysts and RNA-seq was performed. Gene Ontology (GO) analysis was performed by DAVID (Database for Annotation, Visualization and Integrated Discovery). A higher average blastocyst rate was obtained in the pubertal group than in the prepubertal group ($p < 0.05$). There were no differences in the quality of the obtained blastocysts between the two examined groups ($p > 0.05$). We identified 436 differentially expressed genes (DEGs) between embryos derived from prepubertal and pubertal heifers. Kyoto Encyclopedia of Genes (KEGG) pathway analysis suggested that the genes involved in mitochondrial function including oxidative phosphorylation were different in two examined groups. Differences of the level of expression of genes associated with mitochondrial function were found: ATP synthases (ATP5MF-ATP synthase membrane subunit f, ATP5PD-ATP synthase peripheral stalk subunit d, ATP12A-ATPase H⁺/K⁺ transporting non-gastric alpha2 subunit), NADH dehydrogenases (NDUFS3-NADH:ubiquinone oxidoreductase subunit core subunit S3, NDUFA13-NADH:ubiquinone oxidoreductase subunit A13, NDUFA3-NADH:ubiquinone oxidoreductase subunit A3), cytochrome c oxidase (COX17), cytochrome c somatic (CYCS) and ubiquinol cytochrome c reductase core protein 1 (UQCRC1). This study indicated that genes involved in OXPHOS could be predictive indicators of embryo quality, developmental competence and implantation ability.

P-89 - Postovulatory and Maternal Aging Do Not Affect the Biomechanical Properties of Trophectodermal Cells in Mouse Embryos

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As literature data indicate, postovulatory aging - resulting from the extended time between ovulation and fertilization, and maternal aging - related to the mother's age, are among the most important causes of low oocyte quality, which in turn results in lower developmental potential. It

has been reported that different types of aging may affect fertilizability, the ability to form a blastocyst, and postimplantation embryo development. However, there is no data on the impact of aging on the functionality of the trophectoderm, which is crucial for the proper implantation process. Some of the most important determinants of trophectoderm functionality are its biomechanical properties, which are reflected, among others, as the velocity of cytoplasmic movement. In our studies, we checked the impact of both maternal and in vitro postovulatory aging on the velocity of cytoplasmic movement in trophectoderm cells of mouse blastocysts. Using time-lapse imaging and the Particle Image Velocimetry method, we analysed cytoplasmic movements in trophectodermal cells of the following groups: 1) control – blastocysts obtained from in vitro fertilization (IVF) of freshly ovulated oocytes from young females, 2) postovulatory aging – blastocysts obtained from IVF of oocytes (obtained from young females) subjected to nine-hour-long in vitro aging, and 3) maternal aging – blastocysts obtained from IVF of freshly ovulated oocytes isolated from old females. First, we found that the velocity of cytoplasmic movement was significantly higher in polar than in mural trophectoderm cells: 10.14 ± 3.89 nm/s and 6.61 ± 2.32 nm/s in control embryos, 10.94 ± 2.42 nm/s and 6.50 ± 1.80 nm/s in postovulatory aging group, 10.29 ± 3.13 nm/s and 7.05 ± 2.36 nm/s in maternal aging group in polar and mural trophectoderm cells, respectively. However, we did not observe the effect of maternal and in vitro postovulatory aging on the velocity of cytoplasmic movement both in the mural and polar trophectoderm cells. Because in our previous experiments, we discovered that the cytoplasmic velocity depends on the keratin cytoskeleton, we checked the impact of maternal aging on mRNA levels for keratin 8/18 (the most abundant in preimplantation embryos) in mouse blastocysts. We found that the mRNA levels for keratin 8/18 are not changed by maternal aging, which is consistent with the lack of differences in cytoplasmic velocity. To sum up, the adverse effects of maternal and in vitro postovulatory aging on the embryos' development most likely do not result from changes in the biomechanical properties of the trophectodermal cells. This research was funded by the National Science Centre (Poland) grant no. UMO-2020/39/B/NZ5/02962 to AA.

P-90 - Comparative analysis of Embryo development According to Goat Oocyte Grade, Fertilization time, In Vitro Culture Method

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Owing to the growing interest in the consumption of health food, public interest in goats has been increasing. An embryonic in vitro culture technique capable of embryo transfer is required to ensure prolific goat genetic resources. Here, we analyzed the effects of goat oocyte grade, fertilization time, and in vitro culture method on in vitro goat oocyte maturation and embryo culturing. The oocyte grade was evaluated according to cytoplasm and cumulus cells using fertilization times of 6, 12, and 18 h. In vitro culture methods were divided into steps and the development rate was analyzed. In vitro maturation rates differed depending on oocyte grade, along with differences in embryo development rates. When analyzed by fertilization time, there was no difference in the cleavage rate between 6 h (87.38%), 12 h (84.85%), and 18 h (88.79%). Furthermore, embryo development rate remained unchanged between 6 h (31.07%) and 18 h (32.71%). However, the blastocyst rate was low at 12 h (25.25%). Embryo development rates did not significantly differ between steps; 26.32% and 23.81% in the first and second steps, respectively. Our study findings indicate that it is essential to use high-grade oocytes for successful embryo

development, whereas fertilization time and in vitro culture steps do not have significant effect. In future studies, additional research on in vitro goat embryo culturing will be necessary. Conclusively, the establishment of a dependable in vitro fertilized goat embryo system will contribute to successful goat husbandry.

P-91 - Chemical Inhibition of IL6ST Compromises the In Vitro Production of Bovine Embryos

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Previous work found that interleukin-6 (IL6) supplementation improves inner cell mass (ICM) cell numbers within in vitro produced bovine embryos. The embryo expresses IL6, and this raises the possibility that embryo-derived IL6 also plays important roles during early embryogenesis. The IL6 receptor complex is comprised of a ligand-specific α subunit (IL6R) and a common IL6-family β subunit (IL6ST). We hypothesized that incubation with the chemical IL6ST inhibitor, SC144, would impair in vitro bovine embryo development. Abattoir-derived cumulus-oocyte complexes were matured and fertilized, and presumptive zygotes were cultured in SOF-BE1 (45-53 embryos/treatment/replicate; 3-4 replicates/study). Development was assessed at days 2, 5, 7 and 8. Embryo cleavage was not affected by supplementing 0.4 μ M SC144 from days 1 to 8, but there was a tendency for reduction when 2 μ M SC144 was supplemented ($P=0.08$) and a reduction ($P=0.002$) when 10 μ M SC144 was supplemented. All SC144 concentrations reduced morulae formation on day 5 ($P<0.05$). There was a tendency for 0.4 μ M SC144 to reduce total blastocyst formation on days 7 ($P=0.13$) and 8 ($P=0.08$) and for 2 μ M to reduce blastocyst development on days 7 ($P=0.02$) and 8 ($P=0.01$). No blastocysts were observed in the 10 μ M treatment on days 7 and 8. The day 3 to 5 SC144 treatment scheme was used to describe whether IL6ST inhibition influences morula development. Embryos were washed and plated in new SOF-BE1 lacking the inhibitor on day 5 to determine if carryover effects on blastulation were evident. None of the SC144 concentrations affected morulae formation at day 5; however, blastocyst development was reduced at days 7 and 8 in the 0.4 μ M and at day 7 in the 2 μ M SC144 treatments ($P<0.01$). Blastocysts were degenerate on day 8 in the 2 μ M treatment group. No blastocysts were observed in the 10 μ M SC144 treatment group on either day ($P<0.0001$). Blastocysts that were present in the 0.4 μ M SC144 group contained the same ICM and TE cell numbers and ICM:TE ratios as the control group. A final study examined how SC144 exposure from day 5 to 8 affects blastocyst development. Blastocyst development was reduced at day 7 and 8 at each SC144 concentration ($P<0.01$), and substantial blastocyst degeneration was observed on day 8 when 2 or 10 μ M SC144 was provided. Blastocysts exposed to 0.4 μ M SC144 contained fewer ICM ($P=0.03$) and tended to contain fewer TE ($P=0.08$) cell numbers, although no changes in the ICM:TE ratio were detected. In summary, chemical inhibition of IL6ST did not compromise the initial cleavage events but did influence morulation and blastulation. Blastocysts contained a greater than normal rate of degeneration, and reduced ICM and TE cell numbers were detected if the inhibitor was present when blastocysts were developing. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2021-67015-34485 from the USDA National Institute of Food and Agriculture.

P-92 - CDX2 is not Essential for Bovine Blastocyst Formation but Plays a Role on First Lineage Specification

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First cell lineage differentiation results in the formation of the trophectoderm (TE) and inner cell mass (ICM) populations that characterizes the early blastocyst stage. CDX2 is a transcription factor essential for proper trophectoderm (TE) differentiation in mice, where its ablation prevents blastocoel maintenance. Its role in other mammals is unknown, as whereas its TE-specific expression is conserved, the CDX2-mediated transcriptional repression of the ICM-specific transcription factor POU5F1 reported in mice is not applicable to other species, where neither CDX2 binds to POU5F1 promoter nor POU5F1 expression is ICM-specific. To gain insight on how first lineage differentiation occurs in bovine, we have analyzed the developmental ability of CDX2 knock-out (KO) bovine embryos. In vitro matured oocytes were allocated into two groups: one (n=168) was microinjected with messenger RNA (mRNA) encoding for cytosine base editor (CBE) and guide RNA (gRNA) designed to generate a premature stop codon on CDX2 (group C+G, containing KO embryos) and other (n=91) was injected with mRNA encoding for CBE alone (group C, composed entirely by unedited WT embryos). Following microinjection, in vitro fertilization (IVF) was performed and embryos were cultured in SOF. Immunohistochemistry (IHC) analysis was performed on 46 blastocysts from C+G group and 31 from C group collected at Day (D) 8 to detect CDX2, and TE (GATA3+) and ICM (SOX2+) cells. Blastocyst rate was similar between microinjection groups and all blastocysts analyzed in C+G group were KO, as confirmed by Sanger sequencing and the absence of CDX2 expression. CDX2 KO blastocyst displayed a similar total cell number (118±8 vs. 105±6, mean±s.e.m. for WT vs. KO), but a reduced number of TE cells (90±7 vs. 64±6, mean±s.e.m. for WT vs. KO, Mann-Whitney $p < 0.05$) and an increased number of ICM cells (29±2 vs. 44±3 mean±s.e.m., for WT vs. KO, Mann-Whitney $p < 0.05$). To determine if the trophectoderm could proliferate beyond blastocyst hatching, D7 blastocyst were cultured in a post-hatching system based on N2B27 medium up to D12. Survival rate from D7 to D12 was unaffected by CDX2 ablation and IHC was conducted to detect TE (GATA3+), hypoblast (SOX17+) and epiblast (SOX2+) cells in 23 WT and 38 KO embryos. D12 CDX2 KO embryos displayed similar hypoblast migration and epiblast survival rates, but displayed a reduced diameter (852±65 vs. 610±41 μm , mean±s.e.m. for WT and KO, Mann-Whitney $p < 0.05$) and number of epiblast cells (82±1 vs. 24±5, mean±s.e.m. for WT vs. KO, Mann-Whitney $p < 0.05$). In conclusion, TE differentiates and develops in the absence of CDX2, but CDX2 ablation increases significantly the ICM:TE proportion leading to larger embryonic discs and smaller extra-embryonic membranes. Supported by projects StG-757886 from ERC and PID2020-117501RB-I00 from MINECO.

P-93 - Conceptus derived PGs are not Essential for Early Bovine Post-Hatching Development

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Prostaglandins (PGs) are lipid signaling molecules playing critical roles on gestation whose concentrations in bovine uterine fluid increase as conceptus elongation progresses. Previous studies have observed that pharmacological inhibition of PGs impairs conceptus length, but it is unclear whether conceptus-derived PGs play an autocrine role on their own development. To solve that question, we have assessed the developmental ability of bovine embryos lacking the rate limiting enzyme for PG synthesis: PTGS2. In vitro matured oocytes were allocated into two groups: one (n=396) was microinjected with messenger RNA (mRNA) encoding for cytosine base editor (CBE) and guide RNA (gRNA) designed to generate a premature stop codon on PTGS2 (group C+G, containing KO embryos) and other (n=234) was injected with mRNA encoding for CBE alone (group C, composed entirely by unedited WT embryos). Following microinjection, in vitro fertilization (IVF) was performed and embryos were cultured in SOF medium up to Day (D) 7 and subsequently transferred to a post-hatching system based on N2B27 medium to allow further development up to D12, when they were fixed for lineage analysis and subsequently genotyped by Sanger sequencing. A subset of D7 embryos were individually cultured from D9 to D12 to determine total PGs concentration in the spent medium by ELISA. Similar developmental rates to D7 blastocyst (25 ± 2 vs 21 ± 3 %, mean $\pm$ s.e.m. for C vs. C+G) and from D7 to D12 (88 ± 5 vs 88 ± 4 %, mean $\pm$ s.e.m. for C vs. C+G, respectively) were observed in both microinjection groups. 65/68 of the D12 embryos analyzed in C+G group were KO for PTGS2 (i.e., contained only KO alleles). PGs concentration in the spent media was analyzed in 16 embryos per group, being significantly reduced by PTGS2 ablation (~500-fold reduction: 32707 ± 8493 vs $65,08 \pm 26.63$, pg/mL, mean $\pm$ s.e.m. for WT and KO, Mann-Whitney test $p < 0.05$). Lineage development was analyzed by immunohistochemistry for trophectoderm (CDX2), hypoblast (SOX17) and epiblast (SOX2) markers in 56 WT embryos and 65 KO. PTGS2 ablation did not affect embryo growth (800 ± 50 vs 790 ± 40 μ m, mean $\pm$ s.e.m. for WT vs. KO), hypoblast migration rate (24/56 (43 %) vs. 24/65 (37 %), WT and KO), embryonic disc formation rate (40/56 (71 %) vs. 42/65 (65 %), WT vs. KO) or the number of epiblast cells (47 ± 6 vs. 60 ± 7 , mean $\pm$ s.e.m. for WT vs. KO). In conclusion, PGs synthesis inhibition by PTGS2 ablation does not affect bovine embryo development up to early post-hatching stages. Supported by projects StG-757886 from ERC and PID2020-117501RB-I00 from MINECO and 21985/JLI/22 from Fundación Séneca.

P-94 - Dynamics of Higher-Order Interactions Define Transcriptomic Trajectories Associated with Human Embryonic Genome Activation

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Introduction: The newly fertilized embryo is transcriptionally quiescent. The embryo transitions from stored maternal transcripts to dependence on its own transcriptome through embryonic genome activation (EGA). EGA is a fundamental developmental milestone and is a dynamic process, however the temporal regulation of the network of genes involved in EGA has not been quantified. In this study we use mathematical approaches to measure i) transcriptomic trajectories through the preimplantation period and ii) the dynamic organisation of the transcriptome

associated with human EGA. **Methods:** Transcriptomic network analysis measures co-ordination between expressed genes providing a model of the whole transcriptome. Networks define transcriptomic coordination at the level of pairwise relationships between genes (first order interactions [FOI]). We can include complex non-linear dynamic relationships between many genes (higher order interactions [HOI]) using a mathematical approach termed hypergraph analysis. Such properties measure wide-scale reorganizations of the transcriptome as happens during EGA. A data set of human preimplantation single cell RNAseq data (n = 2280 cells, oocyte to day 14) was generated by meta-analysis of 4 studies [1-4] with batch correction using COMBAT and clustered using Leiden community detection. Trajectory analysis was performed using three approaches: Pseudotime inference, RNA velocity, and lineage tracing (scVelo and Monocle3). Cells were aligned in developmental order (pseudotime) and gene networks modelled across the dataset using a moving window approach (window size of 10 and step size of 1). Transcriptomic networks were constructed through co-expression analysis within the individual windows. Both FOI and HOI were characterized in the networks using entropy as a measure of transcriptomic network structure. **Results:** A total of 2046 genes were found to have developmental time dependent expression. Combining velocity and pseudotime inference defined trajectories characterised by high velocity at fertilization before dropping. An increase in velocity was observed in the 8-cell to morula transition (1.2-fold, p-value < 1x10⁻⁵). Network entropy followed a similar pattern to velocity, in both the first- and higher-order networks. However, the 8-cell to morula transition demonstrated a significant increase in the entropy of HOI compared to FOI (adjusted p-value < 1x10⁻³). This increase in HOI entropy was associated with autophagy and oxidative phosphorylation pathways demonstrating co-ordinated regulation of these pathways following EGA. HOI were shown to specifically control cadherin pathway regulation. **Conclusion:** These data show that the complex dynamics of the transcriptome in the early embryo can be precisely measured. The developmental trajectory marked by gene expression velocity confirms current understanding, i.e. that accelerated cell cycle during early cleavage results in reduced transcription but extends this by quantifying the dynamic process. The velocity observations provide evidence for this theory as a spike of activity was observed during the 8-cell to morula transition where a pause in proliferation is thought to occur [5]. The relative increase of HOI entropy associated with EGA suggests that the morula transcriptome is more coordinated when compared to the preceding blastomeres, explaining the differences between cleavage patterns at the blastomere and morula stages. Associated gene ontology reveals that the first action of the embryonic transcriptome is to programme metabolism. Understanding transcriptomic dynamics in the early embryo is informative in defining lineage and stem cell specification. [1] Cell, (2016) 165(4), 1012-1026 [2] Nat Struct Mol Biol, (2013) 20(9), 1131-1139. [3] Development, (2015) 142(18), 3151-3165. [4] Nature, (2020) 577(7791), 537-542. [5] Nat Rev Genetics, (2019) 20, 221-234

P-95 - Embryo-Induced Alterations in The Protein Profile of Bovine Oviductal Extracellular Vesicles In Vitro

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The oviduct provides the ideal environment for early embryo development. Maternal-embryonic communication within the oviduct, important for ensuring embryo quality, is partially facilitated through extracellular vesicles (EVs). Bovine oviductal explants offer an ex vivo alternative for studying this communication between the embryo and the female reproductive tracts via EVs. This study aimed to identify the protein profile of EVs resulting from the interaction between embryos and maternal tissue using this model. For this, five synchronized cyclic heifers were slaughtered 3.5 days after heat. Six 0.25 mm² oviductal explants were obtained from each heifer and cultured individually in 750 µL of protein-free synthetic oviduct fluid (SOF): three were cultured in medium alone (Expl), and three were co-cultured with 10 in vitro-produced 8-16-cell stage bovine embryos each (Expl+Emb). Also, a group of 100 in vitro-produced 8-16-cell stage embryos was cultured alone (Emb) in 500 µL of SOF. All groups were cultured for six hours at 5% CO₂, 38.5°C, and conditioned medium (CM) was collected for EV isolation. EVs were isolated using size exclusion chromatography and their presence was confirmed by the detection of CD63, CD81, and CD44 EV markers with flow cytometry. Proteomic analysis was carried out using nanoLC-MS/MS with spectral counting for protein identification and quantification. The EVs from 5 replicates (representing each cyclic heifer) of CM from the “Expl” and “Expl+Emb” groups, as well as 5 replicates from the “Emb” group, were analyzed. Data were compared between pairs of groups using the Student’s t-test, with a p-value threshold of 0.05. Bioinformatic analysis was performed with PANTHER. We identified 859 proteins in the CM-EVs from Expl, 627 in the CM-EVs from Expl+Emb, and 111 proteins in the CM-EVs from Emb. In the qualitative analysis, 81 proteins were detected in the three experimental groups, 485 were common to Expl and Expl+Emb, 17 were common to Expl and Emb, 5 were common to Expl+Emb and Emb (MUC16, LMNB1, TSN, MELTF, TTC26), 5 were unique to Expl (CAP2, LGALS3BP, PLIN4, MOSPD2 and CRP), and none were unique to Emb. Additionally, six unique proteins (UPK3BL2, PTPRD, TOM1L1, S100A11, PTPA, and PRKAB1) were identified as being exclusively present when there is an interaction between the oviduct and the embryo in vitro (Expl+Emb). Among these, S100A11, a member of the calcium-binding protein S100 family, might act as a modulator of the embryonic transcriptome controlling processes such as cell cycle progression and differentiation. Two protein-modifying enzymes (A0A4W2DFR9 and PSMB8) related to metabolic process proteins were overabundant in the Expl group compared to the Expl+Emb group. Twenty-one proteins, mainly associated with cellular processes such as cell motility, adhesion, and communication, were overabundant in Expl compared to Emb. Additionally, when comparing Emb with Expl+Emb, seven proteins were overabundant in Expl+Emb, some of which are related to the Wnt signaling pathway, a regulator of embryonic cell proliferation and

differentiation. Furthermore, the transcriptional factor YBX1, related to mRNA decay during the pre-implantation period, was found to be present only in the Expl+Emb group. Our data indicate that the oviduct can respond to embryos as early as the 8-16-cell stage, exhibiting distinct protein profiles within its EVs when cultured alone or in the presence of embryos. Funded by the Spanish Ministry of Science and Innovation PID2019-111641RB-I00.

P-96 - HAND1 Controls Epithelial Morphogenesis and Fate Specification in a Human Pluripotent Stem Cell-Based Amnion Model

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Amniogenesis is triggered in a collection of pluripotent epiblast cells as the human embryo implants. Previous transcriptomic analyses of stem cell-derived models of amnion showed a potential role of HAND1, a basic helix-loop-helix transcription factor, in amniogenesis, which showed dynamic expression during amnion fate specification. Strikingly, functional studies reveal a key role for HAND1 in amnion morphogenesis and lineage progression, as loss-of-function mutation of HAND1 results in impaired epithelial polarization and halted amnion fate specification. Molecularly, we identified SOX7 as a downstream target of HAND1 activity, and found that SOX7 is critical for epithelial polarization but not for amnion fate specification. Together, this study reveals a novel requirement of the HAND1-SOX7 axis in maintaining epithelial morphogenesis of developing human amnion. This work was supported by NIH grants R01-HD098231 and P51-OD011106 (to the WNPRC), as well as by MCW Start-up funds.

P-97 - ANKRD49 is Required for Peri-implantation Embryogenesis

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Studies of early pregnancy loss in humans indicate that less than 30% of conceptions progress to live birth and approximately 80% of all cases of pregnancy loss occur within the first trimester.

Some cases of early pregnancy loss can be attributed to environmental factors while others may originate from deficiencies in preimplantation or peri-implantation embryo health. At present, the genetic factors required to support embryogenesis have not been fully defined. We previously identified ankyrin repeat domain 49 (Ankrd49) as a transcription factor that is expressed throughout preimplantation embryogenesis. To examine the functional importance of Ankrd49, CRISPR/Cas9 methodology was used to engineer mouse embryos with inactivated alleles. Founders with monoallelic deletions in Ankrd49 were generated, but no biallelic edited offspring were born. In addition, the building of lines from founders of either deletion allele yielded heterozygous Ankrd49+/- offspring at expected Mendelian ratios, but homozygous Ankrd49-/- offspring were never produced. Both heterozygous and wild-type embryos could be detected at E7.5, E9.5, and E12.5 from timed mating of Ankrd49+/- mice, but knockout embryos were absent, suggesting that loss of ANKRD49 function leads to peri- or post-implantation death. In corroboration, an expected Mendelian ratio of 22.5% of blastocyst stage embryos at E3.5 were found to be Ankrd49-/- in heterozygous intercrosses by genotyping analysis. Interestingly, an abnormally high percentage (12.5–37.5%) of implantation sites at E12.5 of Ankrd49+/- intercrosses were found to be empty of the embryo proper but have persistent placental tissue. Additionally, outcomes of outgrowth assays revealed that Ankrd49-/- blastocysts can hatch and form typical inner cell mass and trophectodermal outgrowth colonies in vitro, suggesting that Ankrd49 null blastocysts are competent to initiate implantation. Collectively, these findings demonstrate that ANKRD49 is required for development of the embryo proper or gastrulation, with loss of function in mice leading to embryonic death between E4.5 and E7.5. Ongoing studies focusing on this developmental window will determine which aspects of peri-implantation embryo development are impacted by Ankrd49 ablation to better understand the genetic factors underlying embryogenesis and early pregnancy loss.

P-98 - Paternal Aggresome Content is Associated with Increased Reactive Oxygen Species and Impaired Mitophagy During Preimplantation Development of Bovine Embryos

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Male fertility studies have focused primarily on sperm morphology and fertilization, however, there is little research following paternal effects on embryo development. This knowledge gap often leads to fertility issues being solely attributed to the female, neglecting the potential impact of the male on pregnancy outcomes. Previous data from our group showed that sires have divergent embryo production abilities. Low-producing sires had embryos with increased autophagy throughout development. Moreover, some low-producing sires also had high aggresome (AGG) content in the sperm head, which also increased embryo AGG throughout preimplantation development. Nonetheless, how misfolded protein accumulation affects the embryo remains unclear. Here we hypothesize that high concentrations of AGG introduced by the sperm, trigger oxidative stress, endoplasmic reticulum stress, and compromise embryo development. Also, we propose that the increase in autophagy reported earlier could be explained by the clearance of paternal mitochondria or mitophagy. To test these, four sires classified as high or low AGG were used to

produce embryos in vitro. Embryos were collected at the zygote (n=141), 4-6 cell (n= 139), 8-16 cell (n=138), and blastocyst (n=118) stages across three replicates. Embryos were then evaluated for AGG, ROS, and mitophagy. As previously reported, AGG content was elevated in the high AGG sires' embryos for all developmental stages ($P < 0.001$). It could be that AGG coming from the sperm induces AGG in the embryo and this is sustained through development. This would agree with the difficulty of cells to degrade AGGs through the proteasome due to their increased size and accumulation. In addition, in all the developmental stages, ROS was increased ($P < 0.001$) in the embryos coming from high AGG sires. This could be a result of two scenarios: AGG content causes cell stress and produces ROS, or this is a response mechanism to eliminate the AGG through free radicals. In embryos from low AGG sires, mitophagy goes up by the 4-6 cell stage and decreases by the 8-16 cell stage. In contrast, in embryos from high AGG sires, mitophagy stays up throughout development ($P = 0.0006$). If mitophagy is effectively regulating mitochondria turnover, a decrease in later stages is expected. However, it is possible that in embryos from high AGG sires, mitochondrial clearance is impaired, and the embryo autophagy system prioritizes AGG degradation leading to sperm mitochondria accumulation up to the 8-16 cell stage. This study provides new insights into how high paternal AGG can set off a cascade of events leading to reduced embryo development. Further research is needed to understand AGG origin and composition, and potential treatments to reduce its effects on embryo development. Funding provided by USDA-AFRI: 2022-6701538938.

P-99 - Dissecting the Role of WBP1 in Preimplantation Embryonic Development: Insights into Hippo Signaling and Trophectoderm Formation in the Bovine

Byron Chasi and M. Sofia Ortega

A SNP in WW binding domain protein (WBP1) has been linked to cow conception rate, a fertility trait in dairy cattle, and embryonic development in vitro. WBP1 interacts with the WW domains and serves as a mediator of protein-protein interactions. In the embryo, the Hippo signaling pathway is composed of many proteins that contain WW domains, including the Yes-associated protein (YAP) which has been associated with trophectoderm (TE) formation. Previous research from our group has shown that when the expression of WBP1 is reduced, blastocyst rate decreases and so does its number of trophectoderm cells. This suggests that WBP1 may be involved in trophectoderm differentiation, however, the exact mechanism of action of WBP1 in the embryo is still unknown. The objective of this study was to determine how the functional ablation of WBP1 affects preimplantation embryonic development. To test this, a CRISPR-CAS9 system was used to ablate WBP1. Briefly, two guides spanning intron 1 and exon 2 were annealed to a tracer and to CAS9mRNA to form a solution of 62.5 ng/ul of each guide and 125 ng/ul of CAS9mRNA. The complex of guideRNA-CAS9 was electroporated into presumptive zygotes 10 hours post-insemination for treated embryos and just Cas9mRNA for controls. Zygotes were cultured in SOF-BEII media at 38.5°C with 5%CO₂ and 5%O₂. Cleavage was measured at day 3 and blastocyst at day 7.5 of development. To assess trophectoderm formation, immunolocalization of CDX2, phosphorylated YAP, and YAP translocation to the nucleus was measured at the 8-16 cell and morula stages. Images were analyzed using ImageJ v1.44. Cleavage was not different ($P = 0.1282$) between WBP1-null and control embryos (LSMEANS + SEM: 74.84 + 3.27% and 67.44 + 3.64%, respectively). Control embryos had a blastocyst rate of 31.60 + 3.4% (LSMEANS + SEM), however, WBP1-null embryos did not produce blastocyst, and embryos were arrested at the morula stage (P

< 0.0001). At the 8-16 cell stage, there were fewer ($P = 0.0028$) nuclear YAP-positive cells on WBP1-null embryos ($53.13 \pm 6.32\%$ of total cells), compared to controls ($82.65 \pm 3.83\%$ of total cells). In addition, the mean intensity of phosphorylated YAP was higher ($P = 0.0246$) in WBP1-null embryos (40.06 ± 3.21) compared to controls (24.76 ± 2.78). There was no difference ($P = 0.866$) in the number of CDX2-positive cells between groups at this stage. In contrast, at the morula stage, WBP1-null embryos had fewer ($P = 0.017$) cells with nuclear YAP ($56 \pm 3.75\%$), compared to control embryos ($81.87 \pm 2.95\%$). In addition, WBP1-null morulas had a reduced ($P = 0.0195$) number of CDX2-positive cells ($49.14\% \pm 3.78\%$) than control ($64.33 \pm 3.66\%$). These results indicate that WBP1 has an important role in the hippo signaling pathway by regulating YAP. The absence of WBP1 causes increased YAP phosphorylation and degradation in the cytoplasm impairing TE differentiation which results in embryonic arrest before the blastocyst stage. Further research is required to elucidate if WBP1 acts in other proteins from the hippo signaling or how it regulates YAP function. Funding provided by USDA-AFRI 2022-6701538938.

P-100 - NANOG is Required for Proper Hypoblast Development in Ovine

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By the late blastocyst stage, mammalian embryos have experienced two lineage differentiation events. The first one leads to the formation of the trophectoderm (TE) and the inner cell mass (ICM), and the second differentiates ICM cells into epiblast and hypoblast. The TE and the hypoblast will form the extraembryonic membranes, while the epiblast will give rise to the embryo proper. NANOG is a key transcription factor involved in the second lineage specification. Its role has been extensively studied in mice, where Nanog KO embryos arrest between E5.5 and 7.5, not being able to develop the epiblast and failing to express the hypoblast marker SOX17. In bovine, NANOG ablation reduces Day (D) 8 blastocyst cell number, but does not impair ICM differentiation. However, the role of NANOG during post-hatching development is unknown in any ungulate species. The objective of this study was to analyze the role of NANOG in ovine post-hatching embryo development through CRISPR-mediated gene ablation (knock-out, KO). In vitro matured oocytes were microinjected with Cas9 mRNA and a sgRNA against NANOG (C+G), or with Cas9 alone as microinjection control (C). Microinjected oocytes were fertilized and cultured in vitro up to D8 (in SOF medium) or D12 (in N2B27 medium from D6/7), when pictures were taken and embryos were fixed and immunostained to detect SOX2 and NANOG (epiblast markers), and SOX17 (hypoblast marker). Embryo genotyping in C+G group was performed by deep sequencing. Blastocyst rate was similar between the group containing NANOG KO embryos (C+G) and the control group (C) ($34.6 \pm 5.8\%$; vs. $33.2 \pm 6.5\%$; mean $\pm$ s.e.m; 6 replicates; t-test, $p > 0.05$). In C+G group, 13/18 (72.2%) blastocysts were KO and lacked NANOG protein. No significant differences were detected in the number of TE (negative to NANOG, SOX2 and SOX17) or total cells between KO and WT D8 blastocysts (TE: 188 ± 31.35 vs. 225.6 ± 66.3 ; total: 219.4 ± 32.9 vs. 331 ± 62.3 ; mean $\pm$ s.e.m; t-test, $p > 0.05$). However, the number of ICM cells (positive to NANOG, SOX2 and/or SOX17) was significantly lower in KO blastocysts (44.7 ± 7.5 vs. 116.8 ± 33 ; mean $\pm$ s.e.m.; t-test, p

< 0.05). Within the ICM, no significant differences were detected in the number of SOX2+ cells between KO and WT blastocysts (SOX2+: 23.1±4.9 vs. 14.2±2.9; mean±s.e.m; t-test, p >0.05). However, SOX17+ cell number was significantly lower in KO blastocysts (28.15±7.9 vs. 94.9±21.5; mean±s.e.m; t-test, p < 0.05). Post-hatching embryo survival at D12 was similar between C+G and C groups (80.4±1.3% vs. 88.6±1%; mean±s.e.m; 3 replicates; t-test, p >0.05). In C+G group, 22/36 (61.1%) embryos were NANOG KO. No differences were detected in embryo area (0.36±0.06 vs. 0.37±0.03 mm²; mean±s.e.m; Mann-Whitney test, p >0.05), in the number of embryos showing surviving epiblast cells (17/22 [77.3%] vs. 46/62 [74.2%]; Chi-square test, p >0.05) or in SOX2+ cell number (15.8±4.2 vs. 21.1±3.3; Mann-Whitney test, p >0.05) between KO and WT embryos at D12. However, hypoblast migration, measured as the percentage of inner embryo surface covered by SOX17+ cells, was significantly reduced in KO embryos (34.9±6.9% vs. 51±3.5%; Mann-Whitney test, p >0.05). In conclusion, NANOG is dispensable for ovine blastocyst formation, epiblast development and hypoblast differentiation, but its ablation impairs significantly hypoblast proliferation and migration at post-hatching stages. Work supported by StG 757886-ELONGAN and PID2021-122153NA-I00.

P-101 - Increased Male Sex Ratio and Significantly Altered Gene Expression Coincide with Increased Pace of Development in Bovine Embryos Produced and Cultured In Vitro

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Studies from several mammalian species as mouse, pig, bovine or human indicated a higher male-to-female sex ratio among the fastest developing embryos. These observations suggest differences in the pace of embryonic development after in vitro fertilization (IVF) and culture. However, the outcome of similar studies is still inconclusive in several species. Here we studied the sex ratio in our bovine in vitro production (IVP) system. In order to elucidate possible molecular reasons for differential developmental speed, we focused on the latest in vitro stage, the hatched blastocyst, and analyzed gene expression by RNAseq and subsequent bioinformatics analysis of affected pathways. Sexing of individual blastocysts was performed by a Y-chromosome-specific PCR test. We observed that also in our IVP system, in vitro produced and cultured bovine embryos showed a remarkably different pace of development. Most blastocysts reached the hatching stage eight days (day 8) after fertilization. However, hatching of a considerable number of blastocysts was observed one day earlier (day 7) or one day later (day 9). Sexing revealed that among embryos with the fastest pace of development, reaching the stage of hatching seven days after fertilization, there was an excess number of males (>60%). This excess was still present but showed a decreasing tendency among blastocysts hatching eight or even nine days after fertilization. Transcriptome analysis by RNAseq revealed 256 differentially expressed genes (DEGs) between fast and normally developing embryos, reaching the stage of hatching seven or eight days after fertilization. Among these genes, 79 showed higher and 177 lower abundance in fast developing embryos. Ingenuity pathway analysis revealed Oxidative Phosphorylation, Estrogen Receptor Signaling, Neutrophil Extracellular Trap Signaling Pathway as the most up-regulated and Mitochondrial Dysfunction, Sirtuin Signaling Pathway, Glycolysis I, Endoplasmic Reticulum Stress Pathway as the most down-regulated canonical pathways in fast compared to normally developing embryos. From these data,

we conclude that IVP bovine embryos show quite different paces of development, with males being more frequently in the most advanced group. An increased developmental pace was clearly associated with a differential gene expression pattern. However, it is unclear, if this is the consequence of the unbalanced sex ratio in early hatching embryos or due to the increased developmental pace. This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 815668 (BovReg).

P-103 - A Multimeric Variant of the Human Contraception Antibody (HCA) Demonstrates Enhanced Contraceptive Activity

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HCA is an IgG1 monoclonal antibody (mAb) that potently agglutinates sperm through binding of its Fab regions to the target antigen, CD52g, on the surface of sperm. HCA was also recently shown to block sperm migration through cervical mucus in vitro and in vivo through two Fc-mediated functions: complement-dependent sperm immobilization and mucus trapping. We have engineered a hexameric version of HCA comprised of 6 IgG1 antibodies linked together using the IgM tailpiece (IgGt). Multimerization of IgG antibodies confers exponentially stronger avidity due to combined binding strength of multiple binding domains. In this study, we compared the activity of the parent IgG1 HCA to the hexameric variant in three assays of contraceptive function: sperm agglutination, sperm immobilization and cervical mucus trapping. As reported previously, IgG1 HCA agglutinated 100% of sperm in less than 30 seconds at low ug/ml concentrations. In contrast, the hexameric IgGt achieved the same effect at 10-100-fold enhanced potency. Similarly, the IgGt variant had greatly enhanced complement-mediated immobilization activity. Hexameric HCA more effectively trapped sperm in midcycle cervical mucus than the IgG1 HCA. These results indicate that HCA-IgGt is a promising second generation contraceptive antibody candidate due to its enhanced contraceptive capabilities.

P-104 - Advances in Reproductive Genetic technology: Expanding toward Global Reproductive Health Equity among populations with Sickle Cell Disease in the United States and Africa

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Two curative gene-editing therapies for sickle cell disease (SCD) treatment were officially approved by the Food and Drug Administration (FDA) as of December 2023, utilizing CRISPR to modify DNA for SCD. Worldwide approvals for gene-editing not only mark radical scientific innovation for populations living with SCD across the United States (US) and Africa, but also highlight serious bioethical concerns. One important concern is how new reproductive genetic technology (e.g.,

noninvasive prenatal testing) and gene-editing impacts fertility, pregnancy, and reproductive decision-making among marginalized populations, such as Black American and African women living with SCD. For instance, as gene-editing advances in the US (and clinical trials are being carried out in some parts of sub-Saharan Africa), reproductive health outcomes for those living with SCD is still perpetually dismissed and neglected. Another aspect is how noninvasive prenatal testing (NIPT) also is widely becoming routine prenatal care in the US (and across 14 European countries), but also is in the experimental implementation phase to screen for SCD in the fetus. Most clinical trials on NIPT, however, predominately enroll White women in their sample cohorts, which structurally excludes the subsample variance of Black American and African women with genetic conditions. This proposed study draws from two datasets of Black American and African women living with SCD, including: 1) NIPT dataset (N=40) patient cases of women (18-51 years) living with and without SCD in the US context; and 2) Sickle Women and Girls (SWAG) dataset (N=45) patient cases of women and girls (13-25 years) living with SCD in Ghana, West Africa. Results show overlap as to how Black American and African women navigate SCD and prenatal care; perceptions of NIPT for SCD screening in the fetus; the intersection of reproductive health and SCD; and NIPT decision-making processes, relating to fertility concerns in curative gene-therapies for SCD. As the global landscape of reproductive health is expanding, we must also meet the needs of the global society for populations with genetic conditions. In order to do this, we need a call to action that frames how reproductive health equity and inclusion can be implemented into scientific frameworks, to better improve the integration between reproductive bioethics and reproductive genetic technology among Black American and African women, worldwide.

P-105 - Efficient Simultaneous Knock-In of Multiple Long DNA Sequences in Mouse Embryonic Stem Cells By CRISPR/Cas9 for Generating Gene-Edited Mouse Models.

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Gene targeting of embryonic stem (ES) cells followed by chimera production has been conventionally used for developing gene-manipulated mice. Although direct knock-in (KI) using murine zygote via CRISPR/Cas9-mediated genome editing has been reported, ES cell targeting still has merits, e.g., high throughput work can be performed in vitro. In this study, we first compared the KI efficiency of mouse ES cells with CRISPR/Cas9 expression vector and ribonucleoprotein (RNP), and confirmed that KI efficiency was significantly increased by using RNP. Using CRISPR/Cas9 RNP and circular plasmid with homologous arms as a targeting vector, knock-in within ES cell clones could be obtained efficiently (~50%) without drug selection, thus potentially shortening the vector construction or cell culture period. Moreover, by incorporating a drug-resistant cassette into the targeting vectors, KI of multiple DNA fragments (over several kilo-base-pairs) at independent genomic loci can be simultaneously achieved at high efficiency (up to 100%) by a single

electroporation. This technique will help facilitate the production of genetically modified mouse models that are fundamental for exploring topics related to human and mammalian biology, including reproductive biology.

P-106 - Development of a Low-invasive mRNA Electroporation Method into Immature Mouse Oocytes and Visualization of Oocyte Chromosomes during Early Embryogenesis

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Deterioration of oocyte quality along with aging of mother is a major cause of infertility. Nowadays, abnormalities of oocyte chromosome segregation is known to occur mainly during meiosis I. However, there has been a technical limitation in the introduction of mRNA into premature oocytes. Here, we established a low-invasive electroporation method to introduce mRNA into premature mouse germinal vesicle (GV) oocytes without affecting embryonic developmental ability. The electroporation procedure with an optimized impedance value resulted in the efficient expression of enhanced green fluorescent protein (EGFP) from its mRNA at a oocyte survival rate of 95.0%. Moreover, the introduction of histone H2B-EGFP mRNA into the GV oocytes enabled us to visualize oocyte chromosomal dynamics and integrity during oocyte maturation and embryonic development. The establishment of this low-invasive EP method can offer assessing quality of pre-implantation embryos and assist improvement of embryonic quality.

P-107 - Engineered Gene Therapy Vectors Enable Accelerated In Vivo Reproductive Genetics

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Conventional genetic knock-out (KO) animal models have been essential for understanding gene function in fertility. Generating new transgenic models, however, is technically challenging and is time consuming as it requires breeding. Recent large genomics datasets have implicated several transcripts present in reproductive tissues, the experimental testing for their potential role, however is lacking. We have engineered adeno-associated viruses (AAVs) for gene delivery and editing in the mouse ovary in vivo. We demonstrate that with a single, systemic AAV injection can efficiently edit genes in the ovary and recapitulate known reproductive phenotypes. By targeting *Foxl2*, *Cyp19a1*, *Esr1*, *Cyp17a1*, *Amh*, *Amhr2*, *Kitl*, *Cdkn1b* we observe the expected changes in ovarian histology (i.e. somatic sex conversion, cyst formation, stroma over proliferation, accelerated or halted follicle activation), change in blood serum hormone levels, and in fertility assessed by long term mating trials. This methodology drastically accelerates functional genetics as the production of custom AAV-KO animals requires only a month and additionally also allows for perturbing combinations of genes at the same time. Currently, we are screening a list of candidate transcripts by AAV-KO and assessing their potential role in ovarian follicle development, fertility and ovarian aging.

P-108 - Investigating the Internalization of Hybrid Structures Comprising Cationic Liposomes and Extracellular Vesicles from Follicular Fluid in Bovine Granulosa Cells

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The development of hybrid vesicles combining liposomes and extracellular vesicles (EVs) has garnered significant interest in the field of drug delivery and cellular uptake due to their scalability, versatility and distinct advantages over conventional delivery systems, enabling precise control over payload delivery and dosage. Liposomes are lipid-based synthetic vesicles that can offer stability to EVs, which are natural extracellular vesicles derived from biological fluids, by safeguarding them against degradation in biological environments. The lipid bilayer present in liposomes may also present a formidable barrier for the controlled release of cargo carried by EVs, such as miRNAs, mRNAs and proteins, facilitating their sustained delivery over extended periods. Also, EVs possess inherent biocompatibility, minimizing the risk of immune responses and mitigating the cytotoxicity usually related to liposomes. This study investigated the production and internalization of hybrid vesicles consisting of cationic nanoliposomes and EVs isolated from bovine follicular fluid into bovine granulosa cells. The nanoliposomes were first produced using the thin-film lipid method followed by extrusion through 100-nm polycarbonate membranes (Avanti Polar Lipids, UK). The liposomal composition consisted of phospholipids, cholesterol, and rhodamine-PE, proportioned in an 80:19:1 molar% ratio to ensure optimal stability. Concurrently, EVs were isolated from follicular fluid via serial centrifugation at 4 °C (300 x g for 10 min to remove live cells, 2,000 x g for 10 min to remove residual cells and cell debris, and at 16,500 x g for 30 min to remove large microvesicles) followed by double ultracentrifugation at 119,700 x g for 70 min (Beckman 70Ti rotor, USA). Extracellular vesicles were then labeled with calcein 1 mM for subsequent visualization. The synthesis of hybrid vesicles involved the strategic combination of liposomes and extracellular vesicles in a 4:1 v/v ratio, followed by a series of processing steps including five cycles of freezing and thawing using liquid nitrogen and 15 cycles of extrusion. Samples were maintained at 4 °C until further analysis. Characterization studies employing nanoparticle tracking analysis (NS3000, Malvern Panalytical, UK) confirmed the production of hybrid vesicles with a size diameter of up to 150 nm, and a concentration reaching 109 vesicles/mL. Nanoflow cytometry (CytoFLEX, Beckman Coulter, USA) analysis served as a pivotal tool for validating the hybridization process, revealing a substantial population of hybrid vesicles exhibiting dual staining characteristics for both rhodamine and calcein. Toxicity tests using propidium iodide carried out via cytometry showed that hybrids were not harmful to granulosa cells in a greater extent when added at a concentration of 108 vesicles per well, showing viability of over 90%. Additionally, fluorescence microscopy (THUNDER Imaging System, Leica Microsystems, Germany) provided visual confirmation of the efficient internalization of hybrid vesicles into granulosa cells within a relatively short incubation period of 6h. All the analyses were carried out at least in triplicates. The significance of these findings lies in their potential applications in reproductive medicine and assisted reproductive technologies. By harnessing the synergistic properties of hybrid vesicles, our approach offers a promising avenue for targeted delivery of bioactive molecules to somatic cells, thereby facilitating the modulation of key molecular pathways involved in follicular development and oocyte maturation. Funding: FAPESP 21/06645-0, 22/01433-7, 22/01505-8, 22/01236-7

P-109 - Enhancing ART Efficiency: OoTrap for Oocyte Capture, in Vitro Maturation and Fertilization

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Assisted reproductive technologies (ART) like in vitro maturation (IVM), fertilization (IVF), and embryo production (IVP) are essential for reproductive practices and conservation. However, the manual handling required for each step, including removing gametes and embryos from the incubator and steps for exchanging media, can introduce stress and compromise their quality. To prevent it, we introduce OoTrap as a single device that allows oocyte capture, IVM and IVF, being a compact device with microwells in a dam channel, and operating in both static and perfusion-based functionality. For device fabrication, a two-piece mold was 3D printed using stereolithography, and devices were prepared using Polydimethylsiloxane. The two parts (perfusion channel and microwell dam) were assembled, and inlet reservoir and outlet tube were added. To evaluate the performance of OoTrap, we investigated fluid dynamics, cumulus-oocyte complexes (COCs) entrapment, IVM and IVF efficiency. For this, follicular fluid (FF) was added into the reservoir, COCs were allowed to settle into microwells and cell debris from FF were removed by washing the channel for IVM. Subsequently, fertilization was performed in device and presumptive zygotes were removed for denudation and culture. Data were analysed in R using a generalized linear mixed model, with "Treatment" as fixed and "Replicate" as random effect and a Tukey post-hoc test. A simulation study using COMSOL Multiphysics showed that hydrogen peroxide can be efficiently cleared, with 83% and 98% removal after 100s, under static and perfusion (20 μ l/h) conditions, respectively. These findings suggest that OoTrap could have effective diffusion and clearance capabilities, which promote optimal culture conditions. Evaluation of trapping efficiency showed 88% entrapment (n=6). Next, we analyzed IVM efficiency under static and perfusion (20 μ l/h) conditions, with conventional IVM as control. For this, FF from 5 ovaries were added per device and left for maturation in IVM media (10% FBS, 0.1UI/mL FSH, 25 μ g/ml gentamicin 0.2mM sodium pyruvate in TCM 199) for 22-24 hours. Nuclear staining for the assessment of maturation rate (Hoechst33342, n=5) showed a higher maturation rate in the dynamic OoTrap compared to conventional IVM (70% vs 62%, respectively, p=0.05), while no differences were observed for the static OoTrap (68%. p=0.83). Immunofluorescence for the analysis of spindle morphology and chromosomal alignment showed a tendency for decreased occurrence of abnormality in the perfusion group (7%) compared to both static (22%; p=0.09) and control (25%; p= 0.067). For IVF, following IVM, media was replaced by IVF medium (Stroebech) in the devices, frozen-thawed bull sperm was washed (Sperm Wash Medium, Stroebech), added in the reservoir of OoTrap and incubated for 18-22h under 20 μ l/h perfusion. Presumptive zygotes were then denuded and transferred to wells containing IVC medium (ECS50 as described in Dos Santos et al, 2021) and cultured until day 9. As control, conventional IVP was performed in wells. Although OoTrap group had a significantly lower cleavage rate compared to control (63 vs 73%, respectively, p=0.0046), there was no difference in blastocyst rate (15 vs 18%, respectively, p=0.524). Next, IVM, IVF and IVC in a single device was also tested, however due to the attachment of cumulus cells in the microwells, we did not see embryo development in these devices. Coating of the microwells to prevent such attachment is currently being investigated. OoTrap's streamlined workflow and

elimination of cumbersome handling steps hold promise for improving efficiency, accuracy, and success rates in ARTs.

P-110 - Toward the Generation of Haploid Human Oocytes by Somatic Cell Nuclear Transfer (SCNT)

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In vitro gametogenesis, or experimental production of gametes from somatic cells, could provide the opportunity for infertile families to have genetically related offspring. We present here an alternative method of producing haploid gametes by direct reprogramming using somatic cell nuclear transfer (SCNT). Haploidization of human somatic cells was induced by premature cell division resulting after transplantation of fibroblasts at the G0/G1 stage of the cell cycle (2n2c) into the metaphase cytoplasm of enucleated mature oocytes. Metaphase oocyte cytoplasm induced rapid nuclear envelope breakdown, premature chromosome condensation and formation of bipolar spindles from the somatic cell chromatin in 81% of SCNT oocytes. Artificial activation of such SCNT oocytes resulted in cell division and chromosome segregation into pseudo polar bodies and zygotic pronuclei at 70% efficiency. Next-generation sequencing of individual chromosomes in polar bodies and zygotes revealed that the numbers of chromosomes was reduced close to half (N=19) compared to the normal diploid (N=46) number in starting fibroblasts. Comprehensive sequencing of homologous pairs revealed that on average, half (N=11) of total 23 homologous pairs were properly segregated into a polar body and zygote while remaining chromosome pairs remained together resulting in aneuploidy. No evidence of recombination between somatic cell homologs was detected. These results indicate that experimentally induced premature cell division after SCNT allows ploidy reduction albeit via random chromosome segregation suggesting that additional optimizations are required to aid in homolog pairing and segregation to achieve proper haploidy.

P-111 - Transcrocetin Inhibits Endometriosis Growth by Regulating Autophagy and Perturbing Redox Balance

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Endometriosis is a benign gynecological disease characterized by the abnormal growth of endometrial-like tissues and fragments outside the uterus. Due to lack of alternative non-hormonal therapeutic targets for endometriosis, our focus has been on investigating autophagy regulation and role of mitochondria in progression of endometriosis. Natural compounds have emerged as

promising candidates in recent years for targeting mitochondrial dysfunction and autophagy. Among them, transcrocetin is a carotenoid and retinoic acid known for its potent antioxidant properties and antiproliferative effects in various diseases. In the present study, we examined therapeutic mechanisms of transcrocetin in endometriosis using the End1/E6E7 and VK2/E6E7 cell lines. Our findings reveal that transcrocetin effectively suppresses the viability and proliferation of these cell lines while sparing normal uterine stromal cells. We observed an increase in p21 Waf1/Cip1, a cell cycle regulator and target of p53, upon transcrocetin treatment, leading to G1 phase of cell cycle arrest through inhibition of cyclin-dependent kinase activity and subsequent cell death. Additionally, we identified endoplasmic reticulum stress by activating GRP78, IRE1A, and phosphorylation of eIF2 α and dysregulation of calcium ions in the cytosol and mitochondrial matrix, resulting in disruption of mitochondrial membrane potential by transcrocetin. Also, it was found to suppress mitochondrial bioenergetics and downregulate the expression of oxidative phosphorylation-related genes. Moreover, transcrocetin-induced oxidative stress was implicated in regulating the proliferation of End1/E6E7 and VK2/E6E7 cells. Furthermore, we observed impaired autophagic flux in both endometriotic cells using pre-treatment of chloroquine. These findings collectively suggest that transcrocetin holds promise as a potent therapeutic alternative for endometriosis.

P-112 - Metabolic Characterisation of the Roe Deer Uterine Fluid During Diapause and Elongation

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Embryonic diapause is a peculiar developmental phenomenon in which the usual consecutive progression from fertilization to implantation is halted. In the case of the European roe deer (*Capreolus capreolus*), the blastocysts enter a four-month period of reduced embryonic growth and minimal metabolism, during which the blastocyst floats freely in the uterus surrounded by the nutrient-rich histotroph. Exit from diapause is marked by the elongation of the embryo, setting the stage for implantation. We theorized that the uterine fluid (UF), which undergoes changes to support early embryonic development, creates a nurturing environment that either actively slows embryo development during diapause or accompanies its reduced developmental velocity to facilitate elongation upon reactivation. Therefore, we performed targeted and untargeted metabolomics via mass spectrometry to characterise the metabolome of roe deer UF during diapause and reactivation. By analysing non-polar lipids, polar metabolites, acylcarnitines, and polyamines, we aimed to provide a comprehensive view of the metabolic landscape within the UF before implantation to understand intricate molecular interactions and metabolic changes. Our results indicate that fatty acid degradation increases during elongation, possibly for membrane synthesis rather than energy production. Elevated glycolysis/gluconeogenesis may compensate for energy needs during elongation. We also observed increases in sphingomyelin, prostaglandin precursors, and the amino acids asparagine, glutamine, and methionine during elongation. Metabolomic Pathway Analysis (MetPA) revealed the importance of sphingolipid and glycerophospholipid metabolism pathways during elongation. We particularly found that the

polyamines spermidine and spermine, but not putrescine, significantly increased 7- and 1.3-fold respectively in the UF during elongation, indicating their importance for reactivation and/or proliferation. Indeed, it was previously shown that putrescine is implicated in the diapause of mink. Whether polyamines are cause or result of embryo reactivation in roe deer requires further investigation. Overall, our research uncovered dynamic changes in the roe deer uterine fluid composition and proposed their role in supporting the energy- and resource-intensive process of embryo elongation. It provided insights into the role of the intrauterine environment on roe deer embryo development. This study will not only improve our knowledge of roe deer reproduction, but also shed light into embryonic diapause regulation with broader implications for understanding fertility issues and furthering conservation efforts.

P-114 - Harnessing Amniotic-Derived Cells for Advanced Regenerative Medicine and Tracking Technologies.

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Regenerative medicine using cell therapy is emerging as a potential strategy to address several issues in the field of reproduction. Transplantation of different types of adult and fetal mesenchymal stromal cells (MSCs) have been tested with promising results for treating both female (e.g. dysfunctional endometrium, premature ovarian failure, ovarian aging) and male (e.g. azoospermia, aspermia, oligospermia) conditions. Despite recent advances in this field of clinical medicine, the existing challenges are still enormous. Also, immunogenic, and tumorigenic constraints limit the clinical translation of the current research protocols. Amniotic-derived cells, due to their plasticity, immunomodulatory effects, and homing capabilities, present an alternative cell source with great regenerative potential for reproductive medicine. This study has the aim to present a technological advancement of the current protocols of cell tracking to combine with tissue transplantation to offer a straightforward and non-invasive approach to track and drive regenerative processes. The research develops an effective protocol to label amniotic-derived cells tagged with gold nanorods (AuNRs) to be applied for real-time visualization of the cells' engraftment, survival, and integration into the target tissue, thus exploring the pro-regenerative properties of this placental stem cell source widespread used to date for biomedical regenerative applications even if not exploited with tracking technologies yet. The study utilized as cell model, ovine amniotic epithelial cells (AECs) in their native epithelial state or induced mesenchymal phenotype (mAEC) through epithelial-mesenchymal transition (EMT) in vitro. The first aim was to assess the efficacy of various uptake methods validated for MSCs on mAEC. The standardized protocol was then implemented by introducing the innovative biological strategy of mitocapture to enhance endocytosis before translating the optimized methods to ovine and human AECs. The findings reveal that the most effective, cost-reducing, and straightforward protocol for AuNRs internalization in living mAEC involved the combination of single-step uptake conditions (cells in

suspension) with centrifugation-mediated internalization (G-force), and mitocaption (mitochondria isolated from mAEC). This novel protocol resulted in high AuNRs internalization, providing labeled vital mAECs within minutes for a prompt use in both preclinical and clinical trials. The optimized protocol could potentially yield up to 10 million labeled amniotic-derived cells from a single amniotic membrane for biomedical purposes. Similar or even greater efficiency was observed when applying the protocol to both ovine and human AECs, demonstrating its transferability across different cell phenotypes and species. This validates its significant potential for advancing biomedical applications in cell-based therapy and diagnostic imaging providing great opportunity to tackle infertility challenges. Funding This research was funded by FONDAZIONE TERCAS, Project Title: “New frontiers in stem cell homing” 2021.

P-115 - Study on the Clinical Application of Saline Contrast Sonohysterography(SHG) in Cesarean Section Scar Evaluation

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Study on the clinical application of saline contrast sonohysterography (SHG) in cesarean section scar evaluation Li Zhang*, Jing Ma, Kailin Yan, Chenghua Wang, Shanyu Wang, Haihua Qu
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Objective: To evaluate the diagnostic value of saline contrast sonohysterography (SHG) in cesarean section (CS) scar evaluation. Methods: 98 Patients Who had a history of CS and presented with secondary infertility or postmenstrual spotting were examined by both conventional transvaginal ultrasound (TVS) and SHG. Patients were divided into two groups according to whether the diverticulum presented or not by TVS. The detection rate of diverticulum was compared between TVS and SHG, and the size of diverticulum and the thickness of residual muscle layer were compared before and after SHG. Results: 62 cases with diverticulum were detected by SHG, while 43 cases were found through TVS. The detection rate of diverticulum by SHG was significantly higher than that of TVS ($P < 0.05$). Hysteroscopy was performed in 85 of the 98 patients, and diverticula was found in 74 of them. The diagnostic rate of TVS was 49.4% and 67.1% for SHG with $P < 0.05$. The size of diverticulum measured by TVS was as follows: sagittal, longitudinal and transverse diameters were 0.47 ± 0.03 cm, 0.57 ± 0.05 cm and 0.74 ± 0.06 cm, respectively. The residual muscle thickness is 0.48 ± 0.21 cm. The measurements of sagittal, longitudinal and transverse diameters by SHG were 0.67 ± 0.06 cm, 0.59 ± 0.05 cm and 0.92 ± 0.08 cm, respectively. The residual muscle thickness is 0.53 ± 0.26 cm. 8 cases with endometrial and cervical canal polyps were detected by TVS; while 16 cases with endometrial and cervical canal polyps were detected by SHG, and another 7 cases with intrauterine adhesions and 12 cases with incisional cysts were detected. Conclusion: SHG is superior to TVS in CS scar evaluation in finding hiding diverticulum, evaluating the structure of the diverticulum and other intrauterine lesions.

P-116 - Complement Alternative Pathway Contributes to the Intrauterine Spermicidal Activity in Mice.

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Mammalian fertilization occurs in the female reproductive tract. Although hundreds of millions of sperm are ejected into the female reproductive tract, only a few sperm reach the ampulla and participate in fertilization. During this process, we found that the uterine sperm was killed by uterine fluid without the protectant of seminal protein. The spermicidal effect was observed only in the estrus uterus, but not in the oviduct. We here focused on complement factor(s) as the spermicidal effect in the uterus. The expression of C3 that a central component of complement was increased in the uterus during estrus or in an E2-dependent manner, and treatment of uterine fluid at 56°C for 30 min attenuated the spermicidal effect. When the uterine fluid from C3 KO mice was added to sperm in vitro, sperm viability was not reduced compared to that from WT mice. On the contrary, the addition of only purified C3 did not show the spermicidal effect, suggesting that multiple proteins are involved in this effect. Moreover, we generated complement factor B (CFB)-deficient mice, which is essential for the complement alternative pathway activation. When the uterine fluid from Cfb KO mice was added to sperm in vitro, no spermicidal effect was observed as well as that from C3 KO mice. These results indicate that the female uterine fluid includes the factor(s) of complement alternative pathway and decreases the uterine sperm survival. As we previously reported, sperm without the protectant of seminal protein were dead in the uterus by artificial insemination, which would be due to the activation of complement alternative pathway. This is the first report that involvement of the complement pathway in sperm-uterus interaction. In the future, we would like to analyze in detail the relationship between the complement system and the sperm selection mechanism and immune tolerance in the uterus.

P-117 - Deriving Hormonally Responsive Bovine Endometrial Organoids

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In cattle, communication between endometrium and conceptus during the peri-implantation period is crucial for successful pregnancy. Understanding these interactions is vital as most early pregnancy loss occurs during this time. A major challenge in understanding uterine function and early pregnancy is lack of appropriate in-vitro models. Two-dimensional models are available, but do not recapitulate the endometrium's complex multicellular structure. Here, we describe a hormonally responsive organoid model of the bovine endometrium, developed as a tool for studying endometrial function and early pregnancy. Bovine glandular epithelial cells were isolated from reproductive tracts and cultured in Cultrex 2 extracellular matrix hydrogel at 37°C, 5% CO₂ (n=3). RNA was extracted and qPCR confirmed the presence of gland markers: Leukemia inhibitory factor, mucin-1, insulin-like growth factor binding protein-1, kruppel-like factor-5 and forkhead box

protein-A2. Organoids were imaged at time points to monitor growth and passaged 3 times in 1:2 or 1:3 ratios after growing for a minimum of 10 days per passage. Morphologically, organoids were spherical and fast-growing at passages 0 and 1, but this declines following passage 2. Bovine endometrial organoids (n=3, passage 0) were treated with 10mg/ml progesterone (P4) for 24 hours and analysed by RNASeq to assess hormone responsiveness. Differential expression analysis by DeSeq2 negative binomial distribution model identified 373 transcripts significantly upregulated ($\text{padj} < 0.05$ or $\log_2\text{fold change} > 0.05$) in response to P4 treatment, with downstream analysis showing significant overrepresentation ($\text{FDR} < 0.05$) of genes associated with positive regulation of protein localisation to plasma membrane and cell periphery. Of the 240 genes significantly downregulated by P4 these were significantly overrepresented ($\text{FDR} < 0.05$) in biological processes of cilium and cytoskeleton organisation. This model provides a tool to investigate bovine endometrial function and peri-implantation communication, subsequently allowing species comparison to understand diversity in reproductive strategies.

P-118 - A New Model of Menstruation and Uterine Regeneration

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Menstruation is a complex physiological process found only in a few mammalian species, including humans, where the inner lining of the uterus (endometrium) undergoes cyclic shedding and subsequent regeneration. Despite its clinical significance and relevance to various disorders, like endometriosis, our understanding of menstruation and endometrial regeneration remains limited since there is no established menstruating model organism. In menstruating species, the endometrium undergoes a major transformation, characterized by massive cell proliferation and tissue growth, in response to rising progesterone levels after ovulation. This newly formed tissue, the decidua, is shed and discarded upon the fall of progesterone secretion towards the end of the menstrual cycle, a process accompanied by bleeding. Non-menstruating mammals, including *Mus*, also form decidua, but only during pregnancy, as their natural ovarian cycle does not lead to decidualization. These observations suggest that decidua formation without pregnancy is a prerequisite for menstruation. To test this hypothesis, we developed a new transgenic mouse model to induce decidualization on demand in the non-pregnant mouse by selectively activating the molecular steps required for decidualization. Characterization of this model revealed that these animals have a cycle mimicking human menstruation when progesterone levels decline, including endometrial bleeding and subsequent regeneration. We are currently characterizing the applications of this model for menstrual disorders, including endometriosis. Our novel mouse model of menstruation offers a unique platform for studying the intricate processes involved in endometrial shedding and regeneration, with important implications for advancing reproductive health and improving the quality of life for millions of women worldwide.

P-119 - Comparative Molecular Dynamics of Aromatase in Ancient and Modern Felines

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Aromatase (P450arom, CYP19A1) is a cytochrome P450 enzyme that plays a crucial role in the rate-limiting step of oestrogen biosynthesis from androgens. It undertakes a three-step oxidative process.¹ Despite the importance of this enzyme, only a few comparative studies have investigated

mammalian aromatases for their structural and functional relationship.² Hence, this study conducted a computational comparison of aromatases obtained from some cat family species: *Homotherium*, *Felis catus*, *Puma concolor*, *Acinonyx jubatus*, *Panthera tigris* and *Panthera pardus*. The human aromatase X-ray structure was used as a template to create 3D structural models of feline aromatases and classical Molecular Dynamics simulations undertaken in aqueous or membrane environments. Within the feline family there was high amino acid sequence identity (99%) and also with human aromatase (86%). Comparisons using classical Molecular Dynamics (100 ns) were used to assess the overall stability of aromatases and showed that dimers of feline aromatase were less likely to form based on RMSD/RMSF, surface potentials and hydrogen bonding patterns. Inclusion of a physiological membrane environment to accommodate the transmembrane region of both monomeric and dimeric aromatases provided more stability for these enzymes. Accelerated molecular dynamics was then used to obtain extended (1 ms) simulations of the human aromatase providing the lowest free energy conformations with the dimeric form in a membrane. The access/egress channels were then probed (with steered molecular dynamics in conjunction with umbrella sampling) using both substrate (androstenedione) and product (oestrone) to define the highest probability pathway. Dimerisation provided a new path for androstenedione to move between the membrane and enzyme via the dimer interface which became more significant for oestrone. These simulations provide comparative analyses of aromatase structure and function as well as evolutionary significance. Also, complementary data on environmental stressors that influence aromatase activity, needed to understand species reproduction can be gleaned.¹ Praporski, S., Ng, S., Nguyen, A., Corbin, C.J., Mechler, A., Zheng, J., Conley, A.J., Martin, L.L., Organization of Enzymes Involved in Sex Steroid Synthesis: Protein-protein interactions in lipid layers, *J. Biol. Chem.* (2009), 284(48) 33224-33232.² Martin LL, Holien JK, Mizrahi D, Corbin CJ, Conley AJ, Parker MW, Rodgers RJ, Evolutionary comparisons predict that dimerization of cytochrome P450 aromatase increases enzymatic activity and efficiency, *J Steroid Biochem. Mol. Biol.* (2015) 154, 294-301.

P-120 - Antagonism Between Retinoic Acid and Estrogen Signaling Maintains Uterine Cytodifferentiation

Yan Yin, Meade Haller, Lauren Goldinger, Shivani Bharadwaj, Emily So, Vivian Robles-Pinos, David Chen and Liang Ma

Classical tissue recombination experiments demonstrate that cell-fate determination along the anterior-posterior axis of the Mullerian duct epithelium occurs between postnatal days 5-7 in mice. However, little is known about how these cell types are maintained in adults and whether cellular plasticity can mediate transdifferentiation between uterine simple columnar and cervicovaginal stratified epithelia. In this study, we provide genetic evidence that a balance between antagonistic estrogen and retinoic acid signaling activity is required to maintain simple columnar cell fate in postnatal uterine epithelium. Transformation of simple columnar uterine epithelium into stratified cervicovaginal epithelium was observed in two mouse genetic models, one expressing a dominant-negative RAR α and the other missing all three retinoic acid receptors in the postnatal uterus. Single cell RNA sequencing (scRNA-seq) analysis identified the transformed epithelial cell populations and revealed extensive immune cell infiltration as a result of loss of retinoic acid signaling, which was confirmed by immunostaining. Surprisingly, differential gene expression analysis revealed a group of strongly upregulated estrogen targets in the transformed cell clusters, suggesting that

these two pathways may functionally oppose each other during uterine epithelial cell-fate determination. Consistent with this model, exposure to diethylstilbestrol, a strong synthetic estrogen downregulated a group of retinoic acid target genes, led to epithelial stratification and immune cell infiltration later in life. Fulvestrant, an estrogen antagonist, -treated RAR triple conditional knockout pups reestablished the balance between the two signaling pathways, resulting in the astonishing complete rescue of the metaplastic stratified epithelia back to the normal simple columnar morphology. Together, these results implicate an essential role for retinoic acid signaling in maintaining uterine cytodifferentiation by antagonizing estrogen signaling in the postnatal uterus.

P-122 - A Comparison of RNA- Versus DNA-Based 16S rRNA Amplicon Sequencing Analysis of the Equine Uterine Microbiome

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An increasing number of studies in humans and large animals indicate an important role of the uterine microbiome in endometrial receptivity. However, the study of this low biomass microbiome is challenging and biased by bacterial DNA contaminations introduced from different sources and high background of host DNA in most of the samples used for 16S rRNA amplicon sequencing. To overcome these challenges, this study investigated if i) RNA as input for 16S amplicon generation is more sensitive compared to DNA (higher copy number of ribosomes per bacterial cell compared to gene copies/genome; ~15,000 vs. 1-15) and ii) RNA as input reveals mainly information about bacteria alive while DNA could also be derived from dead bacteria. Based on a previously established 16S rRNA V3-V4 amplicon PCR, this study compared sequencing results starting from RNA or DNA isolated from uterine cytobrush samples as template for amplicon generation. Uterine cytobrush samples were collected in estrus (1-3 days before ovulation) from mares (n=14) of the University of Illinois Urbana-Champaign or client-owned mares presented at the Veterinary Hospital (IACUC approvals 21237, 21238). From the same sample, RNA and DNA were extracted. The 16S rRNA V3-V4 region was amplified using universal prokaryotic primers Pro341F and Pro805R, binding the conserved regions flanking the V3-V4 regions. For RNA-based PCR, RNA samples were first reverse transcribed with Pro805R, and first-strand cDNA was used as input for 16S PCR. PCR products with a size of approx. 465 bp were isolated and purified from agarose gels. Illumina barcodes were added and paired-end 300 bp reads generated on an NextSeq 2000 instrument (Functional Genomics Center Zurich). Processing of fastq files and analysis of microbial composition was done with QIIME2. Amplicon sequence variants (ASV) were clustered at 97% sequence identity, using SILVA database (v. 138). Overall, sufficient PCR product was obtained with 32 cycles for RNA-based compared to 35 cycles DNA-based amplicon PCR. Gel electrophoresis showed higher band intensity of RNA-derived amplicons indicating an at least 10-fold higher sensitivity of RNA-based PCR. Sequencing revealed 1.58 to 3.02 million paired-end reads per sample. After processing and merging the paired reads, about 70% of the reads remained for subsequent analysis. A preliminary data analysis revealed 26,201 ASVs. The predominant phyla

obtained for both RNA and DNA samples were Actinobacteriota, Proteobacteria, Firmicutes, and Bacteroidota confirming results of previous studies in the mare. The alpha diversity was significantly higher in RNA compared to DNA samples (Chao1: $P = 0.0001$, Simpson: $P = 0.003$). In addition, the analysis of beta diversity revealed significant differences of the RNA group compared to the DNA group (Bray-Curtis distances; $P = 0.001$). The analysis of RNA samples detected more operational taxonomic units (OTUs) ($>2,500$) than DNA samples ($>1,100$). A considerable number of OTUs was exclusively/mainly detected in RNA or DNA samples. Most OTUs only detected in RNA samples had counts in more than half of the samples, while OTUs only detected in DNA samples had counts only in a few of the 14 samples. The results of this study indicate that 16S rRNA amplicon sequencing is more sensitive and reveals a higher microbiome diversity when RNA samples are used as starting material. The identification of OTUs only found in DNA samples suggests origin from dead bacteria. This study was supported by Swiss National Science Foundation Project #200534.

P-123 - The Development of a Diet-Induced Obese Mouse Model and its Effect on Fertility

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Obesity is a common chronic disease worldwide affecting both men and women. Obesity is associated with many other diseases such as subfertility, insulin resistance, and diabetes mellitus. Studies have shown that women with higher BMI undergoing IVF had lower implantation and live birth rates. Similarly, high-BMI surrogates receiving healthy embryos had low implantation and pregnancy rates suggesting impairment in the uterus. However, findings from diet-induced obese (DIO) mouse models are inconsistent. Many do not report a reduction in fertility as observed in obese women. Thus, this study aimed to develop a diet-induced obese mouse model that mimics changes seen in obese women. Eight weeks old mice ($n=36$) were randomly divided into three groups and fed 10% (low-fat diet, LFD), 45% (high-fat diet, HFD), or 60% (very high-fat diet, VHFD) kcal from fat for 18 weeks. Weight was monitored weekly. During the 15th week of feeding, mice were fasted overnight, and an intraperitoneal glucose tolerance test (IPGTT) was performed. Glucose (1g/kg of body weight) was injected, and serum glucose concentrations were measured at 0, 30, 60, 90, and 120 minutes. At 18 weeks mice were fasted for 8 hours and sacrificed in proestrus. The serum was separated and stored at -80°C for serum metabolomics and for measuring glucose, insulin, and leptin concentration. The liver, uterus, ovaries, and adipose tissue (gonadal, retroperitoneal, abdominal) were collected, weighed, and fixed with paraformaldehyde or snap-frozen and stored at -80°C . A significant increase in body weight was observed from weeks 4 and 7 of feeding in the VHFD and HFD groups respectively. Additionally, mice from these groups had a high adiposity index and serum leptin concentration compared to the LFD group, indicating the development of metabolic dysfunction. The liver was enlarged in VHFD mice, whereas ovary weight was reduced in both HFD and VHFD groups. Mice in the VHFD group were intolerant to glucose at 30, 60, and 90 minutes indicating a reduction in clearance of glucose from blood, however, there was no significant change in fasting serum glucose and insulin concentrations between the groups at sacrifice. In another set, mice were housed with male mice for 60 days. The day vaginal plug was found in the mouse was considered day 0.5 of pregnancy and mice were

sacrificed at day 18.5 of pregnancy. Mice were considered infertile if a vaginal plug was not observed after 60 days of housing with a male. Both HFD and VHFD groups showed 50% and 60% reduction in fertility respectively. In mice that did get pregnant, there was no change in litter size, but there was a significant reduction in fetal weight in the 45% fat-fed mice group. There was no change in the placental weight in all the groups. Also, the placental efficiency (fetal weight/placental weight) was not different between the groups. In summary, we have developed a diet-induced obese mouse model that mimics features seen in obese women like reduction in metabolic dysfunction, reduced fertility, and intrauterine growth restriction. Future investigations will use this model to examine changes in serum metabolites, glucose, and glycogen metabolism in the uterus, and immunohistochemistry on glycogen metabolism proteins in the uterine section from the three groups. Keywords: Diet-Induced Obesity, Fertility, Adiposity, IPGTT, Placental Efficiency Financial Support: This work is supported by the National Institute of Health [1R01HD111706-01]

P-124 - Embedded IUD/IUCD in multiparous female – Case Report and Retrieval Options

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Embedded IUD/IUCD in multiparous female – Case Report and Retrieval Options AimAn intrauterine device (IUD), also called intrauterine contraceptive device (IUCD), is one of the most effective reversible contraceptive methods available for female contraception worldwide. In competent hands, it is easy to insert, however, an IUD may perforate the uterus or migrate and/or embed in the uterine muscle or nearby structures. While patients may remain asymptomatic, these complications can decrease contraceptive effectiveness, cause damage to adjoining organs or cause abdominal/pelvic pain and necessitate surgical intervention. This is a case of a 33-year-old G3P3 with Class III obesity and no prior history of cesarean section, uterine anomalies, or fibroids who presented with painful, heavy menstruation. She had a copper IUD placed while she was living in Mexico nine year ago. On pelvic examination, the IUD thread was not visible at the internal cervical os. Her pregnancy test was negative and she had normal hemoglobin. Attempts at IUD localization or retrieval with cytobrush, uterine sound or IUD hook were unsuccessful. She was appropriately counseled about her options, alternatives, risks, benefits and she chose to have the IUD removed. We aim to provide result-based decision making for the escalation of care for complex IUD removal. MethodsThis is a case report and exempt from institutional review board (IRB) application. ResultsA pelvic ultrasound demonstrated an anteverted uterus measuring 9 cm in length with normal echotexture, and no parenchymal abnormalities. A linear echogenic structure was observed within the endometrial canal and encroaching on the uterine wall, and a T-portion was located at the distal aspect. No fluid was detected in the posterior Cul-de-sac. Several small cervical nabothian cysts were noted. She had failed other attempts at retrieval of the IUD in the office, subsequently counseled about her options and she chose to have hysteroscopy and removal of IUD with dilation and curettage (D&C). Hysteroscopy was performed under anesthesia, and successful removal of the IUD using endometrial polyp forceps. A postprocedure D&C yielded endometrial tissue with no polyps and no complication. She tolerated the procedure well and was

discharged home in stable condition. She planned to use condoms for contraception. Conclusion: Managing complications associated with embedded IUDs requires a systematic approach due to the potential for migration to areas such as the abdomen, omentum, or tubal migration, alongside risks such as fragmentation and expulsion. Prompt recognition and intervention are vital, especially in cases of heavy menstrual bleeding and symptomatology. A multifactorial strategy, integrating imaging modalities for localization including ultrasound and in difficult cases plain abdominal X-ray or CT-scan may be necessary. However, Copper IUDs are easier to identify than non-Copper IUDs. Initial attempts at retrieving the IUD with cytobrush or IUD hook should be first line treatment. The use of uterine sound is helpful to identify an intrauterine IUD but plays a role if the IUD is extra-uterine or deeply embedded in the uterus. Hysteroscopy facilitates precise visualization and retrieval of the IUD and an opportunity to address any associated endometrial pathology. Tailored care based on patient preferences and family planning considerations is essential. Educating patients about potential complications and management options is imperative for informed decision-making. Prioritizing patient empowerment can mitigate risks and enhance reproductive health outcomes.

P-125 - Elevated Testosterone Attenuates Vaginal Wall Thickness in Rhesus Macaques

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Rhesus macaques exhibit 28-day menstrual cycles similar to those of women and are excellent animal models for assessing the effect of exogenous steroids on the reproductive tract. This study contrasted the effect of high-dose testosterone (T) therapy, similar to that used for gender-affirming care, with low-dose T, similar to the hyperandrogenemia seen in women at risk for polycystic ovary syndrome (PCOS). Our focus was on vaginal wall thickness, as this is implicated in several disorders including risk for vaginal prolapse. In Exp.1, ovariectomized macaques were treated with Silastic capsules releasing estradiol (E) and progesterone (P) to create artificial 28-day menstrual cycles. Similar capsules were employed to administer testosterone (T) to a subset of the monkeys. Vaginal wall samples were collected during the artificial proliferative phases (80-100pg E/ml; n=4) and secretory phases (E plus 5-6 ng/ml P; n=3) and after 28 days of E plus T (serum T =12.2±1.3 ng/ml n=4). In Exp. 2, 9 cycling monkeys received Silastic capsules that produced serum levels of 1.18 ± 0.12 ng T/ml, similar to those reported for women at risk for PCOS and does not disrupt menstrual cycles. Vaginal samples were collected in the proliferative phase (n=4) and in the secretory phase (n=5). In Exp. 3, cycling monkeys were treated with a high dose T (5.3±2.1 ng/ml) for 30 days, which suppresses cyclicity. All vaginal samples were embedded in tissue tek OCT, and frozen in liquid propane. Cryosections were analyzed histologically for epithelial thickness, extent of cornification, and immunohistochemistry (IHC) was performed to analyze ER, PR, AR and Ki-67 (a marker of cell proliferation) expression. ER, PR, and AR staining was quantified by h-score. Ki-67 was quantified by counting positive cells. In the epithelium and subepithelial stroma high expression of ER, PR, and AR was observed, with minimal cyclic changes in tissue/cellular localization. Vaginal wall thickness was maximal (>2 mm) and markedly cornified following E treatment alone/during the follicular phase. Vaginal wall thickness and Ki-67 positive epithelial

cells were reduced in the secretory phase ($P < 0.05$). Mild hyperandrogenemia did not affect vaginal wall thickness, Ki-67 positive cells, or the localization of ER, PR and AR, potentially due to continued menstrual cyclicity during this treatment. In contrast, high dose T ($>5\text{ng/ml}$), which disrupted menstrual cyclicity, resulted in loss of vaginal cornification and a thinning of the epithelium ($P < 0.05$). High-dose T also reduced epithelial cell proliferation (Ki-67; $P < 0.05$) and reduced the abundance of ER and PR-positive epithelial cells ($P < 0.01$). In conclusion, mild hyperandrogenemia, as observed in women with PCOS does not affect vaginal integrity, or steroid receptor expression, whereas high-dose T therapy may result in clinical complications due to vaginal thinning. Supported by NIH OD011092.

P-126 - Dynamics of PDGFR α + Mesenchymal Cells in Oviductal Regeneration

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The oviduct comprises epithelial cell types and a stromal microenvironment that together facilitate oviduct function, including gamete transport, fertilization and early embryonic development. Although it is known that the oviductal epithelium experiences significant damage during ovulatory cycles, key progenitor cells involved in post-ovulatory oviductal regeneration are largely unknown. Defining progenitors and underlying mechanisms involved in oviductal regeneration and homeostasis are pivotal for understanding and addressing infertility and malignancy. In particular, progenitors in the stroma that may participate in oviductal regeneration remain tenuous and underexplored. In the uterus and mammary gland, mesenchymal cells expressing Platelet-Derived Growth Factor Receptor alpha (PDGFR α) are reported to contribute to epithelial lineages during regeneration. The objective of our study is to characterize PDGFR α + cells in the oviduct and determine their fate and function in oviductal regeneration. Through immunofluorescence and flow cytometry studies on isolated oviducts from female Pdgfra reporter mice, and analysis of published single-cell RNA sequencing datasets, we have observed that PDGFR α expression is restricted to mesenchymal cells in the oviductal stromal microenvironment, and absent from epithelial cells including secretory and ciliated cells. PDGFR α + oviduct cells also express sex hormone receptors as detected by immunofluorescence and analysis of single-cell RNA sequencing data, suggesting that these stromal cells are likely hormone-responsive. Further, we found that Pdgfra-expressing oviductal stromal cells fluctuate during the hormone-driven estrous cycle, exhibiting an increase in the post-ovulatory phase. Notably, the Pdgfra-expressing stromal fraction purified by fluorescence-activated cell sorting had significantly more clonogenic capacity compared to Pdgfra-negative stromal cells, indicating that the Pdgfra-expressing population enriches for stromal progenitors in the oviduct. These findings illuminate a hormone-responsive stromal progenitor in the murine oviduct and our ongoing work is addressing the functional contribution of these progenitors to oviductal regeneration. We envision that this work will lay a foundation for the development of novel cell-targeted therapeutics for oviductal regenerative medicine and cancer.

P-127 - Proteomics of the Apical Oviduct Epithelium after Laser Microdissection: Identification of New Candidate Receptors for Sperm Binding In Cattle

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The caudal isthmus part of the oviduct serves as a sperm reservoir before ovulation in many mammalian species. Sperm cells bind to the epithelium and its apical structures, especially to cilia, which prolongs their survival and ensures the availability of sperm able to fertilize until the time of ovulation. However, very few sperm receptors on oviduct cilia have been identified so far. Therefore, our aim was to compare the apical epithelial proteome of bovine oviducts between anatomical regions (ampulla vs. isthmus) before and after ovulation to identify new receptor candidates for sperm binding. Oviducts from cyclic adult cows were collected at a local slaughterhouse. Only pre- and post-ovulatory oviducts ipsilateral to the ovulation site were used (4 cows/stage = 16 samples). After 6 h fixation and paraffin embedding, 6 µm serial sections of the isthmus and ampulla were used for laser capture microdissection of the apical epithelium. Proteins were digested and analyzed by nano liquid chromatography coupled with tandem mass spectrometry. Proteins were identified using the Uniprot Bos taurus database and considering minimum two unique peptides. Proteins were then quantified by precursor intensities-based label-free. Ciliary origin of proteins was assessed with the Human Protein Atlas and the list of proteins previously identified in human airway cilia. Prediction of subcellular localizations and molecular interactions were performed using TMHMM, MBPPred and ProteinPrompt tools. Protein abundance were compared between stages and anatomical regions by ANOVA and t-tests considering a p-value ≤ 0.05. Functional gene ontology enrichment analysis was performed using Metascape online tool. In total, 505 proteins were identified in the microdissected samples, of which 106 were previously identified in cilia. Among those 106 ciliary proteins, 5 were predicted to be transmembrane and 9 predicted to interact with membrane phospholipids, including the annexins (ANXA1, A2, A4, A5) and the voltage-dependent anion-selective channel proteins 1 and 2 (VDAC1, VDAC2). After ANOVA and hierarchical clustering, 66 proteins were differentially abundant and clustered according to the region: 32 proteins were overabundant in the isthmus (group 1) and 34 were overabundant in the ampulla (group 2). Proteins from group 1 were mainly involved in antioxidant processes while the ones from group 2 were mainly involved in protein binding. Among the proteins overabundant in the pre-ovulatory isthmus (the time and place of the sperm reservoir) compared to the pre-ovulatory ampulla, 6 were previously identified in cilia including the peroxiredoxin 6 (PRDX6) and dynein light chain roadblock-type 2 (DYNLRB2). Among the proteins overabundant in the pre-ovulatory isthmus compared to the post-ovulatory isthmus, 5 were previously identified in cilia including the tubulin beta-2B chain (TUBB2B) and peroxiredoxin 2

(PRDX2). Furthermore, 108 identified proteins were predicted to interact with the sperm-coating Bovine Seminal Plasma proteins (BSPs), including ANXA2, ANXA5, DYNLRB2, TUBB2B, VDAC1 and VDAC2 and 32 predicted to interact with the sperm-coating β -defensin 126 protein including ANXA2, PRDX6, VDAC1 and VDAC2. This is the first proteome of the apical oviduct epithelium in mammals. This study highlights changes in apical proteins along the oviduct during the periconception period and reports new candidates potentially involved in the oviduct sperm reservoir.

P-128 - Dissecting the Spatiotemporal Development of the Human Reproductive Ducts through the lens of Single-Cell and Spatial Genomics

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The Müllerian and Wolffian ducts - the precursors of the female and male reproductive tracts, respectively - develop in both male and female embryos. Over the course of gestation, the sexually matched ducts continue to differentiate, while the sexually unmatched ducts regress. The development, differentiation, and regression of the Müllerian and Wolffian ducts are regulated by a complex interplay of paracrine interactions and hormonal signalling, which is essential for the proper development of the reproductive tracts. Defects in these processes can lead to congenital anomalies and infertility. However, our understanding of the Müllerian and Wolffian ducts in humans has been hindered by the lack of reliable in vitro models and the significant differences between humans and mice. Using a combination of single-cell (scRNA-seq, scATAC-seq) and spatial (spatial transcriptomics, In Situ Sequencing) genomics techniques, we characterised for the first time the temporal and spatial distribution of cells of the human reproductive tracts during prenatal development (from 6 to 21 weeks post conception). Our analysis revealed transient upregulation of neuronal/axonal growth genes in the Müllerian epithelium during migration as well as an increase in the expression of WNT inhibition and autophagy markers during regression in male embryos. We then linked histological features with gene expression changes along the cranio-caudal axis of the differentiating Müllerian and Wolffian ducts to pinpoint transcription factors beyond the HOX code likely responsible for mesenchymal patterning (of both the Müllerian and Wolffian ducts). Moreover, we shed light onto the spatially variable mesenchymal ligands that likely signal to the adjacent epithelium how to differentiate accordingly. Finally, we compared our developmental dataset with publicly available post-pubertal datasets of female and male reproductive tissues to evaluate how much of the spatial organisation and cell type diversity observed in adulthood is already established in utero. Overall, our study elucidates the still poorly-understood mechanisms that underlie the healthy emergence, differentiation and regression of the human reproductive ducts, while representing a significant step towards better addressing developmental anomalies leading to infertility and other reproductive disorders later on in life.

P-129 - Local Uterine Drug Delivery in a Mouse Model of Menstruation

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Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and the antifibrinolytic medication tranexamic acid are the only non-hormonal medical treatments for heavy menstrual bleeding. They are effective, achieving up to 50% reduction in menstrual blood loss in women. However, oral administration results in systemic side effects limiting their use in those with co-morbidities. Patient and Public Involvement (PPI) consultations have highlighted the patient's wishes to have at their disposal treatment options restricted to menses, as opposed to follow a continuous medication regime. We hypothesised that local delivery would be possible to maintain treatment efficacy and may limit off target effects. For the preparation of medicated cotton-pellets, ibuprofen (Ib) or tranexamic acid (TA) were first dissolved in ethanol or deionized water, respectively. Either 1.25 μ l of Ib-stock (0.75 mg), 5 μ l of TA-stock (0.75 mg) or 1.25 μ l of Ib-stock + 5 μ l of TA-stock were injected into a cotton-pellet and dried at ambient conditions. The drug release kinetics were monitored via UV/vis spectroscopy, mimicking physiological conditions with a phosphate buffered saline (PBS) solution (pH 4.0) at 37 °C. Wild-type mice (C57BL/6J0laHsd) underwent simulated menses after ovariectomy, sequential administration of exogenous oestradiol and progesterone and decidualisation via transcervical injection of oil. At the time of progesterone withdrawal, mice were fitted with cotton-pellets containing Ib, TA or both. Mice fitted with unmedicated cotton-pellets were used as controls. Uterine tissue was collected 8h (t8, endometrial breakdown) and 24h (t24, endometrial repair) after progesterone withdrawal. Endometrial breakdown/repair (histological grading) and menstrual blood loss (MBL, alkaline-haematin method) were quantified. Inflammatory mediators were examined by RT-qPCR. To verify the benefits of local delivery of ibuprofen and tranexamic acid, medicated cotton-pellets were prepared by depositing the dissolved drugs onto the cotton fibers. After their drying, the resulting drug release kinetics in physiological conditions (PBS, pH 4.0 at 37 °C) revealed a different release behaviour for the two drugs: rapid full tranexamic acid release was monitored within 45 min, whereas a slow continuous ibuprofen release was observed. This can be attributed to the comparably low solubility of ibuprofen in aqueous solutions. Local uterine delivery of ibuprofen and tranexamic acid revealed no significant differences in endometrial breakdown (t8) or repair grade (t24) compared to the unmedicated group. However, a trend towards enhanced repair was observed in the tranexamic only group at t24, with 43.7% of mice reaching full repair compared to the 3.5% achieved in the unmedicated group. The amount of menstrual blood loss was not significantly different between the experimental groups, with trends of decreased MBL on Ib only ($1.2 \pm 0.29 \mu$ l) and TA only ($1.08 \pm 0.57 \mu$ l) compared to controls ($2.7 \pm 1.38 \mu$ l) at t8. Inflammatory mediator mRNA showed no statistically significant differences between the medicated tampons-treated mice compared to the unmedicated group. In conclusion, uterine drug delivery using medicated cotton-pellets is a feasible strategy for local drug administration in our mouse model of simulated menses.

P-130 - Nuclear F-actin Formation

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Human endometrial stromal cells (ESCs) undergo cyclic changes during the menstrual cycle in response to changing levels of steroid hormones. Especially, ESCs morphologically and functionally change their cellular states for preparing pregnancy, referred to as decidualization. Decidualization is essential for implantation and maintenance of pregnancy. During decidualization, ESCs dramatically change their fibroblast-like morphology into the epithelial-like state with the dynamic rearrangement of cytoplasmic actin. Interestingly, this cytoskeletal actin dynamics not only morphologically, but also functionally regulate decidualization. Recent reports have suggested that actin dynamically alters its polymerized state (filamentous actin; F-actin) upon external stimuli not only in the cytoplasm, but also in the nucleus. However, nuclear actin dynamics during decidualization of human ESCs have not been elucidated. This study investigated the nuclear actin dynamics and its role in decidualization of human ESCs. For visualizing nuclear actin dynamics, ESCs expressing nuclear actin-GFP probe were established. Cells were treated with cAMP (0.5 mM) to induce decidualization. Time-lapse imaging revealed a dynamic formation of nuclear F-actin during decidualization. This was disassembled following the withdrawal of the decidualization stimulus, suggesting its reversible process. To investigate whether nuclear F-actin formation is involved in the regulation of decidualization, nuclear F-actin formation was inhibited by overexpressing the nuclear actin mutant (actinR62D). This significantly reduced the number of cells exhibiting the nuclear F-actin induced by decidualization and suppressed the expressions of decidualization markers (IGFBP-1 and PRL). Therefore, nuclear F-actin formation was essential event for decidualization. In order to investigate how the nuclear F-actin formation is involved in decidualization, we performed RNA-sequence analysis. Among the 618 genes that should be repressed in the course of decidualization, the downregulation of 304 genes was not observed when cells were overexpressed actinR62D. These genes were defined as nuclear actin-regulated genes and were related to the regulation of cell proliferation. Overexpression of actinR62D suppressed the decidualization-induced decrease in cell number. Considering that ESCs have to exit the cell cycle for accomplishing their differentiation process towards the decidualized state, nuclear F-actin formation contributes to decidualization through the suppression of cell proliferation. Furthermore, upstream analysis was performed on nuclear actin-regulated genes to identify factors regulating nuclear F-actin formation, which predicted C/EBP β as an upstream factor. Knockdown of C/EBP β suppressed nuclear F-actin formation, cell cycle arrest, and expression of decidualization markers, indicating that C/EBP β induces the cell cycle arrest through the regulation of nuclear F-actin formation during decidualization. In conclusion, we revealed that actin exists in the nucleus of human ESCs and nuclear F-actin formation is induced by C/EBP β during decidualization. This induces cell cycle arrest to differentiate into decidualized ESCs, which is a novel mechanism for the regulation of decidualization.

P-131 - Single Microcystin Exposure Impairs the Hypothalamic-Pituitary-Gonadal Axis at Different Levels in Female Rats

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Microcystin (MC) is the most common cyanobacterial toxin. Few studies have evaluated the MC effects on the hypothalamic-pituitary-gonadal (HPG) axis function. In this study, we assessed whether MC exposure results in HPG axis changes. Female rats were exposed to a single dose of MC at environmentally relevant levels 5, 20 and 40 µg/kg (MC5, 20 and 40 respectively, n=15). After 24 hours, we evaluated reproductive parameters for 15 days. Improper functioning of the HPG axis was observed in MC rats. Specifically, MC reduced hypothalamic gonadotropin-releasing hormone (GnRH) protein expression in MC40 rats and increased the pituitary protein expression of gonadotropin-releasing hormone receptor (GnRHR) in MC20 rats compared to control rats ($p < 0.05$). A borderline increase in pituitary lutropin β was observed in MC20 rats compared to control rats ($p: 0.09$). In addition, MC exposure reduced serum luteinizing hormone (LH) levels in MC40 rats and increased follicle-stimulating hormone (FSH) levels in MC20 and MC40 rats compared to control rats ($p < 0.05$). MC exposure increased pituitary IL-6 and ED1 protein expression (inflammatory markers) in MC5 rats compared to control ($p < 0.05$). Additionally, MC exposure borderline increased hypothalamic inducible nitric oxide synthase (iNOS) expression in MC5 compared to control rats ($p: 0.06$). A negative linear correlation between hypothalamus GnRH and iNOS protein expression was observed ($p < 0.05$). MC exposure reduced the primary follicles in MC5 and MC40 rats and increased the corpora lutea number in MC20 rats compared to control rats ($p < 0.05$). An increase in anti-Müllerian hormone (AMH) and progesterone levels were found in MC20 rats, and a decrease in estrogen levels were found in MC5 and MC40 rats ($p < 0.05$). MC exposure increased ovarian vascular endothelial growth factor receptor (VEGFR), LH receptor (LHR), AMH, ED1, IL-6 and Gp91-phox (oxidative stress marker) protein expression in MC20 rats compared to control rats ($p < 0.05$). MC increased uterine area and reduced endometrial gland number in MC5 and MC40 rats ($p < 0.05$). Blunted estrogen-negative feedback was observed in MC40 rats after ovariectomy, with no changes in LH levels compared to intact MC rats ($p > 0.05$). Therefore, these data suggest that a MC leads to abnormal HPG axis function in female rats.

P-132 - Müllerian Duct Maintenance at the Cranial Region is Dependent Upon GATA2

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Congenital anomalies of the female reproductive tract (FRT), also known as Müllerian anomalies, are a major cause of female infertility. However, our understanding of normal FRT development remains incomplete. The FRT organs including the oviduct, uterus, cervix, and the upper vagina derive from the same embryonic progenitor, the Müllerian duct (MD). The MD is surrounded by multiple layers of undifferentiated connective tissues known as the mesenchyme, which governs the maintenance and differentiation of the MD. During sexual differentiation, in male embryos, the fetal testis produces AMH (Anti-Müllerian hormone), which acts upon its receptor AMHR2 expressed in the mesenchyme to induce MD regression. On the other hand, ovaries in female embryos do not produce AMH; as a result, MDs are maintained. Therefore, it has been believed that

MD maintenance in the female embryo is a passive outcome arising by default in the absence of testicular AMH. In contrast to this prevailing view, we here provided genetic evidence that the maintenance of MD at the cranial region is actively promoted by GATA2 in the mesenchyme. Gata2 belongs to the GATA family of transcription factors that bind to the characteristic “GATA” DNA sequence. Among the six members of the GATA family, GATA2 is the only member that is expressed in MD mesenchyme. GATA2 has been known to play crucial roles in morphogenesis and differentiation of other tubular organs. We therefore hypothesize that mesenchymal Gata2 plays critical roles in MD development. To test this hypothesis, we established a conditional Gata2 knockout mice (Osr2-Cre; Gata2-flox, Gata2cKO), where Gata2 was deleted specifically in MD mesenchyme. At birth, Gata2cKO females had a shorter MD compared to that of the control. More strikingly, Gata2cKO females completely lost the cranial (head) MD, which is the precursor of the oviductal infundibulum essential for oocyte pickup. These observations indicate that Gata2 is essential for promoting the survival of cranial MD. To identify the potential molecular mechanism, we performed bulk RNA-seq on collected cranial MD region from control and Gata2cKO female embryos at the initiation stage of ductal degeneration (E16.5). We obtained 296 differentially expressed genes (DEGs), which did not significantly overlap with the published list of AMH-induced genes. This observation indicated that the regression of the cranial MD in Gata2cKO female embryos was not caused by ectopic AMH signaling. It is established that mesenchyme-derived secreted ligands mediate the instructive role of the mesenchyme in epithelial fate and differentiation. In our list of 296 DEGs, we identified 13 secreted ligands, among which we were particularly interested in the downregulated growth factor Nrg1 (Neuregulin 1). Nrg1 has been known to play critical roles in growth of other organs including heart and testis. Its receptors (ErbB2 and ErbB3) are expressed in MD epithelium. We are now in process of confirming the decreased Nrg1 signaling and investigating whether NRG1 supplementation can rescue the phenotype of cranial MD degeneration. Overall, our study challenges the notion that MD maintenance is a passive outcome, and demonstrates that the maintenance of cranial MD is actively promoted by mesenchymal GATA2 .

P-133 - Inflammatory Environment Regulatory and Anti-oxidant Properties for Endometriosis Treatment with Cymophenol

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Endometriosis, a chronic reproductive disease with pelvic pain, abdominal bloating, fatigue and infertility, affects more than 20% of women in the world. At the environment of the endometriosis lesion, hyper-inflammation and oxidative stress due to vasculogenesis for adhesion are observed. There is no specific way to prevent, and no fundamental cure for endometriosis, so long-term symptomatic treatment must be implemented. Nowadays, numerous phytochemicals have been studied for less side effects and long-term prevention. Cymophenol, a monoterpenoid phenol, is component of essential oil extracts mainly from *Origanum vulgare*. It is a well-known anti-inflammatory, anti-microbial and anti-cancer chemical. In our study, cymophenol exerted therapeutic potential on human endometriosis primary stromal cells and surgery induced mouse model. It induced autophagy and inhibited cell growth through PI3K/AKT down-regulation. This phenomena seems occur through mitochondria dysregulation and calcium homeostasis in endometriosis primary stromal cells. Similarly, Ca²⁺ selective chelator reduced cell viability by a

certain level but, no synergic effects with cymophenol. Moreover, cymophenol showed anti-oxidant effects increasing the expression of antioxidant enzyme such as HO-1, GCLC, SOD1, GSH-PX and NQO1. This therapeutic effects were confirmed in surgery induced endometriosis mouse model. Daily intake of cymophenol for 28 days reduced lesion size and cyst. Surprisingly, cymophenol administration increased CD4 and CD8 positive T-cell population in the spleen rather there was no changes on macrophage. The immune activation related cytokines including Ccl3, Il18 and Il2 were increased in the spleen and Il13, Ccr1, Ccl3, Il21r and Il1b were increased in the lesion. Likewise, cymophenol induced IL13, CCR1, CCL3, IL21r and IL1b mRNA expression in human endometriosis primary stromal cell. Collectively, herbal component cymophenol has inflammation regulatory and antioxidant properties that could treat endometriosis. This study was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI22C1424).

P-134 - Histological Characterisation of the Ovine Cervical Epithelium Along the Length of the Cervix at Different Stages of the Reproductive Cycle

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Cervical artificial insemination (AI) for sheep using frozen-thawed sperm yield unacceptably low (< 30%) pregnancy rates worldwide. A more thorough understanding of the ovine cervical environment is critical to develop strategies to improve sperm transport across the cervix. The aim of this study was to characterise the ovine cervical epithelium along the length of the cervix and to ascertain if it is altered by the stage of the reproductive cycle. Multiparous Texel cross Suffolk ewes were synchronised using a 12-day progestogen pessary followed by 400 IU of equine chorionic gonadotropin at pessary removal. The ewes were then slaughtered at either the follicular (Day 0; n = 6 ewes) or luteal (Day 10; n = 7 ewes) phase of their cycle following which their reproductive tracts were recovered and the cervix isolated. Circular segments (6 mm in length) were taken from each cervix along the entire length of the cervix (up to 10 segments per cervix), fixed using 10% formalin, wax-embedded, and stained with haematoxylin and eosin. Slides were digitally scanned and analysed using a combination of Imagescope, Qupath, and ImageJ (Fiji) software. The aim was to quantify the ratio of squamous vs columnar epithelial cells, assess the height of columnar epithelial cells and quantify the change in cervical fold surface area of the epithelium (measured as cervical fold linearity). All results are presented as mean $\pm$ s.e.m. The cervical epithelium was predominantly comprised of columnar epithelial cells with squamous epithelial cells only found on the periphery of the first cervical segment at the external Os, mainly in the follicular phase. Columnar epithelial cells were exclusively found through segments 2 through 10 (1 = External Os; 10 = Internal Os) in both reproductive phases. The height of the columnar epithelium was greater in the follicular ($33.3 \pm 1.31 \mu\text{m}$) compared to the luteal ($26.7 \pm 1.21 \mu\text{m}$) phase ($P > 0.001$). There was a significant decrease in cervical fold linearity between the segments nearest the external Os and

segments nearest the internal Os ($P < 0.01$). This was represented by the presence of significantly more crypts and folds in the segments nearest the internal Os. In conclusion, this study illustrates that the epithelium of the ovine cervix consists predominantly of columnar epithelium cells and these increase in height during the follicular phase in line with their mucus producing function. In addition, the increased folding of the cervical epithelium deeper in the cervix has implications for understanding the barriers to sperm transport across the cervix.

P-135 - Subacute Exposure to a Mixture of Tributyltin plus Mercury Impairs Reproductive Axis Function, Exacerbating Premature Ovarian Insufficiency Features and Reducing Fertility in Female Rats.

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Tributyltin (TBT) and mercury (Hg) are known endocrine-disrupting chemicals (EDCs), which individually cause reproductive complications. However, the reproductive consequences of exposure to a mixture of TBT plus Hg are not well known. We hypothesized that exposure to a mixture of TBT plus Hg alters the function of hypothalamic-pituitary-gonadal (HPG) axis components. Adult female rats were exposed daily for 15 days to the following doses: 1-CON rats (vehicle, ethanol 0.4%, by gavage, saline solution 0.9% by im, $n=5$), 2-TBT rats (100 ng/kg/day, by gavage, $n=5$), 3-Hg rats (first dose 4.6 mg/kg, and subsequent doses of 0.07 mg/kg/day, im, $n=5$) and 4-TBT+Hg (similar dose/exposure from TBT and Hg groups, $n=5$). Chemical accumulation in serum and organs, morphology, hormone levels, inflammation, fibrosis, and protein expression of reproductive organs were assessed. Irregular estrous cycles, showing less time in the proestrous phase and longer cycle length were observed in TBT-Hg rats compared with CON rats ($p < 0.05$). Increases in tin and Hg levels were found in the serum, hypothalamus, pituitary, ovary, and uterus of TBT-Hg rats compared with CON rats ($p < 0.05$). TBT-Hg rats showed an increase in gonadotropin-releasing hormone (GnRH) protein expression and serum follicle-stimulating hormone (FSH) levels and, a reduction in luteinizing hormone (LH) levels compared with CON rats ($p < 0.05$). Reduction in ovarian reserve, antral follicles, corpora lutea (CL) number, and estrogen levels, with increases in atretic and cystic follicles, were also found, suggesting that TBT-Hg exposure exacerbated premature ovarian insufficiency (POI) features compared with CON rats ($p < 0.05$). Further, in TBT-Hg rats, there was an increase in ovarian mast cell number, IL6 (inflammation marker), FSH receptor protein expression, and collagen deposition compared with CON rats ($p < 0.05$). In the uterus, apoptosis, reduction in uterine glands, and glycogen levels were observed in TBT-Hg rats compared with the CON rats ($p < 0.05$). Reduction in litters and pups/litters for 90 days were found in TBT-Hg rats, suggesting impairment in fertility compared with the CON rats ($p < 0.05$). Collectively, these data suggest that TBT plus Hg exposure leads to HPG axis abnormalities, exacerbating POI features and reducing in fertility of female rats.

P-136 - Transplantation of Abnormal White Adipose Tissue Induced by Obesogen Tributyltin Leads to Abnormalities in the Reproductive Tract of Female Rats

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Tributyltin (TBT) is a well-known endocrine disrupting chemical associated with metabolic and reproductive dysfunctions. However, no studies have explored the effects of abnormal white adipose tissue (WAT) transplantation induced by TBT in the reproductive tract function in the female rats. To assess whether an abnormal WAT transplantation as result of TBT exposure impairs the reproductive parameters in the female rats. Female Wistar rats were treated daily with vehicle (ethanol 0.4%, CON rats, n = 5-8) or low dose of TBT (100 ng/kg/day, TBT rats, n = 5-8) for 15 days by gavage. The metabolic and estrous cycles were assessed during the exposure time. Further, serum samples were obtained to determined basal levels of luteinizing hormone, follicle-stimulating hormone, estrogen, progesterone, Anti-mullerian hormone and testosterone were by ELISA assays. WAT samples were collected to determined basal levels testosterone by ELISA assays and to analyze gene expression of steroidogenic factors (AR, star, cyp11a1, cyp17a1, cyp19a1, hsd17b1, and 5 α -reductase), an obesogenic marker (ppary) and histology. Other set of littermate rats was transplanted subcutaneously, with gonadal fat pads from CON and TBT rats (C-CON and C-TBT, respectively, n=5). We also performed SHAM controls (n=5). After 7 days following surgical procedure recovery, metabolic and reproductive parameters were assessed for 15 days. All the protocols were approved by the CEUA/UFES (Nº 16/2019). CON and TBT rat donors showed similar metabolic parameters, such as body weight, adiposity and glucose metabolism ($p > 0.05$). Testosterone levels increased in the WAT of TBT rats compared to control rats ($p < 0.05$). TBT exposure induced a borderline increase in 17 β -hsd and ppary gene expression compared to control ($p = 0.05$ and $p = 0.05$). A borderline reduction in cyp19a1 and 5 α -reductase gene expression was observed in TBT WAT tissue compared to CON ($p = 0.05$ and $p = 0.05$). Hypertrophy, oxidative stress and fibrosis in adipocytes were observed in TBT WAT tissue compared to control ($p < 0.05$). After WAT transplantation, C-TBT showed the reproductive tract abnormalities. Specifically, C-TBT rats presented irregular estrous cyclicity, spending more time in the proestrus and metestrus-diestrus phase, with longer cycle length compared with C-CON rats ($p < 0.05$). A Reduction in preantral and antral follicles and corpora lutea number were noted in C-TBT rats compared to C-CON rats ($p < 0.05$). Increased in cystic and atretic follicles were observed in C-TBT rats compared to C-CON rats ($p < 0.05$). A borderline reduction of serum progesterone levels was found in C-TBT rats compared to C-CON rats ($p < 0.05$). Reduction in endometrium and myometrium area are observed in C-TBT rats compared to C-CON rats ($p < 0.05$). Reduction in uterine gland numbers were also noted in C-TBT rats compared to C-CON rats ($p < 0.05$). An increase in presence of mast cells in ovary and uterine of C-TBT rats were observed compared to C-CON rats ($p < 0.05$). An increase in collagen deposition in ovary and uterine of C-TBT rats were observed in C-CON rats ($p < 0.05$). No changes reproductive and metabolic were observed between sham and C-CON rats ($p > 0.05$). Collectively, these data suggest that an abnormal WAT transplantation as result of TBT exposure for short time impairs reproductive tract parameters in the female rats. Funding: FAPES [#19/2022-TO 981/2022; # Nº 03/2021 - TO: 486/2021) CNPQ [# 307224/2021-0]. LHT, LHMI.

P-137 - The Development of a Pre-clinical Swine Endometriosis Model

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Endometriosis is a benign gynecological condition where endometrial-like tissue grows outside of uterus. However, the exact cause and pathogenesis of endometriosis is unclear. Currently, the gold standard for the diagnosis of endometriosis is laparoscopic surgery which is invasive. Pig is an ideal animal model for human health and diseases because their anatomy and physiology is similar to humans and their genetics is highly conserved to the human. Developing a swine model mimicking pathophysiology of endometriosis and being able to easily distinguish endometriotic lesions from surrounding normal tissues will offer a novel large animal model that assist designing an effective cure. The swine model of endometriosis was generated by using ex vivo labeling of endometrial tissues with Fluorescein isothiocyanate (FITC) dye-doped silica nanoparticles. First, approximately 6 cm of swine uterine tissue was excised from a cycling adult pig under anesthesia. After removing myometrium, the endometrial tissue was then minced (5g of small fragments) and labeled by FITC dye-doped silica nanoparticles. An optimal ex vivo labeling condition in swine endometrial tissue was determined by exposing endometrial tissues to different concentrations of FITC dye-doped silica nanoparticles and incubation times; the optimal labeling condition was identified as incubating the nanoparticles at 60% concentration for 90 minutes. To establish swine endometriosis model, FITC dye-doped silica nanoparticle-labeled endometrial fragments were inoculated into the peritoneum of the same pig as autologous transplantation. To examine the development of endometriotic lesions, we euthanized the pig at 6 weeks post-transplantation and examined endometriosis development. We observed ± 20 FITC-positive ectopic lesions in peritoneal wall as well as outside of the uterus and small intestine using FITC fluorescence detector goggles with a UV flashlight. Histological analysis of the ectopic lesions revealed endometrial-like epithelial and stromal cells from the pig. They were morphologically comparable to human endometriotic lesions. Furthermore, immunohistochemistry detected E-cadherin in the epithelial cells and vimentin in stromal cells of the ectopic lesions. Our results suggest that we were able to induce endometriosis in swine like that in human beings. This will provide a new model to further endometriosis research. Coming up with a swine endometriosis model may help improve not only in the discovery of pathogenesis but also in the development of a non-invasive diagnostic tool.

P-138 - TGF- β 1 stimulates migration/proliferation in immortalised Fallopian tube epithelial cells

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Tubal ectopic pregnancy (tEP) occurs when an embryo implants in the Fallopian tube instead of the uterus. tEP occurs in approximately 1-2 in every 100 pregnancies, and is a major health burden which can lead to morbidity and, in rare cases, mortality. There is evidence to suggest that epithelial-to-mesenchymal transition (EMT) is involved in intrauterine embryo implantation, however, the role of EMT in tEP is unknown. We have unpublished data showing that TGF- β 1, which can induce EMT, increases blastocyst attachment in an in vitro model of tEP. Here, we set out to test the hypothesis that exposure to TGF- β 1 promotes EMT in Fallopian tube epithelial cells, resulting in increased proliferation and/or migration. The experiments used a wound healing scratch assay to test the effect of TGF- β 1 and of its inhibitor on cultures of an immortalised human Fallopian tube epithelial cell line (OE-E6/E7). OE-E6/E7 cells (sourced from KF Lee, Hong Kong) were seeded in 6-

well cell culture plates at 3×10^5 cells/ml per well and cultured to 100% confluency in DMEM/F-12 (ThermoFisher) supplemented with 5% charcoal-stripped serum (ThermoFisher) and 2mM non-essential amino acids (ThermoFisher). 24 hrs prior to the scratch wound assays, the culture medium was replaced with either the control (serum-free DMEM/F-12; ThermoFisher), 20ng/ml TGF- β 1 (Invitrogen), or 10 μ M TGF- β 1 inhibitor (SB431542; Stemcell technologies) in serum-free DMEM/F-12 in duplicate wells. Before scratch wounds were performed, medium was replaced with fresh DMEM/F-12 medium. Using a p200 pipette tip, a uniform scratch wound was created in the middle of each cell monolayer, using a spare culture plate lid as a guide to create an even scratch line. Scratch wounds were then imaged at 0, 4, 8, 12, and 24 hr time points. The scratch wound width was analysed using the ImageJ Wound Healing Size Tool Plugin. After 24 hours, there was a significant increase in the percentage of wound gap closure in OE-E6/E7 cells exposed to TGF- β 1 compared to the control ($p < 0.01$). Likewise, there was a significant decrease in percentage gap closure after exposure to the TGF- β 1 inhibitor at that same timepoint, compared to either controls or to cells exposed to TGF- β 1 ($p < 0.01$ and $p < 0.001$ respectively). There was a significant reduction in the rate of migration (μ m/hr) in cells exposed to TGF- β 1 inhibitor compared to cells exposed to TGF- β 1 ($p < 0.05$). However, no difference was observed in the rate of migration of cells after exposure to TGF- β 1 compared to the control. In conclusion, TGF- β 1 increases wound gap closure, while its inhibitor decreases it, in immortalised Fallopian tube epithelial cells. TGF- β 1 inhibition also reduced the rate of migration compared to cells exposed to TGF- β 1. Together, the results suggest that the TGF- β 1 pathway could be involved in the proliferative and/or migratory capacity of Fallopian tube epithelial cells, potentially affecting the likelihood of embryo attachment at that site. Work was funded joint by a joint Medical Research Council/Ectopic Pregnancy Trust PhD Fellowship to HF.

P-139 - In Vitro Reminiscence: Uterine Disturbances in Vivo Affect Respective Luminal Epithelial Cells Function in Vitro

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In cattle, adequate programming of uterine function is required to support pregnancy. The endometrium during diestrus and early pregnancy displays cellular responses that are consequences of prior long term and transient stimuli. Here, we tested a novel study paradigm to characterize endometrial cellular memory. Such paradigm consists of three steps: (1) treating cows, (2) collecting endometrial epithelial cells (BUECs) using a cytology brush (cytobrush) from the same cows, and (3) culturing and measuring responses from cow-specific BUECs. The hypothesis is that stimuli given to endometrium in vivo are retained as a cellular memory that remains after BUECs are isolated, cultured, and further stimulated in vitro. Objectives were to

measure BUEC proliferation/migration and responsiveness to recombinant bovine interferon-tau (rbIFNT) in vitro: among cows that showed estrus (experiment 1, N=12; Exp1), cows that became or not pregnant to artificial insemination (AI; Exp2, N=21), cows that received (high) or not (normal) supplemental progesterone (P4; Exp3, N=24) and cows that received or not a cyclooxygenase 1/2 inhibitor (Flunixin; Exp4, N=12). Only cows that displayed estrus were included. BUECs were collected four days after estrus via cytobrush. The BUECs were cultured, propagated. Responsiveness to rbIFNT was measured by the expression of interferon stimulated genes (ISGs). Proliferation/migration was assessed by a wound healing assay. Confluent BUECs were mechanically displaced from a straight line running diametrically in the center of the well. Micrographs were taken every 2 hours for 24 hours using light microscopy. The horizontal distance between the two borders created by the displacement was recorded. The time point in which 50% of the horizontal distance was repopulated by BUECs was used for analysis. In Exp1, cows clustered according to the time interval to reach 50% of cellular repopulation. In Cluster A (N=5) 50% cellular repopulation happened 10 hours earlier than for Cluster B (N=7; P=0.001). rbIFNT responsiveness measured by ISG15 was 11-fold greater on Cluster A than B (P=0.042). Expression of IFNT receptor (IFNAR1) was similar between clusters. In Exp2 there was no effect of pregnancy on the time to reach 50% of cellular repopulation. Pregnant cows had a greater proportion (19.5%) of proliferating cells (Ki67 positive) in the border of repopulation than non-pregnant cows (6.8%; P=0.012). BUEC from pregnant cows had 116.6% greater rbIFNT responsiveness than non-pregnant (P=0.09). In Exp3, the proportion of cows from high-P4 (50%; 6/12) whose BUECs proliferated in culture was lower than normal-P4 (84%; 10/12; P=0.083). P4 did not affect the rbIFNT responsiveness in BUEC (P4*rbIFNT: P=0.3). However, BUECs from high-P4 cows had increased expression of RSAD2 regardless of rbIFNT treatment (P=0.039). In Exp4, Flunixin in vivo did not affect cellular repopulation in vitro. BUECs from Flunixin treated cows had greater rbIFNT responsiveness (Flunixin*rbIFNT) for classical ISGs (ISG15; P=0.08 and RSAD2; P=0.008; respectively) and showed decreased rbIFNT responsiveness for non-classical ISGs (CST3; P=0.05 and CST6; P=0.03 respectively). In conclusion, physiological and pharmacological stimuli received by the endometrium in vivo were retained as cellular memory in BUECs, persisted in culture, and modulated BUECs proliferation/migration and responsiveness to rbIFNT, which are characteristics associated with fertility in cattle. Long-term implications of these findings include the possibility of establishing in vitro assays to understand endometrial biology and predict the fertility potential of cows. This study is supported by USDA NIFA Award Number 2022-67015-36839.

P-140 - The Influence of Phthalates on Ovarian Endometriotic Cell Invasion Patterns Using a 3D In Vitro Model

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Endometriosis is a chronic inflammatory gynecological disease affecting 1 in 10 menstruators. It is identified laparoscopically by the presence of endometrial-like tissue around the pelvic cavity. These lesions cause dysmenorrhea, menorrhagia, fertility issues, and an increased risk of ovarian cancer (with ovarian endometriosis). Often, it takes a decade from symptom onset to diagnosis, thus, not much is known about the early stages of these lesions and their potential causes. Researchers believe that there is a positive correlation between phthalate exposure and endometriosis risk. Phthalates are known endocrine disruptors used in many everyday products to create flexible plastics. Di(2-ethylhexyl) phthalate (DEHP) is a particularly interesting research target for endometriosis as it is one of the most common phthalates and has been observed in animal models to alter estrogen synthesis. Previous in vitro work has examined the impact of DEHP on healthy endometrial cells and cancer-derived endometrial cells. Here we describe a 3D tissue engineering model to encapsulate endometriotic spheroids in physiologically relevant extracellular matrix to investigate the effects of DEHP on cell invasion, a critical early step in endometriotic lesion initiation. We create spheroids using cohorts of epithelial and stromal cells embedded into a 3D methacrylamide-functionalized gelatin (GelMA) hydrogel. Endometriotic cells are azurite-blue tagged human endometrial-endometriotic stromal cells (azb-iEC-ESCs; Fazleabas Lab, Michigan State University) and GFP-transduced human endometriotic epithelial cells (12z; ABM). Spheroids were formed from 5000 cells (1:3 mixture of GFP-12z:azb-iEC-ESCs), encapsulated in a GelMA precursor suspension, then UV polymerized. GelMA was synthesized utilizing a previously established method by the Harley Lab. GelMA was characterized with NMR (Agilent 600MHz NMR), and compressive testing (Instron 5943). 3D hydrogels were prepared with 5wt% or 7wt% GelMA, PBS, and 0.1% w/v lithium phenyl phosphinate photoinitiator. GelMA was photopolymerized under a UV light ($\lambda = 365$ nm, 7.14 mW cm⁻², AccuCure Spot System ULM-3-365) for 30s. Spheroids were cultured in a 50/50 mixture of 12z complete culture medium (CCM) and iEC-ESC CCM. Media (0 μ M DEHP, 1 μ M DEHP, 0.1 μ M DEHP, or 0.01 μ M DEHP treatments) was changed every two days and spheroids were imaged on D0, D2, and D8. GelMA synthesized at 5wt% and UV polymerized for 30s fell within the stiffness of natural, healthy ovarian tissue (3.3 ± 2.5 kPa), with 7wt% hydrogels above this desired stiffness. We identified a ratio of epithelial and stromal endometriotic cells that mimic histological stains of lesions and supports outgrowth of spheroids within our system. We identified physiological doses of DEHP and observed differences in cell outgrowth patterns where control and 0.1 μ M DEHP experienced the most outgrowth by D8. We observed visual differences in spheroid outgrowth of control vs. DEHP treatments, with reduced outgrowth in response to 1 μ M DEHP and 0.01 μ M DEHP treatments. Ongoing studies are extending the timeframe of studies of DEHP exposure and endometriotic cell outgrowth (epithelial vs. stromal). Ongoing efforts will also expand treatments to include MEHP (a primary metabolite of DEHP), increase the number of experimental replicates ($n > 6$), and incorporate further analysis of gene expression and matrix remodeling in this model of endometriotic lesion initiation.

P-141 - Mucus Production in Conditionally Reprogrammed Endocervical Cell Cultures Derived from Human and Baboon Cytobrush Samples

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The endocervix regulates female fertility by producing cervical mucus that can permit or prevent sperm entry into the upper reproductive tract. We previously demonstrated that using conditionally reprogrammed primary endocervical cells from nonhuman primates (NHPs), we can study the regulation of cervical mucus secretion in vitro. However, our prior studies used primary cells harvested from necropsy specimens. Cell cultures produced from non-invasive cytobrush sampling would facilitate translation to human cell collection, allowing the study of unique genetic or disease states. Thus, we sought to determine if conditional reprogramming would also enable production of primary endocervical cell cultures of both NHPs and women, obtained non-invasively through the same endocervical cytobrush sampling technique used in cervical cancer screening. We collected endocervical cytobrush samples from thirteen women, as well as six adult baboons (*Papio hamadryas*). We obtained cells by inserting a cervical cytology brush (CytoSoft, Fisher Scientific) into the endocervix and twirling the brush for approximately 3 seconds. Brush samples were enzymatically digested, cultured in conditionally reprogrammed conditions. Expanded cells were differentiated at an air-liquid interface, and subsequently treated with either vehicle control or estradiol for one week. We evaluated steroid hormone receptor expression, mucus production, and ultrastructural appearance of resultant cultures. We successfully cultured primary cells obtained through cytology brush samples of four (67% success rate) baboon and four (36%) human endocervical cells. Using quantitative PCR, we confirmed the presence of both estradiol receptor (ER) and progesterone receptor (PGR) in our cytology brush generated cultures. Using WGA-lectin, a glycan specific carbohydrate binding protein that corresponds to the gel-forming mucin MUC5B, we found evidence of mucin production in all differentiated samples. Additionally, we compared our differentiated cell cultures to tissues using both TEM and SEM, demonstrating similar ultrastructural appearance. Using conditional reprogramming, we successfully cultured primary endocervical epithelial cells derived from cytological brush samples of both NHP and women. Of note, this is the first study that has produced mucus secreting cell cultures from conditionally reprogrammed human endocervical cells. Endocervical cytology brushings are a non-invasive, universal, screening procedure routinely used with women. A technique to generate cell cultures from the scant starting material of brushings would bypass the need for animal or human organ donation. As previous studies demonstrate that conditionally reprogrammed cells maintain genetic similarity to their parent tissues, this approach provides a possible experimental platform for individual-specific effects of drugs, toxins and pathogens on endocervical cells.

P-142 - Effect of Embryonic Signals on Activity of DNA Methyltransferases in Luminal and Stromal Cells of the Porcine Endometrium

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The peri-implantation period in mammals, including pigs, is critical period of pregnancy due to increased embryo mortality. Pregnancy establishment requires coordinated communication

between developing embryos and the maternal organism. Uterine receptivity is defined as the state in which the endometrium is prepared for embryo implantation. It is regulated by ovarian hormones. During the maternal recognition of pregnancy on days 11-12 of pregnancy, porcine conceptuses signalize their presence by secreting multiple factors, of which estradiol (E2) is considered as the major embryonic signal. Our previous study reported that DNA methylation patterns of some genes in the endometrial tissue changed during early pregnancy and this may be associated with regulation of these gene expression. DNA methylation is catalyzed by DNA methyltransferases (DNMTs). The aim of the research was to study whether estradiol and conceptus can regulate the activity of the DNMTs in luminal epithelial (LE) and stroma cells (ST) of the porcine endometrium in vitro. LE and ST cells were isolated from porcine uterus collected from gilts (n=6) on day 11-12 of the estrous cycle. LE and ST cells (at confluency of 60%) were incubated with control medium, medium containing E2 (1,10 and 100 nM) or conceptus-exposed conditioned medium with or without estrogen receptor antagonist (ICI 182,780). The activity of DNMTs in endometrial LE and ST cells was determined in nuclear protein extracts of cells using commercially available colorimetric assays. Statistical analyses were performed using one-way ANOVA, followed by Tukey post-test. Estradiol elevated DNMT activity in ST cells ($p < 0.05$). In the contrast, estradiol and the conceptus-exposed conditioned medium decreased DNMT activity in LE cells ($p < 0.05$). Co-treatment with ICI182780 reversed the effect of E2 on DNMT activity in LE cells. Results of the present study indicate that E2 and products secreted by conceptus differentially regulate DNMT activity in LE and ST cells in the porcine endometrium. The results suggest that putative changes in DNA methylation in LE and ST cells in response to conceptus could be important during pregnancy establishment and development. The study was funded by the National Science Centre in Poland, grant no. 2022/47/B/N29/02776.

P-143 - Bovine Endometrial Response to Different Concentrations of Progesterone Before Estrus and Different Intensities of Estrous Expression

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This study aimed to evaluate if different concentrations of progesterone before estrus and different intensities of estrous expression impact endometrial gene expression in lactating Holstein cows. A total of 125 lactating Holstein cows were randomly assigned into two experimental groups: Low progesterone (n = 64) and High progesterone (n = 61). Cows received a pre-synchronization protocol followed by the progesterone treatment (Low progesterone = received extra injections of PGF2 α). Ovarian ultrasonography and blood samples were performed to monitor ovarian structures and progesterone levels. Physical activity was monitored by an activity monitor, and the duration and intensity of estrus were measured. A subsample of 33 cows (High progesterone: n = 16; Low progesterone: n = 17) were submitted to uterine biopsy 7 and 14 d after identified estrous event. Total RNA was extracted from each sample and NanoString nCounter system was used to

determine the abundance of specific mRNA molecules for 92 genes. The data was normalized to housekeeping genes and analyzed using mixed linear regression models including the progesterone levels post estrus as fixed effect and cow as random effect nested within day. Adjusted P-values were calculated using Bonferroni correction. Preliminary analysis suggested that greater progesterone concentrations before estrus as well as greater intensity and duration of estrus were associated with changes in endometrial gene expression of lactating Holstein cows. These modifications were linked to significant changes in the normalized number of reads for a total of 26 and 47 gene transcripts on days 7 and 14, respectively ($P < 0.05$). On day 7, molecular functions represented often in the list of increased gene transcripts included: eicosanoid metabolic process (AKR1C4, HPGD, PTGIS, AKR1B1), growth factor signalling (IGF1, IGF2, MEP1B, EGF), steroid signalling (PGR) and Wnt signalling (WNT5A, WNT6, WIF1). On day 14, genes with an increased number of reads and their functions included: growth factor signalling (IGF1, IGF2, MEP1B), steroid signalling (PGR, PGRMC1, PGRMC2) and nutrients/solute transport (FOLR1, FABP, SLC27A6, SSLP1, SLC27A6, CYP26A1). A considerable number of gene transcripts associated with immune response were included in the list of genes with decreased expression on day 14 (IL1B, CCL2, NFKB2, CCL4, CXCL8, IL1A, CXCL12, PTX3, CCL17, CCL22). Interestingly, a significant interaction between estrous expression and the progesterone treatment was found for several gene transcripts ($P < 0.05$). On day 7, greater intensity and duration of estrus were found to increase the number of reads for genes related to growth factor signalling (IGF2, MEP1B) and nutrients/solute transport (FOLR1, SLC1A4, CYP26A1) only in low progesterone treatment. Similarly on day 14, greater intensity and duration of estrus increased the number of reads for genes related to steroid signalling (PGR, PGRMC2) and nutrients/solute transport (FOLR1, FABP, SLC1A4, SSLP1, SLC6A20), whereas it decreased the number of reads for genes associated with the immune response (CCL2, CCL4, CXCL8, IL1A, CCL17) only in low progesterone treatment. Together, greater progesterone concentrations before estrus and greater intensity and duration of estrous expression seem to be promoting changes in endometrial gene expression that might be related to the uterine support for early embryonic development. Additionally, when the levels of progesterone before estrus is low, estrous expression seems to be able to promote changes in endometrial gene expression that may favour the early development of the embryo. This study was supported by the 'Resilient Dairy Genome Project' and NSERC.

P-144 - The Activated Platelet-rich Plasma-Fibrin Hydrogel Attenuates Uterine Endometrial Fibrogenesis after Acute Injury via Modulation of Senescence, Inflammation, and Epithelial-Mesenchymal Transition

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In fibrotic diseases, cellular senescence plays a major part by exhibiting healthy or pathologic features, and these characteristics determine whether an organ will maintain its health or progress to disease. Although several studies on fibrotic senescence-related mechanisms have been conducted in organs such as the lungs and liver, research on endometrial fibrotic diseases remains poorly understood. We identified senescence in intrauterine adhesion, a typical fibrotic disease of the uterine endometrium, as fibrosis progressed. Additionally, we applied platelet-rich plasma-fibrin hydrogel (PFH), which demonstrated beneficial effects in our previous study, to prevent fibrosis formation. After inducing endometrial fibrosis through acute exposure to hydrochloric acid, senescence in the endometrium showed a tendency to increase over time along with fibrosis formation. In the experimental group, PFH was infused into the endometrial cavity immediately after damage, and it was confirmed that senescent cells and fibrotic tissues were significantly reduced. These results indicate that when PFH is administered promptly after endometrial damage, pathologic fibrosis is delayed by preventing senescent cell fate, leading to proper wound healing.

P-145 - Association of the rs17576 Matrix Metalloproteinase 9 (MMP-9) polymorphism with endometriosis.

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Association of the rs17576 Matrix Metalloproteinase 9 (MMP-9) Polymorphism With Endometriosis. Karachle Georgia¹, Mavrogianni Despoina¹, Drakaki Eirini¹, Drakakis Peter², Stavros Sofoklis² 1. University of Athens School of Medicine, First Department of Obstetrics and Gynecology, Athens, Greece. 2. University of Athens School of Medicine, Third Department of Obstetrics and Gynecology, Athens, Greece. Introduction Endometriosis is characterized by the presence of endometrial tissue (stroma or glands) outside the uterine cavity. Metalloproteinases constitute a group of proteolytic enzymes which are vital for Extracellular Matrix (ECM) degradation and tissue remodeling which are the basic mechanisms for ectopic endometrial migration. As it has been demonstrated by previous studies, the eutopic endometrium of women with endometriosis intrinsically exhibits an increased production of molecules, among which metalloproteinases are found. The purpose of this study is to investigate the association of the rs17576 Matrix Metalloproteinase 9 (MMP-9) polymorphism with endometriosis. Participants /Methods In this study 18 samples from women with endometriosis were included. Genetic material (DNA) from both eutopic and ectopic endometrium was extracted, and subsequently was amplified using PCR with gene specific primers. Incubation with BsoBI depicted in agarose gel the rs17576 polymorphism detection. Results Among women with endometriosis 50% have Wild Type genotype, 39% are heterozygous for the polymorphism and the other 11% is homozygous for the rs17576. Moreover, it is worth noting that it seems to be no difference in the detection and the genotype of the SNP

polymorphism between the eutopic and ectopic endometrial tissue from the same patient.

Discussion A larger number of samples could confirm the results above. Moreover an interesting approach would be to investigate the rs17576 polymorphism in different ectopic sites such as the fallopian tubes, the pelvic peritoneum, the urinary bladder or the chest. Also, the effect of different MMP-9 polymorphisms can be studied and possibly be associated with endometriosis.

P-146 - SMRT Plays an Important Role for Implantation and Decidualization.

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Infertility is a major health issue that affects an estimated 10–15% of couples worldwide and has been growing at an alarming rate. Over 75% of failed pregnancies involve implantation defects. Epigenetic regulation has indeed gained significant attention for its role in implantation and decidualization during early pregnancy. However, little is known about the role of epigenetic regulation that contributes to early pregnancy establishment. Silencing Mediator of Retinoid and Thyroid hormone receptors (SMRT) is a transcriptional co-regulator of a variety of nuclear receptors and transcription factors. Our immunohistochemical analysis revealed that the expression of SMRT was significantly decreased in endometrial epithelial and stromal cells from infertile women with endometriosis compared to controls. Since mouse embryos with homozygous deletion of SMRT are embryonically lethal, we generated a mouse model with *Smrt* conditionally ablated in *Pgr*-positive cells (*Pgrcre/+Smrtf/f; SmrtKO*) to determine the effect of SMRT loss in the uterus. Ablation of *Smrt* in the uterus was confirmed by RT-qPCR, Western blot and IHC analyses. In fertility tests of female *Smrtf/f* and *SmrtKO* mice over 6 months, *Smrtf/f* mice were fertile, whereas *SmrtKO* mice were sterile. The number of implantation sites at GD4.5 revealed normal in both *Smrtf/f* and *SmrtKO* mice and histological analysis indicated normal embryo attachment to the uterine horn in *Smrtf/f* and *SmrtKO* mice. However, we did not detect implantation sites in the uterine horns of *SmrtKO* mice at GD5.5. Our histological analysis indicated that uterine luminal epithelial cells in *Smrtf/f* mice were disappeared, and embryos were attached to the uterine horn and surrounded by decidualized cells. However, in *SmrtKO* mice, uterine luminal epithelial cells were intact, and decidual cells were not evident. These results suggest that *SmrtKO* mice were infertile due to defective embryo implantation. We then used an artificial decidualization model to examine how *Smrt* ablation affects decidualization. *Smrtf/f* mice displayed a decidual uterine horn that responded well to this decidual induction. However, *SmrtKO* mice had a significant defect in the decidual response. We confirmed this decidual defect by histological analysis and reduced expression of the decidualization markers *Bmp2* and *Wnt4* in the *SmrtKO* mouse uterus. These results demonstrate that *SmrtKO* mice were infertile due to a decidualization defect. This work was supported by NIH P01HD106485 and R01HD102170.

P-147 - Both Obesity and DMBA Exposure Alter Ovarian Follicle Number, ZP3 And PPP2CA in Prepubertal Female

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Obesity in adult females is associated with poor oocyte quality which limits female reproductive capacity. 7,12-dimethylbenz[a]anthracene (DMBA) is a polycyclic aromatic hydrocarbon which depletes the ovarian oocyte reserve through genotoxicity. Previously observed altered zona pellucida (ZP3) formation and Protein Phosphatase 2 Catalytic Subunit Alpha (PPP2CA) abundance in oocytes of adult female mice due to obesity and DMBA exposure led to the hypothesis that prepubertal obesity would alter oocyte ZP3 and PPP2CA basally and during DMBA exposure. Nine-week-old female C57BL6J mice were bred to proven males and plug checked daily. On gestational day 17, half the pregnant females were switched from a normal rodent diet containing 3.5% kCal from fat (lean) to a high fat diet comprised of 60% kCal from fat (HFD) which continued during lactation. At post-natal day (PND) 21, female offspring were weaned and received the same diet as their respective dams (lean n = 26, HFD n = 12), while the male offspring were removed from the study. All female offspring were fed ad libitum and puberty onset was monitored daily. At PND 35, each diet group was divided and injected intraperitoneally with corn oil as a vehicle control or 1 mg/kg DMBA for 7 d. Mice were euthanized on PND 42 and one ovary from each mouse was utilized for follicle counts and immunofluorescence staining. Body weight was increased ($P < 0.05$) by HFD but DMBA exposure had no effect. The age at puberty onset was reduced ($P < 0.05$) by the HFD but not altered by DMBA exposure. Ovarian weight tended to be increased ($P = 0.07$) in HFD mice, with no differences in DMBA-treated mice. A tendency for increased number of primary follicles ($P = 0.06$) in HFD mice was observed with no impact of DMBA exposure. Corpora lutea number tended to be increased ($P = 0.1$) by HFD DMBA exposed mice, but there was no effect of HFD alone. There was no impact of HFD or DMBA exposure on ZP3 abundance in developing follicles, however the ZP3 stain in primary follicles of HFD mice had a disconnected and underdeveloped appearance compared to lean control treated mice. PPP2CA was reduced ($P < 0.05$) in primordial follicles of HFD compared to lean mice. PPP2CA in primary follicles was reduced ($P < 0.05$) by DMBA and HFD both alone and in combination. Preantral and antral follicles had reduced PPP2CA ($P < 0.05$) due to DMBA exposure in HFD mice, with no differences noted in lean mice. These findings support that HFD and DMBA exposure during adolescence both impact protein abundance in early folliculogenesis at puberty. Supported by 1R01ES030341-01 from NIEHS.

P-148 - Uterus Healing After Cesarean: Development of a Rabbit Model

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Abnormal scarring tissue after Caesarean in humans often result in complications such as scar defect, and has been suspected to induce multiple gynecological and obstetrical complications. The objective of this study was to develop an in-vivo model of uterus healing after cesarean in order to analyze histological phenomena controlling scarring tissue development and potential cause of defects. Knowing the complex process of uterus healing could help improve the management of scarred uterus. Methods: The study was conducted in a public research institute after agreement of ethical authorities. Eighteen pregnant primiparous female rabbits were bred naturally. At 28 days of gestation (G28), under general anesthesia, a cesarean was practiced. Fetuses were either extracted through a longitudinal incision in one of the uterine horns ("C-section") or via a single incision at the tip of the contralateral horn ("control"). Uterine horns were sutured by single layer, all by the same surgeon. They were mated again 14 days later and euthanized at G28. Genital tracts were collected for histological and biomechanical analyses. Results: Macroscopically, 4/18 C-section horns presented a dehiscence or a spontaneous rupture. After inclusion in paraffin, 5 µm cut, and Hematoxylin, eosin and safran stain, mean thickness of the scarred area was significantly lower vs non-scarring area on C-section horns vs control horns (respectively 1.01 ± 0.65 mm vs 1.73 ± 0.45 mm vs 1.46 ± 0.34 mm, $p=0.03$). The percentage of fibrosis in the scar zone, calculated using Image J software, was significantly higher in the scar zone than in the non-scar zone (C-section horn) or in the control horn. Biomechanical characteristics of the C-section versus control horns were different on the stress-strain curves. From a mechanical point of view, the scar was a zone of mechanical weakness. However, the scarred tissue was very hard and stiff (collagen), and could sustain a traction force almost similar to the non-scar tissue. Conclusion: Although C-section conditions were standardized, uterine healing differed between individuals. Biomechanical analyses seem to confirm histologic findings. These results suggest that this model may be useful for studying uterine healing efficiency according to individual characteristics and suture mode.

P-149 - Lineage Tracing of Endometrial ALDH1A1+cells Reveals their Contribution to Uterine Development

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Endometrial stem cells are thought to confer the endometrium with its unique regenerative potential, however, the identity and growth factors controlling stem cell regeneration are not well understood. The aldehyde dehydrogenase 1 (ALDH1A) family synthesizes retinoic acid and plays important roles during the embryonic development of the uterus. ALDH1A1+ cells are putative stem cell markers in normal and tumor tissues, however, their contribution to adult endometrial regeneration is not well characterized. Here, we examined ALDH1A1 expression in different ages of wild-type (WT) mice to visualize its expression during key timepoints in the uterus using

immunohistochemistry (IHC). We also generated a new tamoxifen-inducible cre reporter mouse, *Aldh1a1creERT2*; *Gt(ROSA)26SortdTomato* (“*Aldh1a1creERT2*-tdTomato reporter mice”) to trace the fate of ALDH1A1+ cells during various timepoints in the neonatal and adult mouse uterus. IHC showed that ALDH1A1 was widely expressed in the WT mouse endometrial luminal epithelia at postnatal day 7 and further expanded to glandular epithelia at postnatal day 14; at 3 weeks, ALDH1A1 was restricted to the crypts of glands. In the adult uterus, ALDH1A1 was widely expressed in luminal and glandular epithelia during diestrus and was restricted to glandular crypts during estrus. Tracing in the *Aldh1a1creERT2*-tdTomato reporter mice from PND8 to PND14 detected ALDH1A1+ cells in the glandular crypts and luminal epithelium. Tracing in the adult uterus showed that ALDH1A1+ cells localized to the glandular crypts, luminal epithelium, and to a population of sub-epithelial stromal cells. These results suggest that ALDH1A1+ cells, which were enriched in the glandular crypts or sub-epithelial stroma, give rise to mature luminal epithelial cells in the adult. We also compared the organoid formation of ALDH^{Low} and ALDH^{High} cell populations of endometrial organoids from human endometrium. Human endometrial organoids contained approximately 7-18% of ALDH^{High} cells (n=5) and ALDH^{High} cells formed more organoids with larger diameters compared to ALDH^{Low} cells, indicating that ALDH^{High} endometrial cells are more proliferative and regenerative than ALDH^{Low} cells. Overall, our results in mice and humans indicate that ALDH1A1+ cells are endometrial stem/progenitor cells that contribute to the development and regenerative potential of the endometrium. Studies were supported by Eunice Kennedy Shriver National Institute of Child Health and Human Development (R01-HD105800) and the Burroughs Wellcome Fund Next Gen Pregnancy Award (NGP10125).

P-150 - Investigating the Enzymatic Activity of Endometrial DPPIV in the Molecular Adhesive Pathways at Embryo Implantation Using an in vitro Co-culture Model

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In the UK, 1 out of 6 couples are infertile and 10% of them suffer from recurrent implantation failures. The clinical success rate has stagnated at 25-30% per cycle for the last decade. The knowledge of molecular events at the embryo implantation remains limited, hindering further increase in clinical pregnancy success rates. The endometrial dipeptidyl peptidase IV (DPPIV) was identified to be upregulated during the window of implantation in fertile women and downregulated in women experiencing recurrent implantation failure. However, its enzymatic function remains unclear at implantation, and we aim to investigate it at the embryo-endometrial interface. In endometrial cells, DPPIV was heterogeneously expressed in endometrial cell membranes using immunocytochemistry, with highly DPPIV-positive cells detected at the site of BeWo spheroid attachment onto endometrial Ishikawa monolayer in our co-culture model. The role of DPPIV enzymatic function in cell adhesion was tested in vitro using sitagliptin and vildagliptin. Using bovine serum albumin-, collagen-1-, poly-L-lysine- or fibronectin- coatings, BCECF-AM-treated endometrial cells were plated for single-cell attachment assays. Fluorescent endometrial cells were pre-treated with inhibitors concentration range (diprotin A: 1 µg/ml-100 µg/ml; sitagliptin: 10 µM-150 µM; vildagliptin: 10 µM-100 µM). The enzymatic activity of DPPIV was measured in vitro and significantly inhibited by a potent DPPIV inhibitor, diprotin A. A 50 µg/ml diprotin A pre-treatment of endometrial cells significantly decreased their cell attachment to fibronectin and in our co-culture model, significantly triggered a reduction in spheroids attachment. Using prescribed DPPIV

inhibitors, only sitagliptin concentrations caused a significant reduction in endometrial cell attachment in fibronectin- and collagen-1-coated wells compared to vildagliptin. Using an in vitro model of implantation, monolayers of Ishikawa cells were pre-treated or not with sitagliptin or vildagliptin and thus, trophoblastic BeWo spheroids were co-cultured onto the monolayer. A decreasing trend in spheroid attachment was observed using 10 μ M and 150 μ M sitagliptin compared to control and vildagliptin concentrations. In this study, the inhibition of endometrial DPPIV by diprotin A impaired its adhesive function in vitro. However, the prescribed DPPIV inhibitors acted differently: sitagliptin impaired endometrial cell attachment and had a limited effect on spheroid attachment, whereas vildagliptin had no impact. These preliminary data suggest the need for further investigations on the inadvertent use of prescribed gliptins at implantation for couples trying to conceive.

P-151 - Loss of Wnt5a Impairs Upper Müllerian Duct Differentiation in Mice.

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Müllerian anomalies (MAs) are anatomical variations of the female reproductive tract resulting from the incomplete development of the Müllerian ducts. MAs are complex gynecological birth defects occurring in 5.5% of the general female population and 8% of infertile women. In addition, MAs are diagnosed in 13.3% of women with miscarriages and 24.5% of those with miscarriages and infertility. Association with other several congenital malformations including renal, skeletal, and cardiovascular anomalies are common. Despite the immense impact on a woman's health, the etiology of MAs remains largely unexplained. WNT5A is a critical regulator of cell migration and polarity through the noncanonical Wnt pathway. Mutations of WNT5A have been associated with Robinow syndrome (OMIM 180700), which is characterized by skeletal and genital anomalies. The most commonly reported reproductive tract malformations in individuals with WNT5A mutations are genital hypoplasia and vaginal/cervical atresia. Studies in the mouse confirmed the requirement of Wnt5a for vaginal formation but analyses involving the upper reproductive tract are lacking. Ablation of Wnt5a in the mouse results in severely hypoplastic uterine horns, suggesting that Wnt5a may have a broader involvement in Müllerian duct development. Wnt5a^{-/-} uterine horns were more than 50% shorter than wild-type, showing a hypertrophic epithelial component while the stromal component was reduced in cellularity. To better understand the role of Wnt5a, we performed single-cell RNA-Sequencing (scRNA-Seq) of developing Müllerian ducts at 18.5 days post coitum (dpc). All identified cell clusters showed a similar cell composition between Wnt5a^{-/-} and wild type Müllerian ducts, except for one cluster that was largely over-represented in Wnt5a^{-/-} samples. To further understand the phenotype, we performed qPCR analysis and immunofluorescence staining, and found that the cranial portion of the uterine horns of Wnt5a^{-/-} mice expressed markers of the fallopian tubes, suggesting a posterior to anterior homeotic transformation. Our results indicate additional roles for Wnt5a during mammalian reproductive

development. These findings help to explain further the pathophysiology of Robinow syndrome and may prompt further investigations into the uterine anatomy of complex clinical cases.

P-152 - Investigating the Female Reproductive Tract Mucosa as a Sex Specific Site for Anti-Citrullinated Protein Antibody Formation

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Rheumatoid Arthritis (RA) is an autoimmune disease characterized by the inflammation and subsequent degradation of synovial joints. While both men and women develop this disease, there is a three-fold higher rate of women suffering from RA. Women often develop the disease earlier and experience worse clinical outcomes compared to men. Years of research has been unable to explain the clear sex disparity seen in RA, despite several known reproductive factors that affect risk in women, including age of menarche, parity, and the post-partum period. Previous studies show that RA autoimmunity develops when citrullinated (cit) proteins are generated at a mucosal surface by peptidylarginine deiminase (PAD) enzymes, which post-translationally covert arginine to a non-coded citrulline residue in target proteins. Auto-antibodies to cit-proteins termed anti-citrullinated protein antibodies (ACPAs) are produced in the mucosa, but can become systemic and lead to joint inflammation. Shared mucosal sites (ie. lung, gingiva, and gastrointestinal tract) have yet to explain the sex disparity, leading us to hypothesize that the female reproductive tract (FRT) mucosa is a female specific site of ACPA formation. Specifically, we are investigating whether PAD enzymes and cit-proteins are present in FRT fluid (FRT-F) and serum across the menstrual cycle in women and estrous cycle in mice. Our results demonstrate that PAD enzymes and cit proteins fluctuate in women's FRT-F across the menstrual cycle and in wild type mouse FRT-F during the estrous cycle. Interestingly, PAD enzymes are not detectable in wild type mouse serum, but are abundant in FRT-F across the estrous cycle. The FRT has a unique immune system, prompting us to next examine if total immunoglobulin G (IgG), ACPAs, and the cytokines IL-6, IL-8, and TNF α are also present in FRT-F and serum in wild type mice. Our results show that they not only fluctuate across the estrous cycle, but their respective levels differ between FRT-F and serum. Our current studies are using mass spectrometry to identify the mouse FRT citrullinome across the estrous cycle. Ultimately, our objective is to identify possible targets for sex-specific diagnostic tests or treatments to improve clinical outcomes for women suffering from RA.

P-153 - Update on Extracellular Vesicles in Porcine Reproductive Fluids: Isolation, Proteomics and Their Interaction with Spermatozoa

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Extracellular vesicles (EVs) are membrane particles produced by various cells, including those of the male and female reproductive tract. EVs ensure intercellular communication and cargo transfer between cells and gametes. Our study is focused on the isolation and characterization of EVs in porcine seminal plasma and oviductal fluid. EVs were isolated from the biofluids by ultracentrifugation and the presence of EVs in a size range between 20 and 500 nm was determined by transmission electron microscopy. The interaction between isolated seminal plasma EVs and spermatozoa (acrosome and flagellum) was uniquely confirmed by structure illumination microscopy (SIM) super-resolution technique (SR-SIM). Since one of the important roles of EVs is the transport of the specific proteins we investigated differences between protein profiles of EVs isolated from seminal plasma and oviductal fluid. These EVs' protein profiles were determined using mass spectrometry (MS), and the Western blot (WB) analysis showed the presence of spermadhesins originating from both aforementioned biofluids. The CD9 and CD81 as major EVs markers were proved by WB analysis, however, MS identified solely CD9 protein marker in the EVs originating from the oviductal fluid. The potential applications of EVs in reproductive biology are significant, as they have been shown to play an essential role in cargo transport to the gametes influencing their maturation and fertilizing ability. In our study for the first time, we confirmed by SR-SIM the interaction of boar sperm with EVs delivered by seminal plasma. Moreover, the presence of spermadhesins in the EVs cargo provides further insight into the EVs biology. Finally, different expressions of CD9 and CD81 markers determined by various methodological approaches introduce important insight into the methods used to study of EVs. Acknowledgment: This work was supported by GACR (GA22-31156S), by institutional support RVO (86652036), and by the BIOCEV project (CZ.1.05/1.1.00/02.0109) from the ERDF. We acknowledge the Imaging Methods Core Facility at BIOCEV, an institution supported by the MEYS CR (LM2018129 Czech-Biolmaging) for their support & assistance in this work, and Dr. Petr Pompach, Ph.D. from the Proteomic Core Facility, IBT CAS, BIOCEV for MS analysis.

P-154 - Peptidylarginine Deiminase 2 and 4 Double Knockout Mice Reveal Citrullination is an Important Post Translational Modification that Regulates Female Reproduction

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Peptidylarginine deiminases (PADs) are a family of enzymes that post-translationally convert positively charged arginine residues into a neutrally charged citrulline, through a reaction termed citrullination. Citrullination, a novel post-translational modification, can alter target protein structure and function. Our previous work discovered that PAD expression is elevated during the estrus phase of the estrous cycle in anterior pituitary gonadotrope cells and citrullination can epigenetically regulate the production of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). To further explore the role of PADs in vivo, we utilized a PAD 2/4 double knock out (DKO) mouse model. Previous work found male PAD2/4 DKO mice have decreased testis

size, altered gonadotropins, and are subfertile compared to wild type mice. Given the complexity of the female reproductive axis, it is unclear if PADs may play a sexually dimorphic role in regulating reproduction. To test this, we first measured pubertal onset in female PAD2/4 DKO mice and found that it takes approximately 5 days longer to initiate puberty. Additionally, female PAD2/4 DKO mice spend significantly longer in the diestrus phase of the estrous cycle and have smaller uteri and ovaries compared to wild type controls. In pituitaries from female PAD2/4 DKO mice, LH β mRNA is decreased compared to controls during estrus, which is consistent with our previous work showing an epigenetic role for PADs in regulating histones around the *lhb* gene promoter. Taken together, our work suggests that PAD expression and activity may be a mechanism that facilitates puberty initiation and the high levels of gonadotropin gene expression necessary for ovulation. Ongoing work is examining PAD2/4 DKO serum gonadotropin levels and single cell transcriptomic profiles across the female reproductive axis.

P-155 - Regulation of Basigin Production by Steroid Hormones and Secretion of Extracellular Vesicles in Bovine Uterine Epithelial Cells

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Basigin (BSG), a transmembrane glycoprotein, is a protein of interest due to its involvement in processes crucial to reproduction including uterine remodeling and implantation. Our objectives were to investigate steroid hormone regulation of BSG in a bovine uterine epithelial cell line (BUTE) and begin to characterize the contents of extracellular vesicles (EVs) shed by these cells. BUTE cells were treated with 0, 30, 300, and 3000 nM of progesterone (P4) 0, 30, 300, and 3000 pM of estradiol (E2), or 0, 10, 50, and 100 ng/ml insulin like growth factor (IGF-1) for 24 hours. Total protein in the cell lysates was measured via BCA. Immunoblotting of cell lysates was carried out to quantify expression of BSG. EVs from BUTE cell-conditioned medium were isolated by ultracentrifugation and characterized by transmission electron microscopy, Nano-Tracker analysis, and immunoblotting for exosome biomarkers CD09 and CD81. Immunoblotting of isolated EVs was carried out to identify the expression of basigin (BSG). BUTE cell EVs were subjected to liquid chromatography mass spectrometry (LC-MS) analysis to identify different biological pathways in which those proteins are involved. BSG expression in BUTE cells was upregulated by 300 and 3000 nM P4 and downregulated by 50 and 100 ng/ml IGF-1. However, E2 had no significant effect. This hormone-dependent regulation of BSG supports a role for BSG in bovine endometrial function. EVs from BUTE cell-conditioned medium were successfully isolated and characterized. The EVs, visualized through transmission electron microscopy, exhibited the typical cup-shaped morphology indicative of exosomes. Nano-Tracker analysis demonstrated the presence of small and large vesicles in the expected size range for exosomes (20-150 nm) and microvesicles (150-500 nm). Immunoblotting confirmed the presence of CD09, CD81, and BSG in isolated EVs. EVs were subjected to LC-MS analysis and a total of 2808 proteins were identified including CD9, CD81, basigin, semaphorin, IGFBP1, MMP-2, galectin-1, and CD44. Immunoblotting results of CD9, CD81, and BSG were consistent with LC/MS results. The identified proteins were involved in different molecular functions, biological processes, and cellular components. Some of them are translation, membrane, extracellular exosome and actin cytoskeleton organization in cellular component category and protein binding, GTP binding, and anion binding in the molecular function category

respectively. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway category showed enrichment of proteasome, carbon metabolism, and nucleocytoplasmic transport. Our study provides insight into the hormonal regulation of BSG expression in BUTE cells, highlighting the impact of P4 on BSG expression in these cells. Isolation, characterization, and LC/MS analysis of proteins in EVs shed by BUTE cells opens opportunities for further investigation into the functions and significance of EVs in the bovine uterus. Key words: Basigin, Steroid hormones, Extracellular vesicles, TEM, Nano-tracker, Western blot

Financial support: This work was supported by a Future Interdisciplinary Research Exploration Grant from the College of ACES, University of Illinois (RAN)

P-156 - Utilising 3D Culture Systems to Investigate the Effect of Conceptus-Derived Factors Upon the Bovine Endometrium

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Conceptus-endometrial bi-lateral communication is essential for successful early pregnancy in mammals including cattle. Interferon Tau (IFNT -the pregnancy recognition signal) is secreted by the bovine conceptus, to block luteolysis- thereby maintaining progesterone secretion from the corpus luteum, to sustain pregnancy. The bovine conceptus also secretes additional proteins, including macrophage capping protein (CAPG) and protein disulphide isomerase (PDI), which we have previously shown using 2D static in vitro culture system to alter the endometrial transcriptome. Our aim was to utilise novel 3D in vitro models (glandular organoids and a 3D microfluidic endometrium-on-a-chip) to better model the endometrial response to CAPG and PDI. Using our 3D bovine endometrium-on-a-chip, we cultured culturing primary bovine stromal (bESCs) or epithelial cells (bEECs) on either side of a porous membrane (n=3). Conceptus-derived factors (recombinant bovine CAPG [rbCAPG] or PDI [rbPDI]) were added to the culture medium (RPMI 1640, 10% FBS, 1% antibiotic antimycotic) at 1 µg/mL and flowed through the chip over the bEECs at 0.8mL/min flow rate for 24 hours, alongside PBS vehicle control samples (n=3). Unconditioned medium samples were also collected (n=2). Conditioned medium was collected and underwent tandem-mass-tag nano-LC mass spectrophotometry analysis, while recovered cells, were sequenced using Illumina NextSeq 500 with a single end 75bp length read. In tandem, bovine endometrial glandular organoids were produced and exposed to conceptus-derived factors (rbCAPG, rbPDI, and recombinant ovine IFNT [roIFNT]) for 24 hours at 1mg/mL (n=5). Organoids were collected and subjected to sequencing using the Illumina Novaseq 6000 with a paired end 150bp length read. All sequencing data underwent analysis at Leeds 'Omics at the University of Leeds to identify differentially expressed genes (DEGs), and downstream enrichment analysis was performed using Webgestalt. All data were also compared to DEGs previously identified in our 2D static culture systems. The addition of rbCAPG in the 3D microfluidic endometrium-on-a-chip system flow through medium resulted in 228 DEGs (padj< 0.05) in epithelial cells compared to

controls. Enriched gene ontologies including signal transduction in the absence of ligand, cell adhesion mediated by integrin, and muscle cell migration, were significantly enriched (FDR< 0.05). Ninety-one DEGs were also differentially expressed in response to rbCAPG in static 2D culture. The addition of rbPDI altered 314 DEGs (padj< 0.05) in epithelial cells, within which signal transduction in absence of ligand, cell adhesion mediated by integrin, and inflammatory response were enriched gene ontologies (FDR< 0.05). One hundred and twenty-five DEGs were also altered in a 2D static culture in response to rbPDI. Exposure to rbCAPG altered abundance of 24 proteins in conditioned medium, and exposure to rbPDI altered abundance of 17 proteins when compared to vehicle control ($p < 0.05$). In bovine glandular organoids, roIFNT elicited 552 DEGs compared to vehicle control samples after 24 hours (padj< 0.05) while 0 DEGs and 7 DEGs (padj< 0.05) were identified in rbCAPG and rbPDI treated organoids respectively. Exposure to conceptus-derived proteins PDI and CAPG altered pathways in the endometrial epithelium in vitro which may promote early pregnancy processes such as attachment during implantation, immune response to conceptus, and altering secretion of proteins into the uterine luminal fluid. In contrast, there was no effect on the deep glandular epithelial organoids, but IFNT elicited a transcriptional response, mostly altering immune-related pathways, in the endometrial glandular organoids. These data demonstrate that the effect of conceptus-derived proteins is cell-type specific.

P-157 - Long-term Consequences of Repeated Superovulation on the Ageing Mouse Female Reproductive Tract

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The use of assisted reproductive technologies and hormonal ovarian stimulations has become widespread in developed countries. While the health of children born from these procedures has been followed through adulthood, the long-term consequences on the parents' or donors' health, especially of their reproductive tissue, remains understudied. This knowledge gap limits our ability to inform patients of the potential long-term risks of such procedures and to provide relevant follow-up monitoring and care. We hypothesize that the increased number of tear-repair events in the ovaries caused by hormonal stimulations could lead to premature ageing of the ovarian tissue. We aged three cohorts of mice up to 18 months: naturally or after five cycles of superovulation at 1 or 9 months. We apply single-cell and spatial transcriptomics to follow the natural ageing of the female reproductive tract, and compare how repeated cycles of superovulation perturb the ageing time course. These experiments allow us to better understand how repeated superovulation impacts the ageing trajectory of the female reproductive tract in terms of morphology, physiology, and genomics and will provide insights into the potential pathologies that superovulation could induce long-term.

P-158 - Intrauterine Administration of Lipiodol® Enhances Fertilization Rates in Mice Inseminated with Low Sperm Numbers

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In the context of couple infertility, hysterosalpingography (HSG) is a common diagnosis used primarily to assess the patency of fallopian tubes in women. The technique consists in visualizing by radiography a contrast media injected into the uterus which fills the fallopian tubes before overflowing in the peritoneal cavity. Interestingly, several reports indicated a therapeutic side effect on fertility after HSG, with the observation of improved pregnancy rates, especially when using oil-based contrast media (Lipiodol®, ethyl esters of iodinated fatty acids of poppy seed oil) compared to water-based contrast media (1). However, the physiological mechanisms underlying this effect remain unknown. In order to understand the beneficial effect of Lipiodol® administration on fertility, we developed a specific protocol using the mouse model. Lipiodol® was administrated into the mouse uterus, followed by intrauterine artificial insemination carried out two weeks later, corresponding to at least two mouse oestrus cycles (representing what is usually found in women, with pregnancy occurring usually two months after HSG). Mice were then sacrificed and all the oocytes/zygotes were collected and cultured in vitro to measure the fertilization rates. In our first attempts, we observed comparable rates of fertilization ($67 \pm 5.4\%$ and $60 \pm 8\%$, $p > 0.05$) from oocytes/zygotes originating from Lipiodol® treated and untreated control mice. This showed that, as practiced, uterine Lipiodol® injection has no deleterious effect but did not induce any improvement in fertilization. We postulated that a potential effect of the administration might be masked by the already optimal reproductive system of young wild type mice. We therefore decided to impair the reproductive efficiency of our model by mimicking oligozoospermia via inseminating the mice with a limiting number of spermatozoa. As expected, the number of fertilized oocytes was decreased but the overall fertilization rate was significantly improved in Lipiodol® administered mice compared to the controls ($29.9 \pm 4.8\%$ and $12.6 \pm 3\%$, $p < 0.05$). Overall, we established an amenable model to study the pro-fertility effect of Lipiodol® intrauterine administration and demonstrated that under a restrictive condition, Lipiodol® administration improved fertilization. 1 - Dreyer, K. et al. Oil-Based or Water-Based Contrast for Hysterosalpingography in Infertile Women. *N Engl J Med* 376, 2043–2052 (2017).

P-159 - The Conserved MicroRNA miR-151 Alters the Ability of the Endometrial Epithelium to Facilitate Implantation

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In order to determine what the regulatory networks are required to facilitate uterine receptivity to implantation in mammals our group has previously identified a core set of microRNAs that arose when placental mammals arose and have been retained in all species studied. As repressors of gene expression and consequentially protein synthesis, miRNA may not only play a role in successful implantation but could also highlight fundamental pathways involved and/or dysregulated in implantation failure. With that in mind we hypothesise that these identified microRNA's hsa-miRNA-151a-3p and hsa-miRNA-151a-5p may function to facilitate implantation in placental mammals. The aims of these experiments were 1) to identify the potential mechanism by which these miRNAs may facilitate receptivity to implantation in vitro, and 2) to investigate if these miRNAs functionally affected implantation. Human endometrial epithelial (Ishikawa) cells were transfected with 1) control, 2) transfection reagent alone, 3) non-targeting mimic, 4) non-targeting inhibitor, or 5-8) mimic or inhibitor for miR-151a-3p, or -151a-5p, alone for 48 hrs. Protein was isolated from samples (n=3 per group) and subjected to tandem mass tag proteomic analysis to determine how these microRNAs alter the proteome of the cell. In parallel, BeWo spheroids were generated and co-cultured with Ishikawa cells transfected with either 1-8 above and the spheroid attachment rate quantified by allowing spheroids to attach to the endometrial monolayer for 30 minutes and calculating the percentage remaining attached, after washing. Proteomic analysis of the endometrial monolayers transfected with miRNA-151a-3p and miRNA-151a-5p mimics/inhibitors against relevant controls, displayed a large shift in protein expression. Dysregulation of miRNA-151a-3p was shown to significantly affect 1738 proteins with 33 of these proteins showing dysregulation by both mimic and inhibitor treatment. Similarly, treatment of cells with miRNA-151a-5p altered 1788 proteins, with 80 of these proteins changed by both miRNA 151a-5p mimic and inhibitor. Interestingly, from these 80 proteins, a total of 46 proteins were dysregulated in opposing directions with transfection of miRNA mimics and inhibitors, indicating a strong likelihood of miRNA targeting. Further DAVID analysis of the proteins significantly affected by our miRNA showed that both miRNAs 151a-3p and 151a-5p regulated cytoskeletal function, microtubule formation, cell division, and mitosis. miRNA 151a-3p regulated proteins involved in; stem cell differentiation, epithelial cell proliferation, actin binding, Wnt pathway signalling, epidermal growth factor stimulated responses and T cell differentiation. Most interestingly of all, miRNA 151a-5p was shown to regulate proteins involved in; in utero embryonic development, cellular differentiation embryonic placenta development, lactation and female pregnancy. There was functional effect of these microRNAs on the ability of the epithelial cells to facilitate attachment as inhibition of miRNA-151a-3p increased the percentage of spheroids that attached, whilst increased abundance of miRNA-151a-5p was shown to increase attachment percentage, when compared to attachment in non-specific miRNA mimic/inhibitor transfected cells. To further develop these findings, we are developing the assay with the addition of a physiological like flow system using the IBIDI μ -Slide VI 0.4 ibiTreat and a more high-throughput assay is also under development using the fluorescent spheroid approach. These data collectively show that miR-151 plays an important role in facilitating implantation in mammals.

P-160 - Characterization of Three-dimensional Scaffold-based Model of the Porcine Endometrium in Vitro

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During the reproductive/estrous cycle, the endometrium of placental mammals undergoes cyclical remodeling. This process allows the endometrium to become receptive for the implantation of the developing embryo and prepare the uterus for pregnancy. Currently, the most popular research models are based on 2D in vitro cultures. However, it has been shown that culture on flat surfaces affects cells by changing their metabolism, gene expression and proliferation. Therefore, it has become important to develop solutions that will allow obtaining results as close as possible to in vivo conditions. One of the most promising solutions to achieve these goals is the use of 3D in vitro models. Three-dimensional endometrial in vitro systems may allow studying physiology aspects of the endometrium by reproducing the micro-environment where the early embryo develops. Therefore, the aim of the present study was to develop a 3D in vitro model of the porcine endometrium using synthetic polymer scaffold (Alvetex®). Endometrial stromal (ST) and luminal epithelial (LE) cells were enzymatically isolated from the uterus of gilts (n=3) in the mid-luteal phase of the estrous cycle. In the first stage, ST cells were seeded onto the Alvetex® scaffold following guidelines provided by manufacturer and cultured for 3 weeks. During this time scaffolds were fixed at 3 time points (day 7, day 14 and day 21) for immunofluorescence and immunohistochemistry analysis. Extracellular matrix proteins (ECM) synthesis by ST cells was assessed and once an appropriate layer of ST cells was obtained, LE cells were seeded on the surface of the scaffold and cultured for another week (day 28) and fixed for analysis. Hematoxylin-eosin staining cross-sections after 7, 14 and 21 days of culture showed that ST cells penetrated homogeneously into the scaffold and showed typical fibroblast morphology. Moreover, ST cells showed positive vimentin staining throughout the culture period. Additionally, the presence of ECM was demonstrated on day 7, 14 and 21 of culture. After 21 days of culture, the stromal layer of endometrial fibroblasts served as a foundation to grow LE cells. On day 28 of culture, LE cells grew on the scaffold surface, showed positive staining with cytokeratin and typical epithelial morphology. The present report demonstrates that Alvetex® scaffolds can support the porcine endometrial cell culture for long periods. Moreover, the reported approach is a promising model for the study of physiology of the endometrium and embryo-maternal interactions during early pregnancy. This research was supported by the National Science Centre in Poland, grant no. 2022/06/X/NZ9/00705.

P-161 - Prevalence of Dysmenorrhea and its association with Hemoglobin Levels Among Saudi Females

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Dysmenorrhea is a gynecological condition characterized by menstrual pain, it is prevalent among Saudi females, impacting their overall well-being. This study aims to explore the prevalence of dysmenorrhea and its association with concentration of blood hemoglobin in this population, providing valuable insights into potential strategies for improving menstrual health. To conduct the study, a cross-sectional pilot was performed at the University of Hafr Albatin in Saudi Arabia involving 136 university students. Participants underwent capillary blood collection to measure

hemoglobin levels and completed a survey detailing demographic information and dysmenorrhea characteristics. Statistical analyses were then used to identify the association between hemoglobin levels and dysmenorrhea severity. Demographic analysis revealed that over half of the participants were aged between 21 and 23, and almost 98% experience varying degree of dysmenorrhea. Nearly 50% of participants were anemic. The mean hemoglobin level was 12.07 g/dl, with anemic participants showing a lower mean of 10.7 g/dl. Further analysis indicated a significant association ($p=0.027$) between hemoglobin levels and the severity of dysmenorrhea. It was observed that females with low hemoglobin level were more likely to experience severe dysmenorrhea compared to non-anemic females who reported mild to moderate menstrual pain. Among anemic participants, dysmenorrhea severity varied, with diverse menstrual patterns and associated symptoms. Monthly pain significantly interfered with daily activities, and concentration during lectures was notably affected. The majority reported a medium level of pain, lasting 1-2 days, and 53% reported 1–3 absences from classes every month. In conclusion, this study highlights a high prevalence of dysmenorrhea among Saudi females affecting as many as 98% of the study participants. The findings also demonstrate a significant association between blood hemoglobin concentration and the severity of dysmenorrhea in this population group. These findings stress the importance of targeted interventions and support systems for improving females' health and quality of life. Future research should focus on exploring strategies and longitudinal approaches to gain an understanding of how hemoglobin levels relate to dysmenorrhea.

P-162 - Transcriptomic Analysis Reveals Effects on Oxidative Phosphorylation and Canonical Wnt Signaling by Form of Selenium in The Intercaruncular Tissue of Beef Heifers at Maternal Recognition of Pregnancy

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Selenium (Se) is supplemented to the diet of grazing beef cattle in regions where the soils and hence forages are deficient in this mineral. When cattle consume forage, organic forms of Se (OSe) are physiologically available, whereas inorganic Se (ISe) is typically used in commercial vitamin-mineral supplements. We have investigated the effects of supplemental Se as ISe or a 1:1 mixture of ISe:OSe (MIX) and have observed significant effects on the corpus luteum (CL) and endometrium in grazing beef cows and heifers. In comparison to ISe, heifers supplemented with the MIX-form Se have an increase in peripheral concentrations of progesterone (P4) during the early luteal phase of the estrous cycle, at a time when increased P4 has been shown to improve embryonic development. Indeed, we have reported that form of Se affects the abundance of mRNA transcripts encoding IFN τ - and P4-induced proteins in the caruncular (CAR) and intercaruncular (ICAR) tissues of the uterine endometrium at maternal recognition of pregnancy (MRP), and that MIX-form Se increases length of the conceptus. To expand upon previous findings, we used RNA-sequencing herein to investigate transcriptomic changes in the ICAR tissue of the endometrium at MRP. Twenty Angus-cross heifers underwent a 45-day period with no supplemental Se (depletion) followed by a 45-day period with ISe alone (repletion). Heifers were then randomly assigned to treatment of a vitamin-mineral mix containing 35-ppm Se as ISe ($n = 10$), or 1:1 mixture of ISe:OSe ($n = 10$, MIX) for 90 days before insemination. Heifers were then killed on d17 of presumed pregnancy. Reproductive tracks and conceptuses were collected and only heifers with a fully intact conceptus were utilized

for further analyses (n = 6 per treatment). RNA-sequencing was conducted on total mRNA from ICAR biopsies to explore the transcriptomic effects in response to form of Se. Differences in gene expression were determined using Differential Expression and Pathway Analysis (iDEP.96), and differentially expressed genes (DEG) were subjected to canonical, functional, and network analyses using QIAGEN's Ingenuity Pathway Analysis (IPA). RT-PCR was used to corroborate RNA-sequencing results. At $P < 0.05$, there were 838 DEGs with 427 transcripts upregulated, and 411 transcripts downregulated in MIX versus ISe heifers. The top canonical pathway upregulated in ICAR tissue of MIX versus ISe heifers was oxidative phosphorylation with upregulated transcripts encoding components of complex I (NADH dehydrogenase), complex III (cytochrome c reductase), and the rate limiting complex IV (cytochrome c oxidase). This suggests an increase in the production of ATP in the ICAR of MIX supplemented heifers compared to ISe. An increase in energy may permit the MIX-form to meet an increase in demand for glucose and nutrients, consistent with the increase in conceptus length previously observed at MRP. Additionally, we observed significant effects on the abundance of transcripts encoding proteins in the canonical Wnt signaling pathway (DKK1, FZD6, LRP6, CTNNB1) in MIX compared to ISe indicating a change in the signal transduction pathway that affects cellular proliferation and functions during this peri-implantation period. Overall, results to date are consistent with our premise that supplementation with MIX as a 1:1 ratio of ISe and OSe, versus ISe as the industry standard, induces a more favorable physiological response at the establishment of pregnancy in beef cattle consuming forages grown on Se-poor soils.

P-163 - Decoding The Biochemical Uterine Environment Essential for Successful Establishment of Pregnancy in Pigs: A Metabolomics View

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Synchronous communication between the uterus and conceptus (embryo/fetus and its extraembryonic membranes) is imperative for establishment and maintenance of pregnancy in pigs, sheep and other ruminants. The biochemical milieu within the uterine lumen, orchestrated by a myriad of metabolic pathways, is pivotal in facilitating cytokine secretion, conceptus elongation, and placentation in pigs. However, little is known about the metabolic dynamics among the endometrium, conceptuses, and their crucial intermediary, histotroph, during the peri-implantation period of pregnancy in pigs. Thus, this study aimed to determine the metabolome in pig endometrium, conceptus, and uterine fluids between days 10 and 16 of pregnancy. We hypothesize that the metabolism status varies distinctly across different tissue types and gestational stages within the uterine environment. Gilts were bred via artificial insemination at 12 and 24 h after onset of estrus (day 0). On gestational day (GD) 10, 11, 12, 13, 14, 15, and 16, uteri (n=5-6 gilts per day) were flushed with 20 ml sterile PBS (pH 7.2) after gilts were subjected to a midventral laparotomy.

Recoverable conceptuses and endometrium tissue were then subjected to metabolomic analyses by Liquid Chromatography-Mass Spectrometry (LC-MS), raw data was extracted and normalized to tissue weight (endometrium and conceptus) or recovered fluid volume (UF) prior to further analysis. A total of 231 metabolites were detected in endometrium and conceptus, and 192 in the UF. Clear separations between the endometrium and conceptus were observed on days 10 to 16 based on principal component analysis (PCA), suggesting different metabolic pathways employed by endometrium and conceptuses. Through longitudinal analyses using KEGG pathways, we revealed significant alterations in metabolite profiles across different days of porcine gestation: specifically, 47, 24 and 18 metabolites were found to be significantly altered (t-test, $P < 0.05$) when comparing days 10 vs. 12, 12 vs 14, and 14 vs. 16, respectively. Notably, these alterations were observed to enrich in key metabolic pathways, with arginine and proline metabolism, vitamin B6 metabolism, and arachidonic acid metabolism emerging as prominent pathways within the endometrium. Moreover, within the conceptus, thiamine metabolism, ether lipid metabolism, and synthesis/degradation of ketone bodies were predominant among the 42, 61, and 20 altered metabolites identified in the respective comparisons. This study also revealed significant changes in amino acid concentrations across different uterine environments during the peri-implantation period. Interestingly, the quantitative trends of amino acids were highly similar in both UF and conceptus, but not in the endometrium. For instance, histidine and glutamine exhibited a significant increase in concentration throughout the peri-implantation period (day 16 vs. day 10) in both UF and conceptus (10.1- and 8.9-fold increase, respectively, $P < 0.05$), while remaining unchanged and decreasing by 2.5-fold in the endometrium, respectively. Conversely, serine and tryptophan showed a significant decrease in UF and conceptus but remained unchanged and increased by 1.5-fold, respectively, in the endometrium ($P < 0.05$). Unexpectedly, glycine decreased in total amount/concentration across all three types of milieus, warranting further functional investigation into metabolism differences within the uterine lumen during the peri-implantation period of pregnancy. Overall, these findings provide comprehensive insights into the biochemical environment within the endometrium, conceptuses, and UF, highlighting unique metabolic needs dictated by spatial and temporal changes and guiding future research directions to facilitate conceptus growth during the critical development stage. This research was supported by Agriculture and Food Research Initiative competitive Grant 2022-67015-36491 from the USDA-NIFA.

P-164 - Loss of Uterine Histone Deacetylase Activity in Neonatal DES Exposed Mice Contributes to Persistent Changes in Gene Expression and the Epigenome

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Estrogen exposure during development results in abnormal uterine differentiation that leads to infertility and cancer. Mice exposed to diethylstilbestrol (DES) exhibit extensive uterine gene expression changes that are accompanied by excessive histone H3K27ac occupancy at nearby enhancers. Histone deacetylase proteins are reduced following neonatal DES exposure and could contribute to this phenotype. To test this idea, we generated a mouse line in which HDAC2 was

conditionally overexpressed in the uterus (HDAC2cOE) to prevent the DES-induced reduction in HDAC activity. HDAC2cOE mice exposed neonatally (postnatal day 1 [PND1] to PND5) to DES (1 mg/kg/day) had 529 differentially expressed uterine genes (DEGs) when compared to wild type (WT) DES-exposed mice on PND5. This number represents ~10% of the 6,328 DEGs between WT DES-exposed and WT control (CON) uteri. In adults, there were 1,495 DEGs when comparing DES-exposed HDAC2cOE and WT; 1,009 (~11%) of these overlapped the 9,174 DES induced DEGs when comparing WT DES versus WT CON. HDAC2 overexpression resulted in extensive removal of acetylated H3K27 but only minor changes in H3K9ac for both CON and DES as determined by CUT&RUN sequencing. Robust epigenetic changes were observed in WT DES compared to WT CON with 4,155 regions of H3K27ac gain; of these, 1,866 (45%) were reduced toward WT CON levels in the HDAC2cOE DES group. These 1,866 locations were near 752 DES-induced DEGs including *Wnt5a*, a gene important for uterine differentiation. An overlap of PND5 and adult DES-induced H3K27ac revealed ~400 locations that persisted over time. We conclude that reduced HDAC activity accounts for a small but significant percentage of the persistent DES induced epigenome and gene expression changes observed following neonatal DES exposure. These findings suggest that alterations in the levels of chromatin remodeling proteins induced by developmental chemical exposures is one mechanism to explain the developmental origins of adult health and disease.

P-165 - Remodeling of the Uterine Luminal Metabolome prior to Embryo Elongation in Beef Cattle

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In cattle, pregnancy losses that occur in the initial three weeks of gestation remain a significant challenge for the industry. For proper embryonic growth during that window, the uterine luminal environment must contain adequate concentration of molecules required by the growing embryo. We hypothesized that luminal concentrations of amino acids and lipids change over time in association with specific milestones of embryonic growth. The objective was to compare the concentrations of amino acids and lipids and evaluate their temporal dynamics of change in the uterine luminal fluid on days of the estrous cycle that coincide with the blastocyst stage (seven days after estrus, D7), embryo hatching (D10) and conceptus initial elongation (D14) in beef cows. The estrus of 22 *Bos indicus*-influenced cross-bred, primiparous, non-lactating, cyclic cows was synchronized by injecting prostaglandin F2alpha twice, 14 days apart. Five animals whose estrus were synchronized remained in the study. On D7, D10 and D14, luminal fluid was collected from the uterine body of each animal using a cytology brush. Fluid was subsequently diluted in PBS, centrifuged, filtered, and stored at -80 °C for analysis. Concentrations of 630 metabolites from 26 biochemical classes were measured on samples by targeted metabolomics. The effect of day was

tested using univariate analysis (one-way ANOVA). Overall, the concentration of 86 metabolites was affected by time ($P < 0.1$). There were 11 amino acids, four amino acids-related, 56 lipids (diacylglycerols, glycerophospholipids, glycosylceramides, sphingolipids and triacylglycerols) and 15 metabolites from other classes. In general, luminal concentrations of amino acids and amino acids-related metabolites increased from D7 to D10, ranging from 210.44% increase for glutamine ($P = 0.006$) to 62.3% increase for asparagine ($P = 0.07$), then decreased from D10 to D14 ranging from 53.78% decrease for glycine ($P = 0.09$) to 3.7% decrease for tryptophane ($P = 0.1$). Amino acid metabolism was also affected over time. Glutaminase activity increased 62.92% between D10 and D14 ($P = 0.06$). Glycine synthesis was greater on D7 (29.72 ± 8.5) than on D10 (9.50 ± 1.94) and D14 (8.05 ± 1.63 ; $P = 0.004$). The concentration of essential amino acids increased 134.96% between D7 and D10 ($P = 0.09$) and ratio of non-essential to essential amino acids decreased from 26.79 ± 4.48 on D7 to 11.60 ± 0.80 on D14 ($P = 0.02$). Lipids, in general, exhibited a gradual increase in concentration from D7 to D14, ranging from 466.66% increase for PC aa C34:3 ($P=0.02$) to 87.77% increase for PC aa C36:1 ($P=0.08$). In summary, the uterine luminal biochemical milieu changes dramatically in composition during the second week of the estrous cycle. Such changes are likely connected functionally with the transition from blastocyst to initial elongation of the conceptus. We speculate that alterations on such dynamics impact embryo development and determine the pregnancy outcome in cattle. This study is supported by USDA NIFA Award Number 2022-67015-36839.

P-166 - Sustained YAP Activity Induces Transdifferentiation of Luteal and Müllerian Mesenchymal Cells in Mice

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The Hippo pathway plays a pivotal role in regulating cell growth and differentiation in the reproductive system throughout embryonic and postnatal stages of development. Deletion of the Hippo pathway kinases Large tumour suppressor (LATS) 1 and 2 in the ovary and Müllerian duct has been shown to lead to the overactivation of the downstream transcriptional coactivators Yes-associated protein (YAP) and the transcriptional coactivator with a PDZ-binding motif (TAZ) and the transdifferentiation of granulosa and Müllerian mesenchymal cells. However, the relative contribution of YAP and/or TAZ (and/or additional effectors downstream of LATS1/2) to these phenotypic changes remains unclear. To assess the impact of YAP on the transcriptome in granulosa cells, RNA-seq analyses were conducted on granulosa cells from eCG-primed immature Rosa26Yap5SA/Yap5SA mice transduced with adenoviruses to drive Cre or eGFP (control) expression. In this model, Cre recombinase permits the expression of a transgene encoding a constitutively active, stable and nuclear mutant YAP protein (YAP5SA), co-expressed with the reporter gene LacZ. The analyses revealed that 129 genes were upregulated and 147 downregulated when the transgene was expressed (P corrected < 0.05 ; fold-change $> |1.5|$), most of which were associated with biological processes related to cell differentiation and angiogenesis. To gain a deeper understanding of YAP's role in gonadotropin-dependent folliculogenesis, Rosa26Yap5SA/+; Cyp19-cre were generated to drive YAP5SA expression in antral follicles. Unexpectedly, X-GAL

staining of Rosa26Yap5SA/+; Cyp19-cre ovaries revealed that Yap5SA was expressed only in a fraction of luteal cells. These LacZ-positive cells later formed roughly spherical nests of poorly-differentiated, vimentin-positive cells embedded in an abundant collagen matrix. BrdU incorporation showed that some of these cells were proliferative, and PECAM1 staining showed the lesions to be highly vascularized. Furthermore, seminiferous tubule-like structures containing cells positive for SOX9 were observed in the ovaries of older mice. Surprisingly, the presence of these lesions did not significantly impact fertility or serum progesterone levels in Rosa26Yap5SA/+; Cyp19-cre mice. Although the Rosa26Yap5SA/+; Cyp19-cre model was not found to be useful for studying the role of YAP during folliculogenesis, it showed that luteal cells are capable of undergoing dedifferentiation and transdifferentiation in response to sustained YAP activity, thereby partially recapitulating the effects of loss of Lats1/2 in granulosa cells. To determine the impact of YAP overactivation on reproductive tract development and function, the Rosa26Yap5SA/+; Amhr2cre/+ mouse model was generated in order to target the Müllerian mesenchyme. Transgene expression was (indirectly) detected by X-GAL staining starting at embryonic day 17.5. Expression was initiated at the rostral end of the Müller ducts and the ovary, and extended caudally to the antimesometrial side of the ducts. Histological analyses revealed disorganization of the Müllerian duct mesenchyme, with the presence of a population of spindle-shaped, X-GAL-positive cells and increased abundance of extracellular matrix. Postnatally, these cells were present in endometrial mesenchyme and myometrium, causing a disorganization of myometrial layers and the deposition of collagen. The development of oviduct was also impeded by the proliferating X-GAL-positive cells, interfering with elongation and coiling, and causing dilations to occur. Ongoing studies are aimed at further characterizing the histopathologic changes observed in the uterus, and to identify the changes in the expression of YAP's target genes that underlie them.

P-167 - Role of NCOR1 in Implantation and Decidualization

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Epigenetic regulation plays an important role for steroid hormone responses in normal uterus. Nuclear receptor corepressor 1 (NCOR1) interacts with SMRT/HDAC3 complexes to repress target gene transcription. Furthermore, NCOR1 is a key regulator of both PGR and ESR1 transcriptional activity. Our previous work has elucidated that HDAC3 is downregulated in the endometrium of infertile women with endometriosis and that loss of HDAC3 results in non-receptive endometrium and female infertility due to dysregulation of PGR and ESR1 signaling. Our immunohistochemical analysis revealed that NCOR1 was significantly decreased in endometrial epithelial and stromal cells from infertile women with endometriosis compared to controls. It suggested that NCOR1 plays a critical role in normal endometrial functions during early pregnancy. To investigate the role of NCOR1 during early pregnancy, we generated a mouse model with Ncor1 conditionally ablated in Pgr-positive cells (Pgrcre/+Ncor1f/f; Ncor1d/d). Fertility test was performed by mating 8-weeks old control (Ncor1f/f) and Ncor1d/d mice with wild-type male in 6-month period. Ncor1f/f mice were

fertile with an average of 4.86 ± 0.34 litter per mouse and 33 ± 2.18 pups per mouse. However, 6 of 7 Ncor1d/d mice (85.7%) were infertile and one Ncor1d/d mice was severe subfebrile with one litter. Ncor1d/d mice showed normal histology of ovary, fertilization, and the serum levels of E2 and P4. To determine whether Ncor1 loss impacts at early pregnancy, high frequency ultrasounds were performed to monitor pregnancy at GD5.5, GD7.5 and GD9.5 in uterus of Ncor1f/f and Ncor1d/d mice using Fujifilm Vevo 3100 Preclinical Imaging System. Although Ncor1d/d mice showed normal number of implantation sites at GD5.5, the sizes of implantation sites in Ncor1d/d mice were significantly smaller compared to Ncor1f/f mice. After that, we could not detect implantation site at GD7.5 and GD9.5 in Ncor1d/d mice. This result suggests that NCOR1 deficiency in the uterus causes early pregnancy loss. To address a defect of embryo implantation in Ncor1d/d mice, mice were dissected at GD4.5. Ncor1f/f mice showed visible implantation sites in uterine, but implantation sites in uterine of Ncor1d/d mice at GD4.5 could be divided into 2 groups: Group #1 with implantation sites were visible (4/10, 40%) and Group #2 with no implantation site (6/10, 60%) in the uterine. Next, we performed artificial decidualization to examine the effect of Ncor1 ablation on decidualization. While Ncor1f/f mice displayed a decidual uterine horn that responded well to this decidual induction, Ncor1d/d mice exhibited a defect of decidualization. These results suggest that Ncor1d/d mice results in early pregnancy lost due to defects of implantation and decidualization. This work was supported by NIH P01HD106485, R01HD101243, and R01HD102170.

P-168 - Molecular Characterisation of the Marsupial Histotroph: A Dynamic Uterine Fluid Microenvironment

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Australian marsupials are facing a biodiversity crisis, requiring quick and decisive action to prevent further extinction. Assisted Reproduction Technologies are a promising frontier in species conservation: however, while embryo culture methodologies have been developed for eutherian species, marsupial embryo culture has not yet been optimized. Given marsupial reliance on histotrophic nutrition, close examination of uterine fluid contents is necessary for optimizing embryo culture. Using a dasyurid animal model, this research identifies key uterine morphological details, metabolites, and proteins of interest within the uterine fluid relevant to embryo culture medium composition, and has developed endometrial epithelial organoids as an in vitro model of the highly secretory marsupial uterus. Uteri from fat-tailed dunnarts (*Sminthopsis crassicaudata*) were collected across gestation. Micro-CT defined the internal structure of the uterus, and cellular detail was resolved using routine histological techniques. Uterine fluid was collected and subjected to metabolomic and proteomic analysis. DAVID analysis identified secreted proteins and their relevant gene ontology pathways. The dunnart uterus is highly glandular with a folded luminal surface, and contains a dynamic fluid microenvironment. Uterine fluid from different developmental stages had unique molecular signatures: >1800 peptides and 180 metabolites were detected. While significant overlap was detected between samples, each developmental stage had a small cohort of unique peptides, likely of significance to the key developmental pathways active in the developing embryo. Fluctuations in metabolic pathways, energy generation, and stress

response are identified between developmental stages using gene ontology analysis. The metabolomic profile of the post-implantation uterine fluid was significantly different to pre-implantation uterine fluid, with enrichment of 57 metabolites observed. The marsupial uterus is a dynamic microenvironment, with modulation of key proteins and metabolic pathways pertinent to the developing embryo. Insights from these molecular characterisations will be key to unlocking marsupial assisted reproductive technologies.

P-169 - The Postcopulatory Transcriptional Response In Different Sections Of The Female Reproductive Tract In Mice

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In internally fertilizing species, the first direct molecular contact between the male and female gene products occurs within the female reproductive tract – a highly complex and rapidly evolving structure in many species. While molecular interactions between sperm and oocyte have received a lot of attention, efforts to gain insight into the crosstalk happening during the sperm cells' journey to the site of fertilization have been limited, especially in mammals. The interactions between sperm cells and the female reproductive tract not only facilitate sperm guiding, storage, and protection against introduced pathogens but also comprise a sophisticated mechanism of female-mediated sperm selection. Molecular interaction partners that are involved directly in sperm assessment and selection, however, are difficult to pinpoint within the massive immunological response happening in the female reproductive tract following copulation. While we have some knowledge of sperm surface proteins interacting with the components of the female reproductive tract, the female counterparts of these interactions are still largely unknown. We characterized the murine female transcriptional response to semen following copulation comparing several lab mouse and wild-derived inbred *Mus domesticus* strains. We mated males and females from different strains and used strain-specific SNPs to distinguish semen-derived from female reproductive tract-derived transcripts. We specifically focused on the different subdivisions of the female reproductive tract, with the aim to identify female molecules directly involved in sperm assessment. Our results show a large number of differentially expressed genes in uterus, uterotubal junction and oviduct compared to unmated control and significant differences in differential expression between the subsections. In addition to the upregulation of genes related to immune activation and tolerance, we found increased expression of transmembrane and cell adhesion genes, as well as receptors involved in cell communication and migration (e.g. *Epha1* and *Adam17*). From these semen-activated genes, we selected the strongest candidates for direct interaction with sperm cells for immunohistochemical validation and sperm surface protein interaction assays. This study ultimately has the aim to test the potential of the discovered candidate proteins in in vitro sperm selection systems involving microfluidic chips. Given the ever-

increasing need for assisted reproduction in humans and the need for specific selective breeding in agriculture, we have to make sure that the natural quality control and selection mechanisms occurring in the female reproductive tract are understood and leveraged to their full potential.

P-170 - GelMA Hydrogels: Towards the Development of an In Vitro Model of the Human Endometrium

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The human endometrium is a complex, multicellular tissue and is the site of embryo implantation. Dysregulated endometrial function can lead to maternal-fetal complications and a variety of reproductive pathologies such as endometriosis preeclampsia and infertility. The mechanisms involved in disrupted endometrial function and perturbed endometrial-embryo communication remain largely unknown due to a lack of appropriate of study models and 3D in vitro culture systems that support multiple endometrial cell types. Our aim was to develop novel biomaterials capable of supporting the in vitro growth of multiple endometrial cell types. Here, we investigate how photocrosslinked GelMA hydrogels can be tailored to the culture of both human endometrial stromal cells (ESCs) and endometrial epithelial organoids (EEOs), which have been shown to recapitulate key features of in vivo endometrial glands. We produced GelMA hydrogels with a range of stiffness degrees by altering the degree of substitution during the synthesis of GelMA and the concentration of GelMA used in hydrogel fabrication. Endometrial biopsies were collected from consenting patients undergoing gynaecological surgery. Endometrial stromal and epithelial gland clumps were isolated from four patient biopsies. ESCs were expanded and passaged in 2D, at passage 2 cells were used experimentally and encapsulated in GelMA hydrogels at a seeding density of 10,000 cells per 5µl gel droplet. ESCs were grown for up to 15 days in DMEM/F12 supplemented with 10% FBS. ESCs were tested in comparison to a commercially available collagen hydrogel control. Epithelial gland clumps were immediately encapsulated in GelMA hydrogels upon isolation and allowed to grow and form organoids over 10 days in expansion medium. Matrigel was used as the control hydrogel for all EEO experiments. To encapsulate primary human endometrial cells in photocrosslinked GelMA hydrogels, we developed a simple protocol employing a cytocompatible photoinitiator (LAP) and a short 3-minute UV exposure time (365 nm). ESCs in both soft and stiff GelMA hydrogels maintained high levels of viability after a 7-day culture period. In soft GelMA hydrogels ESCs adopted an elongated morphology whereas in stiff GelMA hydrogels ESCs adopted a rounded morphology. An increase in cell number, detected by the MTS assay, was seen over a long-term culture period of 15 days in soft GelMA hydrogels indicative of cell proliferation.

ESCs retained their functional ability to adopt a differentiated phenotype and secrete prolactin in response to 4 days treatment with cAMP and MPA. EEOs only form in an appropriate 3D matrix. Soft GelMA hydrogels failed to support organoid formation whereas stiff GelMA hydrogels enabled EEOs to form. The efficiency of organoid formation was enhanced by addition of the basement membrane protein, laminin. 10% DS100 GelMA supplemented with laminin at a ratio of 1-in-10 was determined as the optimal formulation for EO formation. This optimised hydrogel formulation was then tested and confirmed to support an ESC-EEO co-culture. In conclusion, endometrial cells can quickly and simply be encapsulated in GelMA hydrogels and maintain high cell viability. The mechanical and biochemical properties of GelMA hydrogels can be tuned to suit the culture of different cell types. Engineering a platform to study the human endometrium in vitro is the first step towards understanding implantation failure and recurrent pregnancy loss and will pave the way for new treatments.

P-171 - Efficacy and Underling Mechanisms of D-Chiro Inositol in a Mouse Model of Endometriosis

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Endometriosis, a disease affecting 5-10% of women of reproductive age, is characterized by the spread of endometrial-like tissue outside the uterine cavity that produces ectopic endometriotic lesions causing pain and infertility. Surgical intervention and hormonal treatments have no long-term effects. The sensitivity of endometriosis to estrogens is a characteristic that can be used for therapeutic purposes. D-Chiro Inositol (DCI), one of the nine isomers of inositol, is known to decrease the CYP19A1 aromatase gene expression in granulosa cells. Based on these premises, it was suggested that treatment with DCI may counteract endometriosis. To address the study question, a mouse model of endometriosis was generated. Out of 20 CD1 mice, 4 mice were randomly selected as donors of uterine fragments, and the remaining 16 were recipient mice. The first day after transplantation, mice were randomly assigned to four experimental group which received for 28 days 2ml water containing: none (CTRL); DCI 0.4 mg (DCI 0.4); DCI 0.2mg and Dienogest 0.33ng (DCI 0.2+DG 0.33); DG 0.67ng (DG 0.67). Uterine horns were removed from donor mice at the diestrous stage of the reproductive cycle. The tissue cut into fragments was inoculated in recipient mice by intraperitoneal injection. Four weeks after induction, all mice were sacrificed and the endometriotic lesions were excised, measured by number, size and examined for the presence of blood vessels vascularization under stereomicroscope. Then, lesions were processed for immunohistochemical analysis. Endometriotic lesions developed in recipient mice met all criteria for endometriosis, including the presence of endometrial epithelial and stromal cells, and encapsulation in neighboring tissues or organs. The lesions number was reduced in all the treatment groups when compared to CTRL and the rate of vascularized lesions was lower in the treated groups, with more pronounced effect in the DCI group where no vascularized lesions were

observed. The histological analysis revealed a marked reduction of endometriotic foci in all groups. The lesions were then subjected to immunohistochemical analysis aimed at evaluating the proliferative and vascularization status using two specific markers, PCNA and CD34, respectively. PCNA-positive epithelial and stromal cells were detected in the lesions; in the DCI group a decrease in the expression of this marker is observed. CD34 labeling showed similar levels in CTRL compared to DCI+DG and DG treated groups; the DCI group presents a reduction in the expression of this marker. Searching for mechanisms underlying DCI effects, the expression of SIRT1 and E-cadherin as markers of epithelial-mesenchymal transition (EMT) involved in endometriotic lesion development, revealed that DCI negatively affects this process. CYP19A1 transcripts in the ovaries decreased in all groups, especially in the DCI group. This work has made it possible to validate an easily reproducible, minimally invasive model of endometriosis in CD1 mice. Based on this model, we demonstrated that DCI reduces endometriotic lesions development probably by interfering with EMT progression and reducing ovarian estrogens production by aromatase inhibition. In conclusion, the administration of DCI, both alone and in combination with DG, shows promising results in the treatment of endometriosis and opens the doors to further investigations aimed at introducing this substance into clinical practice.

P-172 - miRNA Expression in Equine Follicular Fluid Secretome is Affected by the Clinical Condition and Age of the Mare

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The ovulation of a mature and competent oocyte is the result of intricate molecular interactions between follicular cells and the oocyte. In horses, in vitro maturation (IVM) is essential for in vitro embryo production, but success rates remain limited. The supplementation of IVM media with follicular fluid secretome has been proposed to enhance oocyte maturation rates. Significant progress has been made in unraveling the complete molecular signature of the intrafollicular milieu. However, we have yet to fully comprehend whether specific factors within the follicular fluid (FF) secretome could serve as biomarkers for predicting oocyte maturation rates, subsequent blastocyst formation, and ultimately, pregnancy rates. To analyze if miRNA expression in equine FF secretome is affected by the clinical condition and age of the mare, preovulatory FF was collected from 8 mares aged between 7 and 18 years, 32 hours post hCG stimulation. For secretome isolation, the FF from each mare was diluted 1:3 with PBS and filtered through 10 kDa ultra-centrifugal units at 4000×g for 1 hour at 4°C. miRNA extraction was then conducted, followed by polyadenylation, reverse transcription reactions, and qPCR analysis to examine the expression of a panel of 10 miRNAs (miR-19b, miR-21, miR-23a, miR-24, miR-27b, miR-92a, miR-132, miR-372, miR-378, and miR-382) associated with follicle growth and oocyte maturation. qPCR products were quantified by using the 2- $\Delta\Delta C_t$ method, normalizing against U6 snRNA expression. Data for relative miRNA abundance were compared using the unpaired t-test. Furthermore, equine proteins within

the FF secretome were identified using hybrid trapped ion mobility spectrometry coupled with liquid chromatography. miRNet 2.0 was used to predict the interactions between secretome miRNAs and proteins. Our analysis revealed individual variability in miRNA expression, which correlated with the clinical condition of the mares and their age. Notably, one mare was healthy but aged (17 years), one was clinically obese (7 years), while another suffered from laminitis and was of advanced age (18 years). We identified six upregulated miRNAs (miR-19b, miR-21, miR-27b, miR-92a, miR-132, miR-382, $p < 0.05$) in the group of unhealthy and older mares compared to the healthy and younger ones. Based on our network analysis, the upregulated miRNAs were found to play crucial roles in various biological processes, including autophagy, angiogenesis, chromatin remodeling, and carbohydrate metabolism. Furthermore, these miRNAs were observed to interact with 30 protein-coding genes identified in the proteomic analysis. Interestingly, these targeted genes were implicated in Reactome pathways, such as extracellular matrix organization and the immune system. Despite the potential for the FF secretome composition to reflect the clinical condition and age of the mare, our analysis did not reveal any differences in terms of follicle count or oocyte collection during ovum pick up (OPU). Our study found six upregulated miRNAs in the secretome of unhealthy and old mares, indicating their potential use as biomarkers to predict FF secretome quality and oocyte maturation rates. However, further functional studies are required to elucidate their precise role in oocyte maturation. Funding: Grants FJC2021-047675-I (FM) and PID2021-122507OB-I00 (FM and AGA) by MCIN/AEI/10.13039/501100011033 and European Union NextGenerationEU/PRTR; PID2020-112723RB-I00 by MCIN/AEI/10.13039/501100011033 (BMG); IB20005 by Junta de Extremadura (BMG).

P-173 - Estradiol-17 β Increases Bovine Oviductal Tonus via G Protein Coupled Estrogen Receptor 1 and RhoA/Rho Kinase Signaling Pathway

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According to the World Health Organization (WHO), human infertility is defined as the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse. It is estimated that approximately 48 million couples and 186 million individuals live with infertility in the world. Since the transport of gametes and embryos in the oviduct at an appropriate time is important for the establishment of pregnancy, dysfunction of the oviduct is one of the causes of infertility. Early embryo and sperm transport through the oviductal isthmus depends on the contraction and relaxation of the smooth muscle layers. Estradiol-17 β (E2) is known to indirectly increase contractility by promoting the secretion of oviductal contractile factors such as endothelin. Previous research has shown that the herbal medicine Tokishakuyakusan directly increases bovine oviductal tonus via G protein coupled estrogen receptor 1 (GPER1). In other reports, E2 regulates contractility via RhoA/Rho kinase (ROCK) signaling pathway and increases the expression of RNDs, which act as ROCK inhibitor in small intestine. Therefore, we investigated the direct effect of E2, the original ligand of GPER1, on the contraction and relaxation of bovine oviductal smooth muscle and its mechanism. We used bovine oviductal isthmus tissues at four stages of the estrous cycle: stage I (1-4 days after ovulation), stage II (5-10 days), stage III (11-17

days), stage IV (18-20 days). Isthmic tissues cut into 3-4 pieces of 5 mm length were used for the Magnus method to monitor the longitudinal contractility (contraction frequency, contraction force, and tonus). The effect of E2 (1 or 10 nM) and GPER1 agonist (G-1, 1 or 10 μ M) on oviductal contractility were examined. Similarly, the effect of E2 (1 nM) with pre-treatment of GPER1 antagonist (G-15, 25 or 250 nM) and ROCK inhibitor (Y-27632, 1 μ M) were also examined. The protein expression level of GPER1, RHOA, ROCK II and RND3 in the oviductal smooth muscle tissues of each stage were measured by Western blotting. Furthermore, ROCK II activity in oviductal smooth muscle tissues treated with 1 nM E2 was measured. E2 had no effects on the frequency and contraction force of oviductal isthmus at all stages. However, the tonus was significantly increased by 1 nM E2 in stage I ($P < 0.05$). The tonus in stages II-IV did not change by E2 treatment. The G-1 treatment also increased oviductal tonus similar to E2 at stage I ($P < 0.05$). The treatment of G-15 and Y-27632 significantly suppressed the increase of oviductal tonus in stage I induced by E2 ($P < 0.05$). There was no significant difference in GPER1 and RHOA protein expression among the estrous stages. On the other hands, the protein expression of ROCK II in stage I was higher than that in stage II ($P < 0.05$). ROCK II activity in stage I was also higher than in stage II and IV ($P < 0.05$). Moreover, the protein expression of RND3 in stage IV was higher than that in stage III ($P < 0.05$). In conclusion, 1 nM E2 directly affects oviductal contractility by increasing tonus via GPER1 and ROCK II activation in stage I. The difference in the effect of E2 on oviductal tonus among each stage may be attributed to the differences in the expression of ROCK II and RND3.

P-174 - Distribution of Ovarian Steroid Hormones Across the Oviductal Epithelium: Insights from a Compartmentalized in Vitro Model

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The oviduct epithelium undergoes profound changes driven by variations in ovarian steroid hormone levels throughout the mammalian estrous cycle. However, due to the inherent challenges of conducting in vivo studies, the dynamic distribution profile of these crucial hormones within the oviduct microenvironment remains poorly understood. Therefore, we employed a compartmentalized in vitro model of porcine oviduct epithelial cells (POEC), to elucidate the basal-apical distribution of progesterone (P4) and 17 β -estradiol (E2) across the oviduct epithelium. Primary POEC were plated into 24-well hanging inserts (0.4 μ m pore size) and cultured with medium in both compartments until day 6. Subsequently, the medium in the apical compartment was removed, and the cells were sustained at the air-liquid interface until day 21 using a serum-free medium in the basal compartment. Oviduct fluid surrogates generated on the apical cell side, basal medium, and cellular layers were collected, extracted and subsequently steroids were measured via enzyme immunoassay (EIA). In experiment 1, POEC (2 donors/treatment/duration) were stimulated basolaterally with a single dose of either P4 or E2 representing serum concentrations during diestrus (30 ng P4 /mL medium) or estrus (60 pg E2/mL medium) for 0.5, 1, 3, 6, 12, and 24 hours (h). Basolateral P4 levels exhibited a rapid decrease to

56.01% after 3h, with only 5.19% of the initial P4 content remaining after 24h. Apical P4 levels peaked between 0.5 and 1h (3.19 - 4.13 ng/mL), and then declined to 2.00 ng/mL at 3h and 0.27 ng/mL at 24h, remaining lower than basal levels. Additionally, the total intracellular P4 also reached its peak level between 0.5 and 1h (0.79-1.02 ng), then declined to 0.4 ng by 3h, and further decreased to 0.06 ng, equivalent to 5.9% of the peak level, by 24h. Contrarily, the E2 levels in the basolateral side showed a gradual decline to 59.65% after 6h, with 11.43% of the initial E2 content still observed after 24h. Meanwhile, the total intracellular E2 content peaked between 1 to 3h (11.30-10.40 pg), followed by a gentle descent to 6.15 pg, equivalent to 54.42% of the peak level, by 24h. In experiment 2, POEC (6 donors/treatment/duration) were exposed to either a single dose of 30 ng/mL P4 or 60 pg/mL E2 for 12 and 72h, or subjected to repeated treatment for 72h (hormone refreshment every 12h). In the P4 group, 12h after single treatment, the basal side exhibited a P4 level of 2.86 ng/mL, significantly higher than the apical side (0.62 ng/mL, $p < 0.001$). Comparable levels were observed in the repeated-treatment group after 72h (basal: 3.71 ng/mL; apical: 0.51 ng/mL). 72h after single treatment, the P4 level in the basal side decreased to 0.15 ng/mL, representing only 0.55% of the initial P4 content. In the E2 group, repeated treatment for 72h resulted in an E2 level of 25.05 pg/mL, significantly higher than after single treatment for 12h (16.65 pg/mL, $p < 0.001$), as well as higher than 72h after single treatment (6.42 pg/mL, $p < 0.001$). In summary, E2 and P4 at physiological concentrations exhibit dynamic and divergent distribution patterns in the oviductal epithelium. These results build a basis for further studies on the effect and conversion of steroids in the oviduct epithelium and will thus help to better understand the regulation of oviduct functions. This study was supported by the Deutsche Forschungsgemeinschaft (DFG CH2321/1-1 and Scho1231/7-1).

P-175 - Comparative 3D Cell Culture Models of the Mammalian Endometrium to Understand the Evolution of Uterine Function

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Uterine function is critical to the survival of a species, but the exact mechanisms that ensure successful reproduction differ between mammals. Placental mammals innovated a cellular differentiation of the lining of the uterus called decidualization that prevents early parturition, such as that seen in marsupials. However, the initiation of decidualization differs across species. It can occur spontaneously, as in humans and menstruating primates, or in response to embryo implantation, as in most non-menstruating species. The endometrium, the mucosal layer of the uterus, is a key component of what defines a mammal, providing the interface between the mother and the fetus, but the extent to which its functions and structure are conserved have been underexplored. Establishing the similarities and differences in endometrial hormonal response across species will provide insight into mechanisms of uterine function that will lead to

understanding of uterine dysfunction. Endometrial tissue is dynamic and necessitates sampling at matched time-points to capture cycle-relevant functions such as decidualization. This synchronization of sampling is challenging and potentially confounded by inter-species differences in reproductive or life history traits. Moreover, collecting uterine tissues from multiple mammalian species requires invasive surgery or sacrifice of multiple individuals to appropriately document all time points in the cycle. In this context, cell culture models are a desirable alternative that allow for precise measurements at controlled cycle timepoints, and reduction of the number of animals used for research. Recent 3D cell culture models of the human endometrium that incorporate both stromal and epithelial cell types have been developed, termed “assembloids”. These models recapitulate many structural features of the endometrium in vivo, with epithelial gland-like structures embedded in a stromal matrix, that traditional cell culture models cannot reproduce. Inclusion of both cell types improves the model because stromal-epithelial crosstalk is required for structural changes across the cycle. Human endometrial assembloids can be treated with hormones in culture to induce decidualization. Extending the assembloid model to other mammals allows comparison of hormonal cycles across species with different cycle lengths because hormonal levels can be changed in a controlled manner. To study the function and organization of the epithelial glands and stromal supporting cells, we developed in vitro 3D assembloid models incorporating epithelial organoids and stromal cells across multiple species, including humans, mice, rabbits, and a menstruating species of bat (*Carollia perspicillata*). Here, we report the preliminary assessment of this new cross-species model to study the evolution of uterine functions. We observe qualitative differences in growth rates and internal structures of organoids across the different species. Further experimentation will allow comparative investigation of how changes to the structure and composition of the glands and tissue over time in response to various hormonal treatments will differ in species with spontaneous or induced decidualization.

P-176 - Leveraging Single Cell Transcriptomics to Dissect Cellular Changes in the Late Endometrial Cycle across Primates.

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The mammalian uterine endometrium has developed multiple evolutionary strategies of cellular differentiation to prepare for embryo implantation and to perform its cyclic cellular renewal. In humans, the endometrium differentiates spontaneously into a decidual mucosa in response to progesterone during the window of implantation and breaks down when progesterone level decreases if implantation has not occurred. These cellular transitions have arisen during the evolution of Old World primates, but have also appeared independently in other, more distant mammalian orders. In other primates, the endometrium decidualises only in response to embryo implantation and is resorbed at the end of the cycle in the absence of fecundation through poorly described mechanisms. This phenotypic divergence between related primate clades offers opportunities to dissect the genetic mechanisms underlying the evolution of spontaneous decidualization and menstruation. To identify gene expression changes underlying the evolution of cellular differentiation of the endometrium, we collected samples of 3 menstruating Old World

primates (human, baboon, macaque) and 3 non-menstruating primates (New World primates : saimiri, marmoset – Lemurs : mouse-lemur) at two time points of the endometrial cycle: during the luteal phase when progesterone is maximal, and when the endometrium is shed or resorbed in response to progesterone withdrawal. We performed single-nucleus gene expression sequencing to assemble the first cross-species cartography of endometrial cell populations in the late endometrial cycle. Here, we report the preliminary results of this project. We identified a differentiation of both stromal and epithelial endometrial cells in menstruating species that is not shared with non-menstruating species, and are now pursuing the gene expression changes that control these cellular transitions. This first characterization of endometrial cell types aims to further dissect how endometrial gene expression has evolved in primates, and its functional impact on cellular remodelling in response to progesterone.

P-177 - A Conserved Core of Epididymal-Derived microRNAs May Contribute to Modifying the Endometrial Transcriptome to Facilitate Receptivity to Implantation in Placental Mammals

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Placental mammals show a remarkable diversity in sperm morphology, embryo development, implantation strategies and placental structure, among others. The gene networks that regulate these processes can be either conserved, i.e., the same network regulates a fundamental reproductive process, or species-specific and drive diversity. One of the known mediators of gene networks are microRNAs (miRNAs) which can target a multitude of different pathways. There is evidence in some species that the miRNAs acquired by sperm during the epididymal transit play a significant role in female endometrium by facilitating receptivity to implantation; however, whether these miRNAs are conserved in placental mammals has not yet been elucidated. We hypothesised that a core of conserved epididymal-derived miRNAs may modify the transcriptional landscape in the endometrium to facilitate receptivity to implantation in placental mammals. To test this hypothesis, we accessed publicly available (GEO or publications), epididymal miRNA datasets for representatives of six mammalian families spanning 4 orders, including cow, yak and zebu (Bovidae family, 7 data sources), pig (Suidae, 5), horse and donkey (Equidae, 2), mouse and rat (Muridae, 14), guinea pig (Caviidae, 1), and human (Hominidae, 5). Accessed datasets were generated with RNA-seq or microarray and included epididymal tissue, extracellular vesicles, and sperm. We identified 86 conserved miRNA precursors shared across all families, regardless of the epididymal segment. Of these, 24 precursors were derived from cauda and 69 from caput segment. Interestingly, all 24 cauda precursors were also present in datasets from the caput, indicating potential acquisition of species-specific miRNAs as they transit through the epididymis. These were also detected in ejaculated sperm or seminal plasma from at least one species analysed, suggesting that this set of conserved miRNAs may be the one acting on the endometrium. Therefore, 48 mature miRNAs derived from 24 conserved cauda precursors were used for further analysis. Using RBiomirGS R

package, we retrieved overall enrichment for conserved cauda miRNA targets, which showed a broad coverage of cellular functions and pathways by these miRNAs. We next used multiMiR R package to retrieve both predicted and validated targets resulting in over 16,000 unique genes. To assess whether identified conserved cauda miRNAs may target biologically significant transcripts in endometrium, we accessed datasets of endometrial genes that changed their expression in the presence of the conceptus during pregnancy recognition in cow, pig, and horse. Overall, 290 genes were present in the endometrium and responded to conceptus in all three species, out of which, 271 were targets of 38 conserved cauda miRNAs. Using DAVID tool, we found that these 271 endometrial targets were enriched in functional terms related to innate immunity, extracellular matrix, protein ADP-ribosylation, inflammatory response and TNF signalling pathway. Using STRING app in Cytoscape, 237 conserved targets formed a predicted protein-protein interaction cluster, which could be further divided using Markov Cluster Algorithm into smaller highly interacting groups enriched in: (1) response to stimulus, immune system response and interferon signalling, (2) PPAR signalling, (3) mRNA binding, and (4) Golgi associated vesicle biogenesis. Finally, we built a comprehensive miRNA-mRNA network that can serve as a guide for investigating conserved in-silico interactions of epididymal miRNAs and endometrial targets in placental mammals. Future work will focus on confirming if these predicted conserved miRNAs and their targets modify receptivity to implantation in vitro in species with different implantation strategies.

P-178 - Bovine Oviductal Organoid-Derived Secretions Increase Blastocyst Yield at Day 7 and Day 8 of In Vitro Culture.

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With the aim of mimicking the physiological environment in which fertilization and early embryo development take place, reproductive fluids (RFs) have been used as supplements to in vitro culture media by various research groups over the last decade (Coy et al., 2022). In cattle, embryos produced with RFs showed improved developmental kinetics, a higher number of blastomeres and higher post-vitrification survival rates (Lopera-Vasquez et al., 2017). Due to inter-individual variability in the effect of the RFs, it is necessary to collect, process and test large batches of abattoir material before including them in the IVP media. Additionally, RFs are not immune to sanitary risks, so the detection of bluetongue, BVD or IBR viruses by PCR in each fluid batch must be done before use. The recent development of organoids derivation could contribute to overcome these problems and maintain the high efficiency of bovine culture media with standardized biological additives. As organoids have already been derived from human fallopian tubes and endometrium (Turco et al., 2017), we proposed to generate organoids resembling the bovine oviduct, and derived them to produce fluids similar to those surrounding the embryo during the early developmental stages in the oviduct. Reproductive tracts were collected from a local abattoir,

dissected on ice and the epithelial tissue was minced, centrifuged, and cryopreserved. From this stage, the derivation of oviductal organoids and the collection of organoid-derived secretions (ODS) was performed as described in Turco et al., (2018) and Simintiras et al., (2021). Since organoids respond to hormonal stimulation, we generated 3 different types of organoids: without hormonal stimulation (CODS); stimulated with estradiol resembling the estrus cycle preovulatory phase (E2); and stimulated first with estradiol and then with progesterone, resembling the early postovulatory phase (P4). The protein concentration in the ODS ranged between 2.5 and 3.1 mg/mL. All ODS were resuspended in SOF-BSA medium and added at a final concentration of 5 or 10% (v/v) to the embryo culture media from d1 to d3 post-insemination, when cleavage rate was assessed (D3CL), and embryos were washed in fresh medium and transferred to new droplets until d7 and d8 of culture, when blastocyst yield was assessed (D7BL and D8BL, respectively). Data are expressed as Mean $\pm$ SE. Only statistically significant differences ($p < 0.05$) are described. D3CL was reduced in E2-10% and P4-10% groups ($66.7 \pm 4.6\%$ and $68.0 \pm 4.6\%$, respectively) compared to CODS-5%, P4-5% and CODS-10% ($87.1 \pm 3.5\%$, $87.4 \pm 3.4\%$ and $87.9 \pm 3.2\%$, respectively). D7BL was higher in P4-5% group ($20.0 \pm 4.1\%$) than in Control, E2-5%, CODS-10% and E2-10% groups ($7.1 \pm 2.4\%$, $5.2 \pm 2.3\%$, $7.5 \pm 2.6\%$ and $3.8 \pm 1.9\%$; respectively). D8BL was higher in P4-5% ($26.3 \pm 4.5\%$) than in all groups (11.6 ± 3.0 , 14.0 ± 3.6 , 9.3 ± 3.0 , 13.1 ± 3.3 , 4.8 ± 2.1 and 11.7 ± 3.2 ; for Control, CODS-5%, E2-5%, CODS-10%, E2-10% and P4-10%, respectively). These preliminary results show that ODS, which mimic the early luteal phase of the estrous cycle, can be used as additives in embryo culture media when stimulated first with estrogens and later with progesterone. The 5% concentration was found to be sufficient in increasing the IVP blastocyst yield. To the authors' knowledge this is the first study to report the use of this standardized biological additives in bovine embryo culture. However, to complement this work, it is necessary to add ODS from uterine tissues that resemble the mid-luteal phase of the estrous cycle to the embryo culture media from d3 to d8. Grants PLEC2022-009246 funded by MCIN/AEI/ 10.13039/501100011033 and by the "European Union NextGenerationEU/PRTR", and PID2020-113366RB-I00 funded by MCIN/AEI/10.13039/501100011033/ and "FEDER Una manera de hacer Europa".

P-179 - Investigating the Impact of Ovariectomy- and Chemotherapy-induced Estrogen Loss in the Mouse Vagina

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The vagina is a complex tissue composed of an epithelium and a fibromuscular layer. When estrogen is lost during menopause, ovariectomy or gonadotoxic treatment such as chemotherapy, the vaginal anatomy and function is significantly altered resulting in sexual dysfunction, risk of infection and pain. The vaginal epithelium expands and shrinks during the course of the menstrual cycle (human) or estrous cycle (mice) and displays repair after childbirth. These functions indicate

the vagina is a proliferative tissue and may contain stem cells that support vaginal regeneration. We hypothesize that chemotherapy has direct cytotoxic effects on the vagina, independent of ovarian toxicity, that cause estrogen-dependent degeneration of the vaginal epithelium. C57BL/6 mice (6 weeks old; n=60) were divided between four treatment groups (n=15/group): 1) ovariectomy (OVX) + chemotherapy (Chemo); 2) OVXVehicle; 3) ShamChemo; 4) ShamVehicle. Vaginal lavage was performed on all animals for 14 days to assess baseline estrus cyclicity prior to intervention. At 8 weeks, ovariectomy or sham surgery was performed. Ovary intact cohorts maintained normal estrous cycling, 2.53 ± 0.74 and 2.60 ± 0.74 cycles/14 days, and OVX animals lost cyclicity, 0.00 regular cycles/14 days, respectively ($p < 0.05$). OVX animals also displayed significant weight gain that persisted throughout the trial ($p < 0.05$). At 11 weeks of age, mice were injected with busulfan (24 mg/kg) and cyclophosphamide (200 mg/kg) or vehicle (DMSO + water). Reproductive tissues (n=5 mice/cohort) including vagina, cervix, ovary and uterus and serum were harvested at 7, 30, and 90 days post-injection (chemotherapy or vehicle). Chemotherapy did not have an immediate effect on estrous cyclicity in Sham animals (0.87 ± 0.35 cycles/5 days). At 7 days post-injection, OVXVehicle and OVXChemo animals exhibited a significant decrease in reproductive tract weight compared with ShamVehicle and ShamChemo (2.88 ± 0.38 and 2.57 ± 0.32 vs. 7.65 ± 1.56 and 7.43 ± 1.63 tract weight(mg)/body weight(g), $p < 0.05$). In addition, there were histologic differences in the OVX vaginas including alterations of the stroma, a loss of epithelial crypts, a significant reduction in epithelium area compared with ShamVehicle and ShamChemo ($p < 0.05$). While there was no difference in the number of cells lining the basement membrane in the vaginal epithelium, we observed a significant reduction in Ki67+/DAPI+ nuclei on the basement membrane in OVXVehicle and OVXChemo animals (0.03 ± 0.04 and 0.15 ± 0.13 Ki67+/DAPI+ nuclei) compared with ShamVehicle and ShamChemo animals (0.54 ± 0.25 and 0.57 ± 0.05 , $p < 0.05$) suggesting cell proliferation is affected by OVX. Nerve growth factor receptor (NGFR) signal intensity in the basal epithelia was higher in the OVX animals (44.5 ± 5.03 , $37.0 \pm 9.75\%$ area positive for NGFR signal) than the Sham animals (19.9 ± 3.34 , $27.2 \pm 14.7\%$). In addition, NGFR positive cells in the stroma were significantly reduced in OVX animals (0.52 ± 0.79 , $0.14 \pm 0.14\%$ area positive for NGFR signal) compared to Sham animals (15.2 ± 7.5 , $18.2 \pm 6.2\%$, $p < 0.05$). Taken together, we observed immediate effects of ovariectomy and associated estrogen loss to the reproductive tract, body weight and the epithelial and stromal compartments of the vagina. Data collection and analysis is ongoing for the 30 and 90 day timepoints; however, preliminary findings show a significant reduction in the ShamChemo reproductive tract weight (5.05 ± 0.68) compared to ShamVehicle (7.48 ± 1.66) with no difference to the OVX cohorts (3.17 ± 0.86 and 3.23 ± 1.49 tract weight(mg)/body weight(g), respectively), suggesting chemotherapy impacts tissue integrity after 30 days ($p < 0.05$). This work is supported by the Magee-Womens Research Institute and Foundation and the Richard King Mellon Foundation.

P-180 - Elevated Methylglyoxal Concentrations Induces Cellular Senescence in Endometrial Cells in Dairy Cows

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Excessive sugar in the body of dairy cows due to high grain rations may bind to proteins and accelerate the production of advanced glycation end products (AGEs). Methylglyoxal (MGO) is a precursor of AGEs that induces neurodegenerative diseases and vascular endothelial damage via intracellular reactive oxygen species (ROS) production. Reactive oxygen species induce DNA damage and promote cellular senescence by arresting cell cycle via elevated expression of cyclin-dependent kinase inhibitors p16 and p21. Senescent cells secrete inflammatory cytokines and chemokines, called a senescence-associated secretory phenotype (SASP) which causes inflammation. Based on these findings, we hypothesized that increased blood MGO in dairy cows causes cellular senescence via ROS production in the endometrial cells, resulting in reduced fertility. Initially, blood MGO concentrations in beef and dairy cows were compared. Blood MGO concentrations in Holstein dairy cows (n=20, fed corn silage) were significantly higher ($p < 0.05$) compared with Japanese Black beef cows (n=20, fed grass silage). Subsequently, we examined the effects of MGO on bovine endometrial cells and its mechanism. Methylglyoxal was added to bovine endometrial cells (passage 2) cultured at 38°C and 5% CO₂ in the air at 0, 0.1, and 1 mM, respectively. Reactive oxygen species production was measured 6 h after MGO treatment. The cell proliferation rate, DNA damage level, senescence-associated β -galactosidase (SA β -gal)-positive cell rate, and p21 mRNA expression levels were measured 12 h after MGO treatment. IL-8 secretion was measured 12 h after MGO treatment followed by 24 h of culture in a normal medium. Reactive oxygen species production after 1 mM MGO treatment significantly increased ($p < 0.05$) compared to the control. The expression level of γ H2AX protein (a DNA damage marker) after 1 mM MGO treatment was significantly higher ($p < 0.05$) than that of the control. The cell proliferation rate was significantly decreased ($p < 0.05$) by 1 mM MGO treatment compared to the control, and the ratio of SA- β gal positive cells was significantly increased ($p < 0.05$) compared to the control. p21 mRNA expression significantly increased ($p < 0.05$) after 1 mM MGO treatment compared to the control. These results suggest that MGO causes DNA damage via increased ROS production and induces cellular senescence in bovine endometrial cells. Furthermore, IL-8 production tended to increase ($p < 0.1$) in bovine endometrial cells after 1 mM MGO treatment compared to the control, suggesting that cytokines secreted by MGO-induced senescent cells may cause a decrease in uterine function, resulting in reduced fertility in dairy cows.

P-181 - Effect of Unsaturated N-3 Fatty Acids From The Camelina Sativa L Cake on Fatty Acid and Adipokine Contents in The Serum of HF Heifers

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Polyunsaturated fatty acids (PUFAs) stimulate reproductive functions in cattle. We investigated the effect of Camelina sativa L cake, a rich source of n-3 fatty acids (FAs), on selected parameters related to reproduction and metabolism (serum profile and concentration of FAs and adipokines, number and size of the ovarian follicles). The 71-day experiment included 16 HF heifers (11-12 months old) divided into control (8) and experimental groups (8). Animals received a standard diet,

but the experimental group's diet was supplemented with the Camelina sativa L cake (1 kg per day). The blood samples were collected 3 times whereas the USG examination was performed 7 times during the experiment. The serum of experimental heifers contained more total unsaturated n-3 FAs, and α -linoleic acid (C18:3n-3) on days 46 and 48 whereas no difference was observed for PUFAs and n-6 FAs. The n6:n3 ratio was reduced (40 and 46 d). Regarding adipokines, only the concentration of apelin was decreased in the experimental group on day 20. However, interactions were noticed for 5 out of 7 adipokines (adropin, apelin, chemerin, leptin, and visfatin). The number and size of the ovarian follicles did not differ between the groups. We found linear interactions between the treatment effect and experiment duration for total n-3 FAs and α -linoleic acid concentrations as well as n6:n3 ratio. Our results indicated the impact of the Camelina sativa L cake on the levels of the analyzed parameters, indicating that the addition of n-3 FAs to the diet, could also affect adipokine concentrations.

P-183 - Understanding the Role of Cilia in Preimplantation Oviductal Transport

Understanding the Role of Cilia in Preimplantation Oviductal Transport. Deirdre M. Scully¹; Irina V. Larina¹

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Motile cilia lining the lumen of the oviduct are known to play an important role in reproduction. Motile ciliopathies, such as primary ciliary dyskinesia, are associated with subfertility and infertility, as well as being linked to a higher incidence of ectopic pregnancies. However, the specific role of cilia in the oviduct remains unknown. Traditionally, it has been presumed that cilia facilitate the directional transfer of eggs and embryos through the oviduct to the uterus for implantation. However, imaging of the reproductive organs in their native state is challenging, and there is little information on the dynamic aspects of cilia transport processes in the oviduct. Therefore, this study aims to investigate the role of cilia in preimplantation oviductal transport through genetic and live imaging approaches. To determine the potential role of cilia in fertility, we generated a mouse model with conditional deletion of ciliary motor protein-encoding gene *Dnah5* in the oviductal epithelium and performed a six-month fertility trial. A phenotype of subfertility was established, with litter sizes being significantly reduced in *Dnah5* cKO mice. To confirm the loss of ciliary function, oviductal mucosal surfaces were visualized with digital videomicroscopy. The location and frequency of beating cilia were analyzed by fast Fourier transformation of the pixel intensity fluctuations. The percentage area of actively beating cilia decreased from nearly full coverage to small, isolated patches in *Dnah5* cKO oviducts. In vivo 3D imaging of the reproductive organs using optical coherence tomography revealed that loss of *Dnah5* in oviductal epithelial cells leads to impaired egg transport, with 50% of ovulated eggs trapped in the ovarian bursa cavity at 0.5 days post coitus compared to control mice, where all eggs were successfully transported to the ampulla. These results suggest that oviductal cilia play a significant role in maintaining timed, preimplantation transport. Additionally, we hypothesized that cilia drive circulatory flows in the ampulla by the non-uniform spatial distribution of motile cilia throughout the oviductal mucosa. To test this hypothesis, we investigated the distribution of cilia through a combination of light-sheet and confocal fluorescence microscopies using acetylated α -tubulin immunostaining to label the cilia. Volumetric light-sheet microscopy revealed that the mucosal lining of the infundibulum, upper ampulla, and lower ampulla was densely ciliated, while the isthmus contained pockets of

ciliated cells. Furthermore, light-sheet rendering revealed a dramatic reduction in mucosal fold number within the lower ampulla where cumulus oocyte complexes are known to display circulatory movements. Confocal microscopy of oviductal cross-sections at specific locations revealed non-uniform radial distribution of cilia. Cilia coverage is uniform along the infundibulum region but dramatically changes in the upper and lower ampulla, such that the luminal surface of the outer curvature is more densely ciliated than the inner curvature. Interestingly, this distinct ciliary pattern coincides with the portion of the ampulla where cumulus oocyte complexes are known to display circulatory movements. These results support the hypothesis that the morphology and ciliary distribution of the lower ampulla may play a role in facilitating the circulatory flows in this region of the oviduct, which might be critical for luminal mixing and denudation of cumulus oocyte complexes. This study reveals specific functions of cilia and highlights the important role that cilia play in overall reproduction. This work will contribute to a better understanding of oviduct transport pathologies and could lead to improved management of reproductive ciliopathies.

P-184 - Formation of Bovine Uterine Gland-Like Structures in a 3D Culture System.

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Embryonic mortality is one of the major reasons for the decrease in the conception rate of cattle. A high frequency of embryonic loss occurs between 8-16 days after insemination. During this period, the bovine conceptus rapidly elongates due to a variety of secretions from the uterine glands. Uterine gland knock-out ewes have been reported to show a conceptus elongation failure due to the lack of uterine gland secretions; therefore, the secretory function of uterine glands is important for the establishment of pregnancy. However, the secretory functions of uterine glands are not fully understood. Three-dimensional (3D) culture systems are widely used to maintain the morphology and function of epithelial cells in vitro. It has been reported that salivary and mammary glands elongate by Wnt proteins and growth factors in the 3D culture system, and show the structures resemble in vivo counterparts. The aim of this study was to investigate the effects of Wnt proteins and a growth factor on the morphology of 3D-cultured bovine uterine glands. Bovine uteri were transported from a local abattoir to the laboratory after exsanguination, and uteri at 11-17 days after ovulation were used. The intercaruncular region of the endometrium was collected and, uterine gland fragments were isolated by enzymatic digestion. The uterine gland fragments were embedded in Matrigel for the 3D culture system and treated with or without Wnt proteins (Wnt3a, Wnt5a, and Wnt7a) and epidermal growth factor (EGF) as described below: (1) without Wnt proteins and EGF (control), (2) with Wnt proteins and EGF, (3) only with Wnt proteins, and (4) only with EGF. During 5 days of 3D culture, the morphology of uterine glands was observed in each culture condition. Furthermore, uterine glands before and after 3D culture were stained with a proliferation marker (Ki67) and an apico-basal polarity marker (ZO-1), and analyzed gene expression levels of secretory proteins from the uterine glands (SERPINA14, MEP1B, and SPP1) by qRT-PCR. In the control, the fragments formed spherical structures with cavity (cysts) after the 3-5 days of 3D culture. On the other hand, in the presence of Wnt proteins and EGF, the cells of the fragments lost lumen and

formed aggregates after 1-2 days of culture, and the aggregates showed the structures which resemble in vivo counterparts (uterine gland-like structures) accompanying elongation with twisting after the 3-5 days of culture. The presence of Wnt proteins was not important for the formation of uterine gland-like structures. During the elongation, proliferating cells were detected in the whole area of uterine gland-like structures. Although uterine glands isolated from endometria consisted of only simple layers, uterine gland-like structures contained both simple and stratified layers. Meanwhile, ZO-1 was detected at the apical side of uterine gland-like structures same as uterine glands isolated from endometria. Compared to the cysts, the gene expression level of SERPINA14 was significantly lower in uterine gland-like structures and in vivo counterparts. On the other hand, gene expression levels of MEP1B and SPP1 did not significantly change among these structures. In summary, only EGF but not Wnt proteins contributed to the stratification and elongation of bovine uterine glands in the 3D culture system.

P-185 - Effect of oxytocin administration on luteal function, blood flow and luteolysis in Nili-Ravi buffaloes

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The objective of the study was to evaluate the effect of repeated oxytocin administration on luteal function, blood flow, and luteolysis during the luteal phase in Nili Ravi buffaloes. It was hypothesized that daily administration of oxytocin during luteal phase would induce early luteolysis and shorten the cycle length. Twelve cyclic, non-pregnant, and dry Nili-Ravi buffaloes were treated with normal saline (n=6, NS; control, 5 ml, i.m., b.i.d) and oxytocin (n=6, OT; 5 I.U, i.m., b.i.d) from Day 8 of the cycle until Day 18 after ovulation (Day 0) induction using two doses of prostaglandin 11 days apart. Follicular and luteal dynamics of buffaloes were monitored using transrectal ultrasound (7.5 MHz, MyLab 5, Esaote, Italy) daily while luteal blood flow was monitored using Doppler ultrasound from Days 14 to 18. Blood samples (Jugular venipuncture, 5 ml, EDAT tubes) were taken from each buffalo to determine the concentrations of plasma progesterone from Days 10 to 18 and prostaglandin metabolite at 0, 15, 30, 45, and 60 min after oxytocin administration (0 min) relative to the day of luteolysis (Days 14-18). Results showed no significant differences between OT and NS buffaloes regarding corpus luteum (CL) and ovulatory follicle diameters, luteal blood flow estrous cycle duration, standing estrus, onset of luteolysis, or follicular wave emergence. However, OT buffaloes exhibited larger follicle diameters on Days 9, 10, and 11, and a lower mean growth rate of CL compared to controls (0.54 ± 0.25 vs 0.78 ± 0.18 ; $P=0.046$). OT buffaloes tended to achieve peak

PGFM concentration earlier than controls, with higher PGFM concentration at 0 min ($P=0.07$). Additionally, an earlier rise in PGFM concentration was observed 15 minutes before the peak in OT buffaloes ($P<0.05$). In conclusion, twice-daily oxytocin administration during the luteal phase did not result in an earlier onset of luteolysis.

P-186 - Spatial Transcriptomics of The Neonatal Bovine Uterus Reveals Potential Markers of Epithelial Differentiation Driving Endometrial Adenogenesis

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The endometrial glandular epithelium (GE) plays a critical role in fertility of mammalian species. In a recent study (Sellmer Ramos et al. 2024; Biol. Reprod. 110:1-4), we observed that the earliest phase of glandular development (adenogenesis) in the neonatal bovine uterus was characterized by the patch-like expression of FOXA2 within the luminal epithelium (LE; postnatal day 0, PND0) that was sustained until PND 28. FOXA2 is a pioneer transcription factor, expressed specifically in the GE of the adult, and required for adenogenesis. The unexpected results led us to further investigate the mechanisms that lead to the spatially-constrained induction of FOXA2 within the LE that precede adenogenesis. Spatial transcriptomics was performed on uterine cross-sections to evaluate gene expression within calves at distinct stages of adenogenesis including stage 0 (absence of glandular epithelium, and little to no expression of FOXA2 within the LE, $n=2$) and developmental stage 2 (presence of GE and islands of FOXA2⁺ cells within the LE, $n=2$) for PND0 ($n=2$ stage 0, $n=1$ stage 2) and PND7 ($n=1$ stage 2). Uterine cross sections were collected, embedded in O.C.T., frozen and stored in a -80°C . Tissue blocks were sectioned and placed in a Visium (10X Genomics) slide containing 4 capture areas and sequenced in an Illumina NovaFlowSeq 6000 (sequencing depth of 50,000 read pairs per 50 mm spot). Each cross-section was H&E stained and subsequently imaged. Images and raw FASTQ files were processed using the Space Ranger pipeline and aligned to the Bos taurus genome. Raw counts were normalized using SCTransform in Seurat v.5.0 (R Statistical Software v.4.3.1). Normalized data were merged and integrated using Harmony v.1.2 and submitted to the standard Seurat clustering pipeline prior to downstream analysis. Differential gene expression analysis was performed using a Wilcoxon rank sum test (FindAllMarkers) and clusters were annotated using the PanglaoDB database and manually confirmed based on spatial localization. We found 3 epithelial clusters present in both developmental stages using the FindSubCluster function: early-stage LE (EarlyLE), GE (FOXA2⁺) and late-stage LE (LateLE). Differential expression analysis revealed distinct markers for each cell type. EarlyLE was characterized by expression of IGFBP1, CLDN7, GHRH, PGA5, SAA3 and FXYP4. When compared with LateLE, the EarlyLE cluster had greater SOX17, MMP7, OVOL1, FUT6, WNK1, SPON2, DKKL1 and LGR4. The GE cluster was characterized by FOXA2, ALDH1A1, TUBA1D, IHH, SLC2A5 and FOXJ1. When compared with LE clusters, the GE cluster had greater AXIN2, WNT5B, PTCH2, PTCH1, PENK, CXCL12, FZD2 and CDH11. The LateLE cluster was characterized by KLRJ1, SBSPON, CSF1, GSTT2 and RIMS2. When compared with EarlyLE, the LateLE had increased expression of CDH1, JUN, TFF3, GGT5, IGFBP4, TGFBI, MKI67. Importantly, at developmental stage 0, UMAP plots revealed that all epithelial populations clustered in close proximity, with fewer

unique mapping identity (UMI) spots representing LateLE and GE clusters. At developmental stage 2, these cell populations clustered separately, indicating a potential timeline for determination and specification of epithelial phenotypes. We characterized the EarlyLE phenotype across developmental time points using the FindConservedMarkers function, and found that markers OVOL2, KLF5, ITGB4, ITGA6, SOX17, SPDEF, OVOL1 and TERT were conserved, suggesting that a progenitor-like epithelial population is maintained within the endometrium. These findings shed light on potential markers that might drive adenogenesis and highlight distinct epithelial populations in the neonatal bovine uterus. Supported by the NICHD award R01HD092254.

P-187 - Steroidomic Profiling to Assess Follicular and Luteal Dynamics Across Species Using Semi-Targeted High Resolution Mass Spectrometry

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Steroid hormones including glucocorticoids, mineralocorticoids, androgens, estrogens, and progestogens produced by the adrenal cortex, the gonads, the placenta are small lipophilic compounds with important biological functions including sexual differentiation and reproduction, growth, metabolism regulation, and immune system modulation. Metabolism of steroids includes oxidation and reduction reactions (phase I) and esterification reactions (phase II). During phase II metabolism, polar moieties, commonly a sulfate or glucuronide group, are added to the steroid skeleton. Analytics of steroids is often challenging due to isomeric structures and the low endogenous concentrations in body fluids. Mass spectrometry (MS)-based techniques have evolved as a gold standard for steroid analytics providing increased specificity compared to immunoassays. We aimed at developing a plasma/serum steroidome high-throughput MS based platform to study follicular and luteal dynamics across species including women. We extended our previously published ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) approach for progestogen and glucocorticoid profiling (Rehm et al. 2024) by adding androgens, estrogens and steroid conjugates. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) were employed to extract low-polarity (free steroids) and relatively more polar steroids (conjugated steroids), respectively. We utilized a semi-targeted approach to determine the steroidome in bovine and human plasma, employing a hybrid quadrupole-orbitrap mass spectrometer operating in full scan MS1/data dependent acquisition (DDA) MS2 mode to perform both targeted and untargeted analysis in a single sample injection. Specifically, thirteen progestogens, eleven glucocorticoids, seventeen androgens, nine estrogens and six steroid conjugates were of interest. Optimized ionization of the steroids was performed by polarity switching and the addition of different additives to the mobile phases of LC improving method sensitivity. While progestogens ionized best in positive mode using formic acid (FA) as an additive, resulting in limits of quantification (LOQs) between 50-500 pg/ml, steroid conjugates performed best in negative mode using FA as an additive with a LOQ at 50 pg/ml. Estrogens obtained improved sensitivity when using ammonium fluoride or ammonium hydroxide instead of FA as an additive. Among the 56 target steroids, 14 and 15 displayed changes across the female and bovine cycle, respectively. Of these, several bioactive steroid metabolites have not been described in detail in the context of follicular and luteal dynamics. Taken together, semi-targeted steroidomics enables to

simultaneously monitor a large panel of steroid compounds and thereby allows to study steroid-related feedback mechanisms, elucidate biological functions of active steroid metabolites across species and identify biomarkers for endocrine physiological and pathological conditions such as reproductive health.

P-188 - Single Cell Insights into Epithelial Morphogenesis in the Developing Neonatal Mouse Uterus

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The uterus is vital for successful reproduction in mammals, and two different types of epithelia (luminal and glandular) are essential for embryo implantation and pregnancy establishment. At birth, the uterus is neither fully developed nor differentiated in domestic and laboratory animals as well as humans. On postnatal day (PND) 0, the mouse uterine horns are characterized by a central lumen lined by simple epithelium that is surrounded and supported by undifferentiated mesenchyme cells. Between PNDs 0 and 5, epithelial invaginations (buds) appear in the epithelium that are gland primordia, and the mesenchyme segregates into two layers with the inner forming the stroma of endometrium and the outer forming the myometrium. By PND 10, the epithelium undergoes specification, delineating into uterine-type luminal epithelium (LE), alongside the emergence of bud- and teardrop-shaped epithelial invaginations, marking the initiation of epithelial cell bifurcation and differentiation into FOXA2-positive glandular epithelium (GE) cells. Subsequently, these FOXA2-positive GE cells develop into the stroma, forming mature glands by PND15. Despite extensive characterization of the uterus, the intrinsic cellular and molecular mechanisms governing epithelial morphogenesis in the developing neonatal uterus are little understood. Here, a combination of single-cell RNA-sequencing (scRNA-seq) and fluorescent in-situ hybridization (ISH) was utilized to discover intrinsic cellular and molecular mechanisms directing uterine epithelium morphogenesis during a critical window of postnatal development. The undifferentiated epithelium (PNDs 1 and 5) and determined uterine epithelium (PND 12 and 15) were heterogeneous and contained several different cell clusters based on single cell transcription profiles. Characterization of cell types were based on marker genes for LE (Calb1, Gsto1, Tacstd2), GE (Cxcl15, Foxa2, Sox9), mesenchyme/stroma (Vim), proliferation (Hmgb2, Mki67, Pcna), and putative stem cells (Axin2, Gstm7, Lgr5, Top2a). The expression pattern of putative markers (Aldh1a1, Aldh1a3, and Cited4) of GE progenitor cells identified by scRNA-seq were validated by ISH. Aldh1a1 and Aldh1a3 expression began on PNDs 1 and 5 localized to the epithelia lining the lumen, and by PNDs 12 and 15 expression was localized specifically to GE cells. Expression of Cited4 was detected specifically in the GE at all developmental time points (after PND 5). Interestingly, Cited4 is not regulated by Foxa2 and is expressed in the LE of Foxa2 conditional knockout mice, indicating a role in uterine epithelial development. Taken together, these findings reveal dynamic expression patterns suggesting the specification of epithelial cells down the GE lineage prior to the expression of Foxa2. These findings provided foundational insights for ongoing mechanistic studies on epithelial specification and GE development using mouse genetic models. This work was supported by NIH Grant R01HD096266.

P-189 - The Effects of Bull-Specific Scents on The Reproductive Performances of The Postpartum Hanwoo Cows

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The effects of a bull's presence on cows' reproductive physiology and performance through sensory cues have been studied. The results indicate that the chemical communication between bulls and cows is one of the key factors for normal reproductive functions. We recently extracted the specific chemical compounds from bull's urine and combined them in the air freshener (on a patent). This study aimed to investigate the effect of bull-specific scents on the reproductive efficiency of Hanwoo cows. The study used 90 postpartum Hanwoo cows at four Hanwoo breeding farms from August 2022 to August 2023 in the Republic of Korea. The air fresheners containing bull-specific scents were attached to the pole approximately at 2.5 m height for the treatment group, while such stimuli were absent in the control group. Results revealed significant differences in various reproductive parameters between the treatment and control groups. Specifically, cows exposed to bull-specific scent exhibited shorter intervals (47.6 ± 3.26 days) from calving to first behavioral estrus (ICE) compared to the control group (62.3 ± 3.76 days). The interval from calving to the final artificial insemination (IFAI) from the cows in the treatment group was also shorter than that of cows in the control group (84.1 ± 5.60 days). Additionally, the number of artificial inseminations until conception (NAC) was lower for the cows in the treatment group (1.3 ± 0.07) compared to that of cows in the control group (1.6 ± 0.13). These findings suggest that bull-specific scent-contained air freshener positively influences the reproductive efficiency of Hanwoo cows. In conclusion, the chemical communication between bulls and cows is essential for normal reproductive performance. Acknowledgment: This research was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, and Forestry (1545026914) and the Korea Institute of Science and Technology.

P-190 - The Impact of Micro-Nanoplastics on Female Reproduction

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In recent decades, industrial activities have led to a dramatic increase in the global production of plastic, with millions of tons produced every year. Under the action of various physical, chemical,

or biological agents, plastic waste breaks down into nano and microplastic particles (NMPs) that spread in the environment, posing a threat to human health. Once absorbed, NMPs migrate from the digestive tract to circulatory systems, reaching organs and cells, including female gonads. However, knowledge of their effects on mammalian reproductive functions is insufficient to allow for an accurate risk assessment and management of potential risks in animals and humans. The purpose of the study was to investigate whether NMPs have effects on ovarian follicles, examining potential intracellular mitochondrial dysfunctions and alterations in the sirtuin pathway in a model of human granulosa cells. Human ovarian granulosa cells (KGN cell line) were exposed to NMPs of different sizes (40 nm, 70 nm, 100 nm, and 200 nm) at concentrations ranging from 5 to 1000 µg/ml for 24 hours. Using fluorescent NMPs and observation under confocal laser scanning microscopy, we demonstrated that NMPs are absorbed by granulosa cells at all tested concentrations and doses. Exposure to NMPs was associated with a significant decrease in cell viability, regardless of the sizes and concentrations used. Real-time analysis of oxygen consumption in response to mitochondrial inhibitors (MitoStress kit, Seahorse Xfe96, Agilent) revealed altered bioenergetic profiles in cells exposed to NMPs. Then, we assessed the gene and protein expression of key antioxidant enzymes using real-time Taqman PCR and Western blotting, respectively. Gene and protein expression of catalase (CAT), superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2) was increased in cells exposed to NMPs, while the transcript and protein level of sirtuins (SIRT1 and SIRT3) was decreased. Reduced levels of phosphorylated NRF2, the substrate of SIRT1 that acts as a transcription factor that activates gene expression mediated by antioxidant response elements (ARE), were observed. In this study, we have shown that NMPs enter human granulosa cells and influence their vitality, altering mitochondrial bioenergetics and sirtuin mediated antioxidant response. Our results contribute to understanding the effects of NMPs on mammalian fertility in order to find possible protective approaches. Considering that the susceptibility level of mammalian granulosa cells to NMPs is still unknown, the current results will improve knowledge on the subject and enable living and working in a healthier environment.

P-191 - Progesterone Receptor Component (PGRMC) 1 and 2 Regulates Primordial Follicle Formation

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The ovarian reserve consisting of primordial follicles at the time of birth defines fertility in most female mammals. Each primordial follicle consists of one oocyte surrounded by a layer of granulosa cells. These oocytes start off as primordial germ cells which divide mitotically but with incomplete cytokinesis so that cells are connected by intercellular bridges, forming cysts. The germ cells enter meiosis and progress through the prophase 1 to the diplotene substage. Next, cysts break apart, releasing individual oocytes which are each then surrounded by a layer of granulosa cells forming a primordial follicle containing a diplotene arrested oocyte. The mechanisms controlling these processes remain unclear. Our model is that normally maternal progesterone (P4) prevents premature cyst breakdown and primordial follicle formation. Yet, how the developing ovary receives the P4 signals is unknown. Though P4 signals through both nuclear and membrane receptors, studies have demonstrated active P4-membrane signaling during cyst breakdown. Therefore, our work explores whether the P4 signals are received through P4 membrane receptors, progesterone receptor component (PGRMC) 1 and 2. We also ask whether PGRMC 1 and 2 have roles independent of P4 signaling. Here we used antibodies against PGRMC 1 and 2 in whole mount

immunohistochemistry to determine if both receptors are present prior to and during primordial follicle formation. Ovaries were harvested at 15.5 and 17.5 days post coitum (dpc) as well as postnatal day (PND) 1, 3 and 5. Our results showed PGRMC1 and 2 expression in both oocytes and somatic cells before birth and gradually becoming localized in the cytoplasm of oocytes over time. Furthermore, we used an organ culture system which is a well-established technique that permits us to directly observe the roles of PGRMC 1 and 2 on primordial follicles similar to in vivo. Ovaries were collected at 17.5 dpc and PND 1, placed in culture, and treated with control media, with IgG or 20ug/ml of PGRMC 2 function blocking antibody for 4 to 5 days. Our results showed a significant decrease in percent single oocytes, our measure of primordial follicle formation in PND 1 cultured PGRMC 2 treated ovaries compared to the control. There was no significant change in the percent single oocytes of 17.5 dpc cultured ovaries of both PGRMC 2 treated and control. These results suggest PGRMC 2 may either function independently after birth or requires P4 binding prior to birth to prevent cyst breakdown. PND 1 ovaries were also treated with control media or both PGRMC 1 and 2 function blocking antibodies together, for 5 days to test whether PGRMC 1 and 2 work together to prevent cyst breakdown. there was no significant change in the percent single oocytes in both control and PGRMC 1 and 2 treated ovaries. Our findings suggest a role for PGRMC 2 in the establishment of the ovarian reserve. Currently, we are also testing the role of PGRMC 1 alone during primordial follicle formation.

P-193 - A Remarkable Finding for Embryologists to Choose the Best Protocol in IVF Procedures

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Since the developmental stage of oocyte is a challenging issue in the success of vitrification, this study investigated the effects of vitrification, before and after in vitro maturation, on the survival and maturation rates, developmental competence and the expression levels of genes involved in apoptosis, oxidative stress and epigenetic modifications. Mouse germinal vesicle oocytes were divided into four groups: fresh in vitro matured oocytes without vitrification (fIVM), in vitro matured oocytes after vitrification (vIVM), in vitro matured oocytes before vitrification (IVMv). In addition, in vivo matured oocytes (MII) were used as control. After oocytes collection, maturation and survival rates as well as the intracellular reactive oxygen species (ROS) level were evaluated. Also, the expression level of various genes was analyzed by qRT-PCR. In addition, following artificial activation (parthenogenesis), the developmental competence of oocytes to the blastocyst stage was evaluated. A significant decrease in maturation rate and survival of vIVM oocytes was observed compared to fIVM and IVMv oocytes. Intracellular ROS levels were significantly increased in both vitrified groups compared to the fIVM group, and no significant difference between vitrified groups. pro-apoptotic genes; BAX and Bcl2 as well as genes related to oxidative stress response Hsp1a, Hsp1b and SOD1 were significantly increased in the vIVM group compared to the IVMv

group. Interestingly, epigenetic regulators genes DNMT1, DNMT3a and DNMT3b were highly expressed in IVMv oocytes along with a decrease in the artificial activation rate compared to the vIVM oocytes. Our results indicated that despite observing more negative effects of vitrification before IVM on the survival rate and maturation as well as apoptosis status, less epigenetic changes in vIVM oocytes can make this process a better option in the treatment of infertility than IVM of oocytes followed by vitrification, a hypothesis that needs to be investigation in human oocytes.

P-194 - Exploring The Effect of PRP On Tissue Vascularization and Survival of Follicles in Human Ovarian Tissue Transplanted to Immunodeficient Mice.

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Ovarian tissue cryopreservation (OTC) is a method to preserve fertility in females undergoing gonadotoxic treatment. Despite successful outcomes in restoring endocrine function and achieving pregnancies following ovarian tissue transplantation, a substantial loss of follicles following transplantation reduces the clinical efficacy of the procedure. This study explores the potential of platelet-rich plasma (PRP), specifically human platelet lysate (hPL) and umbilical cord plasma (UCP), to enhance vascularization and follicular survival in human ovarian tissue transplanted to immunodeficient mice. After a grafting period of 3- and 6 days, vascularization was assessed using CD-31 quantification and gene expression of angiogenic markers (VEGF/Vegf) together with apoptosis-related genes (BAX/BCL-2), oxidative stress markers (HMOX/Hmox) and the pro-inflammatory markers (IL-1 β /IL-6/Tnf- α) were quantitatively analysed. Follicle density was analysed in grafts after 4 weeks transplantation. Results indicate that while there was a significant increase in the CD-31 area from day 3 post grafting to day 6, there were no significant differences between the grafts from mice treated with hPL and the control group at day 3 nor day 6, suggesting no added benefit of hPL in enhancing vascularization. Gene expression analysis revealed significant downregulation of VEGF and BAX/BCL-2 from day 3 to day 6 for both hPL and control group and significant upregulation of BAX/BCL-2 in the hPL group compared to the control. Additionally, a small pilot study exploring the suitability of ultrasound scanning of mice or graft survival and vascularization assessment was conducted. Ultrasound biomicroscopy provided valuable insights into graft morphology, necrotic areas, and blood flow, suggesting its potential as a monitoring tool. The follicle density showed a non-significant increase in the hPL group and UCP groups compared to the control. Despite, the angiogenic properties of PRP, this study was unable to demonstrate a significant impact of hPL on vascularization nor of hPL and UCP on follicular survival in xenotransplanted human ovarian tissue. Further research is needed to elucidate the role of PRP in optimizing the outcomes of ovarian tissue transplantation outcomes. Funding Statement: This

project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 860960"

P-195 - Improving Trehalose Uptake and Release via Cold-responsive Nanoparticles to Promote Dehydration Tolerance of Cumulus-Oocyte Complexes during Microwave-assisted Drying

Pei-Chih Lee, Heather Willis, Samantha Stewart, Xiaoming He, and Pierre Comizzoli

As global demand for fertility preservation rises, developing dry-preservation strategies for simpler and more economical long-term storage at non-freezing temperature becomes increasingly appealing. The crucial step in stably maintaining viable gametes at a dried state involves an incorporation of trehalose, a non-permeable disaccharide known to shield anhydrobiotic organisms against dehydration stress. Our previous work demonstrated that cold-responsive nanoparticles (CRNPs) can transport trehalose into cat cumulus-oocyte complexes (COCs) within a 4-hour co-incubation at 38.5°C, followed by a cold-triggered release at 4°C for 15 min. Building on this foundation, this study aimed to (1) optimize the uptake and release of CRNPs to allow safe incorporation of more intracellular protectants, and (2) examine the effect of increased trehalose uptake on membrane and DNA integrity of oocyte after drying and rehydration. COCs were incubated with CRNPs in culture media with different concentrations of bovine serum albumin (BSA) and for different duration. Removing BSA from the culture medium and extending CRNP exposure time from 4 hours to overnight improved CRNP uptake by up to 8-fold. Additionally, an alternative cold treatment at 14 °C for 15 min effectively triggered the release of trehalose without compromising COC competency. Under these enhanced trehalose-delivery conditions, we assessed the effect of intracellular trehalose on desiccation tolerance of COCs. COCs were co-incubated with either blank CRNPs or trehalose-encapsulated CRNPs, followed by cold treatment. COCs then were placed in 0.3 M trehalose and dehydrated via microwave-assisted drying for 0, 5, 10, or 15 min (approximately corresponding to removal of 0%, 35%, 74%, and 96% of water, respectively). Dehydrated COCs were immediately rehydrated in culture medium for 30 min before evaluating membrane (propidium iodide staining; N = 153) and DNA (TUNEL assay; N = 104) integrity. After 10 min of drying, percentages of oocytes with intact membrane significantly increased ($P < 0.05$) from 29% (blank control) to 63% in the presence of intracellular trehalose. No significant advantages were observed at other time points. DNA integrity was maintained in the majority of oocytes ($\geq 93\%$) across all drying times, irrespective of trehalose delivery. Collectively, results suggest that intracellular delivery of trehalose via CRNPs enhances the desiccation tolerance of COCs and contributes to the improved preservation of oocyte membrane integrity during partial dehydration. Our findings offer valuable insights into developing effective dry-preservation strategies to meet the growing demand for fertility preservation.

P-196 - Impact of Long In Vitro Culture on Oocytes from Sheep Early Antral Follicles: A Comparison of Mitochondrial Activity and ROS Levels Between Single and Group Culture

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It has been demonstrated that long in vitro culture (LIVC) of cumulus-oocyte complexes (COCs) from early antral follicles (EAFs) is a promising approach for increasing the number of available oocytes for assisted reproductive technologies. However, suitable culture conditions must be clarified and further optimized. In this study, we compared the efficacy of single versus group cultures of COCs using LIVC. Additionally, we analyzed the mitochondrial function and reactive oxygen species (ROS) levels in cultured oocytes to evaluate the system's efficiency. For this purpose, sheep ovaries were collected from the slaughterhouse, and COCs were retrieved from EAFs (350-450 μ m). The COCs were then cultured either individually (n=327) or in groups (n=312) in TCM199 supplemented with 0.15 μ g/mL zinc sulfate, 10-4 IU/mL FSH, 10 ng/mL estradiol, 50 ng/mL testosterone, 50 ng/mL progesterone, and 5 μ M Cilostamide. Chromatin configuration, mitochondrial activity, mitochondrial distribution, and oocyte ROS levels were analyzed using confocal microscopy and fluorescent probes before and after 5 days of LIVC. Additionally, meiotic competence was assessed after in vitro maturation (IVM). Our findings indicated no statistically significant differences in chromatin configuration, intracellular ROS levels, mitochondrial activity, and distribution pattern after LIVC ($P \geq 0.05$) between treatments. However, following IVM, the group culture showed a significant increase in oocyte maturation capability (34.78 vs. 16.67, $P \leq 0.01$) and a tendency toward lower levels of ROS compared to the single culture ($P > 0.086$). Additionally, mitochondrial distribution patterns showed a noticeable shift from a 'fine' to a 'granular' pattern in the group culture ($P \leq 0.01$), which may benefit oocyte competence. In conclusion, the study demonstrated that both single and group culture conditions effectively supported chromatin configuration changes and mitochondrial activity while decreasing ROS levels in COCs collected from sheep EAFs. However, COCs cultured in groups showed higher rates of meiosis resumption, lower levels of ROS, and a more granular mitochondrial pattern compared to the single culture ones. These findings suggest insights for optimizing the culture conditions of COCs from EAFs, thus widening the availability of competent gametes for assisted reproductive technologies applied to animal breeding and endangered species conservation.

P-197 - Pharmacoprotection of the Ovaries During Chemotherapy Using MicroRNAs Technology Delivered by Gold Nanoparticles

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MiRNA-based therapy is tested as an innovative pharmacological approach to prevent chemotherapy-induced ovarian damage and preserve the fertility of female cancer patients. Previously, we showed that let-7a replacement therapy using liposomes was able to prevent cyclophosphamide metabolites (4HC)-induced apoptosis in PND3 ovaries in vitro but in vivo

delivery of miRNAs remains a major challenge. Gold nanoparticles (AuNPs) system appears to be attractive for their biocompatibility and their ability to be multi-functionalized, allowing the grating of the desired cargo and a future selected ligand for the recognition of a targeted tissue. The synthesis of the AuNPs is concentrated on the chemical bond that link let-7a to the surface of the AuNP. Classically, thiol (sulfur-hydrogen) was used in most of the studies that grafted miRNAs to AuNPs (AuNPs-S-Let-7a). Here, we exploited the macrocyclic molecule calix[4]arene for their robust carbon-gold bond and the possibility to add functional groups, allowing the attachment of a potential control number of miRNAs (AuNPs-X4-Let-7a) and other biomolecules. First, we showed that 3 nM of AuNPs-S-let-7a and AuNPs-X4-Let-7a were not toxic compared to the control (no AuNPs) in PND3 ovaries culture in vitro for 48h by the analysis of the expression of caspase-3 (RT-qPCR), TUNEL assay (double strand DNA breaks detection) and LDH assay (relative secretion of lactate dehydrogenase) (one way ANOVA, $n \geq 4$). Then, AuNPs-X4-Let-7a were functionalized with a fluorochrome that allow the imaging of internalized AuNPs in the PND3 ovaries after 48h of transfection. After observing the safety and the ability of AuNPs-X4-Let-7a to enter the tissue, we transfected PND3 ovaries with these AuNPs 24h prior to a 24h-treatment with 4-HC (AuNPs+4-HC group) to study the protective effect of let-7a delivered by AuNPs. This condition was compared to untreated ovaries (control), ovaries treated with 4-HC for 24h and ovaries cultured with 3 nM of AuNPs-X4-Let-7a for 48h. Using the three same techniques to assess the toxicity of the 4-HC (caspase-3 expression level, TUNEL assay and LDH assay; $n \geq 4$), only the TUNEL assay reported a significant higher apoptosis rate in the 4-HC group compared to control ($p < 0.05$, one-way ANOVA, $n = 5$), although a trend of a higher relative LDH secretion, released by death cells, was observed for the 4-HC group compared to the control ($n = 4$). Regarding the effect of let-7a to protect the ovaries from 4-HC toxicity, no significant difference was reported between the 4-HC group and the AuNPs+4-HC group, suggesting a need for improving let-7 a delivery as not optimal based on let-7 and two of its target genes expression analysis (Hmga2 and Stat3) ($n = 4$). To facilitate access to let-7a, 2 different strategies are currently tested (i) the addition of a spacer (10 uracils) to the passenger strand (ii) the combination of adding a spacer and introducing mismatches in the double strand of let-7a that are endogenously presented. The let-7a delivery is compared to a negative control with a non-targeting sequence. Finally, additional techniques is used to better assess the toxic effect of 4-HC and the potential protective effect of let-7a such as the analysis of other apoptotic genes expression levels and the evaluation of proteins involved in DNA repair, apoptosis and follicles activation.

P-198 - Testosterone and IL-1b Induced Spermatogonial Cells from Normal and Busulfan-Treated Immature Mice to Develop Different Stages of Spermatogenesis in 3D In Vitro Culture.

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Spermatogenesis is a process of sperm generation. During this process, spermatogonial cells proliferate and differentiate into meiotic, post-meiotic stages that continue in spermiogenesis to generate mature sperm. Interleukin-1 is one of the cytokines produced by different testicular cells

under physiological conditions. These microenvironmental factors are modified following pathological conditions, such as chemotherapy, which may lead to subfertility or sterility. The process of spermatogenesis is also regulated by testosterone which is secreted by Leydig cells in response to LH. Different in vitro culture systems were used to induce the development of complete spermatogenesis in vitro; however, this was not yet achieved. We aimed to examine the effect of IL-1b and testosterone alone and in combination on the development of spermatogonial cells from normal and busulfan-treated immature mice to different stages of spermatogenesis in vitro using a methylcellulose culture system (MCS). Sexually immature mice (7-day-old) were used as normal or were intraperitoneally (i.p) injected with busulfan (45 mg/kg) to isolate cells from their seminiferous tubules (STs). Cells were enzymatically isolated from the STs of the mice and were cultured in a methylcellulose culture system (MCS), as a 3-dimensional in vitro system. Fresh media without (CT) or with IL-1b were added to the cultures from the beginning. After two weeks fresh media alone (CT) or containing IL-1b or testosterone were added to the cultures. The cultures were determined after 4 weeks. Cells were enzymatically isolated from the seminiferous tubules and cultured (2×10^5 /well/0.5ml) in MCS that contained StemPro-34 medium, KSR, rEGF, rGDNF, rLIF, and r-bFGF. The cultures were grown in the presence/absence of IL-1b for 2 weeks and thereafter fresh media without or with IL-1b or testosterone (10^{-7} M) were added for an additional 2 weeks. The cultures were incubated in a CO₂ incubator at 37°C. The developed cells and colonies/spheroids were examined microscopically. The developing cells of the pre-meiotic, meiotic, and post-meiotic stages of spermatogenesis were quantified by immunofluorescence staining (IF) and/or qPCR analyses. The expression levels of factors generated by Sertoli cells were examined by qPCR analyses. Our results from normal and busulfan-treated immature mice demonstrated the development of colonies/spheroids that contain pre-meiotic (VASA), meiotic (BOULE), and post-meiotic (ACROSIN) cells in MCS, as examined by IF and or qPCR analyses. The percentage and the expression levels of VASA, BOULE, and ACROSIN were significantly increased following treatment of the cultures (from both types of mice) with IL-1b or testosterone compared to control. However, the percentages and the expression levels of the examined markers were significantly lower in cultures from busulfan-treated mice compared to normal mice under all treatments. Furthermore, the expression levels of 3b-hydroxysteroid dehydrogenase (activity of Leydig cells) and androgen receptor, FSH-R, androgen binding protein, and transferrin (functionality of Sertoli cells) were distinctly expressed in cultures of normal and busulfan-treated immature mice, and following IL-1b or testosterone treatment of both cultures. Our findings provide evidence that busulfan treatment of immature mice reduces the spermatogonial cells and negatively affects their development in vitro. Furthermore, IL-1b and testosterone could improve spermatogonial cells' maturation from normal and busulfan-treated mice in-vitro. This effect of busulfan in vivo and IL-1b and testosterone in vitro on the development of spermatogenesis in vitro is regulated by Sertoli and Leydig cells.

P-199 - A Possible Role of Estrogen Receptors in Primordial Follicle Activation in 3D Culture Model

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Increased primordial follicle activation induces immoderate depletion of ovarian reserve and premature ovarian insufficiency, so the regulation of primordial follicle activation is one of the

crucial steps. Steroid hormones, especially estrogen, are known to play an important role in relation to nest breakdown and subsequent follicle development. Although a relationship between the primordial follicle activation and E2 concentration is predicted in many studies, it is not well evaluated. In this study, the anti-Müllerian hormone (Amh) knockout mice and in vitro culture were employed to treat specific antagonist and agonist of the estrogen receptors and to compare the degree of activation. As expected, the delivery rate of Amh null mice by aging was significantly decreased but the level of phosphate Akt was significantly high. Interestingly the expression levels of Amh mRNA was significantly low but the protein level was significantly high in the ovary of Amh null mice. The expression levels of the Esr1 mRNA and protein were significantly low in Amh KO ovary but not Esr2 and Gper1. In some follicles, ESR1 and FOXO3 were colocalized in nucleus. The expression of genes could be clustered by the culture periods. Although further studies are needed, the results suggest that Esr may have roles in follicle dormancy. Grant: This study was supported by the NRF-2021K1A4A7A02098794 and NRF-2023R1A2C3007223

P-200 - Vaccination against Foot-and-Mouth Disease Deteriorates Acute-Phase Inflammation with Anovulation in Hanwoo (*Bos taurus coreanae*)

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Foot-and-mouth disease (FMD) is a common disease in cattle accompanied with blisters and loss of appetite which leads to economic loss. Vaccination is used to prevent livestock from FMD virus. However, vaccination against FMD has side effects including miscarriage, early embryo death, lower production of milk and decreased growth of fattening cattle. Although, FMD virus vaccines have been reported that loss of early embryo is increased after artificial insemination in cattle, no in vivo studies on the effects of FMDV vaccines within acute-phase inflammation and anovulation in Korean cattle. 100 Hanwoo (*Bos taurus coreanae*) cows from the Gyeongsangbuk-do Livestock Research Institute synchronized with ovulation were used; only individuals with estrus confirmed by ovarian ultrasound were selected for the test. All test axes are inseminated artificially 21 days after the previous estrus period. The control group was administered normal saline, the negative control was injected intramuscularly with lipopolysaccharide (LPS), and the test group was administered a foot-and-mouth disease virus vaccine 2, 9, and 16 days before artificial insemination. Neutrophils and white blood cells significantly increased 1 day after vaccination. In addition, the ovulation rates decreased especially in FMDV vaccine (-2d) group. FMDV vaccine group took 9 days after inoculation for recovering to the normal range of main acute immune response factors. In summary, FMDV vaccine increased expression of acute-phase inflammation response proteins and delayed ovulation with inflammatory cytokines.

P-201 - Effects of Aqueous Plant Extracts on Growth Performance and Haematology of Pure and Crossbred Chickens

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Effects of Aqueous Plant Extracts on Growth Performance and Haematology of Pure and Crossbred Chickens Nnenna W. Anizoba¹; Nnanna N. Ikeh¹; Bright C. Amaefule¹; Chekwube M. Ugwu¹; Celestine Ezenwosu¹; Ndubuisi S. Machebe¹ 1. Department of Animal Science, Faculty of Agriculture, University of Nigeria, Nsukka Enugu State, 410001, Nigeria In the pursuit of solving food insecurity especially with respect to animal protein intake in developing countries, this study investigates novel approaches that promise to bridge the protein gap of the ever-rising population. Nnenna W. Anizoba, Nnanna N. Ikeh, and Ndubuisi S. Machebe, affiliated with the Department of Animal Science at University of Nigeria, Nsukka, join forces with Bright C. Amaefule, Chekwube M. Ugwu and Celestine Ezenwosu from the same institute. This study aimed to evaluate the effects of aqueous plant extracts on growth performance and haematology of pure and crossbred chickens. A total of 360 one-day-old chicks from 3 genetic groups consisting; 120 noiler chicks, 120 heavy ecotype chicks and 120 main cross chicks were randomly distributed after weighing in 4 groups each (3 replicates in each, n=10 chickens). A 3×4 factorial arrangement of 4 dietary treatments were constituted as follows: T1 - Birds on 0 ml of extract; T2 - Birds on 200 ml of neem extract; T3 - Birds on 200 ml of ginger extract and T4 - Birds on 200 ml of neem + ginger extract. Growth parameters such as Hatching weight (HW), Final weight (FW), average daily gain (ADG), average feed intake (AFI) and feed conversion ratio (FCR) showed significant ($P < 0.05$) effect among genotype. Birds on T3 and T4 had the best growth performance with improved feed conversion ratio. Final weight (FW) and average daily gain (ADG) showed significant ($P < 0.05$) effect among the genotype and treatment effect. The results of the haematological indices revealed that the effect of genotype and treatment on haematological indices was significantly ($P < 0.05$) different for haemoglobin (Hb), packed cell volume (PCV), white blood cell (WBC) and platelet (P). Some of the haematological indices were better ($P < 0.05$) for birds on T3 and T4. Based on obtained result, it was concluded that variation in the growth performance and haematological parameters between 3 chicken genotypes in Nigeria is due to differences in their genetic makeup in combination with plant extracts and that T3 (200 mg of ginger) and T4 (100 mg neem + 100 mg ginger) were appropriate without exhibiting deleterious effects on the physiological characteristics of birds.

P-202 - Expression of Receptors for Progesterone in Blood Leukocytes of Cattle

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Contradicting evidence exists about the presence of nuclear progesterone receptor (PGR) in immune cells in cattle. Nonetheless, previous literature identified that bovine peripheral blood mononuclear cells (PBMC) express membrane-associated molecules from the family of Progesterin and AdipoQ receptors (PAQR) and progesterone receptor membrane components (PGRMC). Here we determined expression of progesterone and estradiol receptors in peripheral blood leukocytes (PBL) and effects of reproductive status (cyclic vs pregnant), parity (heifers vs cows), or day of pregnancy on expression. Nulliparous heifers and lactating cows were assigned to be bred or remain non-pregnant. Blood was collected on day 18 of the estrous cycle and pregnancy in cows and heifers, and on days 30 and 60 of pregnancy in cows. PBL were isolated from blood and RT-qPCR analysis determined expression of PGR, ESR1, PAQR7, PAQR8, PAQR6, PAQR5, PGRMC1, and PGRMC2. PGR expression was undetected using RT-qPCR in PBL. On day 18 of the estrous cycle and pregnancy, ESR1, PAQR8, PGRMC1, and PGRMC2 mRNA were detected in PBL of heifers and

cows. On days 30 and 60 of pregnancy, ESR1, PAQR7, PAQR8, PAQR6, PAQR5, PGRMC1, and PGRMC2 were detectable in PBL of cows. In heifers, PGRMC1 expression was less in pregnant than cyclic animals while in cows the inverse relationship was observed. PAQR8 mRNA tended to be greater in cows than heifers while PGRMC2 mRNA tended to be greater in pregnant than cyclic animals. Results support previous reports, and extend those findings to include neutrophils, revealing that bovine PBL do not express PGR. Instead, membrane progesterone receptors (mPGR) of the PAQR and PGRMC family are present and could mediate progesterone effects on leukocyte function. Additional research is required to determine effects of membrane progesterone receptors in leukocyte subsets. Supported in part by USDA NIFA grant # 2017-67015-26455 to TLO and NIH-T32 Fellowship grant #T32GM108563.

P-203 - Dietary Inclusion of African-bread Fruit (*Treculia africana*) Pulp on Growth Performance and Haematological Indices of Weaner Rabbits

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In the pursuit to achieve food security and address the challenge of dietary protein deficiency in Sub-Saharan African countries, this study investigates novel approaches to ensure year-round, cost-effective meat production. Chekwube M. Ugwu and Augustine O. Ani affiliated with the Department of Animal Science, Faculty of Agriculture, University of Nigeria Nsukka, join forces with Nnenna W. Anizoba, Celestine Ezenwosu and Amarachi L. Obinna in the same University. This study investigated the response in growth performance and haematological parameters of rabbits fed dietary inclusion of dried African bread-fruit pulp (DABP). A total of sixty 6-weeks-old hybrid (New Zealand white) rabbits with an average weight of 759.5 g were randomly assigned to 5 dietary groups with 4 replicates of 3 rabbits each for 8 weeks in a completely randomized design. The groups were fed diets containing dried African bread-fruit pulp (DABP) at 0, 5, 10, 15, and 20% levels (coded as (DABP 0, DABP 5, DABP 10, and DABP 20). Results showed that dietary dried African bread-fruit pulp supplementation at 15 and 20% decreased ($P < 0.05$) the feed intake (FI) and body weight gain (BWG). However, rabbit at 15 and 20% showed decreased ($P < 0.05$) packed cell volume (PCV), white blood cell (WBC) and haemoglobin concentration (Hb). Based on the results obtained from the present study, it was concluded that up to 5-10% inclusion level of dried African bread-fruit pulp can be incorporated into rabbit diets without negatively affecting growth performance and haematological traits of rabbits.

P-204 - Sperm Interact with Human Cervical Epithelial Cells to Modulate Cytokine Synthesis

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Seminal fluid introduced into the female reproductive tract at coitus induces an inflammation-like response within the cervical tissues of women. The response involves induction of cytokines and chemokines to drive recruitment and activation of leukocytes that in turn selectively edit the sperm population able to access oocytes, and promote immune tolerance towards male alloantigens to potentially facilitate embryo implantation. Soluble factors in seminal plasma are important

mediators of seminal fluid signalling. Recently we showed in mice that sperm also facilitate the female response – but whether sperm contribute to the cervical immune response in humans is unclear. In this study, we evaluated the hypothesis that human sperm interact with Ect1 ectocervical epithelial cells to induce cytokine production and alter gene expression in vitro. Ten normozoospermic men (according to WHO VI guidelines) of reproductive age provided semen samples for the study. Ect1 cells were incubated with 10% (v/v) whole semen, 10% seminal plasma, 10% washed sperm, or washed sperm at fixed concentrations of 1, 5, and 10 x 10⁶ sperm/mL, then gene expression and cytokine secretion were assessed in 8- and 24-hour supernatants by TaqMan Human inflammation panel OpenArray® and multiplex microbead assay, respectively. Scanning and transmission electron microscopy was used to visualize physical interactions between sperm and Ect1 cells. Washed sperm (10% v/v equivalent), as well as whole semen (10%) and seminal plasma (10%), all induced Ect1 cell production of CSF1, CXCL8, and CXCL11, whilst only semen and seminal plasma induced production of CSF2, CSF3, IL1B, IL6, IL20, CXCL1, CXCL16, CCL3, and CCL20 (n=10, all P < 0.05). Sperm-mediated induction occurred in a dose-dependent manner with the highest dose (10 x 10⁶ sperm/mL) inducing maximal increase in Ect1 cell production of CSF2, CSF3, IL1A, CXCL8, CXCL11, CXCL16, CCL3 (n=10, all P < 0.05). IL1Ra production was inhibited by both whole semen and sperm, whilst CXCL10 was inhibited by whole semen and seminal plasma (n=10, P < 0.05). Gene expression analysis revealed a total of 178 genes as differentially regulated by treatment with seminal fluid components, with 128 genes upregulated and 50 genes downregulated (n=6, P < 0.05). Many cytokine and chemokine genes were upregulated after contact with washed sperm, as well as whole semen and seminal plasma, with CSF2, IL6, IL33, CXCL2, CXCL3, and CXCL8 all amongst the top 20 most upregulated genes across all three treatments. Principal component analysis identified different expression patterns induced by washed sperm compared to the similar expression patterns elicited by whole semen and seminal plasma. Electron microscopy showed evidence of sperm head attachment to microvilli on the Ect1 cell surface, and in some instances, complete engulfment by Ect1 cells. Collectively this data shows that human sperm have capacity to interact closely with cervical epithelial cells and can act to modulate their cytokine production and gene expression profile. Understanding the mechanisms that mediate this interaction will progress understanding of biological processes by which maternal immune tolerance is generated and may aid in the development of interventions that improve immune receptivity to embryo implantation.

P-205 - Evaluating Pregnancy Induced Changes to Peripheral Immune Cell Populations in Cattle Using Single Cell RNA Sequencing.

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Describing the role of the maternal immune system during pregnancy is critical to our understanding of physiology and pregnancy complications. While these evaluations have been performed in humans and rodents using single cell RNA sequencing technology, information pertaining to reproductive immune system changes in cattle are lacking. We hypothesized that peripheral blood immune cell populations would be altered by pregnancy in cattle. Whole blood was collected from two pregnant Holstein heifers on day 33 of gestation, and two virgin heifers on

the day of estrous synchronization immediately before removal of a controlled intravaginal drug release device containing progesterone. All heifers were later confirmed to have carried a pregnancy to term following a single insemination. CD45 positive cells from each heifer were isolated from whole blood using FACS and approximately 10,000 cells from each heifer were subjected to 10x Chromium library preparation and sequenced using an Illumina NovaSeq X Plus. After initial data preparation and cleaning, each heifer had an average of 6,653 cells, 49,693 reads per cell, and 1,091 genes per cell analyzed. When the data for all four heifers was integrated a total of 11 cell clusters were identified in peripheral blood of cattle. Each cluster was manually annotated using expression of cell receptors and transcription factors to identify the immune cell types corresponding to each cell cluster. Pregnancy at d 33 increased the absolute number and relative proportion of activated B-cells, naïve B-cells and gamma delta T-cells in circulation compared to virgin heifers. Pregnancy modulated the expression of several transcripts in specific cell types, including an upregulation of JSP.1 in B-cells, CD4 T-cells, CD8 T-cells, gamma delta T-cells, granulocytes, NK cells, and monocytes. Similarly, pregnancy increased the expression of CCL5, IDO and IL7R in CD4 T-cells, while the expression of IFIT1 and MX2 were increased and IL18 was decreased by pregnancy in granulocytes. Pathway analysis was performed for transcripts effected by pregnancy from each cell cluster. The eukaryotic initiation factor 2 (eIF2) signaling pathway, involved in translational stress responses, is downregulated by pregnancy in B-cells, CD4 T-cells, CD8 T-cells, gamma delta T-cells, NK cells and monocytes. Conversely, the estrogen receptor signaling pathway is upregulated by pregnancy in B-cells, CD4 T-cells, CD8 T-cells, gamma delta T-cells, NK cells and monocytes. As expected, interferon signaling was increased by pregnancy in granulocytes, likely due to the secretion of trophoblast derived interferon tau. Further analysis suggests the involvement of numerous cytokines, kinases, transcription factors, receptors, and enzymes in controlling pregnancy regulated genes in each cell cluster. Of interest is the potential involvement of IL-4 and ESR1 in regulating differentially expressed genes in B-cells, CD4 T-cells, gamma delta T-cells, CD8 T-cells and NK cells. While all the data obtained from single cell RNAseq is too numerous to report here, these analyses reveal clear proportional and transcriptional changes to peripheral immune cell populations due to pregnancy at d 33 of gestation. By using single cell resolution, we have a greater appreciation for the immune cells present in peripheral blood of cattle and their potential involvement during pregnancy. Evaluating pregnancy induced changes to maternal immune cell populations will allow us to better understand the role of the immune system in pregnancy and pregnancy complications, while potentially developing new tools for early pregnancy diagnosis and interventional strategies in cattle.

P-206 - MZB1 Regulates the Immune Microenvironment and Promotes Ovarian Cancer Cell Migration

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Objective: The immune microenvironment of ovarian cancer plays a pivotal and indispensable role in driving its progression, and the emerging recognition of MZB1's substantial involvement in shaping the tumor immune microenvironment (TIME) underscores its growing significance.

However, the role of MZB1 in ovarian cancer remains mysterious. We endeavor to elucidate the role and mechanism of MZB1 in ovarian cancer, thereby promoting the clinical application of MZB1. **Design:** This study explores the role and mechanism of MZB1 in ovarian cancer through an integrated approach combining bioinformatics analysis with cell and molecular biology experiments. **Method:** A total of 381 samples from the TCGA dataset were analyzed. Differentially expressed genes (DEGs) were compared using DEseq2. Multiple algorithms were employed to assess the relevance between MZB1 and the TIME. In both conditions of MZB1 overexpression and knockout, cell proliferation was evaluated using the CCK-8 assay, while cell migration was assessed through the wound healing assay. Subsequently, a nomogram was developed for predicting 3- and 5-year overall survival based on multivariable Cox regression results. The performance of these nomograms was evaluated using a C-index and calibration plot to determine their discriminative ability and calibration. **Results:** Our findings revealed a positive correlation between high MZB1 expression and improved clinical prognosis. Elevated MZB1 promotes migration of SKOV3 cells, but has no effect on proliferation. Furthermore, higher MZB1 expression was associated with increased infiltration of immune cells within the tumor microenvironment. The C-index was 0.702 (0.680-0.724), and the nomogram demonstrated good calibration through the calibration curve, indicating satisfactory discrimination and calibration in predicting patient outcomes. **Conclusions:** This study offers a comprehensive understanding of MZB1 and its implications, paving the way for novel avenues in personalized treatment approaches.

P-207 - Single Cell 'Omics Technologies for Contraceptive Discovery

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Around half of all pregnancies occur unintentionally. While female hormonal pills and barrier-based contraceptive options for men are currently available, there is a critical need to identify new, effective, and safe non-hormonal contraceptive alternatives. Meiosis is a specialized reductive division during gametogenesis that results in the production of haploid gametes. Recent work from our lab has shown that meiosis represents a suitable time point to target for non-hormonal contraception. As meiosis is critical for proper gamete formation in both sexes, we hypothesize that this stage is a promising timepoint to target for novel male and female non-hormonal contraceptives. In males, spermatogonia enter meiosis at around the time of puberty and this continues in waves throughout life. A complex gene regulation network exists in cells undergoing meiotic entry, providing us with many possible gene targets. By contrast, females undergo meiotic entry and progression through prophase I in utero, but oocytes arrest in an extended diplotene phase of meiosis I (known as “dictyate arrest”). Importantly, throughout dictyate arrest, which can last years to decades in women, the oocyte is transcriptionally inert. At puberty, meiosis is resumed in a cohort of oocytes in response to hormonal stimuli that result in a cascade of protein signaling pathways between the somatic cells and the oocytes, leading to the completion of meiosis I and

subsequent ovulation. Given this considerable sexual dimorphism in meiosis, there is a need for differing technologies to study these processes and to identify potential contraceptive targets at single-cell resolution. As we are interested in targeting meiotic entry in males, we have employed cutting-edge spatial transcriptomics platforms that provide information at a single-cell resolution. In this study, we optimized Slide-seq and Stereo-seq for Spatial Total RNA Sequencing, which provides us with the entire repertoire of RNA species (coding and non-coding RNAs) that are involved in meiotic regulation. By contrast, since meiotic entry is inaccessible in female meiosis, we are interested in maintaining dictyate arrest as a non-hormonal contraceptive strategy. Owing to the low transcriptional abundance during this phase, we instead focused on identifying proteins present during dictyate arrest at single-cell resolution, reasoning that only a few oocytes at each cycle can respond to stimuli that lead to ovulation. Thus, we want to understand the proteins involved in meiotic resumption following dictyate arrest. We hope targeting these proteins achieves a contraceptive effect by maintaining oocytes in arrest. As oocytes undergo meiotic resumption in cyclical cohorts, the number of cells undergoing these complex mechanisms is very few. In addition, it is unknown what contributes to the selection of certain oocytes into resuming meiosis during these cycles. Current proteomics techniques require significantly large input material to obtain sufficient peptide information, which has hitherto prohibited the study of these cells for their differences. To overcome these limitations, we are developing a novel proteomics technology that enables the identification of thousands of peptides with as few as ten oocytes. This technology will allow us to identify novel protein targets for the use of non-hormonal contraception, far outcompeting existing methods in the field. This study outlines our efforts to develop state-of-the-art single-cell techniques to further our understanding of possible targets in meiosis in both males and females, which will advance options for non-hormonal contraceptives.

P-208 - Identifying Chemical Features that Favor or Block the Passage across the Blood-Testis Barrier in Mice

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Understanding the chemical features that influence the ability for xenobiotics to cross the blood-testis barrier (BTB) will facilitate the discovery and development of new male contraceptives since many male contraceptive targets are localized to the adluminal compartment of the seminiferous epithelium. We identified key chemical features that facilitate or block the passage of drugs across the BTB by measuring tissue distribution of a total of 300 compounds and their major metabolites using liquid chromatography mass spectrometry (LC-MS) and inferring the properties that influence accumulation in the male genital tract. We have also determined if a compound passes the BTB by monitoring its spatial distribution in the testis using imaging mass spectrometry. Our tissue distribution data indicate that compounds containing N-dialkyl-, N-monoalkyl-, naphthol-, and

biphenyl- chemotypes facilitate mouse testis uptake and accumulation. Conversely, compounds bearing carboxylic acid, glucuronic acid, nitrogen oxide, phenol or primary alcohol oppose testis uptake and compound accumulation in the testis. We are using cheminformatics to correlate structures and molecular descriptors with the experimental tissue distribution data to identify features that facilitate or impede testis accumulation, and we are employing these data to develop explainable Machine Learning to predict drug accumulation in testis. Overall, this work will establish predictive uptake rules applicable to drugs that must act behind BTB, which facilitate the development of new male contraceptives for which accumulation in the testis. This work was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (R61/R33HD099995) to Feng Li; R01 HD110038 to Dr. Martin. M. Matzuk.

P-209 - Exploring QRICH2 as a Potential Male Contraceptive Target

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Currently, the contraceptive options available to men are limited to condoms and vasectomy. This highlights the pressing need for the development of alternative contraceptive methods. In this regard, identifying and characterizing reproductive tract specific proteins that can be targeted by natural or chemical compounds is crucial. We propose that QRICH2 could serve as a potential target as it appears to be testis-enriched and mutations (in humans and bulls) or knockout (in mice) of this protein result in complete infertility without evident accompanying symptoms (Shen et al. 2019, Nat. Commun. 10(1): 433; Kherraf et al. 2019, Clin. Genet. 96(5): 394–401). The objective of this study is to better characterize human QRICH2 to determine if it could be used as a male contraceptive target. We examined QRICH2 expression in 10 different organs by immunofluorescence and by analyzing published proteomes and showed that the protein is restricted to the testes. It is expressed at the level of the nucleus across all germ cell stages, except in elongated spermatids and spermatozoa, where it localizes in the flagellum. It is also expressed around the nucleus in Sertoli cells. In silico studies revealed that QRICH2 lacks sequence homology with other human proteins and exhibits high conservation among mammals, particularly in its three functional domains (Glutenin hmw superfamily, SMC_N superfamily, and DUF4795), the functions of which remain unknown. We also established a protocol for extracting and analyzing native QRICH2 from human spermatozoa. Notably, only strong denaturing buffers (containing high concentrations of SDS or urea) allowed to extract the protein, suggesting its high stabilization in the flagellum, possibly through interactions with cytoskeletal proteins. Indeed, interactions between QRICH2 and AKAP3, AKAP4, ODF2, and TSSK4 have been reported in the literature. However, these proteins are likely just a subset of QRICH2 partners, and we are currently exploring all potential partners through yeast two-hybrid analysis. Although QRICH2 is not a classic target for inhibitor molecules (i.e., not an enzyme or channel), molecules targeting its interactions with protein partners could potentially lead to inhibition of its function.

P-210 - NICHD's Contract Facilities for Contraceptive and Reproductive Health Research Development

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The priorities of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) include development of novel, safe, and effective non-hormonal contraception for both men and women and the promotion of reproductive health. The NICHD has two state-of-the-art facilities: the Biological Testing Facility (BTF) and Chemical Screening and Optimization Facility (CSOF). To advance contraceptive and reproductive health product development, these facilities have the capabilities and capacity for preclinical and Investigational Device Exemption (IDE)- or Investigational New Drug (IND)-enabling studies. The long-term objective is to enable a preclinical candidate for IND/IDE studies that offers a safe therapeutic option in the field of contraception and/or reproductive health related indications as outlined in the scope of the facilities' PARs. NICHD seeks innovative and validated methods for future clinical development. The goal of this poster is to provide potential applicants with information on a mechanism to request services from these NICHD-contracted research facilities that would advance their development program with an emphasis on facilitating early-stage development research, bridging studies, pivot studies and entry into clinical studies. Potential in vitro and in vivo services available to support that goal include but are not limited to protein generation, X-ray crystallography, high throughput screening, structure activity relationships, hit-to-lead generation, drug metabolism in the CSOF and fertility studies, pharmacology, toxicology, formulation, reproductive tract histopathology, sperm morphology and motility, and other studies in the BTF in pursuit of an IND or IDE application for submission to the US Food and Drug Administration or analogous submissions to other regulatory agencies. The facility has the capability to advance multiple projects simultaneously across the entire product development continuum, is open to foreign institutions, and is not limited to programs with current NICHD support.

P-211 - Phosphorylation does not regulate the activity of steroidogenic acute regulatory protein (STAR): Disputing the STAR phosphoprotein narrative

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The Steroidogenic Acute Regulatory (STAR) protein is a cholesterol shuttle in the mitochondrial inter-membrane space crucial for all de novo steroidogenesis. In demonstrating this mechanism of action in recent studies, we were puzzled how a protein that undergoes co-translational import and ultimately folds in the mitochondrial intermembrane space can encounter phosphorylation by the cytoplasmic enzymes: cAMP-dependent protein kinase (PKA) or the mitogen-activated protein kinases (MAPKs), that have been implicated in what is considered a key regulatory step in STAR function. In prior literature, points of STAR phosphorylation were determined based on conserved consensus sites to be the Serine (Ser) amino acids: Ser55, Ser56, and Ser194. Functional assessments for each of these possibilities were carried out in an induced-steroidogenic system using COS-1 cells by expressing STAR mutants that replaced Ser with Alanine (Ala). From these experiments it was suggested that mutations eliminating the ability of STAR phosphorylation at

Ser194Ala resulted in hypomorphic STAR with reduced steroidogenic function. Mutations at Ser55Ala and Ser56Ala had no effects. In analyzing this literature carefully, we identified that although Ala lacks a side-chain that can be phosphorylated, it is a hydrophobic amino acid (compared to the hydrophilic Ser that it replaces), a shift could disrupt the protein's structure/function. Therefore, we decided to reexamine these mutations by replacing the Ser with another hydrophilic amino acid, Glycine (Gly), that would not only lack a side-chain for phosphorylation but also avoid any inadvertent disruptions to the STAR secondary structure. For our experiments, we used MA10 Leydig STAR knockout cells (MA10STKO) for precisely testing functional recovery after reconstituting expression of wildtype STAR or different STAR mutants constructed in lentiviral vectors using precise site-directed mutagenesis. Our findings showed that all the STAR mutants (Ser55Gly, Ser56Gly and Ser194Gly) were able to fully support steroidogenesis in MA10STKO cells. This raised the question whether previous conclusions that were purported to indicate phosphorylation with Ser194 were in fact indicative of a structural/functional interference caused by Ala mutation. These findings allow us to conclude that the premise of STAR being regulated by phosphorylation is unsubstantiated. This is reinforced by the fact that there is no direct mass spectrometry evidence for a STAR phosphoprotein. Previous work on ³²P incorporation in expressed STAR was performed using COS1 cells co-expressing a designer protein fusion (termed F2) that integrated CYP11A1, adrenodoxin and adrenodoxin reductase, a system that has supported cytoplasmic STAR activity without mitochondrial targeting. Our results also challenge the signaling assumptions that PKA and MAPKs can directly target and regulate STAR, thereby highlighting the need to reevaluate upstream signaling mechanisms relevant for the acute regulation of steroidogenesis.

P-212 - Acute Regulation of Steroidogenesis: Endocytosis-mediated Mitochondrial Cholesterol Supply is an Imperative Step that Enables STAR Function

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Recent findings that STAR functions as a cholesterol shuttle in the mitochondrial intermembrane space has brought renewed focus on upstream steps that enable colossal levels of cholesterol supply to the outer mitochondrial membrane (OMM). The current model is built on probable endoplasmic reticulum (ER) to OMM cholesterol transfer mediated by points of close apposition, termed the mitochondria associated membrane (MAM). However, two new observations from MA10 Leydig cells led us to question this model in that: (1) Induced expression of functional STAR by itself could not initiate any steroidogenesis, a finding that conflicted with the widely-accepted assumption about STAR being the rate-limiting step in steroidogenesis. (2) Blocking sterol-associated endocytosis using filipin, a macrolide antibiotic that in live cells binds plasma membrane (PM) free cholesterol, could completely abolish trophic hormone induced steroidogenesis. On this basis, we investigated the role of endocytosis in mitochondrial cholesterol supply. Inhibition of sterol-associated endocytosis by using nystatin, another free cholesterol binding drug, provided results identical to that observed with filipin. In dose dependent responses, both filipin and nystatin treatments resulted in decreases that led to a complete cessation of progesterone biosynthesis at the high doses. This finding demonstrated that without endocytosis and delivery of PM free cholesterol, steroidogenesis is unable to proceed. Endocytosis of cholesterol-enriched regions in the PM can occur through different modalities that include:

Clathrin-mediated, Caveolae-mediated, RhoA-mediated, Endophilin-mediated, and Cdc42-mediated endocytosis. Among these, the first four types of endocytosis are dependent on dynamin, a protein that assembles as helical polymers at the neck of invaginating vesicles and induces fission to generate free endosomes. Therefore, we examined the effect of Dynasore and Dyngo 4a, two small molecules that inhibit dynamin. Our results showed significant declines to steroidogenic function approaching a complete cessation of steroidogenesis with dynamin inhibition. To confirm this pharmacology using a genetic model, we developed a doxycycline inducible lentiviral system to express a dominant negative (DN) form of dynamin 2 in MA10 cells. Induction of DN dynamin 2 significantly suppressed trophic hormone induced steroidogenesis by >80%. These findings not only confirmed that dynamin-dependent endocytosis is an essential step in channeling PM cholesterol to mitochondria, but also indicated that MAMs do not support ER to mitochondrial cholesterol transport for steroidogenesis. As clathrin-mediated endocytosis is triggered for luteinizing hormone receptor (LHR) turnover after trophic stimulation, we evaluated whether the PM cholesterol mobilization is integrated with LHR signaling. When we used chlorpromazine, a small molecule that inhibits clathrin organization, we found significant declines (>80%) to steroidogenic capacity. This finding indicated that PM cholesterol mobilization is mechanistically coupled to trophic hormone signaling in steroidogenic cells. These results are remarkable for three reasons: First, they indicate that all intracellular and extracellular sources need to supply cholesterol to the PM first, before transport to the mitochondria. This principle provides the means for a cell to regulate mitochondrial cholesterol supply by starting and stopping this process via a single channel. Second, they demonstrate that MAMs do not have a role in mitochondrial cholesterol supply for steroidogenesis, which is contrary to the prevailing view. Third, they reveal that STAR function cannot be rate-limiting as OMM cholesterol delivery is an independently regulated first and essential step, that enables the second step of OMM to inner mitochondrial membrane cholesterol translocation by STAR.

P-213 - GATA2 is an Essential Transcription Factor for Epididymal Morphogenesis

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Androgen actions have been known as the predominant driver of male reproductive tract development. During fetal development, testicular androgens promote the elongation and coiling of the Wolffian duct, the precursor of the epididymis. Proper Wolffian duct morphogenesis is important for the epididymal function in supporting sperm transport, maturation, and storage. The Wolffian duct is surrounded by undifferentiated connective tissues also known as the mesenchyme, which governs the morphogenesis and differentiation of ductal epithelium in developing organs. We recently discovered that GATA2 was specifically expressed in Wolffian duct mesenchyme during the fetal masculinization window. GATA2 belongs to the GATA family of transcription factors that bind to the characteristic “GATA” DNA sequence. Among the six members of the GATA family, GATA2 is the only member that is highly expressed in the Wolffian duct mesenchyme. GATA2 has been known to play crucial roles in cell differentiation and tubular

morphogenesis of the mammalian ureter and inner ear. We therefore hypothesize that mesenchymal GATA2 is essential for Wolffian duct development. To test this hypothesis, we used the tissue-specific Cre-LoxP mouse model (Osr2-Cre; Gata2-flox) to delete Gata2 in the Wolffian duct mesenchyme. When Gata2 was absent in the mesenchyme, the Wolffian duct became shortened and failed to coil in the corpus and cauda regions. Wolffian duct elongation and coiling have been predominantly linked with androgen actions; however, we didn't observe any deficiency in androgen production or androgen receptor expression. Then, we turned our attention to androgen-regulated *Inhba*, a mesenchyme-derived morphogen that is essential for epididymal elongation and coiling. By performing RNAscope and RT-qPCR, we discovered that *Inhba* expression was downregulated, and its expression localization was shifted from inner mesenchyme to the outer mesenchymal layer surrounding the Wolffian duct. Consistent with the reduction of *Inhba* expression, we observed reduced percentage of Ki67+ proliferating cells in the epithelium of Gata2cKO Wolffian ducts. These results indicate that the defective coiling observed in Gata2cKO is at least in part due to decreased *Inhba* expression and/or inappropriate localization of *Inhba*. To gain more insights into the mechanisms of GATA2 action, we compared transcriptomes between control and Gata2cKO Wolffian duct by bulk RNA-seq. Among 698 differentially expressed genes, we focused on the epididymal tissue marker, *Itga4*. *Itga4* encodes the integrin subunit alpha 4, a subunit of the receptor for fibronectin. In the control tissue, ITGA4 is strongly expressed in the mesenchyme of the epididymal region. However, its expression was significantly reduced in the Gata2cKO, suggesting that GATA2 might control the identity of the epididymal mesenchyme. Therefore, our discovery not only demonstrates that GATA2 regulates paracrine signaling and identity of epididymal mesenchyme in promoting epididymal morphogenesis, but also prompts the investigation of the potential effects of human GATA2 mutations on epididymal morphogenesis and the predisposition to male infertility.

P-214 - The Aryl Hydrocarbon Receptor Agonist ITE Reduces Inflammation and Urinary Dysfunction in a Mouse Model of Autoimmune Prostatitis

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Prostate inflammation is linked to lower urinary tract dysfunction and is a key factor in chronic prostatitis / chronic pelvic pain syndrome. Autoimmunity was recently identified as a driver of prostate inflammation. Agonists of the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, have been used to suppress autoimmunity in mouse models of colitis, rhinitis, and dermatitis, but whether AHR agonists suppress prostate autoimmunity has not been examined. Here, we test whether ITE (2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester), an

AHR agonist, suppresses inflammation, allodynia, and urinary dysfunction in a mouse model of Experimental Autoimmune Prostatitis (EAP). C57BL/6J adult male mice were immunized with rat prostate antigen to induce Experimental Autoimmune Prostatitis (EAP) or TiterMax Gold® adjuvant (uninflamed control). Mice were also treated with ITE (10 mg/kg/day IP for 6 d) or DMSO (5 mg/kg/day) for 6d (vehicle). EAP heightened histological inflammation in the dorsal prostate, induced tactile allodynia, and increased the frequency of non-voiding bladder contractions. ITE significantly mitigated the actions of EAP. Using the Nanostring nCounter Inflammation Panel, we evaluated the impact of EAP and ITE on prostatic RNA abundance. EAP changed abundance of 40 inflammation-related RNAs, while ITE changed abundance of 28 inflammation-related RNAs. We identified a cluster of RNAs for which ITE protected against EAP-induced changes in the abundance of H2-Ab1, S100a8, and S100a9. ITE also increased the abundance of the AHR-responsive Cyp1b1 RNA. These findings support the hypothesis that ITE activates the AHR and reduces autoimmune-mediated prostatitis in mice.

P-215 - Deciphering the Contribution of Non-Genomic Estrogen Signaling in Steroidogenesis in Perinatal Mouse Testis

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Estrogens, and more specifically 17 β -estradiol (E2), play critical roles in male reproduction. E2 action is in part driven by the estrogen receptor 1 (ESR1) that mediates both a nuclear signaling pathway and a cell membrane-associated non-genomic pathway. ESR1KO mice revealed the critical role of ESR1 for proper development of the male reproductive tract and regulation of perinatal testicular testosterone production. Another mouse model expressing only the nuclear form of ESR1 (Nuclear Only Estrogen Receptor, NOER) highlighted the importance of the non-genomic pathway in the male reproductive tract. However, the contribution of the non-genomic (or membrane) pathway in fetal Leydig cells and perinatal testosterone secretion remained unexplored. Since Leydig cells are hypertrophied in 2-day old ESR1KO testes, we first examined fetal Leydig cell morphology in 2-day old NOER testis. Surface measurement of fetal Leydig cells after 3 β HSD immunostaining did not show any difference between NOER and wild-type (WT) testes (n=3), suggesting that the effect previously observed in ESR1KO was due to the genomic pathway. Next, total RNA was extracted from 2-day old testis collected from 3 WT, 3 NOER, and 2 ESR1KO mice, and used for polyA RNA-Seq. Analysis revealed 11 differentially expressed genes (DEG) in NOER (4 down and 7 up; FDR<0.05; FC2) and 192 DEG in ESR1KO (99 down and 93 up) when compared to WT. Hmga1 and Cyp17a1 were the only two common DEG between NOER and ERKO. Compared to WT mice, Hmga1 mRNA levels were significantly increased in ESR1KO while in NOER

animals Hmga1 mRNA levels were decreased below WT levels. This indicates that Hmga1 expression is antagonistically regulated by the genomic pathway but positively by the non-genomic pathway. Cyp17a1 mRNA levels were increased in both NOER and ESR1KO indicating that expression of this gene is controlled mainly by the non-genomic pathway. Targeted analysis of additional genes important for Leydig cell function revealed a similar increase in Star, Hsb3b1, Cyp11a1, and Ins13 in both ESR1KO and NOER suggesting a regulation by the non-genomic pathway. These data provide a better understanding of the regulation of perinatal testicular steroidogenesis by estrogens and reveal new roles for the cell membrane-associated non-genomic ESR1 pathway.

P-216 - Enhanced Identification and Autophagic Clearance of Aging Spermatozoa Through a Dual-Response Logic Gate Probe

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Background: With increasing paternal age, concerns regarding the repercussions of sperm aging have grown. These encompass diminished male fertility, declined sperm quality, heightened spermatogenic mosaicism, and an elevated susceptibility to hereditary diseases in offspring. Notably, the manifestations and issues stemming from sperm aging extend beyond older populations to include men of reproductive age diagnosed with infertility. Therefore, the identification and rational intervention to eradicate aging spermatozoa within sperm populations emerge as a significant issue warranting exploration. **Results:** To accurately discern aging spermatozoa, we utilized β -galactosidase as the primary marker and hypochlorous acid accumulation during aging as the secondary marker, devising an "And" type dual-response logic gate probe, L5. Laser confocal microscopy revealed L5's effective recognition of H₂O₂-induced aging-like spermatozoa, with β -galactosidase predominantly localized in the sperm head and hypochlorous acid in the neck region. Assessing samples from 20 clinically diagnosed infertile men across 20–60-year ages and contrasting them with 20 fertile men of ages of same range, L5 achieved a recognition accuracy of 69.84% (AUC value from the ROC curve). Furthermore, flow cytometry analysis indicated significant clustering in sperm post-L5 staining. Subsequently segregating sperm subgroups post-L5 staining into positive and negative populations, and proteomic and metabolomic analyses suggested aberrant glycerophospholipid metabolism in the L5 positive group, indicative of compromised cell membrane function. Propidium iodide (PI) and DIR (a long-chain carbocyanine dye) staining confirmed a decrease in lipid affinity and an increase in permeability in the L5 positive sperm group. Consequently, we leveraged differential cell membrane permeability to introduce a toxic fatty acid in aging cells for "self-clearance." This led to the development of L5-OA, designed to introduce oleic acid into aging spermatozoa, thereby inducing autophagic clearance. Compared to sperm samples

from men under 35 ages with normal fertility outcomes, L5-OA exhibited enhanced clearance of H₂O₂-induced aging spermatozoa and those from infertile men of varying ages. Computer-aided sperm analysis (CASA) results demonstrated that treatment with L5-OA at 10 μ M for 1.5 hours significantly improved forward motility ($p < 0.05$), indicating the potential of L5-OA to significantly eliminate aging spermatozoa and enhance their fertilizing ability. Future Directions: Building on these promising outcomes, future research will focus on optimizing L5-OA to further restore sperm motility and increase the success rate of assisted reproductive technologies. This innovative approach holds the potential to significantly improve reproductive health and outcomes in the context of increasing paternal ages, ultimately benefiting individuals facing fertility problem. Conclusion: This study presents a novel strategy to identify aging sperm and target autophagic clearance of aging spermatozoa, providing insights into addressing male infertility and potentially improving reproductive outcomes amidst increasing paternal ages.

P-217 - C-X-C Motif Chemokine Ligand 5 is a WNT-1-Induced Oncogene with Proinflammatory and Antioxidation Roles in Human Prostate Cancer Cells

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The C-X-C motif chemokine ligand 5 (CXCL5), also known as epithelial neutrophil-activating protein 78 (ENA78), is part of the chemotactic cytokines superfamily. CXCL5 is a well-known inflammatory mediator and a potent attractant for immune cells, but its role in the human prostate is mainly unexplored. Serum and glucocorticoid-regulated kinase 1 (SGK1) is a canonical factor affecting multiple genes' expression and signal transduction. Heme oxygenase-1 (HO-1) is a tissue protector with anti-inflammatory and anti-oxidant properties. In this study, we investigated the expression, functions, and regulatory mechanisms of CXCL5 in prostate cancer cells. Overexpression of WNT family 1 (WNT1) instigates active β -catenin translocation into the nucleus, subsequently inducing CXCL5 expression in prostate cancer cells. CXCL5 provokes neutrophil migration and enhances prostate cancer cell proliferation and invasion in vitro. The xenograft animal model results reveal that CXCL5-knockdown attenuates tumor growth in vivo. Treatment with SGK650394, an SGK1 inhibitor, blocks the gene expression of CXCL5 in human prostate cancer PC-3 cells. Additionally, CXCL5 positively regulates IL-6 expression in prostate cancer cells through a feedback loop and heightens the gene expression of prostate-specific antigen (PSA) in a CXCR2-dependent manner. Moreover, CXCL5 upregulates HO-1 expression to counteract the H₂O₂-induced reactive oxygen species (ROS), suggesting a role in antioxidation. Our results identify CXCL5 as an oncogene with proinflammatory and antioxidative properties in human prostate cancer cells and as a downstream gene of WNT1. This study also uncovers a linkage between the SGK1 signal pathway and the expression of CXCL5 in prostate cancer cells. Therefore, targeting the CXCL5/CXCR2 signaling pathway may offer a novel therapeutic strategy for prostate cancer.

P-218 - Regulation of NHE3-Dependent Proton Secretion in Epididymal Principal Cells: pH, cAMP, and Adenosine Signaling

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The epididymis, a crucial male reproductive organ, consists of a highly convoluted, single tubule that connects the testis to the vas deferens. Structurally, it is divided into four distinct regions in rodents: the initial segment, caput, corpus, and cauda, each serving unique anatomical and functional roles. Spermatozoa acquire the ability to fertilize an oocyte as they transit in the epididymis, a process called epididymal maturation. The epididymal lumen is lined by a pseudostratified epithelium that plays a pivotal role in the establishment and maintenance of a unique acidic luminal environment (pH 6.6), which is essential for the maturation and storage of sperm cells. Epididymal principal cells (PC) contribute to luminal acidification via the sodium/hydrogen exchanger type 3 (NHE3). NHE3-dependent proton secretion is regulated via recycling mechanisms that control its localization in sub-apical endosomes and apical stereocilia. PC also secrete ATP, which is rapidly hydrolyzed into adenosine by ectonucleotidases. Adenosine exhibits opposing effects based on the receptors it activates. A1 and A3 receptor activation reduces intracellular cAMP (cAMPi), while A2A and A2B receptor activation increases cAMPi. In other epithelia, cAMPi triggers NHE3 internalization from the apical membrane. We showed high expression of A2B and A3 receptors in the apical membrane of PC by immunofluorescence. We then investigated the roles of pH, cAMP, and adenosine (via A3 and A2B receptors) in the subcellular localization of NHE3 in PC. Adult C57Bl/6Ncr1 (aged 10-12 weeks) male mice were used for in vivo luminal perfusion of the cauda epididymidis. All experiments were performed in at least 6 mice for each group. Immunofluorescence NHE3 labeling and phase contrast images (to visualize PC stereocilia) were obtained using a Zeiss LSM 900 confocal microscope. The localization of NHE3 in stereocilia was quantified using Fiji (ImageJ) software. When the cauda epididymidis was perfused at an alkaline luminal pH of 7.8, NHE3 was mainly localized in PC stereocilia. In contrast, an acidic luminal pH of 6.0 induced NHE3 internalization. cpt-cAMP, a permeant analog of cAMP, and the A2B receptor agonist BAY-60-6583 prevented NHE3 stereocilia accumulation induced at pH 7.8. Adenosine or the A3 agonist 2-Cl-IB-MECA induced NHE3 stereocilia accumulation at pH 6.0. The A3 antagonist MRS1523 prevented the accumulation of NHE3 in stereocilia induced by adenosine at pH 6.0, while the A2B antagonist PSB1115 had no effect. Our findings suggest that NHE3 subcellular localization in epididymal PC is modulated by luminal pH and adenosine. We propose that activation of A3 by luminal adenosine, leading to a decrease in cAMPi, maintains NHE3 on the cell surface. Conversely, A2B activation by adenosine, followed by an increase in cAMPi, induces NHE3 internalization. This intricate interplay of pH and adenosine highlights some of the regulatory mechanisms influencing the establishment of an optimal acidic environment for sperm maturation and storage in the epididymis.

P-219 - Unveiling Interleukin-13 Receptor Subunit Alpha 2 in Prostate Biology: Expression Patterns, Functions, and Therapeutic Prospects

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Interleukin (IL)-13, an immunoregulatory cytokine produced by various immune cells, is known to inhibit the production of inflammatory cytokines. Its receptor, IL13RA2, lacks an intracellular kinase domain, despite serving as a high-affinity membrane receptor for IL-13. Initially considered a decoy receptor to prevent excessive IL-13 signaling, IL13RA2 has been found to be overexpressed in

numerous human tumors. However, its specific roles and functions can vary based on the cellular context. The functions of IL13RA2 in the human prostate remain largely unexplored. Thus, our study investigates the expression, functions, and regulatory mechanisms of IL13RA2 in prostate-associated cells. In silico analysis of TCGA data reveals downregulation of IL13RA2 in prostate cancers, and low expression of IL13RA2 correlates with poor overall survival. Furthermore, overexpression of IL13RA2 inhibits proliferation, migration, invasion, and suppresses AKT and ERK phosphorylation in prostate cancer cells in vitro. Xenograft animal studies demonstrate that IL13RA2 overexpression inhibits angiogenesis and reduces tumor growth in vivo. Notably, treatment with TGF- β or TNF- α downregulates IL13RA2 expression in human prostate fibroblasts (HPPrF) cells in vitro. Our findings suggest that IL13RA2, identified as a tumor suppressor gene, plays significant roles in tumor progression and may serve as a potential therapeutic target for prostate cancer.

P-220 - Wnt9b/ β -Catenin Signaling Controls Wolffian Duct Maintenance

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The maintenance and differentiation of the Wolffian duct (WD), the precursor of the male reproductive tract, is predominantly controlled by the androgen action. During sexual differentiation, under the control of testicular androgens, the Wolffian duct differentiates into the epididymis, vas deferens and seminal vesicle in the male embryos. By contrast, the Wolffian duct degenerates in the absence of testicular androgens in the female embryo. Although disruptions of androgen signaling by environmental and genetic factors during male sex differentiation can cause male reproductive disorders, the etiology of such birth defects remains unknown in many cases. Here, we uncovered the critical role of Wnt9b/ β -catenin pathway in Wolffian duct maintenance and morphogenesis in mice. Wnt9b belongs to the WNT family of secreted glycoproteins, which are known to be involved in every aspect of embryonic development. The loss of Wnt9b caused Wolffian duct degeneration in male embryos that had normal testicular androgen production. We observed comparable expression of two critical steroidogenic enzymes (Hsd3 β and Cyp17a1) and comparable testosterone level between Wnt9b^{+/+} and Wnt9b^{-/-} testes. Since the androgen action is mediated by its receptor AR in the mesenchyme, we next investigated whether mesenchymal AR expression in the Wolffian duct was impaired in the absence of Wnt9b by immunohistochemistry. In the normal condition, AR was normally expressed in most of mesenchymal cells at the initiation of Wolffian duct maintenance (E14.5), which promoted Wolffian duct epithelial proliferation. However, in the absence of Wnt9b, the percentage of mesenchymal cell expressing AR was significantly decreased. Consistent with reduced AR⁺ mesenchymal cells, epithelial and mesenchymal proliferation was also significantly decreased in the absence of Wnt9b. These observations demonstrate that Wnt9b promotes the number of AR⁺ mesenchymal cells in Wolffian duct maintenance. The epithelium-derived WNT9B can activate two intracellular pathways in the mesenchyme: β -catenin dependent and independent pathways. We detected nuclear localization of β -catenin in the Wolffian duct mesenchyme, which indicated the activation of β -catenin-mediated WNT pathway. We therefore investigated whether β -catenin operated downstream of

WNT9B in the Wolffian duct mesenchyme. When β -catenin was deleted in the Wolffian duct mesenchyme, the caudal region of the Wolffian duct degenerated while the WD at the cranial region was still maintained. These observations indicate that mesenchymal β -catenin is required for promoting Wolffian duct maintenance at the caudal region. Taken together, our study demonstrates that WNT9B/ β -catenin pathway facilitates epithelial-mesenchymal crosstalk in promoting Wolffian duct survival.

P-221 - In silico Molecular Studies of Bioactive Compound from *Massularia acuminata* Extracts as a Potential Inhibitor of Phosphodiesterase-5-Enzymes in the Prophylaxis and Management of Erectile Dysfunction

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Erectile dysfunction (ED), often colloquially referred to as impotence, is a prevalent medical condition characterized by the consistent inability to achieve or maintain an erection sufficient for satisfactory sexual performance. While occasional difficulty with erections is normal, persistent challenges in achieving or sustaining an erection can be indicative of an underlying health issue. The enzyme phosphodiesterase-5 (PDE5) is responsible for cGMP degradation; inhibiting PDE5 sustains cGMP levels, prolonging vasodilation and maintaining erections. Endothelial dysfunction, associated with reduced NO production, and factors like neurotransmitters, hormonal regulation, and inflammatory/oxidative stress contribute to the intricate biochemical landscape influencing erectile function. Understanding these pathways has led to pharmacological advancements, such as PDE5 inhibitors, providing effective interventions for ED. *Massularia acuminata*, a plant native to certain regions of Africa, has garnered attention as a potential source of therapeutic bioactive compounds due to its diverse pharmacological properties. The primary aim of this research is to conduct in-silico molecular studies on the High-Performance Liquid Chromatography (HPLC) bioactive compounds derived from *Massularia acuminata* with a focus on evaluating their potential as novel inhibitors of Phosphodiesterase-5 (PDE5) for the effective management of penile erectile dysfunction. Five lead compounds Demethoxycurcumin (-8.586), Hexadiene (-8.129), Curcumin (-7.264), Beta-Tumerone (-7.047), and Alpha Tumerone (-7.028) were shown to have better binding affinity to PDE5 than Testosterone (-6.076). Thus, from the ADMET studies it may be concluded that the studies showed that the lead compounds from *Massularia acuminata* have good drug-like properties that make them potential therapeutic inhibitors of PDE5. These findings warrant further experimental investigations and emphasize the potential of these compounds in drug development efforts for penile erectile dysfunction. Keywords: Erectile dysfunction, *Massularia acuminata*, High-Performance Liquid Chromatography,

P-222 - Investigating the Role of Extracellular Vesicles in Paracrine Communication Between Epithelial Cells from Different Regions of the Domestic Cat Epididymis

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Sperm maturation is dependent upon exposure to the proper luminal environment within the epididymis, but the mechanisms that control how this environment is created and maintained are poorly understood. Epididymal extracellular vesicles (EVs) have been hypothesized to play a role in this process by acting as paracrine communicators between epithelial cells from different regions of the epididymis. The objectives of this study were to (1) describe the development and validation of an in vitro culture system for domestic cat epididymal tissue and (2) investigate the effect of caput-derived EVs on the gene expression profiles of cultured corpus explants. Fresh domestic cat epididymal tissue was separated into caput, corpus, and cauda. EVs were isolated from the caput from pools of tissue from 5 individuals (n= 13 pools of 5 individuals each) using previously published methods. Explants were obtained from separate individuals (n=5, 1 individual per experimental replicate) by cutting 1 mm³ pieces of corpus tissue. Explant culture was performed on agarose inserts for a period of 3 days and was supplemented with fresh culture medium and EVs every 24 hours. Controls were explants from the same individual that were treated identically but without EV supplementation. Validation that culture conditions were not differentially affecting the explants was performed by assessing tissue morphology (H&E staining), apoptosis (In Situ Cell Death Detection Kit, Fluorescein, Roche), and transcription (Click-iT RNA AlexaFluor 594 Imaging Kit, Invitrogen) in treatment and control groups. Using optimal culture conditions, RNA sequencing at a depth of 30M stranded reads then was performed to compare transcriptional profiles of explants supplemented with EVs and control explants. Preliminary culture experiments determined that supplemental fetal bovine serum (FBS) and androgens were necessary to maintain intact tubule epithelium and stereocilia in the cultured explants. Final culture conditions with FBS and androgens also led to levels of apoptosis and transcription that were similar to fresh tissue controls. Analysis of RNA sequencing results using DEseq2 revealed that EV supplementation caused 384 protein coding genes to be differentially expressed with a p-adjusted value < 0.05 and a fold change of 1.5 or greater. Of these genes, 260 were upregulated and 124 were down regulated. Functional analysis of the upregulated genes using the STRING network revealed hub genes that encode for cytokines, including IL6, IL1A, IL1B, and CSF3. This indicates that EVs may play a role in maintaining production of cytokines in the epididymis, which are also produced by non-immune cells in the testis and are important for normal testis function. Additionally, we identified upregulation of the genes WNT9B, SUSD2, TMEM100, and JAG1 which are involved in the Gene Ontology biological process of regulation of epithelial cell differentiation. These results suggested that EVs produced by the caput epididymis can control gene expression in downstream epithelial cells from the corpus epididymis. These findings lay a foundation for further studies on the role of EVs in paracrine communication within the epididymis.

P-223 - Postejaculatory Sperm Exposure to Tributyltin Chloride Induces Novel Structural, Genomic and Epigenomic Alterations in Mammalian Sperm.

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A global top priority for ameliorating male factor infertility includes identification of environmental factors and mechanisms that impact sperm function. Detection of endocrine disruptors (EDs) in seminal plasma and within the female reproductive tract has created an urgent need to understand how environmental stressors alter postejaculatory sperm function. Tributyltin chloride (TBT) is a model organotin, ED and epigenetic modifier that causes reproductive disorders in testicular sperm; however, consequences of TBT exposure on postejaculatory sperm prior to fertilization remain unknown. Our previous data indicate that TBT-exposed sperm does not affect fertilization but lowers embryo development potential. However, identifying reasons for idiopathic male factor subfertility is challenged by lack of routine clinical assessment for subtle phenotypes that compromise sperm function and therefore remain undetected with current diagnostic tools. The present study was aimed at identifying structural, genomic and epigenomic consequences of TBT exposure to postejaculatory sperm in a temporal manner reflective of sperm transport prior to fertilization within the female reproductive tract. Cryopreserved bovine sperm from two different bulls were independently exposed to TBT (0, 1, 10, 100nM; n = 4) for 24 hr under non-capacitating conditions at 25°C followed by quantification of sperm kinematics at 37°C, DNA integrity, and methylation status of select loci. Samples of TBT-exposed sperm and vehicle control (0.1% DMSO) were collected in replicate for all analyses following immediate addition of TBT (0 hr) and again at 24 hr. Data were analyzed by logistic regression with a generalized linear mixed effect model. Computer-assisted sperm assessment of total motility, progressive motility and average-path velocity did not detect any differences in TBT-exposed sperm compared to vehicle-control after 24 hr of exposure. Capacitation status of TBT-exposed sperm also remained unaffected following anti-tyrosine phosphorylation staining. However, acrosome integrity was compromised in an acute and dose dependent manner as detected by FITC-PSA ($P \leq 0.05$). DNA isolation and recovery for genomic and epigenomic analyses was negatively affected following TBT exposure (10, 100nM) after 24 hr ($P < 0.05$). TUNEL assay did not reveal an acute effect on DNA integrity from TBT at 0 hr, but DNA integrity was negatively compromised in a dose-dependent manner after 24 hr ($P \leq 0.05$). For epigenetic studies, DNA was isolated after 24 hr of TBT exposure followed by bisulfite conversion and PCR amplification of select loci (PTK2B, HDAC11, PAK1, SNRPN, H19, HDAC11, KCNQ1). The DNA methylation of CpG sites in these amplicons were measured by PacBio single-molecule real-time (SMRT) long-read high-fidelity sequencing. Preliminary findings indicate TBT-induced demethylation in the promoter region of PTK2B and the imprinted H19 locus while increasing the methylation profile of PAK1 and HDAC11 loci. Confirmation of these findings will be determined by Enzymatic Methyl-Seq conversion and highly optimized PCR amplification following different exposure concentrations of TBT. In conclusion, postejaculatory mammalian sperm exposure to TBT affected acrosome integrity, DNA damage and the methylation status of open chromatin at regions important for early embryonic development suggesting a novel phenotype and paradigm shift to epigenetic modifications of transcriptionally quiescent and translationally repressed mature sperm. The consequences of TBT exposure to mature sperm are masked by unaffected motility but may affect gamete interaction and subsequent embryo development. These findings provided new insight towards environmental impacts on postejaculatory sperm function.

P-224 - FHL2 Deletion Reduces Male Fertility by Impairing the Sperm Meiosis

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To improving the fertility decline worldwide, this study investigated the spermatogenesis regulating mechanism. Guohua Hua affiliated with the college of Animal Science and Technology, is a faculty at Huazhong Agricultural University. Fertility decline is a raising issue and about 1 in 6 people worldwide would be affected by infertility. The idiopathic azoospermia and oligospermia are the common cause of male infertility, which displays as spermatogenesis disorders with unknown underlying molecular mechanisms. Spermatogenesis refers to the process in which spermatogonia proliferate and differentiate in the testicular seminiferous tubules, which determines male reproductive physiology and fertility. Our previous study showed that FHL2 deficiency reduced female fertility by impairing follicular development. Here, we reported that FHL2 deletion also impaired male mice fertility. The female mice mated with FHL2^{-/-} male mice displayed smaller litter size and less total offspring number in a 1-month breeding trail, comparing with age matched wildtype (WT) mice. Furthermore, the number of embryo implanted in female mice mated with FHL2^{-/-} male mice was also significantly lower than that in the WT group. Although no significant difference was found in circulation serum testosterone and inhibin B from 5- to 18-week-old mice, FHL2 deletion induced smaller testicular size and organ index, as well as lower epididymis weight and epididymis organ index. Consistently, the sperm density in the epididymis tail of FHL2^{-/-} mice was significantly lower than that of WT mice. But the sperm abnormality rate of FHL2^{-/-} mice significantly increased. These data indicated that FHL2 deletion induced poor sperm quality which may related with spermatogenesis disorders. HE staining was then performed to exploring the histological morphology of testicular seminiferous tubules isolated from 3 to 12-week old mice. Both the seminiferous tubules diameter and the seminiferous epithelium thickness of FHL2^{-/-} mice were smaller than those in WT mice, indicating an impaired spermatogenic ability by FHL2 deficiency. Importantly, round spermatids emerged at 3 weeks, and elongated spermatozoa showed at 5 weeks in WT mice seminiferous tubules, however, round spermatids and elongated spermatozoa emerged 1 week later in FHL2^{-/-} mice. HE staining of caput epididymides further confirmed that FHL2^{-/-} mice showed a delayed ejaculation time about 1 week. Further, Immunofluorescence staining of seminiferous tubules isolated from different ages of mice testis were performed to investigated the spermatogenesis. These results showed that, no difference was found in the expression of c-kit (spermatogonia marker) and PLZF (spermatogonial stem cell maker) between P10 and P14 FHL2^{-/-} mice and age matched WT mice, which indicated FHL2 deletion showed imperceptible effect on the spermatogonia stage. However, the total spermatogenic cells (indicated by DDX4 staining) in each seminiferous tubule was significantly decreased in FHL2^{-/-} mice. The expression of SCP3, which involved in the formation of synaptosome complexes was also downregulated on days 10, 14, and 21 in FHL2^{-/-} mice. Besides, the expression of Stra8, which symbolizes the initiation of meiosis, was decreased in the FHL2^{-/-} testes on day 21. Consequently, round sperm (indicated by ACRV1) and elongated sperm (indicated by TNP1) were significantly less in FHL2^{-/-} seminiferous tubule compared with WT mice. Collectively, these data illuminated that FHL2 deletion impaired meiosis and delayed spermatogenesis, which leading to idiopathic

azoospermia and oligospermia of male mice. FHL2 may serve as a new potential target to improve of male spermatogenesis.

P-225 - Glyphosate Presence in Human Sperm: First Report and Positive Correlation with Oxidative Stress in an Infertile French Population

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Environmental exposure to endocrine disruptors, such as pesticides, is suspected to have contributed to a decline of human fertility. Glyphosate (GLY) is the main component of Glyphosate Based Herbicides (GBHs) which are the most commonly herbicides used in the world. Various animal model studies demonstrated its reprotoxicity. In Europe, GLY authorization in agriculture is extended until 2033 and the toxicity of GLY in humans is still in debate. The aims of our study were firstly to analyse the concentration of GLY and its main metabolite, amino-methyl-phosphonic acid (AMPA) by LC/MS-MS in the seminal and blood plasma in an infertile French men population (n=128). We secondly determined some oxidative stress biomarkers including malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) by ELISA assays. We next analysed potential correlation between GLY and oxidative stress biomarkers concentration and sperm parameters (sperm concentration, progressive speed, anormal forms). Here, we detected for the first time GLY in the human seminal plasma in significant proportion and we showed that its concentration was four times higher than those observed in blood plasma. At the opposite, AMPA was undetectable. We also observed a strong positive correlation between plasma blood GLY concentrations and plasma seminal GLY and 8-OHdG concentrations, the latter reflecting DNA impact. In addition, MDA blood and seminal plasma concentrations were significantly higher in men with glyphosate in blood and seminal plasma, respectively. Taken together, our results suggest a negative impact of GLY on the man reproductive health and possibly on his progeny. A precaution principle should be applied at the time of the actual discussion of GLY and GBHs formulants uses in Europe by the authorities.

P-226 - Comparison of Prostanoid Concentrations in Extended Boar Seminal Plasma to Pregnancy Status of Sows after Artificial Insemination

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Quantification of semen quality in relation to subsequent successful pregnancy remains elusive; therefore, additional indicators are required. Non-enzymatic [isoprostanes (IsoP) and neuroprostanes (NeuroP)] and enzymatic (prostaglandins; PGF) lipid peroxidation products may be viable indicators of male fertility. However, their use as biomarkers in boars is currently inconclusive. This pilot study evaluated prostanoids, including IsoP, NeuroP, PGF, and their metabolites (n=13), in boar seminal plasma (SP) to identify differences in female pregnancy status after insemination. It is hypothesised that specific prostanoid concentrations in semen would

influence pregnancy rates. Females (Parity 0-4; n=46) located in South Australia were given fenceline contact with a boar on Days 3 to 7 from weaning (Parity 1-4) or Days 4 to 7 from cessation of altrenogest (Parity 0) in October 2022, December 2022, and May 2023. Upon oestrus detection, females were inseminated with single use pouches containing pooled extended semen (80ml) collected from boars housed in Victoria, Australia. A second insemination occurred approximately 16h later in animals displaying receptive behaviour. Semen was scavenged from discarded pouches after insemination for analysis. Spermatozoa concentration and total live spermatozoa were determined using a nucleocounter (Chemometec, New South Wales, Australia). SP rich supernatant was obtained from a semen aliquot by centrifugation (1000g, 5 min) and stored at -80°C until analysis. Prostanoids were quantified in frozen-thawed samples using liquid-chromatography tandem mass-spectrometry. All non-parametric data were log transformed prior to respective one-sided independent t-test analyses to compare prostanoid data in SP that did (n = 43) or did not (n = 7) result in pregnancy. SP prostanoid data were compared to spermatozoa viability (%; live spermatozoa/concentration) using Spearman's correlation analyses. Alpha was set to 0.05. Data are presented in Table 1. PGF2 α affects uterine contractility which may improve sperm transport in the female reproductive tract, as exogenous administration of PGF2 α induces myometrial contractions and increases pregnancy rates after artificial insemination in sows. In this study, SP concentrations of PGF2 α from non-pregnant sows were similar to published results that were unable to induce uterine contractions in vitro, while PGF2 α concentrations in SP that resulted in pregnancy were significantly higher. Various NeuroPs and IsoPs have been associated with spermatozoa quality in other species. While no significance differences were detected between pregnant and non-pregnant groups, 20-F4t-NeuroP, 4(RS)-4-F4t-NeuroP, and 17-F2t-dihomo-IsoP displayed mean differences in concentrations between pregnancy status groups that were outside the standard error, suggesting the sample size may not be high enough in the non-pregnant group to achieve significance. Prostanoids could be additional biomarkers to characterise boar semen quality; however, larger studies are required to determine if they are accurate predictors of semen fertility regarding pregnancy success after insemination.

P-227 - Interactions between Estrogens and Cyclin D1 are Crucial for Epithelial Cell Recovery in the Initial Segment of the Mouse Epididymis.

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The estrogen receptor (ER) plays a crucial role in regulating growth and differentiation in various types of epithelium. Recent evidence suggests that a deficiency of ER is related to the failure of recovery in the mouse epididymal epithelium. However, little is known about the detailed molecular mechanisms that link ER and cyclin D1 in the recovery of epithelial cells in the mouse epididymis. In this study, we utilized two mouse models: the efferent duct ligation mouse model and the estrogen receptor-alpha knockout (ER α KO) model. These models were used to induce cell death programs in the epididymal epithelium and to gain insight into the roles of estrogens in the mechanism of epithelial repair, respectively. Deprivation of testicular luminal factors through efferent duct ligation (EDL) induces degeneration of epithelial cells in the initial segment (IS) of the epididymis, followed by regeneration. EDL was performed when the mice were 10-12 weeks old, and apoptotic and proliferating epithelial cells were quantified at 24 hours, and at days 2 and 3 post-EDL, respectively. All collected epididymides were fixed and evaluated using

immunofluorescence (IF) with specific markers, including caspase3 (cell death), Ki67 (cell proliferation), B1-VATPase (clear cells), and cytokeratin 5 (basal cells). A significant increase in apoptotic basal cells (BCs) and principal cells (PCs) was observed in both the control and ERαKO groups at 24 hours after EDL. There were no differences in the rate of apoptosis between the groups. However, ERαKO mice exhibited a significant decrease in the number of proliferating BCs and PCs compared to the control group at 2 and 3 days after EDL, respectively. Additionally, ERαKO mice showed a low level of cyclin D1 protein. Treatment with anti-cdk4/6 after EDL resulted in a complete cessation of cell proliferation in both the control and ERαKO groups. In summary, 1) the epididymal epithelium can be repaired and regenerated through the proliferation of BCs and PCs at 2 days and 3 days after EDL, respectively. 2) The interaction between estrogens and cyclin D1 is crucial for the repair of BCs and PCs after injury in the mouse epididymis. These results indicate that ERα plays a crucial role in the repair and regeneration phase by activating the cell cycle, rather than apoptosis, through its interactions with cyclin D1 in the initial segment of the mouse epididymis.

P-228 - A Newly Identified Role for Endocrine Disrupting Chemicals in Erectile Dysfunction

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Erectile dysfunction is an extremely prevalent condition globally and has been estimated to more than double in the past thirty years. Genetic mutations or increased reporting alone cannot account for this trend, and thus environmental factors must play a major role in its aetiology. Endocrine Disrupting Chemicals (EDCs) are one likely candidate, given that development and function of the erectile tissues are hormonally dependent. Previously we have shown that exposure to estrogenic EDCs during mouse penis development impacted genes that may impact erection physiology during adulthood. Therefore, this study investigated a role for estrogenic EDCs in the aetiology of erectile dysfunction. Functional testing of the primary erectile tissue in mice was performed following chronic and acute estrogenic EDC exposures. Male mice chronically exposed to the potent estrogen diethylstilbestrol (DES) exhibit abnormal contractility of the erectile tissue, indicative of ED. The treatment did not affect systemic testosterone production yet significantly increased estrogen receptor α (Esr1) expression in the primary erectile tissues, suggesting EDCs directly impacted penis function. In response, we isolated erectile tissues from mice and briefly incubated them with DES or the phytoestrogen genistein. These acute-direct exposures similarly caused a significant reduction in erectile tissue contractility, again indicative of erectile dysfunction. Overall, these findings demonstrate a direct link between estrogenic EDCs and erectile dysfunction and show that both chronic and acute estrogenic exposures are likely risk factors for this condition.

P-229 - Identifying Testosterone Producing Hydroxysteroid Dehydrogenase Enzymes in Hsd17b3-Deficient Mice

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Testosterone is essential for the regulation of androgen-dependent functions including male sexual development and spermatogenesis. In the adult testis, androgens are synthesized by highly specialized cells, known as the Leydig cells. Adult Leydig cells synthesise testosterone via the canonical androgen biosynthesis pathway, where the HSD17B3 enzyme catalyses the conversion of the androgen precursor androstenedione into testosterone. Consequently, loss of function mutations in the human HSD17B3 gene results in a disorder of sexual development. 46,XY HSD17B3-deficient individuals retain internal Wolffian structures, however the external genitalia is undermasculinised, appearing as female or ambiguous. Two independent research groups have generated Hsd17b3-deficient mouse models. Surprisingly, and in contrast to human cases of HSD17B3-deficiency, male Hsd17b3 knockout mice are masculinized from birth and are fertile in adulthood. Although Hsd17b3 knockout mice exhibit high androstenedione/testosterone ratios (indicative of HSD17B3 dysfunction), intratesticular testosterone remains normal. This data suggests that mice have compensatory mechanisms/alternative enzymes which enable the continued production of testosterone in the absence of HSD17B3. We aimed to identify alternative hydroxysteroid dehydrogenase (HSD) enzymes that may be responsible for continued testosterone biosynthesis in Hsd17b3 knockout mice. We have identified mouse HSD enzymes that can convert the precursor androstenedione into testosterone and validated this conversion in vitro. We have demonstrated that a key amino acid in a particular HSD allows it to synthesize testosterone, in contrast to the human enzyme which has a different amino acid and is unable to produce testosterone. To model human androgen production in mice, we developed a humanized transgenic mouse line expressing the HSD that is altered to express the human amino acid and is thus unable to produce testosterone. To determine if this mutated HSD enzyme is unable to compensate for the lack of Hsd17b3 we have cross bred this humanized HSD mouse line with Hsd17b3 knockout mice. The humanized mutation in mice has been validated by genomic sequencing and the phenotype has been characterized. Intratesticular and circulating steroid analysis is being performed and will indicate if this HSD plays an important functional role in testosterone biosynthesis in Hsd17b3 knockout mice. In conclusion, we are generating mouse models that can be used to better understand human disorders of androgen biosynthesis and can be exploited to identify novel therapies for androgen deficiency.

P-230 - Impact of Yoga-Conditioned Serum from infertile men on Prostate cancer Cell Characteristics

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Introduction Infertility affects about 15% of couples worldwide. Additionally, there is an increased likelihood among infertile men to develop both gonadal and extra-gonadal cancers. Oxidative stress (OS) & associated DNA damage, accumulation of DNA adduct (8-OHdG) may lead to de novo germ line mutations. 8-OHdG result in global hypo-methylation & genome instability. Loss of genomic integrity, hypermethylation of tumor suppressor genes & hypomethylation oncogene may enhance the risk of cancer in infertile men. OS induced by lifestyle factors, stress, smoking, obesity or

infection. Yoga, a mind-body therapy, could improve genomic integrity & factors may reduce susceptibility to cancer. Does yoga intervention reduce the risk of developing cancer that is associated with male infertile men? **Material and methods** A single-arm interventional study was conducted over a three-year period. 40 primary infertile men (mean age 34 ± 2.5) were enrolled from the Andrology Clinic, All India Institute of Medical Science, New Delhi, India and 40 fertile men (mean age 31 ± 2.8) served as fertile control. They underwent a 12-week (1h daily) Yoga intervention. The yoga-primed serum from subjects was used to treat prostate cancer cells (PC-3 cells and LNCap cells) in vitro, which was then further assessed for cell proliferation by MTT assay and apoptosis using Annexin-V and PI stain, quantified by FACS. Transwell migration assay and Matrigel invasion assays were done. 8-OHdG, global methylation (5mC) & hydroxyl-methylation (5hmC) levels by ELISA and DFI by SCSA. The sperm Telomere length (TL) and selected genes expression measured using Q-PCR. **Results** The yoga primed serum was used for in-vitro cell culture experiment to assess parameters like cell proliferation, migration, invasion and apoptosis. A 72-hour incubation of Prostate cancer cells, the Post-yoga serum from infertile men reduced viable cancer cells by 25% compared to pre-yoga serum. Flow cytometry analysis showed a significant increase ($P < 0.007$) in apoptotic cells in both PC-3 and LNCap cell lines as compared to pre yoga, similarly a significant reduction in cell invasion and migration ($P < 0.005$) of prostate cancer cells indicating that post-yoga serum may have an anti-carcinogenic effect on cancer cells. Additionally, the infertile men showed higher levels of seminal ROS, sperm DFI, and 8-OHdG ($p < 0.001$); however, these levels were significantly decreased after yoga. Also, following a yoga intervention, there was a rise in the percentage of 5mC and TL ($p < 0.004$) in infertile individuals while levels of 5-hmC have decreased. The expression level of gene participating in DNA repair mechanism were assessed and an upregulated expression of MLH1, WT1, ERCC1, RET & SMAD4 was observed after Yoga intervention. **Conclusion** Lifestyle interventions like yoga could improve genomic integrity and increased expression of DNA repair gene and cell-check point genes. Thus, may reduce enhanced susceptibility to develop cancer in infertile men. Enhances the understanding of the link between male infertility and cancer susceptibility, shedding light on shared mechanisms and contributing factors. Yoga primed serum significantly reduced hallmarks of cancer, like cell proliferation, invasion, and migration in prostate cancer cell lines demonstrating the anti-carcinogenic effect of Yoga.

P-231 - Expression of PPP1r Protein Regulators of Smooth Muscle Relaxation is Altered in Prostates of Aged Mouse Models of LUTS/BPH and in Human Cells Cultured from BPH Diseased Prostate

William H. Walker

Benign prostate hyperplasia (BPH) incidence increases with age and is common in older men. Quality of life is severely impacted by BPH, which leads to bladder outlet obstruction (BOO) and lower urinary tract symptoms (LUTS). Sustained contraction of prostrate smooth muscle around the bladder and urethra is a major contributor to lower urinary tract symptoms (LUTS) associated with benign prostate hyperplasia (BPH). Smooth muscle contraction requires phosphorylation of myosin light chain (MLC) by myosin light chain kinase (MLCK), whereas dephosphorylation of MLC and muscle relaxation in the prostate is promoted by protein phosphatase 1c (PP1c). PP1c forms a complex with the poorly characterized M20 protein and a member of the family of protein phosphatase 1 regulators (PPP1Rs) including PPP1R12a/b/c, PPP1R14a/b/c and PPP1R16a/b that

increase or decrease PP1c activity. The relative expression and activity of PPP1r proteins in normal and BPH prostate tissue is not well characterized. Our objective was to understand how smooth muscle relaxation is regulated in normal and BPH/LUTS prostate by assaying PPP1R protein expression in mouse and human prostate models. Direct comparisons of Ppp1r mRNA quantities by RT-qPCR in mouse prostate extracts revealed that Ppp1r12c (a PP1c activator) and Ppp1r16b (a PP1c inhibitor) were the highest expressed Ppp1r mRNAs in mature mice (6 months-old) and that Ppp1r12c and Ppp1r16b mRNA levels were induced a further 2.0- and 3.6-fold (n=5), respectively in prostates from aged (21-month-old) mice that have LUTS characteristics. These results are the first characterization of relative PPP1r mRNA expression in prostate tissue and identify Ppp1r12c and Ppp1r16b as the major regulators of PP1c in normal and aged mouse prostate. In human stromal cells cultured from normal or BPH diseased regions of the same prostate we found that most cells were positive for smooth muscle cell markers. We also found phosphorylated MLC immunostaining intensity (marker for increased cell contraction) was greater in BPH than control prostate cultures. This finding supports the hypothesis that the SM relaxation pathway is less active in BPH cultures. Studies of Ppp1r mRNAs in human prostate cell cultures determined that expression of mRNA encoding PPP1R14A, an inhibitor of PP1c, was 28.7-fold higher in BPH than normal prostate cells (n=3). This observation suggests that PPP1R14a-mediated inhibition of PP1c may contribute to initiation and/or maintenance of LUTs in humans. Mining of published RNA-seq studies revealed PPP1R14c as well as PPP1R16b inhibitors of PP1c are induced 3.7- and 3.4-fold, respectively in human BPH tissue. Future studies will focus determining whether decreasing androgen:estrogen ratios that occur in aging men and in association with BPH causes altered expression of PPP1R proteins and their interactions with PP1c to decrease smooth muscle relaxation. In conclusion, our data indicate that in 1) aged mouse models of BPH/LUTS, 2) cultured cells from human BPH and 3) human BPH tissue, expression of PPP1r regulators of PP1c and SM relaxation is altered to promote sustained SM contraction, a cause of LUTS. Our studies have identified the most likely PPP1R proteins responsible for regulation of PP1c and SM relaxation in prostate. Our results also provide a mechanistic explanation for sustained SM contraction around the prostatic urethra, restriction of urine flow and LUTS including voiding dysfunction that occurs with aging-associated altered hormone levels. Our findings may provide new targets for LUTS therapies to reverse aging associated LUTS. Supported by 1R21AG077740-01A1.

P-232 - In Vivo Dynamic Volumetric Imaging of Mouse Testis and Epididymis

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The implementation of live imaging in reproductive research is crucial for studying the physiological dynamics. Sperm transport is a highly dynamic process regulated by tubular contractions and luminal flows within the male reproductive tract. However, due to the lack of imaging techniques to capture these dynamics in vivo, there is little information on the physiological and biomechanical regulation of sperm transport through the male reproductive tract. Towards this technical limitation, we develop a functional in vivo imaging approach using optical coherence tomography, enabling live, label-free, depth-resolved, three-dimensional, high-resolution visualization of the mouse testis and epididymis. With this approach, we spatiotemporally captured tubular contractility in mouse testis and epididymis, as well as microstructures of these reproductive organs. Our findings demonstrated that the contraction frequency varies significantly depending on the epididymal regions, suggesting the spatial regulation of epididymal contractility. Furthermore,

we implemented quantitative measurements of the contraction wave and luminal transport through the epididymal duct, revealing the physiological dynamics within the male reproductive tract. The results show that the contraction wave propagates along the epididymal duct and the wave propagation velocity was estimated in vivo. In conclusion, this is the first study to develop intravital dynamic volumetric imaging of the male reproductive tract, which allows for quantitative analysis of the dynamics associated with sperm transport. Our findings set a platform for various studies investigating normal and abnormal male reproductive physiology as well as the pharmacological and environmental effects on reproductive functions in mouse models, ultimately contributing to a comprehensive understanding of male reproductive disorders.

P-233 - Human INHBB Gene Variant (C.1079T>C:P.Met360Thr) Is Disruptive for Pregnancy and Labouring in Female Mice

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Ovarian-derived inhibin A and inhibin B (α/β dimers) suppress follicle-stimulating hormone (FSH) production at the pituitary by blunting receptor activation by the activins (β/β dimers). This hypothalamic-pituitary-gonadal (HPG) loop is integral to reproductive function, and consequently, imbalances in inhibin/activin can negatively impact fertility. In a recent study, human INHBB gene variant (c.1079T > C:p.Met360Thr), identified in an infertile man, was shown to significantly reduce serum activin B levels and alter testis germ cell content in corresponding *Inhbb*M364T/M364T male mice. Here, we aimed to determine if the identified INHBB gene variation also altered female reproductive function. To address this, we examined ovarian and uterine function in *Inhbb*M364T/M364T adult female mice and *Inhbb*WT/WT littermate controls. As observed in the *Inhbb*M364T/M364T male mice, female *Inhbb*M364T/M364T mice tended to have reduced ($p=0.62$) circulating levels of activin B as well as significantly reduced ($p<0.01$) activin A levels relative to *Inhbb*WT/WT littermates. Despite the reduction in serum activins, serum FSH levels and ovulation rates were comparable between *Inhbb*M364T/M364T and *Inhbb*WT/WT. However, pregnant *Inhbb*M364T/M364T dams were found to carry significantly more ($p<0.01$) and significantly smaller ($p<0.01$) fetuses to late gestation (17.5 days post coitus) relative to *Inhbb*WT/WT pregnant controls. Furthermore, *Inhbb*M364T/M364T females were found to experience dystocia, with significantly extended gestation periods ($p<0.05$) and labour ($p<0.01$) relative to *Inhbb*WT/WT pregnant controls. In these female mice, dystocia is attributed to weakened uterine contractility.

Our findings support that the inhibin β B-subunit is essential for maintenance of pregnancy and normal labouring in females.

P-234 - Sexual dimorphisms in Alzheimer's disease-related disruptions of circadian entrainment and a potential role for gonadotropin receptor signaling

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Alzheimer's disease (AD) is associated with progressive disruption of entrained circadian rhythms, and sexual dimorphisms in both AD and circadian function have been well established. However, whether sex differences in AD and circadian function share a common mechanism is unknown. Gonadotropin [follicle stimulating hormone (FSH) and luteinizing hormone (LH)] levels increase with menopause and have been suggested to contribute to the fact that two thirds of AD patients are women. Yet, it is unclear whether increased gonadotropin receptor signaling also contributes to sex differences in AD-related circadian dysfunction. In the TAPP (APPSwe-Tau) mouse model of AD, we recently showed that females develop AD-related pathology and disruptions of entrained circadian rhythms much earlier than males. Markers of circadian dysfunction were strongly associated with the development of hyperphosphorylated Tau (pTau) pathology in lateral parabrachial (LPB) neurons that project to the circadian system, including the suprachiasmatic nucleus (SCN, the master circadian pacemaker) and its primary axonal relay, the adjacent subparaventricular zone (SPZ). In this current study, we examine the sexually dimorphic role of gonadotropin receptor expression and signaling on entrained rhythms of core body temperature (Tb) and locomotor activity (LMA) in wild-type (WT) and TAPP mice. Additionally, we performed gonadectomy (GDX) at ages relevant for the development of AD pathology in TAPP mice. Our preliminary findings suggest that GDX increases variability in markers of circadian entrainment in WT mice compared to shams and increases circadian disruption and LPB pTau in GDX compared to sham TAPP mice. We also collected tissue from the LPB and SCN/SPZ regions and are using quantitative PCR to determine changes in gonadotropin receptor expression and how these differences correlate to circadian disruption. We hypothesize that there will be sexually dimorphic changes in gonadotropin receptor expression in the LPB, as both FSH and LH concentrations significantly increase with GDX and have been associated with increased deposition of AD pathology.

P-235 - Seasonal Changes in the Short Form of Leptin Receptor Expression and the VEGF System in the Choroid Plexus, Arcuate Nucleus and Anterior Pituitary In Leptin/MTS-Leptin and Resistin-Treated Sheep

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Seasonal reproduction is strongly influenced by metabolic status. Matching reproductive activity to nutritional reserves is fundamental to the survival of a species. Many factors that affect reproduction, appetite, and energy expenditure have been described. Many of these factors are adipokines, hormones produced mainly by white adipose tissue. New roles for adipokines in fertility, reproduction, and metabolism have recently emerged, particularly since the description of leptin, and resistin. The effects of many of these peptides are dependent on the photoperiod. In

spring and summer, when food is plentiful and readily available, sheep show an increased appetite and seem to be resistant to the high concentrations of leptin that result from increased adiposity. Leptin resistance can either be a pathological state, for example, in diet-induced obesity, or a hyperleptinaemia state, or it can be an adaptive response and a physiological phenomenon to allow for shifts in the body weight set point, for example, in seasonal animals. Although the cellular and molecular mechanisms underlying the maintenance of energy balance in seasonal sheep have been studied extensively, how the photoperiod and metabolic status impact the interaction between leptin and resistin has not yet been explored. The blood-brain barrier (BBB) is a key player in adipokine signaling from the periphery to the central nervous system (CNS). The short form of the leptin receptor (LeptRa) plays a key role in the transport of leptin to the central nervous system CNS. Here, MTS-leptin and recombinant ovine (ro) leptin-mediated expression of LeptRa and VEGFA and VEGFR2 in selected hypothalamic nuclei, the choroid plexus (ChP), and anterior pituitary (AP) were analyzed considering the photoperiod and acute fasting (experiment 1) and nutritional status (experiment 2) of ewes. In experiment 1, sixty sheep were fed normally or fasted for 72 h and received one injection of saline, MTS-leptin or roleptin 1 h prior to euthanasia. LeptRa mRNA transcript levels and VEGF system protein concentrations were detected in the arcuate nucleus (ARC) and ChP predominantly in the short day (SD) period and in the AP in the long day (LD) period without detection of LeptRa in the preoptic area (POA) and ventro- and dorsomedial nuclei (VMH/DMH). In experiment 2, an altered diet for 5 months created lean or fat sheep. Twenty sheep were divided into four groups: the lean and fat groups were given saline, while the lean-R and fat-R groups received resistin 1 h prior to euthanasia. The results of the experiments showed for the first time the effect of MTS-leptin/roleptin on the expression of LeptRa, protein concentrations of VEGFA, and VEGFR2 in selected brain regions, namely, the AP, ChP, and ARC, in the context of nutritional status, resistin, and photoperiod. Changes in adiposity influenced the lowering effect of resistin on the expression of LeptRa and VEGF system protein concentrations. There were no differences between the expression of mRNA for LeptRa and concentrations of the VEGF system in lean and fat sheep. Overall, both photoperiodic and nutritional signals influence the effects of MTS-leptin/roleptin and resistin-mediated leptin transport to the CNS via LeptRa, and resistin seems to be another adipokine involved in the adaptive and/or pathological phenomenon of not only central but also peripheral leptin

P-236 - Omentin-1 increases cell proliferation and decreases apoptosis in cultured porcine anterior pituitary cells: a description of the potential signaling pathways involved

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Omentin-1 (OMNT-1) is an adipokine involved in the regulation of energy metabolism and ovarian functions. Our previous study showed OMNT-1 expression in the porcine anterior pituitary and its influences on in vitro endocrine functions including gonadotropins secretion. However, the specific role of OMNT-1 on anterior pituitary cell proliferation and apoptosis is not thoroughly established. Hence, the research goal is to understand the influence of OMNT-1 on cell proliferation and programmed cell death, while also clarifying the related molecular pathways. Pituitary glands were isolated from sexually mature female pigs (Large White breed) in the mid-luteal phase. Next, cultured anterior pituitary cells were treated with OMNT-1 at increasing doses of 10, 50, and 100 ng/ml alone and with the combination of GnRH (100 ng/ml) for 24, 48, and 72 h. Next, we assessed cell viability (AlamarBlue), cell cycle (flow cytometry), and executive caspase activity (Caspase-Glo 3/7 assay). Additionally, we examined the gene and protein expressions of cyclins A, B, D, and E, proliferating cell nuclear antigen PCNA, and key components involved in programmed cell death pathways (caspase-3, -8, -9, Bax, Bcl2, p53, Fas, Fadd, XIAP) using quantitative reverse transcription polymerase chain reaction and western blot. Also, we studied the influence of OMNT-1 (50 ng/ml) on extracellular signal-regulated kinases, protein kinase B, 5'AMP-activated protein kinase α , and signal transducer and activator of transcription 3 phosphorylation at various time points (5, 15, 30, 60 min). Pharmacological blockers (PD098059 (25 μ M), LY294002 (15 μ M), AG490 (3 μ M), and Compound C (1 μ M), respectively) for these kinases were used, and OMNT-1 impact on cell viability and caspase activity was assessed. Statistical analyses included Student's t-test, one-way analysis of variance, and Tukey's post-hoc test ($p < 0.05$) with six repetitions for each group. Our results affirmed the mitogenic properties of OMNT-1, promoting increased cell growth in the G2/M phase and upregulating cyclins A, B, D, E, and PCNA expression, particularly at a concentration of 50 ng/ml, also in combination with GnRH after 24 h of incubation. OMNT-1 stimulated time-dependent phosphorylation of selected kinases, while inhibiting levels of executive caspases (-3, -7) at doses 10 and 50 ng/ml, suggesting an anti-apoptotic effect in pituitary cells. Additionally, we observed a modulatory effect of OMNT-1 on proteins involved in the process of programmed cell death and confirmed the mitogenic and anti-apoptotic effects of OMNT-1 using pharmacological inhibitors targeting kinases. In summary, our studies demonstrated for the first time a stimulatory effect of OMNT-1 on proliferation and an inhibitory effect on apoptosis in porcine anterior pituitary cells by the regulation of different signaling pathways. Due to the intricate regulation of apoptosis and the crucial balance between cell proliferation and death that maintains tissue homeostasis, OMNT-1 may apply a significant physiological influence. Thus, OMNT-1 might be a new regulator of the balance proliferation/apoptosis of the porcine anterior pituitary cells. Supported by National Science Centre, Poland, 2020/37/B/NZ9/01154.

P-237 - Serum Concentrations of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) in KISS1 Knockout Gilts Treated with Neurokinin B, Kisspeptin, and GnRH hormone analogs

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Kisspeptin knockout (KISS1^{-/-}) pigs exhibit hypogonadotropic hypogonadism. Hormone analogs stimulate gonadotropin secretion and ovulation in mice and humans with induced or naturally occurring mutations in the KISS1 gene. It is unknown how such agonists affect gonadotropin secretion in KISS1^{-/-} gilts. The goal was to characterize LH and FSH secretion in gilts treated with hormone analogs activating the hypothalamic-pituitary-gonadal axis. Gilts (254 d of age) were jugular catheterized for serial blood collection before and after treatments. Concentrations of LH and FSH were quantified with RIA. Data were analyzed as repeated measures with genotype and time as fixed effects. In Experiment 1, baseline hormone secretion was established for 6 h, then gilts were treated with a neurokinin 3 receptor (NK3R) agonist (senktide; 10 mg/kg, i.v.) to activate KISS1 neurons. In Experiment 2, gilts were estrus synchronized with altrenogest (18 mg/d, 14 d) and treated with PG600 (1000 IU hCG, 2000 IU PMSG, i.m.) 12 h after altrenogest withdrawal to synchronize follicular development. At 112 h after PG600, a kisspeptin receptor agonist (C6; 0.3 nMol/kg, i.m.) was administered to activate GnRH neurons. In Experiment 3, gilts were treated with GnRH (Cystorelin; 150 ng/kg, i.v.) to stimulate gonadotroph cells. In Experiment 4, gilts received an estradiol implant and GnRH with increasing frequency every 2 wk (1500 ng/kg, 1/d; 1000 ng/kg, 2/d; 500 ng/kg, 4/d, i.v.) followed with PMSG (1000 IU) and hCG (1000 IU) 78 h apart to induce follicular development and ovulation. LH and FSH pulse amplitude, but not pulse frequency, was greater in KISS1^{+/-} and KISS1^{+/+} gilts compared with KISS1^{-/-} gilts, which had reduced serum LH and FSH concentrations ($P < 0.01$). LH but not FSH concentrations in KISS1^{+/-} and KISS1^{+/+} gilts were increased for 60 min following senktide ($P < 0.001$), which did not affect LH or FSH in KISS1^{-/-} gilts. C6 treatment did not affect FSH but increased circulating LH in KISS1^{+/-} and KISS1^{+/+} gilts for approximately 24 h ($P < 0.05$), but C6 did not affect LH in KISS1^{-/-} gilts. The C6 treatment induced ovulation in all KISS1^{+/+} gilts but none of the KISS1^{-/-} gilts. A single acute injection of GnRH increased LH in KISS1^{+/-} and KISS1^{+/+} gilts but not in KISS1^{-/-} gilts ($P < 0.001$). LH secretion in KISS1^{-/-} gilts was increased modestly with a greater dose and frequency of GnRH ($P < 0.05$). One KISS1^{-/-} gilt ovulated 2 follicles after hCG and one had luteinized follicles, but the rest failed to ovulate. A single functional KISS1 allele is sufficient to confer normal reproductive endocrine function in pigs. This is the first report to confirm NK3R regulates LH secretion in gilts. Lack of GnRH-induced LH secretion in KISS1^{-/-} gilts may result from insufficient releasable pools of LH due to lack of sufficient GnRH priming. Optimization of treatments will be required to induce full ovulation in KISS1^{-/-} gilts. Funding FFAR 552176 and CRIS 3040-31320-001-000D. The USDA is an equal opportunity provider and employer.

P-238 - DHED, A Brain Specific 17 β E2 Prodrug, Affects Gonadal Steroid Receptor Expression but not Metabolic Function.

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Gonadal steroid hormones including estrogen and progesterone play an important role in reproductive and metabolic function over the lifespan. The decline of circulating sex hormones during menopause is associated with many symptoms including weight gain and hot flushes, thought to be mediated through the central nervous system. Hormone replacement therapy (HRT) is the clinical gold standard to alleviate these symptoms and contains estrogens such as 17 Beta estradiol ($17\beta\text{E}_2$). However, peripheral estrogen receptor activation by HRT can increase the risk of reproductive cancers in some patients. Specifically in the context of weight gain, $17\beta\text{E}_2$ is known to exert protective effects against metabolic dysfunction, mediated by the arcuate nucleus of the hypothalamus. Therefore, restricting $17\beta\text{E}_2$ actions to the brain could serve as a safer mechanism of HRT in the treatment of metabolic dysfunction. 10b,17B-dihydroxyestra-1,4-dien-3-one (DHED), is a prodrug of $17\beta\text{E}_2$ which is enzymatically converted to estradiol exclusively within the brain. DHED has demonstrated positive benefit in rodent models of hot flushes, cognitive decline and stroke and critically does not act on estrogen sensitive tissues in the periphery. We hypothesised that DHED treatment in female mice would act within the hypothalamus to provide the same beneficial metabolic effects as $17\beta\text{E}_2$, while avoiding peripheral actions. Female mice placed on a high fat diet to induce metabolic dysfunction were split into either control, DHED, or $17\beta\text{E}_2$ treatment groups. Uterus weight, body weight, food intake and glucose tolerance was recorded along with estrogen and progesterone receptor expression in the brain. Findings to date indicate that while DHED influences the expression of steroid receptors in the hypothalamus and avoids uterine proliferation in periphery, the prodrug does not elicit the same protective metabolic effects as $17\beta\text{E}_2$. Further optimisation of delivery route and drug dosage may be required to fully establish whether DHED can provide protection against metabolic dysfunction.

P-239 - Immortalized Ovine KNDy Neuronal Cell Lines Reveal Mechanisms Underlying Regulation of Reproduction by Sex Steroids and Seasonal Cues

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The multiphenotypic hypothalamic KNDy neurons play a critical role in the neuroendocrine regulation of reproduction via their stimulation of GnRH release, and are requisite for fertility and pubertal progression. In vivo evidence in mouse models has accumulated identifying the Arcuate population of Kisspeptin neurons in mediating predominantly negative feedback effects of gonadal steroids, and their ability to synthesize and secrete kisspeptin (KP), neurokinin B (NKB/Tac3), and dynorphin (Dyn) implicates them as crucial regulators of GnRH pulse generation. We previously generated murine immortalized arcuate KNDy neuronal cell lines to explore molecular mechanisms underlying the steroid hormone regulation of these cells, and in the current study, we characterize novel immortalized ovine neuronal cell line model of KNDy (oKNDy) cells derived from fetal female sheep brain. Clonal lines were derived from isolated mediobasal hypothalamic explants using lentiviral infection of neurons with plasmids encoding SV40 large T-antigen. These neurons express ovine kiss1, tac3, and pdyn, as well as steroid receptors esr1, esr2, and pgr. Similar to KNDy neurons in vivo, receptors for Tac3 (tacr3) and Dyn (oprk1) were also found to be expressed in our oKNDy cells. Further, low physiological concentrations (5-50pM) of 17β -estradiol (E2) were found to

significantly repress kiss1 and tac3 expression, while having no effect on tacr3. Additionally, concentrations of E2 and progesterone (P4) together approximating varying estrous cycle levels modulated kiss1 and tac3 expression. While cells exposed to higher E2 levels exhibited greater kiss1/tac3 repression, P4 exposure alone revealed stimulation of expression with increasing P4. Since these neurons were derived from the short-day seasonally breeding sheep, they can also be used to explore how circannual timing cues derived from the pituitary pars tuberalis may exert gating signals on the reproductive axis as previously modeled in vivo. Consistent with this, we have found high levels of thyroid hormone receptor α (thra) expression in our oKNDy neurons, and found kiss1/tac3 expression inhibited by exogenous levothyroxine, suggesting they can respond to locally-synthesized T3. In conjunction with the above, we have also generated a kiss1- and tac3-expressing neuronal line from the ovine preoptic area (oPOA), which exhibit different morphology and E2-responsiveness from our oMBH-derived neurons. We are continuing to characterize these ovine neuronal cells in culture in order to provide insight into GnRH pulse generation and construct molecular models of neuronal responsiveness to steroids and other regulatory inputs.

P-240 - Knockout of the FSH Receptor in Adipocytes Does Not Impact Body Composition in a Mouse Model of Post-Menopause

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Post-menopausal women are at higher risk of developing obesity and associated comorbidities, such as diabetes and metabolic disorder, than premenopausal women. Hormonal changes, such as reduced estrogens, are likely to be contributing factors. Menopause is also characterized by increases in pituitary-derived follicle-stimulating hormone (FSH). Recent data suggested that FSH might directly stimulate adipogenesis and block thermogenesis (energy expenditure) in adipocytes. FSH reportedly decreased thermogenic gene expression (Cox8b and Ucp1) in murine 3T3-L1 cells differentiated into adipocytes. These effects were not observed in cells co-treated with an FSH-neutralizing antibody. The same antibody reduced fat mass and increased lean mass in ovariectomized female mice (OVX), a rodent model of post-menopause, on a standard chow diet or in intact male and female mice on a high-fat diet. In these studies, it was not established whether FSH acted directly through its canonical receptor (FSHR) in adipocytes to mediate its effects. To address this gap, we first differentiated 3T3-L1 cells to adipocytes and followed by treatment with FSH. In contrast to the earlier report, FSH did not alter the expression of markers of lipogenesis (Cebpa and Pparg2) or thermogenesis (Ucp1 and Cox8b) nor did it impact mitochondrial respiration or lipid accumulation. We also did not detect Fshr mRNA in these cells. We next differentiated primary pre-adipocytes from inguinal adipose tissue of adult female mice into white adipocytes. FSH treatment did not alter expression of several markers of adipogenesis or thermogenesis, and did not affect lipid accumulation in these cells. To assess FSH actions in fat in vivo, we generated adipocyte-specific Fshr knockout mice (Fshrfx/fx;Adipoq-Cre, hereafter cKO). Adult cKO and control female mice were OVX at 10 weeks and maintained on standard chow. Eight to 10 weeks later, we performed glucose tolerance and insulin tolerance tests, and determined body

composition by EchoMRI. Cre-mediated recombination of the floxed Fshr locus in adipocytes was successful, but did not alter body weight, fat or lean mass, or glucose metabolism in OVX cKO relative to control mice. We next performed OVX on control and cKO females at 10 weeks of age, followed by pair-feeding on a high-fat diet for 8 weeks. Again, there were no genotype differences in glucose or insulin tolerance, body weight, lean mass or fat mass. Finally, using a newly developed FSHR-3xHA knockin mouse model, we detected FSHR protein expression in ovarian granulosa cells, but not in inguinal and perigonadal white adipose depots or in intrascapular brown adipose tissue of adult female mice. We similarly failed to detect Fshr mRNA in various fat depots. Collectively, our results fail to show FSH effects on adipogenesis in vitro and challenge the hypothesis that FSH acts through the canonical FSHR in adipocytes in vivo. FSH may therefore not represent a relevant therapeutic target for post-menopausal weight gain.

P-241 - Hormonal Havoc: Dysregulation of Hippo Signaling in Murine Gonadotropes Leads to Morphological Alterations and Dysfunction of the Anterior Pituitary Gland

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The Hippo transcriptional coactivators, YAP and TAZ, are expressed in cells throughout the anterior pituitary gland in mice. Although the potential physiological roles of YAP and TAZ in gonadotrope cells was unknown, we recently reported that conditional deletion of YAP and TAZ in gonadotrope cells results in increased circulating levels of LH (in males and females) and FSH (males only), and female hyperfertility in mice. Here, to further study the effects of Hippo signaling in gonadotropes, we conditionally expressed a constitutively active YAP (5SA) in these cells using the Cre-lox system with one of two Cre-driver strains, steroidogenic factor-1 (SF-1)-Cre or GnRH receptor-IRES-Cre (GRIC). Such crosses generated two distinct mouse models, which are referred to herein as YAP5SA;SF1-Cre and YAP5SA;GRIC, respectively. Expression of total YAP (YAP5SA and endogenous Yap) and the classic YAP-TEAD target genes, Ankrd1, Ctgf and Cyr61, and was significantly increased in pituitaries of 6-week-old mutant males and females in both the YAP5SA;SF1-Cre and YAP5SA;GRIC models. Animals from both models also showed significantly reduced serum LH and FSH levels as well as lower body mass and gonadosomatic indices when compared to respective controls. Six-week-old male and female mutants from both models showed significant morphological alterations in the pituitary. YAP5SA;SF1-Cre mutants exhibited proliferation of poorly differentiated cells, with high cellular and nuclear atypia with several mitoses. Similarly, YAP5SA;GRIC pituitaries were characterized by an increased number of poorly differentiated cells as well as follicle or papillary-like structure formation and increased cell proliferation and necrosis. Together, these preliminary findings strongly suggest pituitary blastoma or choriocarcinoma, and confirmatory experiments are underway. In light of gross morphological changes that appeared to

extend beyond gonadotropes, we performed qPCR analysis of other cell lineages in 6-week-old YAP5SA;GRIC mutants and corresponding controls. Gh, Pomc, Prl and Tshb mRNA levels were decreased in pituitaries of mutant animals. In addition, GH and ACTH protein expression, as assessed by immunohistochemistry, was nearly undetectable in pituitaries in mutant animals, suggesting compromised presence or function of somatotropes and corticotropes. To better understand the onset of this pathology, we performed histopathological analysis of YAP5SA;GRIC mutants in early post-natal life. Although pituitaries from 1-day-old mutant animals seemed normal in comparison to controls, we found signs of cellular necrosis and vacuolar degeneration in 1-week-old mutants. Together, our results indicate that constitutively active YAP in murine gonadotropes leads to significant morphological and functional alterations throughout the pituitary.

P-242 - Raphe Glucose-Sensing Serotonergic Neurons Stimulate KNDy Neurons to Enhance LH Pulses via Stimulatory Serotonin-2C Receptor in Female Rats and Goats

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Dysfunction of central serotonergic neurons is known to cause depressive disorders in humans, who often show reproductive and/or glucose metabolism disorders. Previous studies suggest that raphe serotonergic neurons may sense an increase in glucose availability to regulate glucose metabolism, but it is unclear whether serotonergic neurons sense and mediate energy status to regulate reproductive function. This study, therefore, examined whether dorsal raphe (DR) serotonergic neurons sense high glucose availability to upregulate reproductive function via activating hypothalamic arcuate (ARC) kisspeptin neurons (also known as KNDy neurons), a source of gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) pulse generator, using female rats and goats. RNA-seq analysis and double in situ hybridization revealed that stimulatory serotonin-2C receptor (5HT2CR) was mainly expressed in the KNDy neurons in female rats. The administration of fluoxetine, a selective serotonin reuptake inhibitor, into the mediobasal hypothalamus (MBH), including the ARC, significantly blocked glucoprivic suppression of LH pulses and hyperglycemia in female rats treated with intravenous 2-deoxy-D-glucose (2DG) administration as an experimental model of malnutrition. A direct infusion of glucose into the DR significantly increased in vivo serotonin release in the MBH and partly restored LH pulses and hyperglycemia in the 2DG-treated female rats. Furthermore, using an electrophysiological technique recording multiple-unit activity (MUA) volleys, as an indicator of GnRH pulse generator activity, in the ARC of female goats showed that central administration of serotonin or a 5HT2CR agonist immediately evoked the MUA volley, and central 5HT2CR antagonism blocked the serotonin-induced facilitation of GnRH pulse generator activity in ovariectomized goats. These results suggest that DR serotonergic neurons sense high glucose availability to reduce gluconeogenesis and upregulate reproductive function by activating GnRH pulse generator activity in mammals.

P-243 - Biological Pathway Analysis of Mass Spectrometry Results Reveals a Temporally Induced Citrullinome That Regulates Gonadotrope Cell Function

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Peptidylarginine deiminases (PADs or PADIs) catalyze the conversion of positively charged arginine to neutral citrulline, which alters target protein structure and function. Our previous work established that gonadotropin releasing hormone agonist (GnRHa) stimulates PAD2-catalyzed histone citrullination to epigenetically regulate gonadotropin gene expression in the gonadotrope derived LβT2 cell line. However, PADs are also found in the cytoplasm. Given this, we used mass spectrometry (MS) to identify additional non-histone proteins that are citrullinated following GnRHa stimulation and characterized the temporal dynamics of this modification. Our preliminary data reveals temporally citrullinated proteins associated with distinct cellular pathways following 10 and 30 minutes of GnRHa stimulation. We found citrullinated proteins associated in cell proliferation, transcriptional activation, and the RAF/MEK/ERK signaling cascade, all of which are vital to gonadotrope function. Not only do we see signaling cascades, but we also find the cytoskeleton and proteins facilitating vesicle release as targets of citrullination within 10 minutes of GnRHa stimulation. Our previous results in primary pituitaries suggest engagement of the cytoskeleton is necessary for the spatial positioning of gonadotropes towards vasculature and LH release. To confirm specificity, we utilized the pan-PAD inhibitor biphenyl-benzimidazole-Cl-amidine (BB-CIA), which results in decreased levels of citrullination in our identified protein targets. Taken together, our data has identified novel PAD substrates in gonadotropes and provides a temporal spectrum of the collective cellular processes regulated by PADs.

P-244 - Essential Role of DNMT3A in Neuronal Programming of Induced-Pluripotent Stem Cells

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DNMT3A (DNA methyltransferase 3A) is essential for establishing DNA methylation; an epigenetic mechanism implicated in gene regulation and crucial for development and cellular identity. During brain development, DNMT3A adds DNA methylation marks to the promoters of germline- and

pluripotency- specific genes, repressing their expression in progenitor cells. Conversely, DNA methylation is lost at promoters of neuron-specific genes, activating their expression. Alterations in these processes are linked to many neurological diseases. In mice, complete lack of Dnmt3a is lethal, whereas brain samples of mice with heterozygous Dnmt3a loss-of-function mutations show alterations in DNA methylation and transcription profiles. In humans, heterozygous loss-of-function mutations in the DNMT3A gene are associated with a range of conditions linked to overgrowth and neurodevelopmental disorders. Interestingly, altered DNA methylation patterns and changes in the expression of genes crucial for neural development and differentiation was found in blood cells from individuals with DNMT3A loss-of-function mutations. This intriguing connection underscores the intricate relationship between DNMT3A function, genetic regulation, and neurodevelopmental outcomes. Despite existing knowledge about the role of DNMT3A in brain development, our understanding of how DNMT3A loss-of-function mutations impact the differentiation of neural progenitor cells into specific neuronal cell types and how that contributes to neurological disorders is almost inexistent because of the lack of targeted mechanistic studies, especially in patient-derived brain cells. We hypothesize that DNMT3A loss-of-function disrupts DNA methylation marks and affects the normal programming of progenitors into terminally differentiated neurons, ultimately leading to neurodevelopmental disorders. To test this, we generated induced pluripotent stem cells (iPSC) from an individual presenting with a DNMT3A loss-of-function mutation leading to overgrowth and intellectual disabilities. We differentiated these human iPSC into neuronal progenitor cells (NPC), cortical neurons and 3D organoids. Our study reveals that DNMT3A loss-of-function in iPSC does not compromise pluripotency or the capacity to differentiate into all three germ layers. Although this loss-of-function does induce substantial changes in DNA methylation profiles within iPSC, the subsequent gene expression alterations are limited in number at this stage. Nevertheless, as cells traverse the neurogenesis pathway, transitioning from iPSC to NPC and cortical neurons, discernible changes in both DNA methylation and gene expression become increasingly apparent. Throughout our dataset, DNMT3A loss-of-function induces a reduction in DNA methylation at gene promoters in iPSC, setting the stage for the loss of H3K27me3 and an increase in gene expression during the NPC and neuron stages. Notably, we discern that DNMT3A loss-of-function prompts alterations in the expression of genes pivotal for neurogenesis and cell fate commitment (e.g., WNT7A, NEUROD1), reminiscent of characteristic phenotypes observed in various neurodevelopmental disorders. Interestingly, DNMT3A loss-of-function leads to larger expansion of derived 3D cortical organoids, which show cortical disorganization due to dysregulated fate specification. Our study reveals that DNMT3A loss-of-function in human pluripotent stem cells disrupts the establishment of precise DNA methylation profiles during neural lineage specification. This interference triggers alterations in gene expression, impacting cell fate commitment and potentially resulting in aberrant functioning of brain cells.

P-245 - The Age-Associated Increase in Ovarian Stiffness Impairs Follicle Development and Oocyte Quality Through Early Modulation of Follicles' Transcriptome

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Oocyte quantity and quality are severely impacted by multiple extrinsic factors, with female reproductive aging being among the most prevalent in our society. We and others reported that with

aging the mouse ovary assumes a pro-fibrotic milieu, which is associated with ovarian stiffness. However, the role of stiffness on ovarian function and oocyte quality is unknown. The objective of this work is to use an alginate-encapsulated in vitro follicle culture system that mimics age-associated changes in the ovarian microenvironment to identify whether stiffness is a novel mechanism mediating the progressive decline in oocyte quality observed with advanced maternal age. To evaluate how stiffness impacts follicle development and oocyte quality, we synthesized hydrogel that replicated the soft (young: 0.5%, 1.79 ± 0.08 kPa) and stiff (old: 2%, 4.56 ± 2.03 kPa) environments. Secondary follicles from CD1 (D12-D13, N=3 replicates) were cultured in 0.5% or 2% alginate for up to 12 days. Follicles cultured in stiff environments showed significant reduction in follicle size compared to follicles in soft environments (0.5% $226.9 \pm 17.4 \mu\text{m}$, 2% $160.8 \pm 9.9 \mu\text{m}$, $p < 0.0001$). These differences were triggered by granulosa layer since no changes were detected in oocyte size (oocytes: 0.5% $66.19 \pm 5.7 \mu\text{m}$, 2% $60.6 \pm 4.5 \mu\text{m}$, $p = 0.401$; granulosa cells: 0.5% $160.04 \pm 13.4 \mu\text{m}$, 2% $103.37 \pm 16.5 \mu\text{m}$, $p < 0.0001$) suggesting that granulosa cells are not proliferating in a stiff environment. To explore this, we assessed estradiol synthesis and granulosa cell death. Estradiol levels were reduced in 2% (0.5% $11.3 \pm 15.9 \text{ ng/ml}$, 2% $0.3 \pm 0.5 \text{ ng/ml}$, $p = 0.296$) while CC3 (apoptosis-marker) intensity was increased (CC3 intensity/area, 0.5% $20.4 \pm 5.8 \text{ a.u.}$, 2% $27.1 \pm 4.3 \text{ a.u.}$, $p < 0.05$), confirming that stiff environments impact granulosa cells viability. We then evaluated whether stiff environment impact gamete quality. Oocytes from follicles at D12 were isolated and inspected morphologically. Oocyte quality significantly declined in 2%, with $68.9 \pm 16.8\%$ of oocytes degenerated, compared to $23.6 \pm 9.2\%$ in 0.5%. We repeated all the analyses using the CB6F1 strain yielding comparable results. Since the effects of stiffness on follicle growth were already evident at day 4, we investigated early-changes in follicles' transcriptome that might mediate the observed phenotypes. 32 secondary follicles were cultured in 0.5% or 2%, and analyzed by RNAseq at 3h, 6h, 12h and 24h. We analyzed follicles' gene expression (DESeq2, R/Bioconductor) at each timepoint comparing 0.5% and 2% (significance at $\text{padj} < 0.05$; $\log_2\text{FoldChange} \geq 2$). Surprisingly, we found few differentially expressed genes (DEG) at 3h, a peak of DEG at 6h, followed by a dramatic decrease which reached a plateau at 12h and 24h. DEGs at 6h were involved in response to external stimuli and ERK1/2 cascade, pathways of proliferation and differentiation, suggesting that at 6h follicles' transcriptome is more sensitive to changes in ovarian biomechanics. To further evaluate how stiffness impacts ovarian transcriptome, we analyzed DEGs in a time-dependent manner. We identified 1033 DEGs in follicles cultured in 2% at 24h compared to 3h. Most of the upregulated genes were associated with apoptosis and matrix remodeling. In the 0.5% condition, 1295 DEGs were identified, with upregulation of genes related to metabolism and cell cycle at 24h. Overall, we demonstrated that the age-associated increase in ovarian stiffness impacts follicle development and oocyte quality by impairing granulosa cell viability. In addition, our transcriptome analysis reveals that follicles quickly respond to stiffness triggering the expression of well-known genes associated with advanced maternal age when exposed to a stiff environment. These results show that stiffness might be a novel regulator of folliculogenesis and oocyte quality. Work supported by NIH-K99/R00-HD108424 to FAR.

P-246 - RAB3A and RAB27A Interact with Rabphilin 3A to Promote Cortical Granule Exocytosis in Mouse Eggs

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Cortical granule exocytosis, or the “slow block to polyspermy,” is an important event that occurs shortly after sperm-egg fusion to prevent entry of more than one sperm into the egg, which is not compatible with further development. Proteins in the Rab family, particularly RAB3A and RAB27A, are well known to participate in exocytic events in secretory cells and there is evidence for the involvement of these Rabs in cortical granule exocytosis (CGE) in mouse and sea urchin eggs; however, the role of RAB3A in particular needs some clarification because it has also been demonstrated to affect spindle migration during oocyte maturation. Using fluorescently tagged proteins, dominant negative proteins, and Trim-away to specifically deplete proteins, we show that both RABs 3A and 27A are required for this process. RAB3A is primarily expressed in the cytoplasm, with some cortical localization. RAB27A is localized both at the plasma membrane (PM) and in the cytoplasm, whereas Rabphilin 3A (RPH3A) is localized exclusively in the cortex opposite the meiotic spindle. All 3 proteins are rapidly removed from the PM following a Ca^{2+} stimulus. Total RAB3A and RAB27A protein remains the same after egg activation, suggesting that these proteins are recycled rather than lost during the process of exocytosis. Depletion of both RAB3A and RAB27A using Trim-away does not affect cortical granule localization at the PM but prevents the cleavage of ZP2 following egg activation. In addition, a dominant negative form of RAB27A potentially blocks exocytosis without affecting cortical granule placement at the PM. Cortical granules become dispersed in response to a Ca^{2+} stimulus in eggs expressing dominant negative RAB27, suggesting that RAB27A functions at a very late stage in exocytosis. Depletion of RAB3A and/or inhibition of RAB27A prevents placement of RPH3A at the PM. However, a dominant negative form of RPH3A does not block CGE, suggesting that more than one RAB effector is important for CGE. These results demonstrate that RAB3A and RAB27A are both required for cortical granule exocytosis and work through RPH3A, though not exclusively.

P-247 - Long-Term Dietary Metformin Supplementation Alters Liver Metabolism to Sustain Egg Production in the Aging Broiler Breeder Hen

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Broiler breeder hens, the parent stock of commercial broiler chickens, have poor reproductive efficiency due to the selection pressure for superior growth related traits in broiler progeny. Decades of genetic selection for growth-related traits negatively associated with reproductive function have led to severe ovarian dysfunction in the broiler breeder hen. A previous study in our lab found that dietary metformin supplementation resulted in increased and prolonged egg production, however the mechanism(s) of action remain undetermined. The liver plays a significant role in reproduction in the laying hen as it is the main site of de novo lipogenesis and yolk-precursor synthesis and previous studies have shown that the beneficial effects of metformin are mainly through its action in the liver and its ability to improve insulin sensitivity and reduce hepatic glucose production. Therefore, the main objective of this study was to characterize the transcriptomic

profile of liver tissue collected from hens supplemented with metformin in the diet. Broiler breeder hens were supplemented with 0, 25, 50, or 75 mg/kg body weight of metformin mixed in the diet for 40 weeks (n= 45 hens/treatment; 25-65 weeks of age). At the end of the treatment period, liver tissue samples were collected from a subset of hens in each treatment group (n=12 hens/treatment group). Total RNA was extracted from liver tissue and RNA-Seq was used to perform differential gene abundance analysis. We observed that the hens which received the highest dose of metformin (75 mg/kg body weight) had the largest number of significant differentially abundant genes when compared to the control. The pairwise statistical analysis of transcriptomic data revealed that 1165 genes were differentially abundant (FDR < 0.05) in the liver of hens which received the highest dose of metformin in the diet compared to the hens which received no metformin. Transcriptomic analysis revealed an increase in the abundance of genes related to estrogen-stimulated yolk precursor synthesis, insulin-stimulated de novo lipogenesis, and AMPK-mediated glucose homeostasis. In agreement, Ingenuity Pathway Analysis predicted increased signaling through insulin receptor and estrogen receptor signaling pathways. Furthermore, transcriptomic data were confirmed through quantitative PCR analyses of genes involved in yolk-precursor synthesis and de novo lipogenesis. Based on these findings, we concluded that metformin increases and prolongs egg production in the broiler breeder hen by maintaining yolk-precursor and fatty acid synthesis, previously observed to decline in the aging hen.

P-248 - Gene Therapy Delivery of Anti-Müllerian Hormone in Kittens Induces Long-term Contraception Without Impairing Puberty

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The uncontrolled reproduction of free-roaming domestic cats is the major driver of stray and feral cat overpopulation. Without proper access to veterinary care and with the inherent risks of living outdoors, these unowned felines typically experience a shortened lifespan. The most recent estimates indicate that feral cats have contributed to 13.9% of worldwide insular bird, mammal, and reptile extinctions. Surgical sterilization of females is a lengthy procedure requiring highly trained personnel. For this reason and because of logistics and economic constraints, the current Trap-Neuter-Return approach cannot scale to control the reproduction of the hundreds of millions of stray and feral cats worldwide. We recently reported long-term contraception in adult female cats receiving an intramuscular injection of an anti-Müllerian hormone (AMH) transgene delivered with an adeno-associated viral vector. The objective of this study was to evaluate the safety and efficacy of this novel non-surgical contraceptive, termed AAV9-fcMISv2, when administered to prepubertal male (M) and female (F) domestic cats. We injected 2-3 months old kittens with either 5x10¹² (1M, 4F) or 1x10¹³ (1M, 3F) viral genomes per kilogram of body weight (vg/kg) of AAV9-

fcMISv2 or 5×10^{12} empty AAV9 particles per kg (vp/kg) (1M, 2F). Injection site monitoring, regular physical examinations, and hematologic and biochemical analyses did not reveal any sustained abnormalities. Supraphysiological ($>100\times$ basal) levels of AMH persisted throughout the 16-month-long study. Importantly, every monthly AMH reading of a treated kitten was higher than the lowest reading that induced contraception in our previous adult study. The average adult body weight of treated females (3.07 kg) was similar to that of controls (2.76 kg). In males, testosterone production and spermatogenesis were unaltered indicating that testicular function is unaffected by supraphysiological AMH. Monthly ultrasonographic measurements revealed that the prepubertal injection of AAV9-fcMISv2 impacted uterine growth as treated cats had a significantly lower average uterine cross-section area compared to controls (4.4 and 7.8 cm², respectively. $P < 0.0001$). Average fecal excretion of estrogen metabolites was lower in females from the higher dose group when compared to controls (398.4 and 523.5 ng/g of feces, respectively. $P < 0.0001$). Compared to controls, serum LH levels were higher in females from both the lower and higher dose groups (5.27, 7.23 and 7.68 ng/ml, respectively. $P = 0.0494$ and $P = 0.0134$). All but one treated female bred repeatedly during a mating trial that spanned the last four months of the study. Although the six females that bred did it significantly more often than controls (bred on 48.2 and 18.5% of days in trial, respectively. $P = 0.0286$), none of them achieved clinical pregnancy while both controls did. They presented an altered estrous behavior with multiple occurrences of single-day breeding and prolonged breeding bouts lasting >20 days, while every breeding bout in controls lasted 7-10 days. Consecutive days of steady breeding in treated females never resulted in a luteal phase indicating that ovulation was impaired. Together, health, hormonal, behavioral, and fertility data indicate that the vectored delivery of an AMH transgene prevented ovulation and luteal phases and is a safe and effective contraceptive strategy in prepubertal female domestic cats. This work was supported by The Michelson Found Animals Foundation, the Joanie Bernard Foundation, the Sudna Gar Foundation, and the Department of Surgery of the Massachusetts General Hospital. P.G. is supported by a Postdoctoral Research Scholarship (B3X) from the Fonds de recherche du Québec – Nature et Technologies (FRQNT).

P-249 - SkQ1 Treatment Preserves Ovarian Reserve in Mice.

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Key words: Ovarian reserve; antioxidant treatment; DNA repair in mammals, oocyte development and maturation are critical processes for female fertility. Women are born with a defined number of follicles, the ovarian reserve, which decreases during their reproductive lifespan, and, finally, when it is depleted, menopause begins. Previously reported data from our lab revealed that the age-associated decrease in primordial follicles is linked to the activation of the DDR. Furthermore, these studies highlighted the importance of oxidative stress in regulating natural menopause in women. Considering all of the above, we attempted to evaluate the effect of an antioxidant treatment that could reverse the effects of ROS in the ovarian reserve preservation. To address this hypothesis, we administered, through the drinking water, the mitochondria-targeted antioxidant, SkQ1, to young C57BL/6 female mice for 14 weeks. To evaluate SKQ1 efficacy, we administered

ethanol, an inductor of ROS linked to a diminished ovarian reserve in humans and co-administered SkQ1 and ethanol. Our preliminary findings reveal a significant reduction in primordial follicles in mice exposed to ethanol, suggesting that ethanol consumption diminishes the ovarian reserve. In addition, the SkQ1 treatment could revert these effects on the ovarian reserve and even increase the number of primordial follicles to control levels. To study the effects of the SkQ1 treatment in natural aging, we administered SkQ1 dissolved in DMSO into the drinking water of young C57BL/6 female mice for 14 weeks. Untreated mice lost around 45% of their primordial follicles, contrary to the SkQ1-treated animals, in which the loss was just 22%. So, SkQ1 consumption rescued approximately 40% of the primordial follicles loss during natural aging. Based on these findings, we propose that the daily consumption of ethanol, even in small doses, could significantly affect the fertility status of mammalian females. However, an SkQ1 treatment could counterbalance the ethanol effects and, also, revert the natural aging effects and highly preserve the mammalian ovarian reserve. Financial Support This work received support from the Spanish Ministerio de Ciencia e Innovación grants (BFU2016-80370-P and PID 2019-107082RB-I00), as well as a Fundación Dexeus Mujer 2019 Fellowship. NN was also awarded a FPI fellowship from the Spanish Ministerio de Ciencia e Innovación (PRE-C-2020-0084).

P-250 - Selective Autophagic Degradation of ACLY (ATP citrate lyase) Maintains Citrate Homeostasis and Promotes Oocyte Maturation

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Macroautophagy, also known as autophagy, serves as a critical cellular and energy regulatory mechanism, contributing to the preservation of primordial follicles, germ cell viability, and counteracting ovarian aging. However, it remains unclear whether autophagy in granulosa cells influences oocyte maturation. Our study demonstrates a noticeable decrease in autophagy levels in human granulosa cells obtained from older women, suggesting a potential inverse relationship between autophagy levels and oocyte quality. Through the establishment of a co-culture system, we observed that either pharmacological inhibition or genetic knockout of autophagy in granulosa cells detrimentally impacts oocyte quality and fertilization potential. Metabolomics analysis further revealed that the deleterious effects of autophagy deficiency on oocyte quality are mediated by reduced citrate levels, with exogenous citrate supplementation significantly enhancing oocyte maturation. Mechanistically, we identified ATP citrate lyase (ACLY), a key enzyme involved in citrate cleavage, as preferentially targeted for selective autophagic degradation via its association with K63-linked ubiquitin chains and recognition by the autophagy receptor protein SQSTM1/p62. In human follicles, autophagy levels in granulosa cells decline with maternal aging, concomitant with decreased citrate levels in the follicular fluid, suggesting a potential link between citrate metabolism and oocyte quality. Furthermore, elevated citrate levels in porcine follicular fluid promote oocyte maturation. Taken together, our findings elucidate the role of autophagy in granulosa cells in maintaining optimal citrate levels during oocyte maturation, highlighting its importance as a protective mechanism for reproductive health.

P-251 - Effect of Maternal Age on the Oocyte Cortical Actin Layer and Associated Endoplasmic Reticulum Clusters

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Mature oocytes in many mammalian systems produce specialized endoplasmic reticulum 'clusters' unique to this cell type that are thought to play a critical element in the Ca²⁺ oscillations generated at fertilization. Endoplasmic reticulum clusters may also be important in human fertility, as dysmorphisms in smooth endoplasmic reticulum clusters are associated with lower oocyte viability. Here we compare structural differences in ER clusters and their interaction with the cortical actin layer in oocytes from young mice (5-6 weeks; n=60) and aged (55-58 weeks, n=57) C57BL/6 mice obtained from Jackson Laboratory or the National Institute for Aging. Mice were superovulated and oocytes were fixed in situ in the oviducts, isolated, and then labeled with anti-human calreticulin, Alexa488 goat anti-rabbit, and Alexa568 phalloidin, prior to confocal microscopy. The endoplasmic reticulum clusters in oocytes from young females were consistently localized in close proximity to the 'fenestrae' of the cortical actin layer, excluding the microvillus-free zone over the spindle. In contrast, oocyte endoplasmic reticulum clusters from aged females frequently exhibited disassociation from the actin layer (15%) and structural abnormalities including the presence of vesicles (44%) and enlarged, distorted shapes (6%). Additionally, the 'actin' fenestrae were commonly reduced or absent in the oocytes from aged females, especially when endoplasmic reticulum clusters were not associated with the cortex (29%). These results share many similarities with observational reports in human oocytes from patients of advanced maternal age, suggesting that a systematic study of the mouse system could be used to identify possible functional defects associated with these abnormal structures.

P-252 - Effect of Antioxidant Procyanidin B2 (PCB2) on Ovine Oocyte Developmental Potential in Response to In Vitro Maturation (IVM) and Vitrification Stress

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It was demonstrated that external stress, such as IVM and vitrification process would induce significantly reduced development capacity in oocytes. Previous studies indicated that antioxidants played a pivotal part in the acquisition of adaptation in changed conditions. At present, the role of a natural potent antioxidant PCB2 in response to IVM and vitrification during ovine oocyte manipulation is not explored yet. It aimed to investigate whether PCB2 treatment could improve the developmental potential of ovine oocytes under IVM and vitrification stimuli. The experiment was divided into two parts. Firstly, un-supplemented and 5µg/mL PCB2-supplemented in the IVM solution were considered as control and experimental groups (C + 5µg/mL PCB2). The polar body extrusion (PBE) rate, mitochondrial membrane potential (MMP), ATP, reactive oxygen species (ROS) levels and early apoptosis of oocytes were measured after IVM. Secondly, survival rate, PBE rate and early apoptosis of oocytes were compared within fresh group, vitrified group and 5µg/mL PCB2-supplemented in the IVM solution after vitrification (V + 5µg/mL PCB2). Compared to

the control group, adding PCB2 significantly increased PBE rate (79.40% vs. 62.84%, $P < 0.01$) and MMP level (1.85 vs. 1.30 $\Delta\phi m$, $P < 0.01$), and decreased ROS level (47.07 vs. 145.33 pixels, $P < 0.01$). However, there was no significant difference in ATP content and early apoptosis. Compared to the fresh group, vitrification significantly reduced oocytes viability (43.04% vs. 90.78%, $P < 0.01$) and PBE rate (24.19% vs. 60.57%, $P < 0.05$). However, 5 $\mu g/mL$ PCB2-supplementation during maturation had no effect on survival rate, PBE rate or early apoptosis in vitrified oocytes. PCB2 could effectively antagonist the oxidative stress during IVM stage and promote oocyte development.

P-254 - Later Onset of DNA Methylation in Bovine Oocytes

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The development of a competent oocyte depends on morphological and molecular changes during its growth phase. In cattle, the morphological transitions of the follicle and oocyte have been described in detail. However, knowledge of the underlying molecular modifications remains incomplete. Previously we described the modulation of the oocyte transcriptome across bovine oocyte growth; our current objective was to characterise the establishment of the oocyte methylome. Oocytes were recovered from slaughterhouse-derived ovarian follicles by cortex-slicing. The diameter of single oocytes was measured and they were snap-frozen in RTL-Plus buffer. A total of 179 oocytes were allocated to the following diameter groups: $< 60 \mu m$, 60-69 μm , 70-79 μm , 80-89 μm , 90-99 μm , 100-109 μm , 110-119 μm , and $> 120 \mu m$. Single-cell Bisulphite Conversion Sequencing was performed, and the data was mapped to the ARS-UCD2.1 bovine genome, with the quantification of 39,337,556 cytosines. Global CpG methylation remained low ($< 11.5\%$) in oocytes until they reached 90-99 μm in diameter when the methylation levels increased to $\sim 28\%$. Active gain in methylation continued until oocyte growth was completed ($> 120 \mu m$ in diameter), at which stage methylation stabilised at $> 48\%$. The methylation data was quantified over gene bodies for further analysis. Following clean-up, a total of 18,327 genes were identified for which methylation quantification data was available in all groups. Setting the oocyte diameter group $> 120 \mu m$ as the control, genes with more than a 25% difference in methylation between diameter groups were identified and enriched pathways were determined. Most of the genes presented low to medium methylation levels in the smaller oocytes (< 60 to 80-89 μm) but were highly methylated in the control group ($\sim 12,000$ genes). The genes were related to spindle organisation, cell division, chromosome segregation, protein and histone modification, and cytoplasmic microtubule organisation, among others. As oocyte diameter increased above 90 μm (90-99 μm , 100-110 μm , and 110-119 μm), the number of differentially methylated genes decreased compared to the control group (4,428 to 459 genes), with no enriched pathways assigned to them. However, higher methylation of OOSP2 and ZARL1 in the control oocytes is noteworthy. Next, we evaluated the methylation status of imprinted genes since their methylation will be preserved in future embryos. From a total of 32 identified imprinted genes, 13 were characterised by a continuous methylation gain from the smallest to the largest oocyte group, starting with less than 20% in oocytes $< 60 \mu m$ until more than 70% methylation in oocytes $> 120 \mu m$. A group of 6 imprinted genes presented a medium level of methylation (30 – 70%) in the largest oocytes ($> 120 \mu m$). Interestingly, the

methylation levels of 13 imprinted genes remained low (< 30%), e.g., IGF2, indeed 6 of these genes presented less than 6% methylation, e.g. OOEP. In conclusion, here we present novel information on methylation dynamics during bovine oocyte growth. Our data reveals that methylation onset is later and occurs at different speeds and levels at imprinted genes in bovine compared to other species. This information provides a platform to better understand the epigenetic events during bovine oocyte growth and highlights a potential window of sensitivity when oocyte quality may be enhanced or compromise.

P-255 - Ovarian Infusion of Anti-Müllerian Hormone Does not Improve Fertility Outcomes in Reproductively Aged Rhesus Macaques

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Anti-Müllerian Hormone (AMH) plays an important role in the regulation of follicle growth in mammalian ovaries. At the mechanistic level, expression of AMH by the growing follicle pool is essential for suppressing the activation of primordial follicles and maintaining follicle reserve in rodent models. In non-human primate models, AMH has been shown to promote the growth of secondary and pre-antral follicles in vitro and in vivo, but suppresses the development of antral follicles. Expression of AMH by the growing follicle pool in humans is used as a proxy for follicle reserve, guiding therapeutic approaches for infertility patients. These observations then raise the question: Can manipulation of AMH in humans with low follicle reserve increase the pool of growing follicles, thereby increasing the total number of follicles available for retrieval? In order to answer this question, reproductively aged female rhesus macaques (N=5, range of 17-21 years of age, roughly equivalent to 40-45 yo women) underwent ovarian catheterization in a cross-over longitudinal design to directly infuse ovaries with recombinant human AMH (N=2, 25 ng/hr) or PBS (N=3, 0.25 µl/hr) for 60 days. Animals underwent controlled ovarian stimulation (COS), and ultra-sound guided follicle aspiration for in vitro fertilization (IVF). Animals then underwent 2 menstrual cycles of recovery with saline infusion, followed by a final 60-day infusion of either PBS (N = 2) or AMH (N =3), ending with a final COS-IVF cycle. Overall oocyte yield, maturation, fertilization rate, embryo cleavage rate, and blastocyst formation rate were quantified. Levels of serum estradiol, progesterone, and AMH levels were measured in every cycle. Mean values for each parameter from each treatment group were compared using Wilcoxon matched paired tests. Aggregating the COS and embryo data by treatment group demonstrated no significant differences in the overall numbers of oocytes retrieved, the numbers and proportions of MII oocytes, the number and ratios of fertilized oocytes formed, and the numbers and ratios of cleavage stage embryos. Although not statistically significant (P=0.125), the blastocyst rate with PBS treatment was over 2 times higher than with AMH treatment (59.23% ± 24.86% vs 25% ± 35.36). Additionally, although there were no notable differences in estradiol or progesterone for the COS cycles, there

was a trend ($P = 0.15$, One-Way ANOVA) for decreased serum AMH when animals ($N=4$) were treated with recombinant AMH compared to PBS or saline infusions (2.60 ± 4.24 ng/mL vs. 8.12 ± 6.12 ng/mL vs 7.7 ± 5.4 ng/mL), suggesting negative feedback signaling. The results of these ongoing studies suggest that exogenous administration of AMH to the primate ovary neither increases the total number nor overall quality of the oocytes, and may inhibit blastocyst formation. Further study in nonhuman primates is required to determine if altering the dose or timing of AMH administration can improve fertility outcomes in reproductively aged women. This work is supported by NIH R01HD082208 (JX and AJK) and NIH P51OD011092 (ONPRC Core grant), with excellent support from the ONPRC Surgical Services Unit and the Endocrine Technologies Core.

P-256 - Expression of TGF β Signaling Molecules and Genes in Loci Linked to Polycystic Ovary Syndrome in Human Fetal and Adult Tissues

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Altered signaling of androgens, anti-Müllerian hormone and transforming growth factor beta (TGF β 1) during fetal development have all been implicated in the predisposition to polycystic ovary syndrome (PCOS) in later life. There is also a genetic predisposition to PCOS. In fetal ovarian fibroblasts, we have shown that TGF β 1 regulates androgen signalling and expression of seven genes located in genetic loci associated with PCOS (Hum Reprod. 2022, 37:1244-1254). Since PCOS exhibits a myriad of symptoms, it likely involves many organs other than just ovaries. We aimed to identify the relationships between TGF β signalling molecules and PCOS candidate genes in different human fetal and adult tissues. Using RNA-sequencing data, we examined the expression patterns of TGF β signalling molecules in human ovary, testis, heart, liver, kidney, brain tissue and cerebellum from 4-20 weeks of gestation and postnatally. We also conducted correlation analysis with expression of PCOS candidate genes. TGF β signalling molecules were dynamically expressed in most tissues prenatally or/and postnatally. Many relationships were observed. FBN3, a PCOS candidate gene involved in TGF β signaling, was expressed during fetal development in all tissues examined. The PCOS candidate genes HMGA2, YAP1 and RAD50 correlated significantly ($P < 0.01$) with most TGF β signalling molecules in at least four fetal tissues; and specifically with TGFBR1 in six out of the seven tissues examined. This study suggests that crosstalk is likely to occur between genes in loci associated with PCOS and TGF β signaling molecules in multiple tissues during fetal development. Thus, alteration in TGF β signaling during fetal development could affect many tissues, potentially contributing to the multiple symptoms and phenotypes of PCOS in later life.

P-257 - Culture Conditions and Cumulus-Oocyte Complex Integrity Affect the Program of Oocyte Maturation in Higher-Order Mammals

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In oocytes, resumption and completion of meiosis I are driven by post-transcriptional mechanisms on a background of repressed mRNA transcription. However, there is a lack of in-depth understanding of the regulation of translation in maturing oocytes, particularly in higher-order mammals, primarily due to methodological constraints in isolating polysome-associated mRNAs. Thus, we aimed to shed light on this process and how it can be affected by oocyte in vitro maturation (IVM). This objective was achieved through three sequential steps. First, we applied a bioinformatics approach to gain information on mRNA polysome association (GSE56603 and GSE196484) and polyadenylation (GSE61717) in immature and in vitro matured bovine oocytes. The GEO-retrieved datasets were re-analyzed to determine differentially polyadenylated/polysome-associated genes using edgeR. While the overlap of differentially polyadenylated/polysome-associated genes between datasets was limited when comparing mature oocytes (27 genes), all comparisons conducted in immature oocytes were substantial (372 genes $p < 0.05$). These findings indicate that the experimental approaches potentially yield comparable results, but that this homogeneity is lost with IVM, denoting that culture conditions contribute to changes in mRNA translation patterns. Next, we tested whether the EGF-like growth factors regulate the translation of some transcripts by activating the oocyte PI3K/AKT/mTOR axis in bovine oocytes, as demonstrated for mice. For this, the previously mentioned datasets were compared with polysome-associated transcripts of mouse oocytes exposed to the EGF-like growth factor, amphiregulin (GSE46640). Notably, one bovine dataset showed a significant correlation with EGF network activation, supporting, at least in part, the observation that IVM conditions might affect mRNA translation. To experimentally confirm the role of the EGF network in the bovine, we monitored oocyte AKT during IVM by western blots and immunofluorescence and observed a significant, transient phosphorylation with amphiregulin, indicative of the pathway activation. To understand if AKT activation affected translation, we investigated polyadenylation in target transcripts using two approaches. First, we attempted to indirectly catch differences in the retrotranscription efficiency in association with short or long poly(A) tail. The mRNA was split in half and retrotranscribed using random hexamers or oligo(dT). This assay did not highlight differences between immature and mature oocytes or IVM conditions. However, when the poly(A) tail length was directly assayed, a differential extent of polyadenylation in target genes related to cell cycle progression and protein localization to the centrosome was observed in amphiregulin-supplemented IVM. Furthermore, with this assay a starting length of the poly(A) tail was set at approximately 200 bp in immature oocytes, providing an explanation for the inability of observing differences using random hexamers and oligo(dT). Since the extension of the poly(A) tail is necessary for polysome association and translation, these results confirm that, despite differences in timing and initial tail length, also in higher-order mammals translation of genes crucial for proper oocyte maturation are regulated by the EGF network, likely through PI3K/AKT/mTOR. To further understand if the impossibility to activate the EGF network during IVM could affect downstream signaling, AKT phosphorylation was

monitored during rescue IVM of denuded human immature oocytes obtained after controlled ovarian stimulation. As predicted by our model, AKT failed to activate, providing a putative explanation for the low quality achieved after rescue IVM in humans. This study highlights the importance of providing an appropriate cultural environment and maintaining the structural integrity of the cumulus-oocyte complex for a proper execution of the maturation program.

P-258 - The Role of PARP1 in Female Gametogenesis

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Premature Ovarian Insufficiency (POI), affecting 1% of women, is marked by early onset of menopause, impacting fertility, and increasing vulnerability to cardiovascular issues, osteoporosis, and premature mortality. While the causes remain undefined, in most cases, it is attributed to a small primordial follicle pool, rapid follicle exhaustion, or dysfunction of the ovarian follicles. The follicle pool, comprised of germ cells (oocytes) and surrounding supporting cells, defines a woman's reproductive lifespan. Female gametogenesis, the process involving the generation of germ cells, is crucial for forming the follicle pool (Rudnicka, Kruszewska et al. 2018). In a Genome-Wide Association Study (GWAS), we identified a PARP1 variant (V762A) linked to an earlier onset of menopause (Stankovic, Shekari et al. 2022) and a 40% reduction in PARP1 activity (Wang, Wang et al. 2007). In this project, we aim to unravel the role of PARP1 and its activity in female gametogenesis. Using the CRISPR/Cas9 gene editing system, we generated mouse embryonic stem cells carrying the PARP1 V762A variant. Employing an in-vitro protocol (Hayashi, Ohta et al. 2011), we are able to replicate the differentiation of embryonic stem cells into primordial germ cells, enabling us to examine the specific involvement of PARP1 in early gametogenesis. We found that mouse embryonic stem cells carrying the PARP1 variant display a reduced ability to differentiate into primordial germ cells-like cells when compared to wildtype PARP1. Further, we aim to elucidate the molecular mechanisms underlying PARP1 activity during early gametogenesis.

P-259 - Bovine Follicular Fluid Extracellular Vesicles mRNAs Contents and Effects on Oocyte Maturation and Development When Supplemented During In Vitro Maturation, Preliminary Results

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Oocytes develop and mature in vivo as the cumulus-oocyte complex (COC) which is bathed by fluid within the ovarian follicle (follicular fluid, FF). The FF contains extracellular vesicles (fEVs), which are known to function as a communication system between follicular cells and the oocyte, as they carry several molecules as RNAs, which can regulate the function of cell uptaking such vesicles. However, during in vitro maturation (IVM), the COCs are placed in culture medium without fEVs. The present study aimed to assess effects of fEVs during IVM on oocyte maturation and developmental

competence and to determine their transcript contents related with lipid metabolism, antioxidant enzymes and epigenetic regulators, which are important for oocyte maturation. Bovine FF and COCs were obtained from slaughterhouse ovaries by aspiration of 3-6mm follicles from separate collections. fEVs were isolated from 1 ml FF by size exclusion chromatography (SEC, qEV1 columns 35 nm Gen 2, Izon) followed by ultracentrifugation (100,000 x g for 70 min at 4°C, Beckman Coulter), and used for mRNA analysis content by RT-PCR or for medium supplementation during IVM. Bovine COCs were subjected to IVM in TCM199 containing 0.4 mM glutamine, 0.2 mM pyruvate, 50 mg/mL gentamicin, EGF (20 ng/ml) and supplemented only with 10% fetal calf serum depleted of its own EVs (dFCS, control) or with dFCS plus fEVs (fEVs group), for 24h, at 38.5°C and 5% CO₂ in air. At the end of IVM, cumulus cells (CC) were removed from part of the COCs, and the denuded oocytes evaluated for maturation rates by first polar body extrusion (1st PBE, 5 replicates). CC were assessed, as also the fEVs, for lipid metabolism, antioxidant enzymes and epigenetic regulation transcripts by RT-PCR (3 replicates). The other part of the COCs was submitted to in vitro fertilization and culture, and blastocyst rates on day 7 (D7) were recorded (5 replicates). The Wilcoxon two group test was used to analyze the rates of maturation, blastocyst and Δ Ct values. Maturation rates were not affected by treatments ($P > 0.05$) and 1st PBE rate was 77.2 (n=169) and 79.5 % (n=170), for control and fEVs, respectively. From 224 and 245 COCs matured without or with fEVs, D7 blastocyst rates were also unaffected (47.0 and 44.5%, respectively, $P > 0.05$). fEVs were shown to contain transcripts related with lipid metabolism (PLIN2 Ct=29.05; ACACA Ct=29.92; LDLR Ct=27.93; PLIN2 Ct=29.05), antioxidant enzymes (GPX1 Ct=28.65; GPX4 Ct=29.08; SOD1 Ct=29.04; SOD2 Ct=28.59) and epigenetic regulation (DNMT1 Ct=30.53; DNMT3A Ct=31.29; MAT2A Ct=30.11; SHMT2 Ct=29.05). CC also expressed the same genes found in fEVs, but their expression levels were not affected by maturation with fEVs ($P > 0.05$). In conclusion, fEVs carry mRNAs that could impact important functions for COCs, including lipid metabolism, antioxidant enzymes and epigenetic regulators. Although fEV during IVM did not improve maturation and embryo development and did not affect transcript levels in CC, effects on oocytes cannot be ruled out. These are preliminary results, and more replicates are being performed as well as analysis of expression in oocytes and of other transcripts and miRNAs. Future assessments may bring further knowledge to the area. Financial support: FAPESP (SPEC Grant # 2021/09886-8; AR Grant #2021/06760-3); FS - DS Scholarship (Capes 88887.694635/2022-00); AB - PD Scholarship (FAPESP 2023/01524-5); JRQO - Sci Scholarship (FAPESP 2023/12424-1); LCZJ DS Scholarship (Capes 88887.836321/2023-00); LCM - Sci Scholarship (PUB-USP 2023/83-1).

P-260 - SOX9: A Novel Downstream of PGR and Regulator of Steroidogenesis in Granulosa Cells of Human Ovulatory Follicles

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Progesterone receptor (PGR) is found to be essential for successful ovulation in all species studied thus far. However, the specific action of PGR in the human ovulatory follicle remains largely elusive. We recently performed ChIP-Seq analysis to identify PGR-regulated genes in granulosa cells of human ovulatory follicles collected at the time of oocyte retrieval from patients undergoing IVF as well as primary human granulosa/lutein cells (hGLC) treated with hCG. ChIP-Seq analysis revealed a diverse array of genes directly bound by PGR. Of our particular interest, SOX9 has emerged as a novel direct downstream target of PGR, suggesting its role as a downstream mediator of the P4/PGR-regulated pathway in the human periovulatory follicle. SOX9, a member of the SRY-box transcription factor family, plays a crucial role in regulating a spectrum of genes across various tissues. Its roles have been extensively explored in embryonic development and sex determination in reproductive tissues. However, its expression, regulation, and function in human periovulatory follicles are unknown. To address this, we first characterized the expression of SOX9 *in vivo* using dominant follicles collected from normally cycling women before the LH surge (preovulatory phase) and at defined times after hCG administration (early, late, and post-ovulatory phases). Following hCG administration, SOX9 mRNA levels were transiently upregulated in granulosa cells of dominant follicles; the levels were highest during the early ovulatory phases. Consistently, the positive staining of SOX9 protein increased after hCG stimulation. Its localization was predominantly observed in granulosa cells of dominant follicles throughout all phases following hCG administration, including the early, late, and post-ovulatory phases. Next, to dissect the regulatory mechanisms underlying SOX9 up-regulation in periovulatory granulosa cells, hGLCs were treated without or with hCG in the absence or presence of the PGR antagonist, RU486, for various time points. We found that hCG induced transient increases in the expression of SOX9 (mRNA and protein), mimicking *in vivo* expression patterns. The hCG-induced increase in SOX9 expression was completely reduced by RU486, affirming PGR as an upstream regulator of SOX9. Lastly, to further elucidate the role of SOX9 in the periovulatory follicles, we employed a small interfering RNA (siRNA) approach to knockdown the expression of SOX9 in hGLCs. siRNA-mediated attenuation of SOX9 expression resulted in the reduction of progesterone production. In line with the reduced levels of progesterone, transfection with siRNA for SOX9 decreased the expression of key steroidogenesis-related factors (STAR, CYP11A1, and HSD11B1). In summary, our study unveils, for the first time, that the expression of SOX9 is transiently up-regulated in the granulosa cells of human periovulatory follicles *in vivo* and *in vitro*. ChIP-seq and PGR inhibition studies further demonstrated that PGR acts as a direct transcription mediator of hCG-induced SOX9 expression in human luteinizing granulosa cells. In turn, SOX9 modulates progesterone production by regulating the expression of genes involved in steroidogenesis, thereby playing a crucial role in coordinating the ovulatory process and luteinization in human ovulatory follicles. Supported by R01HD096077.

P-261 - Effect of MAPRE1 on Oocyte Maturation and Preimplantation Embryonic Development in Mice

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Microtubules are assembled from heterodimers of α - and β -tubulin into highly dynamic polymers essential for many intracellular processes. Microtubule-associated RP/EB family member 1 (MAPRE1), also termed EB1, is a member of microtubule plus-end-tracking protein (+TIP) family that plays a crucial role in regulating microtubule dynamics by binding growing microtubule ends and interacting with a network of +TIPs. In this study, we investigated the function of MAPRE1 during mouse oocyte maturation and preimplantation embryonic development. Immunofluorescence analysis revealed that MAPRE1 was distributed in the cytoplasm at germinal vesicle stage and along the spindle at metaphase I and metaphase II stages. The results of Western blotting analysis showed that MAPRE1 was consistently expressed from germinal vesicle stage to metaphase II stage. To investigate the function of MAPRE1, Mapre1 was knocked down by microinjecting of Mapre1 siRNA into germinal vesicle stage oocytes and zygotes respectively, and the effects were examined by Western blotting, immunofluorescence, live-cell imaging, etc., with at least three replicates per experiment. The results showed that Mapre1 knockdown in germinal vesicle stage oocytes caused defects in spindle assembly, chromosome alignment, and kinetochore-microtubule attachment, which subsequently inhibited spindle assembly checkpoint silencing, cyclin B1 degradation, and the release of separase activity and ultimately led to failed anaphase entry and first polar body extrusion. Mapre1 knockdown in zygotes resulted in most of Mapre1-depleted embryos arrested at 2-cell stage. This study demonstrated that MAPRE1 plays an important role during the oocyte maturation and early embryonic development in mice. This study was supported by the National Key Research and Development Program of China (2022YFC2702200) to Z.H.

P-262 - Anthocyanins Present in Dietary Berries Reduce the High Androgen Production of Granulosa Cells by Interfering with the Mitochondrial Population and Lipid Metabolism.

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Infertility is a global health concern affecting 48 million couples and 186 million of persons in the world. Infertility has economic and social consequences for couples who wish to conceive and has been associated with lifestyle factors, including poor diet and physical inactivity. In present-day societies who women choose to have children later in life leads to increase difficulty to conceive by women. The female fertility takes place in ovary, and granulosa cells surrounding the oocyte, support and nurse the developing oocyte. These important cells provide energy for oocyte follicular maturation through carbohydrate, lipid, and protein metabolism, playing an essential role in oocyte maturation and human reproduction. Granulosa cells can be disrupted by environmental factors such pollutants or diets or ageing and addition of anti-stress molecules could improve their activity and the oocyte health. Numerous basic and clinical studies have demonstrated that micronutrients and more specially, anthocyanins, and other polyphenols from various botanical sources, are efficacious in ameliorating endocrine diseases such as: diabetes or obesity. Some approach by using dietary interventions are used to reduce stress in order to improve fertility outcomes. Several

berries characterized by their high content of vitamins, phenolic compounds, and organic acids are used. Blue berries are highly concentrated in anthocyanins which are a bioactive compound, providing many health benefits based on their antioxidant and anti-inflammatory properties, which could improve fertility, and the success in assisted reproductive technology approach. The objective of our study is to understand at the molecular levels by omics strategy the effect of anthocyanins on human granulosa cells functions. In this study, we have evaluated two varieties of anthocyanidins: delphinidin and aronia extract rich in cyanidin. We assayed proliferative, and steroid production aspects of human GC function. Granulosa cells exposed to delphinidin have shown that viability is not affected at the 50 μ M concentration. Expression analysis by RNAseq showed that cholesterol biosynthesis and lipid metabolism pathways are deregulated. The associated lipidomic analyses confirm a change in lipid profiles with an increase in monosaturated lipids after exposure to delphinidin. One of the consequences is the reduction in steroid synthesis. In addition, anthocyanins reduce the mitochondrial population during long exposure with delphinidin. Seahorse analysis confirms that an acute exposure reduces mitochondrial respiration by 11%. Oxidative stress was evaluated. Delphinidin and aronia extract reduce the Reactive Oxygen Species content in granulosa cells, improving the ageing. However, RNAseq analyses also showed several pathways associated with the formation of lysosomes that are upregulated. We confirmed by electron microscopic observations that anthocyanin promotes autophagy-lysosomal pathway. In conclusion, delphinidin or Aronia exposition on granulosa will reduce proliferation and limit hyperandrogenism. The beneficial effect of anthocyanins on pathological situation (i.e. PCOS) is the ability to slow down their growth, limiting their hyperandrogenism and reducing oxidative stress which suggests an anti-ageing effect on the ovarian follicles. Project supported by Region Centre Val de Loire "PEPS"

P-263 - In Vitro Culture Improves the Transcriptome and Chromosome Structure of Secondary Follicles in Aging Mice.

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In recent years, the age of first pregnancy has continued to increase. The problem is that the proportion of aneuploid metaphase II oocytes increases rapidly with the woman's age. However, the mechanisms of aging-related meiotic defects and aneuploidy in oocytes remain to be clarified. The interaction of oocytes with granulosa cells (GCs) and cumulus cells (CCs) during oocyte growth and maturation is essential for obtaining functional oocytes. This study explores how age-related changes in GCs and CCs may impair oocyte development. We analyzed transcriptomic alterations in these cells and oocyte chromosome structures following in vitro growth (IVG). Secondary and antral follicles were collected from young (8-12 weeks) and old (44-54 weeks) C57BL/6J female mice, and GCs and CCs were isolated for RNA-seq. In vivo grown and IVG oocytes were subjected

to in vitro maturation, and spindle and chromosomes were evaluated. Principal component analysis revealed variation between old and young CCs but not GCs. Notably, the expression variability in in vitro-cultured CCs was reduced, indicating altered gene expression dynamics by culture. Aneuploidy rates were comparable in IVG and vivo-grown oocytes. However, IVG oocytes exhibited improved spindle morphology and reduced chromosomal irregularities, suggesting a potential benefit of in vitro culture on chromosomal stability. IVG significantly reduced the distance between sister chromatid kinetochores, enhancing chromosomal alignment and possibly reducing partitioning errors during meiosis. These findings support the potential of in vitro techniques to alleviate age-related defects in oocyte quality.

P-264 - The Impact of Vitrification and In Vitro Culturing on Expression of FOXO3 and Ki-67 Proteins in Bovine Ovarian Tissue

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In cattle, there is spontaneous activation of primordial follicles in ovarian tissue cultured in vitro. This effect is likely achieved by disruption of the HIPPO signaling pathway, which occurs after mechanical fragmentation (Kawamura et al., 2013). However, the molecular pathways regulating the activation of primordial follicles are still unknown. Numerous studies suggest that a key factor in primordial follicle activation is the transcription factor forkhead box O3 (FOXO3). It is presumed that FOXO3 is a suppressor of the activation of primordial follicles (Albamonte et al., 2020). In the mouse primordial follicles FOXO3 is expressed in the nucleus and translocates to the cytoplasm at the next stage of the primary follicle (Castrillon et al. 2003). Moreover, the expression of Ki-67 protein is related with cell proliferation. Ki-67 protein appears during all active phases of the cell cycle (G1, S, 2, and mitosis), but is not present from resting cells (G0). During interphase, the antigen can be only detected in the nucleus, whereas in mitosis most of the protein is translocated to the surface of the chromosomes (Scholzen and Gerdes, 2000). These features of Ki-67 make this protein an excellent marker for determining the proliferative status of ovarian follicles. For the study bovine ovaries from local slaughterhouse were used. The ovarian cortex was separated from medulla, cut into small pieces and divided into 4 groups: control (fresh tissue), in vitro cultured, vitrified-warmed, and vitrified-warmed and in vitro cultured. Ovarian tissue pieces from all groups were used for immunohistochemical analysis of FOXO and Ki-67 proteins. Additionally, apoptosis level was evaluated using TUNEL Assay Kit - HRP-DAB. In ovarian tissue subjected to in vitro culture, apoptosis occurred in some cortex cells, but not in ovarian follicle cells. In vitrified-warmed ovarian tissue and in vitrified-warmed and in vitro cultured tissue, apoptosis occurred in some cortical cells, but not in ovarian follicle cells. We found variable pattern of FOXO3 protein expression in the pool of ovarian follicles. Some primordial follicles expressed FOXO3 in both the nucleus and the cytoplasm and some only in the cytoplasm. Immunoreactive granulosa cells were presented in primordial, primary and secondary follicles in both studied groups. However, at later developmental stages, FOXO3 expression was absent in granulosa cells. In cases of Ki-67, expression occurred in the cytoplasm and granulosa cells. Some follicles showed strong Ki-67 protein expression also in the nucleus. The in vitro culture and cryopreservation protocols used for

the study do not accelerate apoptosis in the cells of ovarian tissue. Additionally, the expression of the examined proteins accounts for the presence of the processes related to activation of ovarian follicles in ovarian tissue.

P-265 - Optimising a Multi-omics Approach for Characterising the Progenitor to Theca Cell Transition in Mice

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The theca cell layer of the ovarian follicle plays a key role during the development and ovulation of a mature oocyte. Until recently, the stromal origins and identity of theca cell progenitors were unknown. In the mammalian ovary, at any given time, each follicle is at a unique stage of development. Thus, theca cells will be present at all stages of development, from progenitors to differentiated theca cells, but will be differentially localised. We exploited this feature of follicular development and a 'multi-omic' approach to identify theca cell progenitors, characterising the transition to differentiated theca cells. Ovaries were collected from two week-old mice and were either dissociated for single-cell RNAseq (scRNAseq) or frozen in optimal cutting temperature compound for spatial transcriptomics. For scRNAseq, dissociated samples with high cell viability ($80 \pm 5\%$; mean $\pm$ SEM) were fixed and prepared as per manufacturer's instructions for the Chromium Fixed RNA Mouse Transcriptome kit (10X Genomics), targetting 10,000 cells per reaction ($n=3$). Libraries were sequenced at a minimum coverage of $\sim 60,000$ reads per cell. For spatial transcriptomics, RNA quality was evaluated by Bioanalyzer and tissue blocks with RIN >7 were systematically sectioned and the ovarian morphology evaluated. Once an appropriate section was identified, the next section was mounted onto the Stereo-seq Chip T Slide (STOmics). Slides ($n=2$) were imaged by confocal microscopy to generate 20X fluorescent images and permeabilised to prepare libraries for sequencing on a DNBSEQ-G400 sequencer (MGI). Herein, the optimised experimental approach is described including preliminary data characterising the progenitor to theca cell transition. Given that some infertility associated with maternal aging and obesity is caused by ovulation failure, understanding the development of this cell type may provide novel insights into ovulation failure-related infertility. This project was supported by a grant to ZC and SS from Research For Life (2023/352).

P-266 - The Effect of Pigment Epithelial-Derived Factor (PEDF) Supplementation on Ovarian Aging

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Ovarian aging is associated with diminished fertility and a decrease in overall health quality in women. Innovative approaches are in progress to elongate the functional lifespan of the ovary,

which ultimately could extend healthy lifespan. The depletion of the follicle pool is the major cause of the onset of menopause. However, the biological mechanisms underlying this physiological process remain poorly understood. Aging in the ovary has been associated with increased mTOR signaling pathway, fibrosis, and inflammation. Our previous research has identified pigment epithelium derived factor (PEDF) as a systemic anti-aging factor that can modulate angiogenesis, age-related fibrosis, and lipid accumulation. This study aims to investigate the potential benefit of PEDF supplementation on ovarian function during aging. Forty-four-week-old mice with irregular estrus cycles were treated with PEDF (1mg/mL) or vehicle for a period of four weeks (N = 10). Ovarian function was evaluated through estrus cycle, histological, gene, and protein expression analyses. Mice's estrus cycles, as well as the proportions of quiescent and activated follicles, were similar between the treatment and control groups. Gene expression assessments on PI3K/AKT/mTOR and Hippo pathways revealed no significant difference among groups. Surprisingly, ovaries from mice treated with PEDF exhibited a lower level of p-RPS6 ($p = 0.006$) compared to the control group. Our results suggest that PEDF may influence the mTORC1 signaling pathway in the aging ovary.

P-267 - In Vivo Dynamics of Mouse Ovulation and Oocyte Transport

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Ovulation is a highly dynamic reproductive event where mature oocytes are released from the ovary into the periovarian space, followed by their transfer to the oviduct. This process is critical to human fertility: ovulation-related disorders are the leading causes of female infertility, accounting for 32% of cases. Therefore, much effort has been put into understanding the genetic and endocrine regulations of ovulatory processes. However, limited imaging access to the site of ovulation has left unclear how oocytes behave within the ovulating follicle and how they travel to the site of fertilization after ovulation. The objective of this study was to investigate the dynamic regulation of ovulation and subsequent oocyte transport using mouse models. Toward this aim, we develop a novel approach for in vivo 3D imaging of ovarian tissues using optical coherence tomography that allows spatiotemporal analysis of mouse ovulation within the periovarian space. With this approach, we volumetrically and quantitatively capture both the process of ovulation as well as the transfer of ovulated oocytes through the periovarian space into the infundibulum, the section of the oviduct closest to the ovary. We observed a slow, gradual release of the oocyte surrounded by the cumulus cell mass after follicular rupture, which coincides with the reduction in follicular volume. The oocyte shows the highest velocity and becomes stretched into an oval shape as it exits the follicle, suggesting that the oocyte may be squeezed through a follicular opening. After ovulation, spatial tracking of the oocyte reveals that the ovulated oocyte remains at the ovulation site for approximately 25 minutes. After leaving the site of ovulation, the oocyte shows directional movement into the infundibulum and reaches the ampulla within a few minutes. In vivo volumetric imaging reveals additional intriguing observations which provide new insight into oocyte transport mechanisms. During ovulation, the infundibulum repeatedly pumps oviductal fluid into the periovarian space. This finding aligns with micro-computed tomography analysis of the ovarian bursa, a membrane surrounding the periovarian space, showing dramatic expansion during ovulation. Another intriguing finding is that spatial tracking of scatters such as loose cumulus cells

and mucus demonstrated bidirectional periovarian flow inside the ovarian bursa. Interestingly, the velocity of periovarian flow was significantly higher during ovulation than before ovulation. These results suggest that the oviductal pumping may cause the ovarian bursa to expand during ovulation and potentially generates the periovarian flow. To investigate whether the oviductal pumping plays a role in ovulated oocyte transport, we by inhibited oviductal smooth muscle contraction during ovulation. The mice treated with the inhibitor showed a lower number of oocytes successfully transported to the ampulla and a higher number of oocytes remaining inside the ovarian bursa, indicating that inhibition of oviductal contraction caused retention of ovulated oocytes in the ovarian bursa. Together, our findings demonstrate that pumping induced by oviductal contraction contributes to oocyte transport from the site of ovulation to the site of fertilization. In conclusion, this study reveals the physiological dynamics of ovulation, captures never-before-seen oocyte behaviors, and proposes a potential mechanism for ovulated oocyte transport through the periovarian space into the oviduct.

P-268 - Identification of a Novel Skp1 Isoform that Encodes a Novel SKP1 Protein in Mouse Oocytes

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GV oocytes are transcriptionally quiescent and rely on stored mRNA that is recruited for translation. Translation of stored mRNA is essential for maintaining oocyte quality, and sustaining early embryonic development. The complexity of the oocyte proteome is driven by the diverse pool of stored mRNA that have undergone alternative splicing. However, the impact of alternative splicing on proteome diversity within the oocyte remains poorly understood. To better understand this relationship we utilized direct RNA-sequencing of cumulus oocyte complexes (COCs) to directly measure transcriptome complexity at a single, full-length molecule resolution, coupled with mass spectrometry. Direct RNA-sequencing was performed using total RNA from 3,751 COCs, generating an average of 467,843 (SEM $\pm$ 33,510.67) full-length reads across three separate sequencing runs, with mean molecule length of 1.08 Kb (SEM $\pm$ 0.012). We identified 45,086 unique transcripts mapping to the mouse genome, of which 85% were single transcript genes and 15% were multi-transcript genes. Within multi-transcript genes, we identified 15,571 alternative splicing events, with alternative exon usage the most predominant (46% of all events). Next we assessed the protein coding potential for the 45,086 unique transcripts. Using the Ensemble database, 82.6% of the transcripts were unannotated, while 13.0% were listed as protein coding. We used in silico translation to estimate protein coding potential and observed that 40.1% of transcripts identified to be protein coding. Next we performed mass spectroscopy using a group of 40 GV oocytes. Identified peptides were searched against a Mouse Swiss Prot database and a custom protein database generated from in silico translation of the direct RNA-sequencing data. We identified a novel Skp1 mRNA isoform that encodes a novel protein. Analysis of the novel protein revealed a loss of the key N-terminal region essential for interaction with the ubiquitin ligase Cullin-1. Skp1 is a

key component of the SCF complex and is essential for maintaining synapses during meiosis. Our studies reveal that alternative splicing within GV oocytes can result in the production of novel proteins that could impact normal oocyte function. Future studies will assess the role of these novel proteins in oocyte physiology.

P-269 - MEIOC Prevents Continued Mitotic Cycling Prior to Meiotic Entry During Mouse Oogenesis

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In multicellular organisms, the germ cell's transformation into a haploid gamete requires that it transitions from mitosis to meiosis, whereby it stops mitotic cycling and enters the meiotic cell cycle. In mammals, the decision to enter the meiotic cell cycle by undergoing the G1-to-meiotic S phase transition is triggered by transcriptional activator STRA8-MEOSIN. However, what prevents continued mitotic cycling before entering the meiotic cell cycle remains unclear. Here, we demonstrate that the transition from mitosis to meiosis in mouse ovarian germ cells occurs as two molecularly distinct steps: first, during premeiotic G1 phase, germ cells are prevented from re-entering the mitotic cell cycle; then, they transition from premeiotic G1 to meiotic S phase to enter the meiotic cell cycle. We demonstrate that MEIOC, previously shown to repress the mitotic program after germ cells have entered meiosis by destabilizing mRNA, prevents re-entry into the mitotic cell cycle during premeiotic G1 phase. Furthermore, MEIOC supports the G1-to-meiotic S phase transition by indirectly increasing Meiosin transcript abundance and thereby activating the STRA8-MEOSIN transcription factor. We conclude that, in mouse oogenesis, MEIOC's post-transcriptional control of mRNA promotes the transition from mitosis to meiosis by preventing continued mitotic cycling and promoting entry into the meiotic cell cycle.

P-270 - Follicle Size Influences Bovine Oocyte Cytoplasmic Maturation and Early Embryonic Development

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Despite recent technological advances and increased use of in vitro embryo production (IVP) in cattle, the outcome of this assisted reproductive technique remains suboptimal. In vitro, up to 80% of oocytes achieve successful nuclear maturation but only 20-30% of oocytes that undergo fertilization result in transferable embryos. Nuclear maturation is a hallmark of oocyte competence, however, during follicular growth and before ovulation, the oocyte undergoes cytoplasmic changes necessary for subsequent embryonic development. Cytoplasmic maturation involves organelle replication and redistribution along with the accumulation of molecules transported from the surrounding cumulus cells into the oocyte via transzonal projections. Oocytes derived from larger

follicles exhibit greater developmental potential than those from smaller follicles due to increased exposure to the follicular environment. Meanwhile, in IVP, oocytes are typically collected from follicles ranging from 1-6 mm in diameter. This study investigates the effects of follicle size on oocyte competence as evaluated by cytoplasmic maturation parameters and early embryonic development. Bovine cumulus-oocyte complexes were aspirated from small (< 6 mm), medium (6-9 mm), and large (10-20 mm) follicles from slaughterhouse-derived ovaries. Mitochondrial membrane potential and lipid content were measured using fluorescence microscopy along with the distribution of mitochondria and lipids at 0, 12, and 24 h of maturation (n = 305). The number of transzonal projections and cortical granule distribution was also assessed at 0, 12, and 24 h of maturation (n = 160). Embryos from oocytes derived from small, medium, and large follicles were evaluated for cleavage rate on day 3 and blastocyst development on day 8 (n = 412). Mitochondrial membrane potential significantly decreases throughout maturation in oocytes from small, medium, and large follicles ($P = 0.0001$). Lipid content of oocytes from large follicles increases mid-maturation compared to oocytes from small ($P = 0.008$) and medium ($P = 0.078$) follicles but decreases at the end of maturation compared to those from small and medium follicles ($P = 0.037$ and 0.008 , respectively). Organelle distribution was described as diffuse, semi-peripheral, or peripheral within the cytoplasm. Mitochondria and lipid distribution in oocytes from small and medium follicles changed from semi-peripheral or peripheral distribution before maturation to predominantly diffuse distribution at the end of maturation. Meanwhile, oocytes from large follicles had continuous diffuse mitochondria and lipid distribution throughout maturation. Oocytes from small follicles had greater transzonal projection count at 0 h of maturation compared to oocytes from medium ($P = 0.002$) and large follicles ($P < 0.0001$). However, the number of transzonal projections did not differ among sizes for the remainder of maturation. Distribution of cortical granules at 0 and 24 h was consistent among follicle sizes, though oocytes from large follicles demonstrated increased transition to peripheral mid-maturation compared to those from small follicles ($P = 0.037$). Furthermore, embryos derived from large follicle oocytes had higher cleavage percentage compared to embryos from small ($P = 0.03$) or medium follicle ($P = 0.04$) oocytes. No significant differences were observed for blastocyst rate from any follicular size ($P = 0.537$). These data suggest that follicle size alone has implications on oocyte cytoplasmic maturation which may affect oocyte competence. Additional understanding of oocyte competence is required for improved cytoplasmic maturation and improvement of IVP. This work was supported by USDA-AFRI 2022-67015-38938 and SciMed Graduate Research Scholars.

P-271 - Notch Signaling Differentially Regulates Proliferation of Cultured Mouse Ovarian Granulosa Cells Dependent on Exogenous Gonadotropin Exposure.

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Ovarian follicle development relies on a complex interplay of signaling events that coordinate granulosa cell specification and proliferation and also support oocyte health. Early stages of folliculogenesis are influenced by myriad local signaling factors. During the pubertal transition in the mouse, these local factors prepare the activated follicle to be receptive to circulating pituitary gonadotropins. Among the many signaling systems involved in these important processes is Notch signaling, an evolutionarily conserved juxtacrine pathway that has pleiotropic effects on tissue patterning and regulates the balance between proliferation and differentiation in many cell types. Our group and others have shown that Notch ligands, receptors, and downstream effectors are expressed in the ovary throughout follicle development. We previously demonstrated that siRNA knockdown of the Notch ligand, Jag1, in cultured granulosa cells harvested from PMSG-primed prepubertal mice suppressed steroidogenesis and enhanced proliferation. In support of this, work presented here shows that knockdown of other Notch components, including the Jag2 ligand and the Notch receptor, Notch3, in PMSG-primed granulosa cells likewise result in increased cell proliferation. However, we have paradoxically found that Notch signaling is positively associated with granulosa cell proliferation in the absence of gonadotropin treatment of the pre-pubertal mice. Using a Transgenic Notch Reporter (TNR) line of mice, we observed that granulosa cells that are Notch active are more proliferative than those that are Notch inactive. We postulate that Notch activation promotes proliferation in using primary cultures of non-PMSG primed granulosa cells co-cultured in the presence of recombinant Jag1 (rJag1), an increase in proliferation was observed. Gene expression analysis of the cell cycle regulators, Ccnd2 and Cdk1, show an increase in their expression as well. When cultured in the presence of DAPT, a Notch signaling inhibitor, granulosa cell proliferation is suppressed, further supporting a pro-proliferative role for Notch in this context. Currently, we are investigating if the same Notch receptors or ligands are responsible for promoting proliferation in granulosa cells naïve to exogenous gonadotropin stimulation as those involved in more differentiated response in cells from PMSG-primed mice. Overall, this study indicates that Notch regulation of granulosa cell proliferation is highly context dependent, adding an important nuance to our current understanding of the network of regulatory mechanisms that drive granulosa cell proliferation and differentiation in the ovarian follicle. Supported by the Eunice Kennedy Shriver NICHD Program Project Grant (NIH P01 HD021921), Northwestern University internal funding, and by the Master's Program in Reproductive Science and Medicine.

P-272 - In Vitro Differentiation of Bovine Embryonic Stem Cells Toward Progenitors of Bipotential Gonad-like Somatic Cells

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Somatic cells of the mammalian gonad originate from the mesoderm, which differentiates into the intermediate mesoderm (IM), coelomic epithelium (CE), and then bipotential gonad (BG) via cell signaling by specific morphogens. Research in human gonadal cell specification remains hampered by difficulty accessing embryos; therefore, stem cell-based models have been used to recapitulate stepwise gonadal cell differentiation in vitro. However, reports of successful cell induction into BG-like cells are still limited. Hence, in vitro differentiation of bovine embryonic stem cells (bESCs) into BG-like cells presents opportunities to understand mechanisms of early gonad development in a large mammal model. We hypothesized that bESCs would differentiate into IM and early CE (eCE) after sustained WNT activation, short duration of activin A (Nodal) and bFGF (FGF) signaling, and low concentrations of BMP4 during in vitro culture. In experiment 1 (n = 3

replicates), bESCs (p19-26) were cultured for 48 h in mesoderm induction medium (MIM) containing 3 μ M CHIR99021 (GSK3 inhibitor/WNT activator) and 70 ng/mL activin A. Cells were then further cultured without or with 10 ng/mL bFGF starting at 48, 72, or 96 h and harvested at 120 h. In experiment 2 (n = 3 replicates), we sought to determine if low bFGF exposure with BMP4 synergistically upregulated IM and eCE markers. As in experiment 1, bESCs were cultured in MIM for 48 h and then supplemented with 0, 1, 10, or 20 ng/mL BMP4 for an additional 72 h with a pulse of 10 ng/mL bFGF during the final 24 h of BMP4 exposure. Cells were harvested at 120 h and subjected to RT-qPCR to examine expression of IM and eCE/bipotential gonad transcripts. After 48 h of mesoderm induction, LHX1 (early IM marker) showed peak expression in both experiments ($P < 0.001$). After 72 h of additional culture, LHX1 became downregulated independently of bFGF or BMP4 supplementation ($P < 0.0001$), aligning with in vivo temporal expression patterns. Likewise, 72 h after mesoderm induction, OSR1 (IM marker) and WT1 (eCE marker) were upregulated similarly across bFGF and BMP4 regimens. We found no difference in GATA4 (CE/BG marker) expression across bFGF regimens, however 10 and 20 ng/mL of BMP4 increased GATA4 compared to bESCs ($P < 0.05$). PAX3 (paraxial mesoderm marker) expression was low and similar to bESCs in all culture conditions in both experiments, indicating appropriate suppression of the paraxial mesoderm lineage. We also found that at the end of the 120 h culture period, FOXF1 [lateral plate mesoderm (LPM) marker] was upregulated in the absence or after short duration exposure to bFGF (24 or 48 hours of culture; $P < 0.05$). There was a dose-dependent increase in FOXF1 ($P < 0.05$) in response to BMP4, indicating a shift towards the LPM in response to an increased gradient of BMP4 as it occurs in vivo. We corroborated our findings of WT1 expression by demonstrating WT1 protein expression in bESC following exposure to BMP4 with bFGF via immunocytochemistry. Together, these data suggest that 1) bESCs are being driven towards the IM and eCE state by 120 h of culture with constant WNT activation, hence show potential to be further driven towards a somatic BG-like state; 2) high BMP4 with short bFGF exposure may induce the LPM; and 3) paraxial mesoderm fate is not induced by these culture conditions. Financial support: NIH F31 Pre-doctoral fellowship.

P-273 - Secretome by Human Mesenchymal Stromal Cells can Mitigate Age-related Ovarian Dysfunction by acting on both Follicle and Stroma Compartments: focus on Signaling Pathways deregulated by released MiRNAs

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Natural ovarian aging is characterized by an ongoing decline in the primordial follicle reserve and an increase in the general inflammation of the ovarian stroma both in human and mouse (Camaioni et al., 2022). In particular, in women this decline is associated with reduced fertility and irregular menstruation that leads to menopause in the early fifties, and it is strongly associated with the onset and progression of several alterations, such as osteoporosis, cardiovascular disease and cognitive dysfunction. The aim of the present study is to determine whether conditioned medium (CM) of human Mesenchymal Stromal Cells (MSCs) derived from Adipose Tissue (ADSCs) and Dental Pulp (DPSCs), generally waste material, is able to mitigate the ovarian aging in 129/Sv

females. In more detail, we analyzed different components of the secretome to which this effect can be attributed. Among them, the microRNAs (miRs) highly (HE) and differentially (DE) expressed between CMs have been evaluated for their biological effects in the aged ovaries. The protocol consisted in intravenous administration of 5 ml/Kg of CMs collected after 3 days of MSC culture in aged 129/Sv females (8 months), every other day for three times, with one repetition after four weeks. One month later, some females were sacrificed and ovaries collected to perform follicle count, morphological analysis and gene expression, while other females were mated to follow their reproductive output. The miRs present in CM-DPSC and CM-ADSC were analyzed using a commercial column purification kit and sequenced on the Illumina platform. After detecting the HE and DE miRs, the TargetScan web tool identified the binding sites of miRs to the UTR region of gene transcripts, while the Enrichr web tool found the pathways deregulated by them. The analysis of histological sections showed that the treatment increased the number of total follicles, mainly primordial and primary follicles, compared to control group, increased the pregnancy rate, the average number of pups delivered per mating cycle and the duration of the entire reproductive life. We then performed a statistical analysis to detect the HE/DE miRs among the 3-day CMs that have been used in *in vivo* experiments and have showed the capacity to counteract ovarian aging. Interestingly, miRs with high expression in both CMs, and those most highly expressed in each of them, showed binding sites for genes involved in signaling pathways related to follicle ovarian reserve (FoxO, PI3K-Akt), follicular atresia (apoptosis, P53), and inflammatory processes (IL-2, TNF). These data indicate that treatment with MSC-CMs may mitigate ovarian aging and improve the functional status of the entire reproductive system, probably acting on the inflammatory state of the ovarian stroma, on the follicle reserve and the oocyte development potential. The analysis of miRs secreted in CMs highlighted their fundamental role in counteracting degenerative processes associated with ovarian aging, but underlined also the possibility of using them as therapeutic agents for the menopause-associated dysfunctions that affect skeletal, cardiovascular and nervous systems.

P-274 - Effect of Short and Long-Term Oestrus Synchronisation Protocols in Merino Ewes during Non-Breeding Season

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The aim of the study was to compare the effect of short and long-term oestrus synchronisation protocols and their combination with or without equine chorionic gonadotropin (eCG) on hormonal levels, oestrous response, onset and duration of South African Merino ewes during non-breeding season. Seventy-six (76) South African Merino ewes aged between 2–5 years were divided into 2 groups (36 for short-term and 40 for long-term oestrus synchronisation protocols). In the short and long-term progesterone treatment groups, controlled internal drug release devices (CIDR) were inserted for 11 and 14 days, respectively. At withdrawal of CIDR, half of each group were injected intramuscularly with 300 IU of eCG. Blood samples were collected to measure the levels of progesterone and oestradiol. Ewes treated with both short or long-term progesterone combined with or without eCG resulted in the same oestrus response, onset and duration. The levels of

progesterone hormone were the same at CIDR insertion, removal and 48 hours post CIDR removal. However, oestradiol hormone was significantly higher at CIDR removal and 48 hours post CIDR removal than at insertion in all treated groups. Therefore, it can be concluded that oestrous synchronisation protocols of both short and long-term progesterone combined with or without eCG during non-breeding season can be used to synchronise oestrus of South African Merino ewes during non-breeding season.

P-275 - Inhibin A and Antral Follicle Numbers Correlate in Mares Subjected to Follicular Growth Manipulation

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The optimal time for a mare's oocytes to be collected for the purposes of in-vitro fertilization (IVF) and efficient in-vitro production (IVP) of embryos is complex and riddled with uncertainty. In order to increase the odds that the greatest number of IVP embryos will be obtained, the current culture within the world of assisted reproduction is to transvaginally aspirate (TVA) oocytes from follicles, but only after peak follicle numbers have been counted following repeated transrectal ultrasonographic examinations (US). A quantifiable biomarker able to identify the ideal time to perform TVA within a mare's estrous cycle, essentially replacing the need for repeated invasive and labor-intensive US exams, is non-existent. Inhibins originating from the gonads are protein complexes that possess an alpha subunit and give rise to inhibin A or inhibin B forms. These glycoprotein hormones play a pivotal role in follicular dynamics by regulating follicle stimulating hormone (FSH) release from the pituitary gland. Historically, these two biologically active forms of inhibin (A and B) have been difficult to individually quantify due to the lack of antisera specificity during inhibin assay development. The aim of our project was to determine if serum concentrations of inhibin A correlated with the number of antral follicles present on mare ovaries as determined by repeated US examinations. If such a correlation exists, identification of the ideal day(s) for TVA of oocytes by real-time measurement of inhibin A may be futuristically feasible. As part of another ongoing trial, six mares underwent treatment with a Proprietary Drug (designed to manipulate follicular growth), while four mares were utilized as untreated Controls. On Day 0, all mares underwent TVA of ovarian follicles for the purposes of ablation/synchronization; thereafter, on Days 1-5 and 8, transrectal US was performed, the number of antral follicles were documented, and serum was collected and stored for inhibin-A ELISA. There was no difference in inhibin A serum concentrations or antral follicle numbers between the Proprietary Drug and the Control mares (T-Test, $p = 0.42$ and $p = 0.48$, respectively); therefore, the following results are reported utilizing pooled data. The mean serum concentration of inhibin A was 22.3 pg/mL (range of 0 - 86.79 pg/mL) and the mean antral follicle count was 2.79 follicles; on Day 1, mare antral follicle numbers ranged from 0-2 and by Day 8 ranged from 2-11. A positive correlation between inhibin A concentration and antral follicle number was identified (Corr SAS, $p = 0.047$). Recent work has speculated that inhibin A is a more accurate biomarker and indicator of the ovarian-pituitary axis response to ovarian gonadotropin stimulation compared to inhibin B and anti-mullerian hormone. Inhibin A peaks around the time of ovulation in the mare and reaches its lowest point around the time of mid-luteal phase (i.e. the two weeks following ovulation); utilizing this information in combination with our results, inhibin A provides promise as a biomarker for identifying the ideal day(s) for TVA of oocytes and increasing the efficiency of IVP of embryos.

P-276 - Status of 25-(OH) Vitamin D3 Levels Among Young Women with Polycystic Ovarian Syndrome (PCOS).

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Vitamin D deficiency (VDD) is seen in 70 to 100% of apparently health population in India. Polycystic ovary syndrome (PCOS) is also in epidemic proportions in the country. VDD is incriminated in insulin resistance (IR), the central driver of PCOS pathophysiology, but data is scarce from our country. We aimed to assess the magnitude of VDD among Indian women with PCOS and also investigated its relation to clinical, biochemical, hormonal and IR markers. Among total of 2792 women aged 18-40 years, 860 were PCOS (Rottardam criteria, 2003), 758 pre-PCOS women and 1174 healthy controls. Detailed medical history, clinical examination, biochemical, hormonal, and IR parameters were assessed. Lips classification was used to assess Vitamin D status and were graded as VD deficient ($< 20\text{ng/ml}$), VD insufficient ($\geq 20\text{-}29\text{ng/ml}$) and VD sufficient ($\geq 30\text{-}100\text{ng/ml}$). Student t-test and ANOVA were used to evaluate the differences in the means of various parameters. Correlations were investigated by Pearson Correlation. The mean age (29.21 ± 6.63 vs. 30 ± 6.13 years) and BMI (24.64 ± 4.34 vs. $24.44 \pm 4.18 \text{Kg/m}^2$) of PCOS cases and controls was comparable. The mean number of menstrual cycles per year (8.57 ± 2.91 vs. 12 ± 0.7 ; $p < 0.001$) was significantly lower while a-smFG scores (5.86 ± 5.76 vs. 1.08 ± 2.13 ; $p < 0.000$) and serum total testosterone (0.52 ± 0.27 vs. $0.27 \pm 0.13 \text{ ng/ml}$; $p < 0.001$) levels were significantly higher among women with PCOS as compared to healthy women. The serum vitamin D3 levels were lowest in the PCOS group (9.11 ± 11.41) followed by Pre-PCOS group (16.11 ± 10.86) and control group (17.20 ± 10.80) ($p < 0.001$). The HOMA-IR (3.74 ± 4.39 vs. 3.40 ± 3.86 ; $p < 0.000$) was significantly elevated among VD deficient PCOS women than VD sufficient PCOS women ($p < 0.021$). The serum 25OHD3 correlates positively with serum calcium ($r = 0.1445$; $p < 0.05$), visceral adipose index ($r = 0.7289$; $p < 0.05$), serum total cholesterol ($r = 0.2701$; $p < 0.05$) and serum total triglyceride ($r = 0.5628$; $p < 0.05$) and negatively with serum testosterone ($r = -0.2131$; $p < 0.05$). VDD is highly prevalent in PCOS women and the magnitude of VDD worsens the biochemical, hormonal and IR parameters among women with PCOS.

P-277 - Spatially Resolved Transcriptomic Profiling of Developing Primary Follicles in the Chicken Ovary Reveal a Potential Developmental Checkpoint

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The female reproductive lifespan relies on the controlled activation of primordial follicles and regulation of primary follicle development. In avian species, a sufficient number of primary follicles need to be recruited into advancing growth phases for the maintenance of the follicular hierarchy. Despite its importance, the process regulating the coordinated activation and development of avian

follicles has received little attention. Elucidating the complex interactions in the ovary responsible for initiating and maintaining primary follicle development would benefit from retaining the original tissue morphology. The objective of this study was to characterize the transcriptional landscape of developing primary follicles ($\approx$ 2 mm diameter) in the chicken ovary. Whole ovaries were harvested from two 42-week-old White Leghorn hens with a normal laying cycle. Large follicles (>3 mm diameter) were removed before processing and the remaining samples were embedded in optimal cutting temperature (OCT) compound and snap frozen. Four 12 μ m frozen sections, one from layer 1 and three from layer 2, containing primary follicles at various stages of development were placed onto a single Visium Spatial Gene Expression Slide (10X Genomics). Sections were fixed in methanol, stained with hematoxylin and eosin, and imaged followed by tissue permeabilization, reverse transcription, and second strand synthesis for cDNA amplification and library construction. The sequenced reads were aligned to the *Gallus gallus* reference genome using cellRanger v7.1.0. Gene expression was normalized across the four tissue sections with individual chickens as covariate followed by principal component analysis for dimensionality reduction and unsupervised spot clustering using Seurat. Differentially expressed genes (DEG; p adj $\approx$ 0.05 and $|\log_2FC| > 0.25$) of relevant clusters were annotated using Gene Ontology (GO) and visualized using Kyoto Encyclopedia of Genes and Genomes (KEGG) to gain insight into their functional significance during early follicle development. Distinct transcriptional signatures associated with small ($\approx$ 600 μ m diameter) and larger (>600 μ m diameter) primary follicles were identified. Notably, smaller primary follicles exhibited enrichment in pathways related to controlled degeneration, or atresia. In contrast, larger primary follicles showed enrichment in pathways involved in mRNA surveillance, transport, and processing. Furthermore, a subset of DEGs were selected and plotted individually across the tissue sections to explore their similarity to the histological features. Genes involved with cell cycle progression (e.g., CCNO, CCNA1, CKS1B), vesicular trafficking (e.g., MARCKSL1, EHD3, DAB2), and maintenance of follicle identity (e.g., FOXL2) were spatially enriched within the oocytes of primary follicles while genes related to oxidative phosphorylation (e.g., COX1, COX2, COX3) were highly enriched in the area directly surrounding large primary follicles. Genes encoding ribosomal proteins (e.g., RPS17, RPS28, RPL35A) showed spatial expression in the surrounding stroma. These findings suggest a potentially important developmental checkpoint during the development of primary follicles that may lead to either their atresia or proceed in preparation for later stages of development. A deeper understanding of these processes not only has implications for improving reproductive success in poultry but also advancing our broader knowledge of avian reproductive biology.

P-278 - In Vitro Cannabis Exposure Alters Human Granulosa Cell Extracellular Vesicle Release

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Extracellular vesicles (EVs) are very important for cell-cell communication in ovarian follicles and exogenous molecules could perturb these exchanges affecting crucial events involved in oocyte

maturation and competence. Indeed, cannabidiol (CBD), sometimes found in cannabis products, has been reported to decrease cancer cell exosome release. We have recently shown that cannabis metabolites (including tetrahydrocannabinol (THC) can be found in the follicular fluid of patients undergoing IVF treatment. Thus, the objectives of this study were to determine if CBD and/or THC affect EVs release by granulosa cells. Granulosa cells were obtained from consenting patients undergoing in vitro fertilization treatments with ethics approval (IRB #16518). 0.5x10⁶ cells/mL were plated in a 6-well plate for 24h and then treated with EV inhibitors (Imidazole (10 μ M), GW4869 (5 μ M)), cannabis compounds (CBD 1 μ M), or a combination of THC metabolites (Δ 9-THC 25ng/mL, 11-OH-THC 5ng/mL and 11-COOH-THC 50ng/mL). The next day, exosome-like particles were enriched using Total Exosome Isolation Reagent from conditioned cell culture media (Invitrogen), quantified using NanoSight NS300 and DynaPro Plate Reader III. Protein concentrations were assessed using Qubit, and exosome-like particle membrane protein content was assessed using the MACSPlex Exosome Kit (Miltenyi). No difference in the number of cells or cell viability was observed after 24h, but a significant decrease in the number of particles and particle size 25-150nm when cells were treated with Imidazole (2.6x10⁷/mL, $p < 0.01$), GW4869 (1.7x10⁷/mL, $p < 0.001$) and CBD (4.2x10⁷/mL, $p < 0.01$), compared to control (11.3x10⁷/mL). In contrast, we observed a significant increase in number of particles when cells were treated with THC (18.5x10⁷/mL, $p < 0.01$), compared to control (11.3x10⁶/mL). When exosome-like particles were enriched, there was no difference in total protein content or exosome marker expression (CD9, CD63, and CD81). We observed a significant decrease in the expression of CD9, CD44, CD142, CD24 and CD41b in EVs enriched from GW4869 treatment ($p < 0.05$), but not for the other treatments ($n=4$). In conclusion, CBD decreased the amount of EVs secreted while THC increased it, but there was no change in their membrane protein content. Investigations into the effect of acute and chronic exposure on EV secretion, as well as differences in protein and miRNA cargo are ongoing.

P-279 - Isolation and Characterisation of Extracellular Vesicle Subpopulations in Human Follicular Fluid

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Extracellular vesicles (EVs) are lipid-bilayered particles present in various body fluids, including follicular fluid (FF). The cells within the ovarian follicle release EVs of endosomal and plasma membrane origin into the extracellular environment. EVs participate in intercellular communication, influence folliculogenesis and oocyte development. Based on their biogenesis, EVs are classified into three subpopulations: exosomes, microvesicles, and apoptotic bodies. The aim of the current study was to optimize EV subpopulation isolation techniques from human FF and to identify protein markers associated with EVs. The primary focus was to optimize the EV yield while ensuring the preservation of their qualitative characteristics. Ethical approval was granted by

the Research Ethics Committee of the University of Tartu, Estonia and follicular fluid samples were collected from preovulatory follicles of 10 patients at Nova Vita Clinic in Tallinn, Estonia. Size-exclusion chromatography was implemented to isolate EVs from FF, followed by tangential flow filtration to separate EV subpopulations based on their size (cut-off value 200 nm). EV subpopulations were concentrated with ultracentrifugal filters and characterized by Western blotting. Additionally, the size and concentration of the isolated particles were determined with nanoparticle tracking analysis to assess the EV yield at each purification step. The presence of EVs was confirmed by transmission electron microscopy. Subsequently, human ovarian granulosa-like tumour cell line KGN was treated with isolated EV subpopulations for 24 hours. Following this, cell viability and proliferation were assessed using a bioluminescent assay. The techniques for isolating EV subpopulations, such as size-exclusion chromatography and concentration, were effectively optimized to achieve the highest possible yield. The average diameter of small nanoparticles was approximately 100 nm, whereas the diameter of large nanoparticles ranged from 250 to 350 nm. Notably, the concentration of small nanoparticles from the same amount of FF was observed to be 15 times higher than the large ones. Immunoblotting confirmed the presence of EV-specific surface markers, such as CD9 and CD81, in both subtypes. The purity of the isolated EVs was validated by the reduced levels of albumin and lipoprotein ApoA1. Isolated EVs, even at high concentrations, demonstrated no toxicity to KGN cells. This makes them suitable for analysing their effect on steroidogenesis and gene expression changes. Furthermore, EVs enter KGN cells without significantly affecting the viability or proliferation in a statistically significant manner. The results set the stage for future functional experiments with EV subpopulations to investigate the effects of follicular fluid EVs on the proliferation, viability, steroidogenesis, and metabolism of various follicular cell types. The optimized isolation procedure does not only establish the groundwork for identifying biomarkers to assess and predict the quality of oocytes but also provides opportunities for applying EVs in the diagnosis and treatment of reproductive diseases.

P-280 - In vitro Effect of Intelectin-1 on the Levels of Adipokines in Porcine Ovarian Cells: Interaction with Estradiol

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White adipose tissue secretes hormones named adipokines like adiponectin (ADIPOQ), apelin (APLN), leptin (LEP), visfatin (NAMPT), chemerin (RARRES2), resistin (RETN) and vaspin (SERPINA12). These adipokines not only regulate energy metabolism but also reproductive functions. Another one is omentin-1, known as intelectin-1 (ITLN1), which improves insulin sensitivity and glucose metabolism. Our previous study showed that ITLN1 is expressed in the porcine ovarian follicles and its plasma levels depend on estrous cycle phase. However, its impact

on ovarian endocrinology and levels of other adipokines is still unknown. Therefore, the porcine ovarian follicle cells (10-12 days of estrous cycle) of Large White pigs were cultured in vitro for 48 h with ITLN1 (10-100 ng/ml) to assess mRNA expression of ADIPOQ, APLN, LEP, NAMPT, RARRES2, RETN, SERPINA12 and their respective receptors as well as aromatase using RT-qPCR (n=5). The concentration of 17 β - estradiol (E2), ADIPOQ, APLN, LEP, NAMPT, RARRES2, RETN and SERPINA12 in the culture medium was determined by ELISA assay (n=5). Also, effect of E2 (10-1000 nM) on ITLN1 protein levels in the ovarian cells was analyzed (n=5). Statistical analysis was performed in Graph Pad Prism 8, using one way ANOVA and Tukey's test ($p < 0.05$). We noted that ITLN1 enhances gene expression of RETN, NAMPT, and SERPINA12, inhibits ADIPOQ and LEP, has no impact on APLN and RARRES2, as well as modulates the levels of their respective receptors. Moreover, our results indicated that ITLN1 increases ADIPOQ, LEP, and NAMPT, decreases APLN, and has no effect on RARRES2, RETN, as well as SERPINA12 concentrations in the culture medium. We also observed the stimulatory effect of ITLN1 on mRNA expression of aromatase and E2 levels in the culture medium and interestingly, the E2 dose-dependently increased ITLN1 levels in ovarian cells. Additionally, a strong negative correlation between E2 and RARRES2 levels was observed. Taken together, our results for the first time showed positive feedback between E2 and ITLN1 levels in porcine ovarian follicle cells. Moreover, ITLN1 by modulating the levels of other adipokines as well as their respective receptors might be a new auto- and paracrine regulator of pig reproduction. Funding: National Science Centre, Poland, OPUS19 project, no. 2020/37/B/NZ9/01154.

P-281 - ADROPIN in Polycystic Ovarian Syndrome: Expression and Impact on Proliferation, Apoptosis, Steroidogenesis and Signaling Pathways in Human Granulosa Cells.

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ADROPIN is a new adipokine, which regulates energy homeostasis and food intake. The serum and follicular fluid levels of adropin are decreased in women suffering from polycystic ovarian syndrome (PCOS), however its role in ovarian function is still unknown. The aims of the study were to determine the expression of adropin and its receptor G protein-coupled receptor 19 (GPR19) in human granulosa cells (Gc), its immunolocalization as well as direct in vitro effect on cell proliferation, apoptosis, steroidogenesis and signaling pathways. The Gc were obtained from healthy women (normal weight (NW) and obese (OB)) and women diagnosed with PCOS (NW and OB) to determine adropin/GPR19 mRNA expression. Paraffin ovarian slides from healthy NW women were used for its immunolocalization. Next, human Gc and KGN cells in vitro culture were

performed to investigate the effects of recombinant adropin on cell proliferation/apoptosis (by alamarBlue and PCNA, cyclin D, BAX/BCL2, caspase3, p53 mRNA and protein level) and steroidogenesis (by E2 secretion and mRNA and protein expression of StAR protein and enzymes: CYP11A1, HSD3B, HSD17B, CYP17A1, CYP19A1). Additionally, adropin effect on MAP3/1, AKT, PKA and STAT3 phosphorylation was analyzed by Western blot. Statistical analyses were performed by Graph Pad Prism 5. The mRNA expression of adropin in Gc between groups was stable, while level of GPR19 mRNA was decreased in Gc from obese and PCOS women; adropin/GPR19 were localized in Gc, theca cells and oocyte. Also, adropin reduced cell proliferation and related gene expression (PCNA and cyclin D) in both human Gc and KGN, while increased cell apoptosis related genes level (caspase3 and BAX/BCL2 ratio). Additionally, adropin had a negative effect on steroidogenesis by inhibiting STAR and CYP19A1 mRNA expression. The negative effect of adropin on ovarian function could be associated with a decrease in phosphorylation of AKT, PKA and STAT3. This effect of adropin was independent of the patient's condition ($P < 0.05$, $n=6$). The obtained data clearly indicate that adropin/GPR19 are present in human ovaries and its negative effect on ovarian physiology may be a medical target to contribute to restore fertility in PCOS suffering women. The project is co-financed by the Polish National Agency for Academic Exchange.

P-282 - Expression of Spexin and GALR2/3 Receptors in Human Granulosa Cells Collected from Polycystic Ovarian Syndrome Women and In Vitro Effect of Spexin on Steroidogenesis, Proliferation and Signaling

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Polycystic ovarian syndrome (PCOS) is the most common endocrine pathology in females of reproductive age and characterized by ovulatory dysfunction, polycystic ovaries, increased LH concentrations, insulin resistance and hyperandrogenic symptoms. PCOS women are frequently overweight or obese with increased risk of type – 2 diabetes, endometrial cancer, impaired glucose tolerance and cardiovascular disease. However, the exact pathophysiology of PCOS remains largely unclear, therefore novel insights into the mechanism of PCOS are imperative to identify potential diagnostic markers and therapeutic targets for this endocrine disease. The aim of this study was to characterize the expression of spexin (SPX) and its receptors, galanin receptors 2 and 3 (GALR2/3) in the human ovary and to study its in vitro effect on granulosa cells (Gc) function. SPX is a novel neuropeptide negatively correlated with obesity and insulin resistance. Follicular fluid (FF) and Gc were obtained from healthy and diagnosed with PCOS women including normal weight and obese. Expression of SPX and GALR2/3 in the ovary was studied by qPCR, western blot, and immunohistochemistry, while level of SPX in FF was assessed by ELISA. The in vitro effect of SPX on Gc steroidogenesis, proliferation, and signaling pathways (MAP3/1, STAT3, AKT, PKA) was analyzed. Moreover, Gc proliferation and estradiol (E2) secretion were measured with and without an siRNA against GALR2/3 and pharmacological inhibition of the above kinases. The results showed that both the SPX concentration in FF and its gene expression were decreased in Gc of PCOS women, while the protein level of GALR2/3 was increased. We observed that SPX reduced Gc proliferation and steroidogenesis; these effects were mediated by GALR2/3 and kinases MAP3/1, AKT and STAT3 for proliferation or kinases MAP3/1 and PKA for E2 secretion. The obtained data clearly documented that SPX is a novel regulator of human ovarian physiology and possibly plays a role in PCOS pathogenesis. Grant support: the Priority Research Area (B.1.7.2021) under the Strategic

Programme Excellence Initiative at Jagiellonian University in Kraków, Poland. The project is co-financed by the Polish National Agency for Academic Exchange.

P-283 - Effect of Supplementation with GDF9 and BMP15 at IVM media of Prepubertal Goat Oocytes on Intracytoplasmic GSH and Parthenogenic Blastocyst Production.

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The oocyte-secreted factors (OSFs) bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) are well recognized to play a pivotal role in the normal proliferation and differentiation of granulosa cumulus cells (CC) and in the acquisition of oocyte developmental competence (reviewed by Belli and Shimasaki, Vitam Horm, 107;317-348, 2018). Oocytes are dependent of CC to perform fundamental metabolic processes on its behalf. The OSFs regulate CC glycolysis and affect antioxidant glutathione (GSH) and ROS of oocytes (reviewed by Richani et al., Hum Reprod Update, 27(1):27-47, 2021). BMP15 and GDF9 present species-specific roles. In goats, we have shown the positive effect of IVM supplementation with BMP15 on blastocyst development after parthenogenetic activation (PA), cumulus-oocyte communication through transzonal projections and EGFR expression both in the oocyte and their cumulus cells (Ferrer-Roda et al., Reprod Fert Devel 35(2):241-241, 2022; Ferrer-Roda et al., Anim Reprod, 20(2):0, 2023). Our aim is to study the effect of adding GDF9, alone or in combination with BMP15, to IVM media of prepubertal goat oocytes on PA-blastocyst production and ROS and GSH levels of IVM-oocytes. Cumulus-oocyte complexes (COCs) from 1-2 months old goats were collected by ovary slicing and matured in TCM-199 with FSH, LH, estradiol, EGF and cysteamine during 24h at 38.5°C with 5% CO₂. IVM media of the experimental group was supplemented with 100 ng/ml of GDF9 (GDF9 group) or in combination with 100 ng/ml of BMP15 (GDF9+BMP15 group). The control group was IVM medium without GDF9 or BMP15. A total of 173-192 matured oocytes per group (4 replicates) were parthenogenically activated. Oocytes were denuded, put on PBS with 5µM ionomycin 4 min and then on TCM-199 with 2mM 6-dimethylamino-purine 4h. Afterwards, activated oocytes were embryo cultured for 8 days at 38.5°C with 5% CO₂ and 5% O₂ in BO-IVC medium (Bioscience, UK). Blastocysts were stained and fixed with 25 µg/ml Hoechst 33258 in pure ethanol overnight. Intra-oocyte ROS and GSH levels of 26-31 oocytes (3 replicates) were quantified after IVM by fluorescence imaging by staining with 10 µM 2',7'- dichlorodihydrofluorescein diacetate (Molecular Probes Inc., USA) and 10 µM Cell Tracker Blue (Molecular Probes Inc.) in PBS for 15 min. Data were statistically analyzed by two-way ANOVA followed by Tukey's correction. There were no significant differences in blastocyst percentages and cell number among groups. Control, GDF9 and GDF9+BMP15 groups showed a blastocyst development of 17.9 % ± 3.6, 16.1 % ± 2.37 and 12.5 % ± 3.7 respectively, and a total blastocyst cell number of 187.1 ± 20.8, 176.6 ± 17.5 and 195.5 ± 29.0 respectively. Neither ROS nor GSH levels were statistically significant. Results of ROS levels were 7.6 ± 2.1, 8.05 ± 1.2 and 5.2 ± 1.0, and GSH levels were 59.3 ± 5.4, 60.9 ± 3.9 and 64.4 ± 6.2 arbitrary units respectively, for control, GDF9 and GDF9+BMP15 groups. In conclusion, GDF9 alone or in combination with BMP15 did not improve PA-blastocyst development or intracytoplasmic antioxidant GSH in prepubertal goats. In cattle, BMP15 alone, GDF9 alone or the two combined increased the proportion of oocytes that reached the blastocyst stage post-insemination from 41%

(controls) to 58%, 50% and 55%, respectively (Hussein et al., Dev Biol, 296(2):514-521, 2006). Study funded by the Spanish Ministry of Science and Innovation (PID2020-113266RB-100).

P-284 - Reproductive Seasonality Influences Follicle Dynamics and the Ovarian Extracellular Matrix Structural Properties in Ewes

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Successful ovarian function, which includes follicular development, ovulation, and corpus luteum formation and regression, requires extensive cyclical tissue remodeling provided by its extracellular matrix (ECM). The ovarian ECM was long considered to be a passive structure that provides anchoring and mechanical support to the cells, but is now increasingly recognized as a pivotal element in the multi-directional communication that occurs between components of the ECM, stroma, theca, granulosa cells, and the oocyte. As new roles for the ECM emerge, in-depth examination of its changes across suspension and resumption of ovarian activity during seasonal breeding may reveal key aspects for improving oocyte yield and embryo production for agricultural purposes or restoring ovarian function in women. The aim of the present study was to investigate the influence of reproductive seasonality on follicle dynamics and the ovarian ECM properties. Ovaries from ewes were obtained from the local abattoir, with eight of them collected during the breeding season (November/December) and eight during anoestrus (April/May). Ovaries were weighed and measured, and ovarian morphometric parameters as well as ECM-related proteins expression (collagen, fibronectin, laminin) were quantified using histological and immunohistological analysis. Ovarian weight and volume were higher during the breeding season compared to the non-breeding season ($P < 0.001$). No difference was observed in the proportion of pre-antral follicles and earlier stages of development, but the percentage of antral follicles was significantly increased during anoestrus compared to oestrus ($P = 0.028$) while 6 out of the 8 ovaries (75%) collected during oestrus contained a corpus luteum, compared to none in those retrieved during anoestrus ($P = 0.010$). In addition, the tunica albuginea, the connective tissue layer located beneath the surface epithelium, thickened during the anoestrus season, rising from $110.3 \mu\text{m} \pm 14.9 \mu\text{m}$ during the breeding season to $121.3 \mu\text{m} \pm 18.8 \mu\text{m}$ during anoestrus ($P = 0.215$). Stromal collagen, fibronectin and laminin content were also quantified. Both collagen and fibronectin peaked during anoestrus compared to oestrus (collagen: $87.0\% \pm 3.3\%$ positive area versus $72.4\% \pm 5.4\%$, respectively [mean $\pm$ SD], $P < 0.0001$; fibronectin: $33.5\% \pm 4.3\%$ positive area versus $25.4\% \pm 6.0\%$, $P = 0.007$), while laminin slightly dropped ($12.4\% \pm 2.8\%$ positive area versus $16.1\% \pm 3.4\%$, $P = 0.032$). Collagen fibres thickness and packing density were further assessed and showed a significant increase of both thick and mid-sized fibres during anoestrus compared to the breeding season ($P = 0.003$ and $P < 0.001$, respectively), while the proportion of thin fibres dropped ($P < 0.001$). Given that collagen confers tissues with rigidity, these results suggest a stiffer microenvironment during anoestrus, corroborated with a thicker tunica albuginea. Similar features have been reported in patients with polycystic ovary syndrome and are likely to prevent ovulation. Ongoing experiments are assessing the mechanical properties of the ECM at each reproductive phase to clarify the consequences of such structural changes. These results offer a first look at the dynamic remodeling that occurs across the reproductive season, modulating the microenvironment to potentially regulate follicle growth and ovulation. They also reinforce the use

of the ovine model, that share physiological characteristics with women, as a model to provide comparative insights into ovarian biology. This study was supported by a grant from the Medical Research Council (MR/T025654/1 to E.E.T.).

P-285 - Ovarian Stromal Cell Conditioned Media Improves Survival in Pre- and Early Antral Cat Follicles

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Ovarian stromal cells act as crucial support and regulators for in vivo folliculogenesis; however, less is known about their effect on in vitro grown follicles. The objective of this study was to investigate the impact of ovarian stromal cell co-culture or conditioned medium (CM) on survival and development of cat pre-, early, and antral follicles in vitro. Ovaries were obtained from cats older than 6 months ($n = 3$), then enzymatically digested to release stromal cells. The ovarian stromal cells were allowed to grow to confluency in a T75 flask, before being cryopreserved for long term storage in liquid nitrogen. Cells were thawed one week prior to follicular culture onset, and passaged once before CM collection. CM was subsequently removed 24 - 48 hours after feeding, and stored at -80°C until used. Ovarian follicles were mechanically isolated from cats older than 6 months ($n = 23$ cats, 155 follicles), encapsulated in 0.5% alginate hydrogel, and cultured for 13 days in Endothelial Cell Growth Medium. The isolated follicles were then divided into five treatment groups (control, ovarian stromal cell co-culture, 20% CM, 50% CM, and 100% CM), and classified based on initial diameter as preantral ($224.4 \pm 4.7 \mu\text{m}$), early antral ($394.8 \pm 7.4 \mu\text{m}$), or antral ($592.2 \pm 18.8 \mu\text{m}$). The survival and growth of isolated follicles were then evaluated on Day 0, 4, 6, 8, 11 and 13. At the culture period endpoint, follicles were assessed via RTPCR for expression of CYP19A, FSHR, and GDF9 to further quantify development. Statistical analysis was done in R software. Follicles in 100% CM had higher survival up to Day 11 of culture as compared to other treatment groups (Cox proportional hazards model, $p \leq 0.01$). Initial stage also influenced survival, with antral follicle survival significantly lower than that of pre- and early antral follicles ($p \leq 0.0001$). However, no differences in growth were detected across the treatment groups, nor across initial size classifications (Kruskal-Wallis test, $p > 0.05$). Post culture RTPCR analysis of the three selected genes showed CYP19A was upregulated in 50% CM follicles compared to the control (ANOVA, $p \leq 0.05$). However, there were no differences in CYP19A expression between the control and other treatment groups, or in GDF9 and FSHR expression among culture groups ($p > 0.05$). In summary, the findings demonstrated that conditioned medium collected from primary culture of ovarian stromal cells improves in vitro survival and modulates CYP19A expression of isolated cat follicles. Further research to identify paracrine factors present in conditioned medium will elucidate the roles of ovarian stromal cells pertaining to follicle survival during in vitro folliculogenesis.

P-286 - CRISPR/dCas9-Tet1-Mediated Targeted DNA Demethylation of MTNR1A in Porcine Cumulus-Oocyte Complexes Enhances Oocyte Maturation

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Oocyte maturation is a crucial aspect of in vitro embryo production (IVEP), as it directly impacts the subsequent fertilization and growth of porcine embryos. Failure to perform maturation accurately and under ideal conditions can negatively affect embryo development. Several studies have demonstrated the positive effects of melatonin on oocyte maturation and subsequent embryonic development in various animal species such as pigs. Melatonin receptors play a critical role in triggering physiological responses through different signal transduction pathways, such as the AKT and ERK pathways, which aid in nuclear maturation and cumulus layer expansion around oocytes. Therefore, overexpression of melatonin receptors could potentially affect the oocyte maturation process in vitro. In this study, we successfully used CRISPR/dCas9-Tet1 to demethylate melatonin receptor 1A (MTNR1A) in porcine oocytes as a novel strategy for in vitro oocyte maturation (IVM). We introduced 1 μ L of both sgRNA and dCas9 into a carefully selected group of porcine oocytes in maturation media by transfection. The diameter of the cumulus-oocyte complexes (COCs) was assessed following a 22-hour incubation at a temperature of 39°C and a carbon dioxide concentration of 5% in wells containing hormones (equine chronic gonadotropin; eCG and hCG). The diameter of cumulus-oocyte complexes (COCs) was significantly increased in the groups treated with dCas9 and sgRNA (491.516 μ m) compared to the control group (419.186 μ m) ($p < 0.0001$). In addition, following an incubation period of 18-20 hours in wells without hormones, the group treated with dCas9 and sgRNA exhibited a greater proportion of grades 3 and 4 (48.173 %) when assessing cumulus cell expansion using a scoring system (Grade 1-4) compared to the control group (37.493 %) ($p < 0.01$). The CRISPR/dCas9-Tet1 system demonstrated efficient demethylation of endogenous MTNR1A in cumulus cells surrounding oocytes. Following in vitro fertilization (IVF) using fresh swine spermatozoa, the rate of nuclear maturation was compared to the percentage of oocytes in the MII phase, which was higher in the treatment group (67.90 % vs 62.78 %; $P < 0.01$). The cleavage and blastocyst rates on days 2 and 7, respectively, were compared between the treatment and control groups using in vitro culture (IVC) conditions of 38 °C, 5% CO₂, and 5% O₂. The results showed that both rates were greater in the treated group (Cleavage rate: 77.87 % vs 71.43 %; Blastocyst rate: 22.45 % vs 17.39 %; $P < 0.05$). In addition, during our assessment of the expression of genes related to cumulus cell expansion, we observed considerable upregulation of several genes involved in the AKT and ERK pathways in the treatment group. Notably, demethylation of MTNR1A using CRISPR/dCas9-Tet1 improved the maturation of oocytes damaged by reactive oxygen species (ROS). These findings suggest the potential use of the CRISPR/dCas9-Tet1 system to enhance the success rate of both normal and damaged oocytes during IVM.

P-287 - Follicle-Stroma Interplay Unravel the Mechanisms Involved in Bovine Early Folliculogenesis

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Exploiting primordial follicle reserve to improve assisted reproductive technologies is still ineffective. So far, no defined culture system has been able to replicate the folliculogenesis in vitro

due to the lack of knowledge of the mechanisms involved. During folliculogenesis, diverse and unique pathways control the fate of primordial follicles, from remaining dormant to undergoing cell death or activation. Morphological and functional changes occur within the follicles and interact with the ovarian stromal environment, particularly the ovarian cortex. The ovarian cortex surrounds the preantral follicles and mediates dialogue through paracrine factors. It is reported that the ovarian cortical matrix can influence the action of local paracrine biochemical signaling pathways to sustain the development of primordial, primary, and secondary follicles. The present study aims to delineate the signaling networks involved in preantral follicle differentiation and the role of the ovarian cortex in guiding early folliculogenesis from the primordial to the secondary follicle stage. A bovine follicle dataset was generated in-house by mechanically isolating pools of primordial (n=3), primary (n=3), and secondary (n=3) follicles and subjecting them to 50bp paired-end bulk RNA sequencing on Illumina NextSeq2000. Sequenced data were trimmed with TrimGalore, mapped to the bovine transcriptome assembly ARS UCD 1.3, and quantified with Salmon. Differential gene expression analyses were performed with DESeq2 on R. Functional enrichment analyses were then conducted to identify overrepresented pathways. The transcriptome analysis of primordial, primary, and secondary follicles reveals an evident clustering of the samples via principal component analysis. Pairwise comparisons of the subsequent follicle stages, i.e., primordial-primary and primary-secondary, identified 1083 and 4596 significant differentially expressed genes ($\text{padj} < 0.1$), respectively. Collectively, overrepresentation analyses of the differentially expressed genes revealed signaling pathways, such as PI3K-Akt, Wnt, mTOR, and cAMP, guiding the transitional phases. To explore preantral follicle gene expression dynamics, we simultaneously compared all three follicle categories using the Likelihood Ratio Test of R(DESeq2). Significant genes ($\text{padj} < 0.1$) were subjected to hierarchical clustering. Four clusters were generated that contained 2030, 1527, 536, and 147 genes in clusters 1-4, respectively, showing distinct expression trends. To better understand the interplay between preantral follicles and the ovarian cortex, a meta-analysis was then performed using the publicly available data (GSE147176), downloaded from GEO, processed and analyzed with the same pipeline described above. The metanalysis between the follicles and the ovarian cortex transcriptomic profiles shortlisted 2479 differentially expressed genes ($\text{padj} < 0.01$) with substantial differences in expression levels ($\log_2\text{FoldChange} < -4$ and > 4). Our preliminary data report key overrepresented pathways between follicles and cortex, such as focal adhesion. Further studies are needed to narrow down key regulators involved in follicle-stroma communication. However, the definition of pathways involved in follicle-cortex crosstalk may translate into effective systems for supporting early folliculogenesis in vitro. These analyses uncover the networks governing the development of follicles, from the primordial to the secondary stage, also by including the signal transduction from the ovarian cortical matrix. Indeed, understanding the support provided by the ovarian cortex during early folliculogenesis is essential for reconstructing 3D culture systems to sustain the growth of follicles in vitro. The in vitro development of preantral follicles can represent a significant achievement in mammalian fertility preservation plans to maximize the exploitation of the ovarian reserve, adding a step to assisted reproductive technologies. Funded by MUR PRIN2020, No. 20209L8BN4 (InfinitEGG). NM funded by PON MUR 2014-2020

P-288 - Identifying Risk Variants for Embryo Aneuploidy Using Ultra-Low Coverage Whole-Genome Sequencing from Preimplantation Genetic Testing

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Aneuploidy frequently arises during human meiosis and is the primary cause of early miscarriage and in vitro fertilization (IVF) failure. Individuals undergoing IVF exhibit significant variability in aneuploidy rates, although the exact genetic causes of the variability in aneuploid egg production remain unclear. Preimplantation genetic testing for aneuploidy (PGT-A) using next-generation sequencing is a standard test for identifying and selecting IVF-derived euploid embryos. The wealth of embryo aneuploidy data and ultra-low coverage whole-genome sequencing (ulc-WGS) data from PGT-A has potential for discovering variants in parental genomes that are associated with aneuploidy risk in their embryos. Using ulc-WGS data from ~10,000 PGT-A biopsies, we imputed genotype likelihoods of genetic variants in embryo genomes. We then used the imputed variants and embryo aneuploidy calls to perform a genome-wide association study of aneuploidy incidence. Finally, we carried out functional evaluation of the identified candidate gene in a mouse oocyte system. We identified one locus on chromosome 3 that is significantly associated with meiotic aneuploidy risk. One candidate gene, *CCDC66*, encompassed by this locus, is involved in chromosome segregation during meiosis. Using mouse oocytes, we showed that *CCDC66* regulates meiotic progression and chromosome segregation fidelity, especially in older mice. Our work extended the research utility of PGT-A ulc-WGS data by allowing robust association testing and improved the understanding of the genetic contribution to maternal meiotic aneuploidy risk. Importantly, we introduce a generalizable method that has potential to be leveraged for similar association studies that use ulc-WGS data.

P-289 - Effect of DMBA Exposure on Ovarian Chemical Metabolism, DNA Damage Sensing and Repair, and Oxidative Stress Proteins in Prepubertal Mice

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The polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene (DMBA), is formed due to combustion of organic material, and environmental exposure occurs through wildfires, cigarette smoke and exhaust fumes. Exposure to DMBA induces ovarian DNA damage, causes ovarian follicle loss and the DNA damage response is partially attenuated in obese relative to lean female mice. In the United States, obesity affects 14.7 million children and 20% of girls, and impairs oocyte quality and fertility, and is associated with precocious puberty in girls. In adult mice, obesity accelerates oxidative stress, alters ovarian chemical metabolism, and disrupts the phosphatidylinositol-3 kinase pathway. This study investigated the effect of DMBA exposure in lean

and obese female prepubertal mice on ovarian proteins involved in DNA damage sensing and repair, chemical metabolism, and oxidative stress. C57BL/6J female pregnant mice were fed with chow diet (lean) or switched to a 60% high fat diet (obese) on gestational day 17 until post-natal day (PND) 21 when pups were weaned. Female pups remained on the same diet and on PND 35, each diet group received either corn oil (CT) or 1 mg/Kg of DMBA (D) for 7 days by intraperitoneal injection. Body weight was recorded twice weekly, and mice were euthanized at PND 42. Final body and tissue weights were recorded. The abundance of ovarian proteins was quantified via western blotting. Statistical analyses were performed using GraphPad Prism 10.1.2 software. Final body weight increased in the obese group ($P < 0.05$) regardless of treatment compared to lean mice. Obesity increased ($P < 0.05$) ovary, uterus, kidney, and spleen weight but decreased liver weight ($P < 0.05$). Obese mice exposed to DMBA had increased ($P < 0.05$) uterus, heart, kidney, and spleen weight ($P < 0.05$) compared to lean DMBA-exposed mice. Ovarian protein abundance of CYP2E1 and BRCA1 tended ($P < 0.1$) to be decreased while CAT, EPHX1 and GSTP1 were decreased ($P < 0.05$) by basal obesity and DMBA exposure in lean mice. In obese mice exposed to DMBA, ovarian protein abundance of EPHX1 was decreased ($P < 0.05$) compared to identically exposed lean mice. There was no effect of obesity or DMBA exposure on the ovarian abundance of γ H2AX ($P > 0.05$), but obesity decreased ovarian SOD1 compared to basal and DMBA-induced levels in lean mice ($P < 0.05$). Taken together, these data suggest that DMBA exposure and changes in physiological status alter organ weight and the ovarian abundance of proteins involved in chemical metabolism, DNA damage sensing and repair and oxidative stress in prepubertal mice leading to negative female reproductive effects at early stages of reproductive life.

P-290 - TRPV2 as a Potential Therapeutic Target of Granulosa Cell Tumors

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We previously identified the ion channel transient receptor potential vanilloid 2 (TRPV2) in cells of the human ovary. In isolated human granulosa cells (GCs), derived from patients undergoing medical reproductive procedures, activation of TRPV2 by cannabidiol (CBD) resulted acutely in increased levels of intracellular calcium and stimulated the secretion of inflammatory factors, including interleukin (IL) 6 and IL 8. We concluded that it is involved in the regulation of inflammatory processes. Granulosa cell tumors (GCTs) are derived from granulosa cells. They amount to 2-5% of all ovarian cancers and are malignant stromal tumor types. If diagnosed at an early stage, treatment includes surgical removal of the tumor tissue followed by chemotherapy, but further treatment options are limited. By employing immunohistochemistry, we found that TRPV2 is expressed in a set of human GCT (in 52% of 29 investigated primary GCT samples). TRPV2 was also found in the well-established GCT-derived cell line, KGN, which was therefore used for further studies. Acutely, the addition of CBD elicited increased intracellular calcium levels (5-30 μ M CBD; $n = 250$ cells from 10 independent experiments), implying channel opening. Within hours (4-6 h), and in striking contrast to primary human GCs, CBD caused cell death in KGN cells, which occurred in a concentration and time-dependent manner. While being a preferred ligand of TRPV2, CBD is known to also interact with other proteins and channels. Therefore, we generated TRPV2-deficient KGN clones using the CRISPR/Cas9 technique and two (clones #4 & #13) were examined more intensively e.g. by cell counting and live cell imaging. The loss of TRPV2 led to several

changes, including increased cell size (mean diameter: 16.7 μm in normal KGN vs. 19.5 μm (#4) and 19.8 μm (#13) in TRPV2-deficient KGN clones; n = 10, each), higher proliferation (mean cell counts after 24 h: 142.8% in normal KGN vs. 206.8% (#4) and 203.8% (#13); n = 9, each) and a higher migration speed (0.32 $\mu\text{m}/\text{min}$ in normal KGN vs. 0.46 $\mu\text{m}/\text{min}$ (#4) and 0.43 $\mu\text{m}/\text{min}$ (#13); n = 28 cells, each). Mass spectrometry (n = 5, each) further supported and complemented the results. Importantly, the absence of TRPV2 made these clones less vulnerable to CBD-induced cell death, as demonstrated e.g. by means of ATP assays, making this ion channel a new therapeutic target for GCTs. This study is funded by DFG MA 1080/31-1, MA4790/5-1 and FR3411/5-1, project 456828204.

P-291 - Development of Bovine Oocytes Cultured with Nicotinamide Monocleotide (NMN) Was Improved via Enhanced Mitochondrial Function

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Nicotinamide adenine dinucleotide (NAD⁺) and its metabolites function as crucial regulatory factors to maintain physiological processes, allowing cells to adapt to environmental changes such as nutritional deficiencies, genotoxic factors, disruptions in circadian rhythms, infections, inflammation, and exogenous substances. In this study, we examined whether an increase of NAD⁺ level in oocytes lead to an improvement of blastulation after IVF. Bovine cumulus-oocyte-complexes (COCs) were matured in vitro in the culture (IVM) medium supplemented with 0-100 μM nicotinamide mononucleotide (NMN, a precursor to NAD⁺). Matured oocytes were inseminated with the washed spermatozoa and cultured to assess the blastulation. Matured oocytes were also used to examine their NAD⁺ content, gene expression pattern, reactive oxygen species (ROS) and ATP levels. The addition of NMN to the in vitro maturation medium of bovine oocytes showed an increase in intracellular NAD⁺ levels, leading to enhanced developmental competence to the blastocyst stage after in vitro fertilization. Transcriptome analysis revealed that the increase in intracellular NAD⁺ levels led to the change of the mitochondrial function. Additionally, NMN supplementation increased ATP levels and decreased reactive oxygen species levels in oocyte. The results suggest that an increase of NAD⁺ level in oocytes improve the post-fertilization developmental competence through mitochondria function.

P-292 - Transcriptomic Analysis of Luteal Tissue Supports the Delayed Timing of Luteolysis in Heifers with Greater Ovarian Reserve

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Antral follicle count (AFC) is reflective of ovarian reserve and linked to reproductive performance in mammalian females. We previously demonstrated a delayed upregulation of endometrial oxytocin receptors in heifers with greater ovarian reserve, indicating delayed induction of luteolysis. Luteal abundance of selected genes of the luteolytic cascade were investigated to further support these findings. We hypothesized that in response to luteolytic signals, mRNA transcript abundance of

thrombospondin-1 (THBS1) will be increased and luteinizing hormone/choriogonadotropin receptor (LH/CG-R) will be decreased in the corpus luteum (CL) of open heifers, with a pronounced effect in heifers with diminished ovarian reserve. Furthermore, we hypothesized that the increase of luteal interferon-stimulated gene 15 (ISG15) and interferon-induced GTP-binding protein Mx1 (MX1) transcript abundance in pregnant heifers is greater in animals with increased ovarian reserve. Based on rectal ultrasonography, beef heifers were chosen from the top 10% (high AFC, 33.5 ± 8.4 , $n=40$) and bottom 10% (low AFC, 14.4 ± 2.9 , $n=40$) of the population distribution. All heifers were inseminated (d0) following a 7d-CO-Synch protocol. On day 15 or 16 after insemination, reproductive tracts were collected and flushed. Pregnancy status was defined by presence or absence of a conceptus. Corpora lutea were isolated, weighed and snap-frozen. Transcript abundance of ISG15, MX1, THBS1 and LH/CG-R was determined by RT-qPCR from heifers with a single CL. Circulating progesterone concentrations were determined via radioimmunoassay. Data were analyzed using the MIXED procedure of SAS with AFC group, pregnancy status and their interaction as fixed effects and day as a covariate, followed by Tukey's test. Progesterone concentrations tended to be greater in high vs low AFC heifers (9.3 ± 1.1 vs 6.5 ± 1.2 ng/ml, $P < 0.09$) and pregnant vs open heifers (9.5 ± 1.1 vs 6.4 ± 1.2 ng/ml, $P < 0.06$), but were not influenced by their interaction. Luteal weights and transcript abundance of ISG15 and MX1 were not affected by pregnancy status, AFC group or their interaction. Transcript abundance of THBS1 was upregulated in open vs pregnant animals (8.4 ± 1.2 vs 3.7 ± 0.5 -fold, $P < 0.0001$) and showed greater abundance in low vs high AFC heifers (7.3 ± 1.1 vs 4.3 ± 0.6 -fold, $P < 0.01$). THBS1 expression was not influenced by the interaction of AFC group and pregnancy status. An interaction of AFC group and pregnancy status was identified ($P < 0.05$) for LH/CG-R abundance, with greater transcript abundance in pregnant vs open (228.5 ± 39.0 vs 88.5 ± 16.2 -fold) and in high vs low AFC heifers (199.5 ± 33.5 -fold vs 101.3 ± 18.7 -fold). Open low AFC heifers showed the lowest LH/CG-R transcript abundance (46.7 ± 13.5 -fold, $P < 0.01$). These results suggest that day 15 and 16 might be too early to detect differences in luteal response to conceptus derived interferon tau in heifers differing in pregnancy status and/or AFC. The transcript patterns of THBS1 and LH/GC-R support the concept of a delayed induction of luteolysis in open heifers with greater ovarian reserve. This may contribute to an extended window for maternal recognition of pregnancy and result in superior reproductive performance in heifers with greater ovarian reserve. USDA is an equal opportunity provider and employer.

P-293 - Environmentally Relevant Maternal Atrazine Exposure Leads to Aberrant Endocrine Effects in Piglets

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Atrazine is a widely used herbicide in United States agriculture. Atrazine is an environmental toxicant that persists in soil and is one of the most commonly detected contaminants in U.S. surface water sources. For drinking water, the U.S. Environmental Protection Agency has set the maximum contaminant level of 3 mg per liter for atrazine. Previous toxicology studies in rodents indicated that atrazine is an endocrine disruptor and disrupts the hypothalamic-pituitary-gonadal axis. Further, a previous study found that atrazine exposure increases luteinizing hormone serum levels in gilts. The effects of environmental atrazine exposure on the female reproductive capacity

of agriculture animals are unknown. This study tested the hypothesis that maternal atrazine exposure leads to aberrant endocrine effects in piglets. Pregnant sows were exposed to either vehicle control (0.002% (v/v) ethanol) or atrazine (20 µg/L) via drinking water administered ad libitum from 28 days of gestation through parturition and lactation (~127 days total). Umbilical cord sera were collected at birth, and atrazine exposed piglets and controls were necropsied on post-natal day 10. Pregnenolone, progesterone, and estradiol cord serum levels were quantified by enzyme-linked immunosorbent assays. Gene expression was assessed by quantitative pcr (qPCR) on pituitaries collected at necropsy. Atrazine exposed piglets had lower levels of cord progesterone than control exposed piglets ($p=0.0043$). In contrast, cord serum levels of pregnenolone and estradiol did not differ between atrazine exposed and control exposed piglets. To examine a possible mechanism of atrazine exposure leading to decreased progesterone serum levels, pituitary mRNA expression of luteinizing hormone beta (Lhb), follicle stimulating hormone beta (Fshb), and thyroid stimulating hormone subunit beta (Tshb) were assessed by qPCR, and the results indicate that pituitary Lhb, Fshb, and Tshb expression levels were similar between control exposed piglets and atrazine exposed piglets. These results suggest that maternal atrazine exposure during gestation and lactation may lead to aberrant ovarian steroid hormone levels in the exposed piglet offspring through mechanisms that do not include changes in pituitary expression of Lhb, Fshb, and Tshb. Supported by the University of Nebraska Collaboration Initiative (to ATD).

P-294 - Naked Mole-Rat as Model of Reproductive Aging: In Vitro Germ Cell Expansion and Oocyte IVM Using the Same Ovary

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Female reproductive aging is characterized by a loss of follicles, the functional units of the ovary, consisting of oocytes surrounded by companion granulosa cells. The quality of the oocytes remaining in the ovary also deteriorates with age. Reproductive aging is associated with adverse reproductive outcomes, including infertility, miscarriages, and birth defects, with such consequences becoming more frequent as women, globally, are delaying childbearing. The adult human ovary is devoid of definitive germline stem cells. As such, female reproductive senescence largely results from the depletion of a finite ovarian follicle pool that is produced during embryonic development. Because the ovarian reserve, which dictates reproductive lifespan, is established in utero, the reproductive aging timeline, in effect, is slated before birth. Research groups around the world have explored different models to study mammalian ovarian development and aging. However, these models only allow us to study specific times in development, and, up to now, it has been impossible to evaluate in the same ovary both the early stages of development and aging. Nevertheless, to each rule, there is an exception. The naked mole-rat (*Heterocephalus glaber*; NMR) is the longest-lived rodent, with a maximum lifespan of >37 years. Female NMRs show no decline in fertility or fecundity during their entire lifespan, meaning that the dominant breeding females (also known as “Queens”) will breed until they die. Herein, we characterize how in the

naked mole-rat ovary it is possible to study the ovarian reserve, mitotic expansion of the germ cell postnatally and, oocyte in vitro maturation using the same ovary. Our results confirm that it is possible to induce oocyte in vitro maturation in the naked mole-rat, but also that spindle morphology and the timeline required for maturation is closer to humans than mice.

P-295 - Interactions Between Addition of Serum and Type of Culture Medium on Gene Expression of Bovine Blastocysts Produced In Vitro

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Serum is commonly added to culture medium used for producing bovine embryos in vitro. It is often suspected that serum can cause abnormal calf development. Here we tested 1) whether addition of bovine serum to embryo culture medium increases the percent of embryos becoming blastocysts and alters blastocyst gene expression and 2) whether actions of serum depend upon culture medium. The media used were synthetic oviduct fluid medium bovine embryo 2 (SOF) or a commercial medium from IVF-Biosciences (IVFB). Three experiments were performed using embryos produced by in vitro fertilization with abattoir-derived oocytes. The design for each experiment was a 2 x 2 factorial design with 10% (v/v) serum or control medium as one main effect and medium type as the other. Fetal bovine serum was added on day 5 of culture in Exp 1 (6 replicates). Adult bovine serum was added at day 5 in Exp 2 (3 replicates) and on day 1 in Exp. 3 (4 replicates). Data were analyzed by logistic regression using the GLIMMIX procedure of SAS. Effects of medium and serum on development were similar for each experiment so data were combined for statistical analysis. The percent of oocytes that cleaved was not affected by medium or serum but the percent of putative zygotes becoming blastocysts was greater for IVFB than SOF ($P=0.0010$) and greater for serum than no serum ($P=0.0004$). Least-squares means were 44.1 (SOF), 54.5 (SOF+serum), IVFB 51.9 (IVFB), and 58.2% (IVFB+serum) ($SEM=0.04\%$). Gene expression for pools of 10 blastocysts collected in Exp. 1 was evaluated by RNA-Seq ($n=4$ pools per treatment). Differentially expressed genes (DEG) were those with an adjusted p-value of < 0.01 and an absolute $\log_2$ fold change $> |2|$. In the absence of serum, there were 99 DEG for IVFB vs SOF (22 upregulated and 67 downregulated). Serum resulted in 80 DEG (21 upregulated and 59 downregulated) for embryos cultured in IVFB and 108 DEG (71 upregulated and 37 downregulated) for embryos cultured in SOF. A total of 11 DEG were regulated by serum similarly for both media including transcription factors ASCL2 (downregulated) and ZSCAN4 (upregulated). ASCL2 is imprinted in the mouse and plays a role in placental formation and fetal growth. Among the DEG downregulated by serum for IVFB only were NANOG, involved in epiblast formation, and three interferon-tau genes (maternal regulation of pregnancy signals). Four genes downregulated by serum in IVFB are imprinted in the cow (MEG3, MEG8, MEG9, and RTL1) but none of the DEG for SOF are known to be imprinted in the cow. In conclusion, actions of serum on gene expression are modest in extent, dependent on culture medium, and include an overrepresentation of imprinted genes. One DEG, ASCL2, has been implicated in fetal growth. Future experiments to evaluate the impact of

disruptions in its expression on fetal development are warranted. (Funding: USAID Egypt, FFAR and Larson Endowment)

P-297 - Interaction of Cumulus Cells and Granulosa Cells During Luteinization Based on Analysis of Transcriptome

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Significant changes of gene expression induce various cellular functions in ovarian follicular cells undergoing luteinization. We previously reported a transcriptome change in mouse granulosa cells (GCs) undergoing luteinization. However, there are no reports on the transcriptome change in mouse cumulus cells (CCs). In this study, we identified up-and down-regulated genes and their associated cellular functions using RNA-sequencing data of CCs and GCs undergoing luteinization. It has been reported that interactions from GCs to CCs are involved in the regulation of cellular functions in CCs during luteinization. However, the comprehensive interactions between CCs and GCs, especially from CCs to GCs, remain unclear. Therefore we also investigated the interactions between these cell types using RNA-sequencing data. Three-week-old mice were intraperitoneally injected with 4 units of equine chorionic gonadotropin, followed by 5 units of human chorionic gonadotropin (hCG) 48 hours later to induce luteinization. The ovaries were obtained before hCG (0), and 4 and 12 hours after hCG injection to collect cumulus and granulosa cells, and RNA-sequencing was performed. The up-and down-regulated genes during the luteinization were identified in CCs and GCs, respectively, and were subjected to Gene Ontology (GO) analysis. A total of 8,888 genes showed significant changes in CCs during luteinization. Among them, 5,302 genes were up-regulated and were associated with cellular functions in glucose metabolism, reactive oxygen species (ROS) metabolism, and activation of the MAPK cascade. Glucose metabolism in CCs contributes to the oocyte maturation. ROS metabolism reflects its role in protecting the oocyte from excess ROS during the ovulatory process. The MAPK cascade contributes to cumulus-oocyte complex (COC) expansion. In granulosa cells, 11,799 genes showed significant changes. Among them, 4,254 genes were up-regulated and were associated with cellular functions in angiogenesis, ovulation, and steroidogenesis. These functions contribute to the transformation of granulosa cells into a vascular-rich corpus luteum, primarily producing progesterone. To determine the activated interactions between GCs and CCs during luteinization, interactome analysis was performed. At 4 hours after hCG injection, twenty-two interactions were identified as the activated pathways acting from CCs towards GCs. They included Mif-CD74_CXCR4 pathway and, Fgf2-Fgfr3 pathway, which is associated with ovulation and angiogenesis. Thirty-nine interactions were identified, acting from GCs to CCs. They included Nrg1-ITGAV_ITGB3 pathway, which is associated with oocyte maturation. In addition, there were 78 bidirectional interactions, including EGFR pathway, which is a key pathway for luteinization in both cells. 4 to 12 hours after hCG injection, twenty interactions were identified, acting from CCs toward GCs. They included Wnt-FZD4_LRP5 pathway, which is associated with steroidogenesis. Twenty-one interactions were identified, acting from GCs toward CCs. They included Wnt5a-Fzd8 pathway, which is associated with a COC expansion. In addition, there were 25 bidirectional interactions associated with lipid metabolism. We revealed the transcription changes and functional alterations in CCs and GCs undergoing luteinization. The cellular functions associated with oocyte protection and COC expansion are activated in CCs whereas steroidogenesis and ovulatory, angiogenesis function are activated in GCs. These cellular functions might be regulated through the interactions between CCs and GCs.

P-298 - ORC 1 is Required for the First Round of DNA Synthesis in the Zygote

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The origin recognition complex (ORC) is a six-subunit complex that identifies origins of replication. It was originally discovered in yeast but is also crucial for mammalian cells. ORC subunit 1 (ORC1) is the final subunit that binds to the complex, and the first to be released as each origin is fired during replication. We have previously demonstrated that one of the other ORC subunits, ORC4, that is part of the complex that binds to origin before ORC1 is absolutely required for zygotic DNA synthesis. However, previous studies have shown that certain cancer cells can replicate DNA in the absence of ORC1, and it was possible that the zygote might replicate DNA without ORC1. We crossed knock-in mice with loxP sites flanking exons with Zp3-Cre mice to delete *Orc1* exons 9-14 during oogenesis to generate *Orc1*-CKO mice that had oocytes that did not contain *Orc1*. These *Orc1*-CKO oocytes were parthenogenetically activated and tested for DNA replication with EdU (5-Ethyl-2'-deoxyuridine) incorporation. We found that 55% of parthenogenetically activated oocytes had positive EdU staining. All parthenogenotes progressed to the 2-cell stage, but only 37% of 2-cell embryos were EdU positive. The large majority of these embryos were arrested at the 2-cell stage, but some developed to 3-cell embryos before arrest. When *Orc1*-CKO oocytes were injected with wild-type sperm, the DNA replication was similar; 55% and 57% of 1 cell zygote and 2-cell embryos were EdU positive, respectively. However, some of these ICSI generated embryos progressed to the blastocyst stage; and only 19% reached blastocyst stage. These data suggest that ORC1 is required for complete DNA replication in the zygote, and clearly required for the progression beyond the 2-cell stage. It also suggests that ICSI can partially rescue ORC1 deletion in the oocyte by providing a new, paternal, *Orc1* gene

P-299 - Treatment of Cumulus-Oocyte-Complexes with Age-Associated FSH Glycoforms Results in Differential Modulation of Meiotic Resumption

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Differences in N-glycosylation of the β -subunit of follicle-stimulating hormone (FSH) results in macroheterogeneity. Of interest, FSH glycoform abundance changes throughout female reproductive aging. The hypoglycosylated form of FSH (FSH21), which is dominant in women < 35 years of age when reproductive potential is high, exhibits increased FSHR binding and bioactivity compared to fully glycosylated FSH (FSH24) which is the major glycoform present in women > 35 years of age. Thus, there is a shift from FSH21 to FSH24 with age that correlates with reduced bioactivity. We previously demonstrated that FSH21 better supports folliculogenesis compared to FSH24 due to enhanced cellular communication. Given that FSH is also important for later stages of oocyte development, we sought to determine whether FSH glycoforms mediate differential effects on cumulus cell expansion and meiotic progression during in vitro maturation (IVM). To do this, we collected intact cumulus-oocyte-complexes (COCs) containing fully grown oocytes

obtained from hyperstimulated adult mice and matured them in the presence or absence of 10 ng/ml FSH21 and FSH24. IVM was performed using an EmbryoScope+ platform with images taken every 10 min at 11 focal planes for a total of 16 hrs which enabled analysis of morphokinetic variables of meiotic progression and cumulus expansion. Both the FSH glycoforms promoted cumulus cell expansion, whereas no expansion was observed in the control without FSH. The velocity of cumulus cell expansion was similar between glycoform treatments. Oocytes within FSH21-treated COCs underwent germinal vesicle breakdown (GVBD) at 0.90 ± 0.18 hrs compared to FSH24-treated COCs which showed a significant delay in GVBD at 1.8 ± 1.23 hrs ($n=16$ oocytes; $p=0.0061$). Polar body extrusion (PBE) showed a delayed trend in the FSH24-treated group compared to FSH21 with PBE occurring at 9.30 ± 1.01 and 8.72 ± 0.62 hrs, respectively ($n=15-16$ oocytes, $p=0.08$). The difference in meiotic progression between FSH21 and FSH24 treatments was not observed in oocytes that were denuded of their cumulus cells prior to IVM ($n=16$ oocytes, $p=0.74$), indicating that the effects are mediated through direct actions of FSH on the cumulus cells. Examination of metaphase-II eggs and subsequent in vitro fertilization will provide insight into whether these differences in maturation kinetics result in differences in egg quality and fertilization potential. This work demonstrates that the effect of age-associated FSH glycoforms is relevant beyond follicle growth, with glycoform-specific modulation of oocyte meiotic resumption.

P-300 - The Inhibition of the NLRP3 Inflammasome via Autophagy Regulation Mitigates Ovarian Fibrosis in Reproductively Aged Mice

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The microenvironment within the ovary plays a crucial role in follicle growth and ovulation but undergoes age-related changes. Fibrosis emerges as a hallmark of aging ovaries, accompanied by the establishment of an inflammatory environment, such as the activation of the NLRP3 inflammasome. Autophagy, an essential cellular homeostatic process for cell survival, is related to fibrosis by regulating NLRP3 inflammasome. However, the exact relationships between age-associated ovarian fibrosis and autophagy are poorly understood. In this study, we investigate the influence of autophagy on ovarian fibrosis through the modulation of the NLRP3 inflammasome. Here, we observed collagen deposition and fibronectin, both fibrosis markers, were increased within the ovarian tissue with advancing age. Also, diminished autophagic flux was observed in aged ovaries, compared to young ovaries, and this implies a functional impairment of autophagy with aging. In reproductively aged mice, the treatment with autophagy inducer reduced collagen deposition and increased the number of ovulated oocytes in aged ovaries, in association with alleviated NLRP3 inflammasome. Additionally, the administration of the NLRP3 inflammasome inhibitor (MCC950) attenuates ovarian fibrosis, concurrently restoring ovulatory function. Our studies demonstrate dysfunctional autophagy as a hallmark of the aging ovarian stroma and suggest that modulation of autophagy may contribute as a potential therapeutic intervention for ovarian function recovery by inhibiting ovarian fibrosis.

P-301 - The Mouse Ovary has the Capacity to Convert the Neonicotinoid Pesticide Imidacloprid to Toxic Metabolites In Vivo

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Imidacloprid (IMI) belongs to a class of neuro-active insecticides called neonicotinoids. Neonicotinoid pesticides are nicotine derivatives that target nicotinic acetylcholine receptors (nAChRs) in the nervous system. IMI is used in large scale agricultural systems, sold for private residential use, and is found in veterinary pharmaceuticals. Because IMI is more water soluble than other insecticides, there is tremendous potential for environmental accumulation and chronic exposure of non-target species. IMI can be converted to desnitro-imidacloprid (DNI) by the liver through phase I biotransformation; both compounds act as an agonist for mammalian nAChRs, with DNI having a significantly higher affinity than IMI for nAChRs. A previous study showed that IMI is converted to toxic metabolites in vitro. However, it was not known whether the whole ovary contains the metabolic enzymes required to convert IMI to toxic metabolites. Thus, this study tested the hypothesis that the ovary contains the enzymes required to metabolize IMI. To test this hypothesis, mice (6 weeks of age) were dosed orally with either vehicle control (dimethyl sulfoxide), a low dose of IMI (0.5 mg/kg body weight), or high dose of IMI (5.7 mg IMI/kg body weight for 30 days. After dosing, the ovaries were collected and ovarian RNA was used for qPCR reactions to quantify gene expression of metabolic enzymes associated with IMI metabolism. The expression of the following six genes was measured: aldehyde oxidase 1 (Aox1), aldehyde oxidase 2 (Aox2), aldehyde oxidase 3 (Aox3), cytochrome P450 family 2 subfamily D member 22 (Cyp2d22), cytochrome P450 family 2 subfamily E member 1 (Cyp2e1), and cytochrome P450 family 4 subfamily F member 18 (Cyp4f18). These genes were selected because they are known to convert IMI to toxic metabolites such as DNI in the liver. The results indicate that the ovaries expressed all six genes. However, exposure to both low and high doses of IMI did not significantly increase expression of Aox1, Aox2, Aox3, Cyp2d22, or Cyp4f18 compared to control. Interestingly, low dose IMI significantly increased the expression of Cyp2e1 compared with control. These data indicate that mouse ovaries metabolize IMI and that IMI can induce ovarian Cyp2e1 expression at a low dose, with IMI following a non-monotonic dose response curve. Cyp2e1 is important in IMI metabolism because it reduces IMI to DNI and other downstream metabolites. Collectively, these data suggest that the mouse ovary has the capacity to metabolize IMI to DNI and other downstream metabolites. Supported by NIH F30 ES033914 and NIH R01 ES028861.

P-302 - IFNT-Dependent and IFNT-Independent Changes in the Corpus Luteum of Early Pregnancy

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The effects of interferon tau (IFNT) on the uterus are critical for luteal rescue during maternal recognition of pregnancy in ruminants. In addition, during the time of IFNT secretion, the corpus luteum (CL) of pregnancy is transiently more resistant to the actions of prostaglandin (PG) F_{2A} than the CL of the cycle. We and others reported changes in the CL of early pregnancy, including mechanisms related to cell survival, immune pathways, PGF_{2A} signaling, and matrix remodeling (527 mRNA changed temporally in day 14, 17, 20, and 23 CL, $P_{adj} < 0.05$. An additional 630 tended to change, $0.15 > P_{adj} > 0.05$; Hughes et al., 2020). The objective of this study was to determine the

extent to which the changes observed in the CL of pregnancy are directly regulated by IFNT. To address this, we performed two experiments. In experiment 1, IFNT (10 ug/ml) in 20 ml of PBS containing BSA (200 ug/ml) or BSA vehicle alone (control) was infused into uteri of nonbred heifers 2X per day from day 14-16 of the cycle. CL were collected on the morning of day 17 and RNAseq was performed (n=4/group). Relative to BSA alone, IFNT infusion resulted upregulation of 87 mRNA and downregulation of 30 (Padj < 0.05). As expected, these genes were primarily involved in interferon signaling and antiviral response. In experiment 2, CL were collected from nonbred, day 10-12 cows, luteal steroidogenic cells were cultured for 24 hours with or without 1 ng/mL of IFNT, and RNAseq was performed (n=4/group). Relative to control, treatment with IFNT resulted in upregulation of 1733 mRNA and downregulation of 1153 (Padj < 0.05). These genes were primarily involved in immune response, cytokine signaling, and antiviral response. In total, 75% of changed genes from experiment 1 also changed in experiment 2, confirming the effectiveness of intrauterine IFNT infusion in inducing interferon-stimulated genes in the CL. Remarkably, only 31% of the genes that changed during early pregnancy were regulated by IFNT treatment in vitro or by IFNT infusion in vivo (Padj < 0.05). Gene ontology analysis revealed, as expected, that these genes were regulators of antiviral and immune responses. Among these genes were three Poly(ADP-Ribose) Polymerase (PARPs), PARP9, 12, and 14, which promote DNA damage response and cell survival, and CD55, an inhibitor of the complement-mediated cell death pathway. In contrast, 50% of the genes that changed (Padj < 0.05) during early pregnancy were neither regulated by IFNT in vivo nor in vitro (Padj > 0.2 and log2 fold change < |1| in both IFNT datasets). These mRNA are associated with cellular proliferation and extracellular matrix organization. Predicted upstream regulators included estradiol, ESR1, and prostaglandin E receptors. A final 19% of transcripts could not be conclusively grouped as either regulated or not regulated by IFNT. In summary, this integrative analysis suggests that most of the temporal changes in the CL during early pregnancy that we previously reported are not regulated directly by IFNT, drawing into question identities of other luteal regulators during early pregnancy or the effect of age of CL independent of embryonic signaling. Supported in part by USDA NIFA grant # 2017-67015-26455 to TLO, NIH-T32 Fellowship grant #T32GM108563, and C. Lee Rumberger and Family Chair in Agricultural Sciences endowment to JLP.

P-303 - Role of Zinc Insufficiency in Bovine Granulosa Cell Viability

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Due to the reproductive similarities between cows and women, and the naturally occurring hyperandrogenism that occurs in subsets of beef and dairy cattle herds, cows are an emerging and promising animal model for the study of polycystic ovary syndrome (PCOS). Recently, serum zinc levels have been found to be abnormal in women with PCOS, with some observing high serum zinc in comparison to healthy controls, but most studies observing low serum zinc in women with PCOS. Despite the robust knowledge on the effects of zinc insufficiency on ovarian function in other animal models, such as mice, the effect of zinc insufficiency on the bovine ovary is limited. To begin to investigate this, we hypothesized that zinc insufficiency would decrease cell viability in bovine mural granulosa cells. Plated bovine mural granulosa cells were incubated with differing

levels of the transition metal chelator, N,N,N',N'-tetrakis (2-pyridylmethyl)ethylenediamine (TPEN, 2.5, 3.5, 5, and 10 μ M) for 24 hours. Cell viability was measured with an MTT assay. A moderate decrease in cell viability was seen at 3.5 μ M TPEN, while a severe decrease was seen at 5 and 10 μ M TPEN ($n = 4$; $p < 0.05$; one-way ANOVA). This decrease in cell viability was rescued with the addition of 10 and 20 μ M ZnSO₄, confirming no off-target effects of TPEN ($n=4$; $p < 0.05$; one-way ANOVA). Because TPEN has a high affinity for not only zinc, but also copper, iron, and manganese, chelators specific for those micronutrients were used to determine if the above-mentioned decrease in cell viability in response to TPEN seen is zinc mediated (iron chelator = 10 μ M 2,2'-Dipyridyl; copper chelator = 10 μ M Bathocuproinedisulfonic acid disodium salt; manganese chelator = 10 μ M Sodium-4-aminosalicylate dihydrate). None of these other chelators demonstrated a decrease in cell viability compared to the vehicle control, confirming zinc insufficiency is responsible for the decrease in cell viability in response to TPEN ($n=4$; $p > 0.05$; one-way ANOVA). These results demonstrate that zinc is an essential micronutrient for bovine mural granulosa cells, a cell type whose proliferation is essential for a growing follicle.

P-304 - Melatonin Improves Porcine Oocyte Maturation through Regulation of F-actin Associated Mitochondrial Fission In Vitro

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Mitochondria play a crucial role in regulating cell survival and apoptosis by facilitating mitochondrial fission through the action of the dynamin-related protein (DRP1) during mammalian meiotic maturation and early embryonic development. Additionally, F-actin supports the migration and fission of mitochondria, essential for energy metabolism, maturation, proliferation, and development in mammalian cells. In our previous study, we demonstrated that melatonin enhances spindle assembly-related functions by reducing mitochondrial-derived superoxide during the maturation of porcine oocytes. In this study, we investigated the unclear relationship between the regulation of F-actin by antioxidants and mitochondrial fission. We confirmed a significant reduction ($p < 0.05$) in the expression levels of proteins related to mitochondrial fission, as well as decreases in mitochondrial activation and fragmentation, following Drp1 knockdown. Afterward, we found that the expressions of genes associated with mitochondrial dynamics, oxidative stress, and cytoskeleton were altered by Drp1 siRNA treatment during meiotic maturation, as revealed by mRNA-sequencing analysis. Changes in genes associated with the response to oxidative stress were particularly notable. Also, the expressions of tubulin formation, DNA repair, actin binding, and responses to oxidative stress-related genes significantly changed ($p < 0.05$), and the rate of abnormal spindle assembly and mitochondrial superoxide production increased ($p < 0.05$) in Drp1 siRNA treated oocytes. Based on these findings, we administered melatonin and MIT-001 to the Drp1 siRNA-treated group to mitigate oxidative stress during porcine oocyte maturation. In the groups treated with melatonin and MIT-001 following Drp1 siRNA administration, we observed a

reduction in the expression of mitochondrial fission-related proteins, and the abnormalities in mitochondrial activation and morphology were restored to levels comparable to those in the untreated group ($p < 0.05$). Furthermore, there was a decrease in the rate of abnormal spindle assembly and mitochondrial superoxide production ($p < 0.05$). Additionally, the expression of genes related to tubulin formation, DNA repair, actin binding, and responses to oxidative stress were largely restored ($p < 0.05$). In conclusion, we suggest that melatonin mediates the regulation of mitochondrial fission by reinstating the cytoskeleton in porcine oocytes with Drp1 knock-down. This cytoskeletal restoration is crucial for the enhancement of porcine oocyte maturation.

P-305 - Oocyte Storage Vesicle and Lipid Droplet Composition and their Role in Oocyte Quality

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Poor oocyte (egg) quality is a leading cause of reduced fertility in humans and animals, but we do not yet understand how to improve oocyte quality nor how to identify good quality oocytes. Oocyte vesicles, also referred to as vacuoles, have been described using transmission electron microscopy (TEM) in many different species and can constitute up to 30% of the cytoplasm. Despite this, the composition and function of these organelles has been largely ignored in the literature. Using a sheep model of oocyte quality, we have shown that the volume of vesicles is lower in poorer quality lamb oocytes when compared to higher quality adult ewe oocytes, and vesicle volume decreases during maturation, suggesting these organelles are important determinants of oocyte competence. The initial aim of our study was to identify the composition of these oocyte vesicles. Using centrifugation to segregate the different oocyte organelles, and live cell confocal microscopy with Nile red staining and different excitation wavelengths, we have shown that vesicles contain lipid that is of a different form to the lipid droplets conserved by conventional EM fixation methods. We then carried out a lipidomic study using mass spectrometry to compare oocytes from lambs (poor quality) and adult ewes to identify lipids that are lacking in the poorer quality oocytes. Data was analyzed in MS-DIAL using the LipidBlast reference library to identify peaks. The area under the peak was normalized and mean abundance of each metabolite in three pools of 20 oocytes each from adults and lambs was analyzed by t-test. The most abundant lipids in both ages were a mixture of saturated and unsaturated fatty acids. A total of 28 metabolites were significantly increased and 68 were decreased ($P < 0.05$) in lamb compared to adult oocytes and the ontology of 60 of these was identified from the reference library. Diacylglycerides and ceramides were the most common subclass to have reduced abundance in lamb oocytes. Interestingly, progesterone was the sixth most abundant lipid in adult oocytes and was significantly reduced in lamb oocytes. In conclusion, oocyte vesicles contain lipids likely to be saturated lipids not preserved by conventional osmium tetroxide fixation and poorer quality lamb oocytes are deficient in several key lipids. Future work will examine the effect of supplementation of lamb oocytes with progesterone, diacylglycerides and ceramides during in vitro maturation and fertilization to determine if oocyte quality (blastocyst rate) can be improved.

P-306 - Deoxynivalenol Induces the Endoplasmic Reticulum Stress-Mediated Apoptosis via the IRE1/JNK/CHOP Signal Pathway in Porcine Embryos

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The cytotoxic mycotoxin deoxynivalenol (DON) has been reported to adversely affect oocyte maturation and embryo development in pigs. The interplay between cell apoptosis and endoplasmic reticulum (ER) stress has been increasingly recognized as important in embryogenesis. However, the role of the inositol-requiring enzyme 1 (IRE1)/c-Jun N-terminal kinase (JNK)/C/EBP-homologous protein (CHOP) pathway within the unfolded protein response (UPR) signaling in DON-induced apoptosis of porcine embryos remains unclear. In our study, we found that exposure to DON (0.25 μ M) led to a significant decrease in cell viability up to the blastocyst stage. This was associated with the onset of cell apoptosis via the IRE1/JNK/CHOP pathways, as a response to ER stress in porcine embryos. Also, our validation via quantitative PCR (qPCR) results confirmed the upregulation of UPR signal related transcription factors in DON-induced porcine blastocysts. And IRE1/JNK/CHOP signal activations by Western blot analysis, indicating the instigation of ER stress-associated apoptosis by DON exposed embryos in pigs. Further experiments have shown that tauroursodeoxycholic acid (TUDCA) mitigates the ER-stress induced by DON in porcine embryos, indicating that TUDCA can counteract the toxicity of DON on early embryonic developmental competence in pigs during the IVC process. In conclusion, DON exposure impairs the embryonic developmental ability by ER-stress mediated apoptosis via IRE1/JNK/CHOP signal in pigs. These results suggest that they can also be a cause of decreased fertility and embryonic development in human.

P-307 - Medaka Mutant of *cyp21a2* with the Loss of Cortisol Reveals Presence of Two Populations of Pomca-Expressing Cells in Pituitary and the Defect in Reproduction – A New Disease Model

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Cortisol is a critical steroid hormone indispensable for sustaining life in various vertebrates. In human being, an impaired synthesis of cortisol has been well documented as Congenital Adrenal Hyperplasia (CAH) which shows manifesting life-threatening symptoms such as salt-wasting and dehydration accompanied with hyperandrogenism. The defect of CYP21A2 is identified as the most common genetic causes of CAH. Using the teleost fish, medaka (*Oryzias latipes*), we have disrupted the gene of *cyp21a2*. The homozygous mutant does not show any apparent defect during embryogenesis, but after hatching, only 10% reaches adulthood. No cortisol was detected in whole embryos and blood samples by liquid chromatography-mass spectrometry. The mutant exhibits hyperplastic interrenal (adrenal-equivalent organ in teleost) and pituitary, which is recovered upon

administration of cortisol during embryogenesis. We found two populations of pomca (precursor transcripts of ACTH)-expressing cells which responds differently to a level of cortisol. Interestingly, the homozygous mutants of adult males (XY) are fertile while no spawning of eggs is observed. We will describe the critical role of cortisol on ovulation and meiotic arrest along with analysis of an HPG axis.

P-308 - Mitochondrial Contribution to the Oocyte and Embryonic Development

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The energy supply through oxidative phosphorylation (OXPHOS) is mainly considered an essential function of mitochondrion. However, there are alternative roles of mitochondria relevant to female reproduction, such as the synthesis of biomolecules, epigenetic regulation, and the storage of the mitochondrial (mt) genome. Indeed, the complex I of OXPHOS is silenced in the oocyte, and the early embryo relies on glycolysis. We hypothesized that mitochondrial insufficiency does not jeopardize fertilization and early embryonic development. Therefore, we developed a mouse model of a deficient mitochondrial population in the oocyte: transcriptional factor A, mitochondrial (Tfam) conditional knock-out (TfamloxPloxP/Zp3-Cre) females as donors of Tfamnull oocytes. We observed that TFAM insufficiency significantly decreases mitochondrial population in Tfamnull oocytes. Surprisingly, a decreased amount of mtDNA does not affect either the fertilization rate of Tfamnull oocytes or the success of early development of Tfam^{+/-} embryos. We observed a negligible amount of TFAM expressed from parental allele after embryonic genome activation and, accordingly, blastocysts were developed in spite of an imperceptible amount of TFAM. Taken together, we suggest the indispensability of mitochondrial population in the oocyte and early embryonic development due to OXPHOS silence due to the late blastocyst. We expect the Warburg effect to cover the energy demand of the oocyte and early preimplantation embryo; on the other hand, the genetic aspect of mitochondrial insufficiency should be considered whereas impaired biogenesis can lead to the accumulation of mtDNA mutations. Funding This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic: the Cooperatio Program, research area MED/DIAG of Charles University; Grant SVV 260 651, and MSTC in the Danube Region (Grant No. 8X23009).

P-309 - In VITRO STUDY of PHOENIXIN-14 ACTION on PORCINE LUTEAL CELLS ANGIOGENESIS and APOPTOSIS: ROLE of GPR173 and KINASES ERK1/2, AKT, AMPK PATHWAYS

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Phoenixin-14 (PNX-14) is a neuropeptide with a well-established function in reproductive processes at the hypothalamic-pituitary-gonadal axis. However, its role in the physiology of the ovary, and especially the corpus luteum (CL) function requires further research. Our previous study showed

that PNx-14 and its putative receptor GPR173 are expressed in the porcine CL during the estrous cycle and PNx-14 has a luteotropic action by regulating the endocrine function of luteal cells. In the current study, we focused on understanding the role of PNx-14 in the process of angiogenesis and apoptosis occurring in the CL. We performed in vitro culture of luteal cells isolated from the porcine CL from days 10-12 of the estrous cycle. Luteal cells were treated with PNx-14 at increasing doses 1-1000 nM and after 24 h of incubation, we checked the transcript level and secretion of angiogenic factors (VEGFA, bFGF2, ANG-1) as well as gene expression of apoptotic factors (caspase-3, -8, -9, Bax, Bcl-2), the activity of caspase 3/7 and level of DNA fragmentation. Additionally, using pharmacological inhibitors of extracellular signal-regulated kinases 1/2 (ERK1/2), protein kinase B (AKT), 5'AMP-activated protein kinase (AMPK) as well as silencing the GPR173 receptor by siRNA, we study the molecular mechanism of PNx-14 action on luteal cells angiogenesis and apoptosis. The results were analyzed by one-way ANOVA in GraphPad Prism 8. We observed that PNx-14 increased the level of angiogenic factors in luteal cells: bFGF2 at all used doses and ANG-1 on gene level at 10 nM and its secretion at 1 nM. The effect of PNx-14 on bFGF secretion was mediated by GPR173 receptor and ERK1/2 and AKT. PNx-14 decreased gene expression of caspase-3, -8, and Bax, while had no effect on caspase-9 and Bcl-2 level. Additionally, caspase 3/7 activity was decreased after PNx-14 treatment, and its effect was mediated by GPR173, AKT, and AMPK. DNA fragmentation was downregulated only upon PNx-14 at 100 nM. The current study confirmed that PNx-14 is a luteotropic factor in the CL of pigs by stimulation the process of angiogenesis and protects luteal cells against apoptosis. Grant support: National Science Center, Poland (PRELUDIUM project no.: 2020/37/N/NZ9/00981).

P-310 - Characterization of Human Follicular Fluid Extracellular Vesicle Subtypes and Their Impact on Human Granulosa-Like Tumor Cell Line KGN Transcriptome

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Extracellular vesicles (EVs) are lipid-bilayered nanoparticles released by all cell types. They carry biologically active cargo, including proteins, lipids and nucleic acids that influence recipient cell function. The presence of extracellular vesicles in follicular fluid (FF) has been established across various species, with indications that FF EVs may play a role in oocyte maturation and granulosa cell proliferation. While previous studies have focused on the collective pool of FF EVs, EV subtypes may serve distinct functions within the ovary. This study aimed to characterize the surface tetraspanin profile of FF EV subtypes and investigate their impact on the transcriptome of the human granulosa-like tumor cell line KGN. Ethical approval was obtained from the Research Ethics Committee of the University of Tartu, Estonia. FF was collected from preovulatory follicles (>18 mm diameter) at Nova Vita Clinic in Tallinn, Estonia. Small and large EVs (SEVs and LEVs, respectively) were purified from FF pools using size exclusion chromatography followed by tangential flow

filtration. EV characterization involved nanoparticle tracking analysis (NTA) with ZetaView PMX110 (Particle Metrix), transmission electron microscopy (TEM) with JEM-1400 (Jeol), and single vesicle analysis with the ExoView R100 platform (NanoView Biosciences). For RNA-sequencing, KGN cells were incubated with 109/ml SEVs (n=6), 108/ml LEVs (n=6) or DPBS (n=6) for 24 hours and then lysed. Total RNA was extracted using the Qiagen miRNeasy kit, and sequencing libraries were prepared from 200 ng of RNA using the Revvity NextFlex Rapid Directional RNA-seq kit. Single-end sequencing was performed with the Illumina NextSeq2000 instrument. Reads were mapped to and annotated according to the human genome version GRCh38 (Ensembl). Differentially expressed genes (DEGs) were identified with the R package DESeq2 version 1.42.0 and DEGs with an adjusted p-value ≤ 0.1 were selected for further analysis. The mean diameter of isolated SEVs and LEVs was 93 nm and 299 nm, respectively ($p = 2.26 \cdot 10^{-11}$). In total, an average of $8.4 \cdot 10^{10}$ SEVs and $2.4 \cdot 10^9$ LEVs were obtained from 20 ml of FF. TEM confirmed the presence of EVs with round morphology. ExoView analysis revealed differences in the surface tetraspanin profile between the EV subtypes, with a higher proportion of CD9+CD63+CD81+ particles in SEVs compared to LEVs ($p < 0.05$). RNA-seq analysis of KGN cells treated with SEVs showed 1006 DEGs, with 642 downregulated and 364 upregulated genes. G:Profiler analysis categorized downregulated genes into pathways related to cholesterol biosynthesis, cell cycle and extracellular matrix organization, while upregulated genes were enriched into translation-related pathways. Treatment with LEVs resulted in 86 DEGs, with 63 downregulated genes enriched into extracellular matrix and adhesion pathways, and 23 upregulated genes enriched into translation-related pathways similarly to SEVs. This study sheds light on distinctive tetraspanin marker compositions among EV subtypes in human FF, suggesting that both subtypes possess unique molecular characteristics. Notably, both SEVs and LEVs alter the expression of genes related to extracellular matrix regulation and translation processes, both essential for ovarian follicle development and oocyte maturation. Furthermore, SEVs, but not LEVs, downregulate genes related to the biosynthesis of cholesterol, a precursor for the synthesis of steroid hormones. This suggests a potential mechanism through which SEVs contribute to the maintenance of the hormonal environment within the follicle. These findings provide novel insights into the functionality of FF EV subtypes and enhance our understanding of the intercellular communication within the ovarian follicle.

P-311 - Double Knockout of NR5A1 and NR5A2 in the Mouse Ovary Disrupts Follicle Development and Ovulation

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The two orphan receptors Nr5a1 (Steroidogenic factor 1; SF-1) and Nr5a2 (Liver receptor homolog receptor-1; LRH-1) are essential for ovarian function. Previous studies have shown that LRH-1 regulates multiple processes necessary for ovulation, while SF-1 plays roles in follicle assembly and subsequent development. Both receptors bind to the same motif sequence to regulate gene transcription, and studies indicate that they act on both common and distinct gene targets. Individual depletion of SF-1 or LRH-1 suggests there is little compensation of one for the other in the ovary. To further understand the roles of these receptors, we established a model of conditional depletion of both SF-1 and LRH-1, using Cre recombinase under the control of the Amhr2 promoter (Amhr2Cre; Nr5a1 f/f; Nr5a2 f/f; dKO). In the resultant mouse strain, the LRH-1 and SF-1 signals were abolished in granulosa cells of dKO ovaries, while SF-1 persisted in cells of the theca and the ovarian stroma. Tabulation of the follicle populations at postnatal day (PND) 4 revealed that the

ovarian reserve was reduced, in that there were five-fold fewer primordial follicles in the dKO ovaries compared to controls, indicating an effect on follicle assembly. The primary follicle population was similarly disrupted at PND4, with an approximate threefold reduction in dKO ovaries relative to control, demonstrating an effect on the activation of primordial follicles. This was further in evidence at PND13, where the population of secondary follicles was half of that in control ovaries. In adult (five months of age) dKO mice, the primordial follicle population was entirely depleted, with near depletion of primary and secondary follicles. As some antral follicles persisted at five months, we sought to determine whether antral follicles in dKO mice were capable of ovulation. Immature (25-28 days of age) dKO and control mice were treated with 5IU eCG followed by 5IU hCG. The control mice displayed a mean of 39 ± 5 ovulations, while only a few of the dKO mice ovulated < 3 oocytes. Analysis of transcript abundance by qPCR in dKO and control ovaries following gonadotropin treatment indicated that concurrent depletion of LRH1 and SF-1 dysregulated genes associated with steroidogenesis, extracellular matrix formation, and cell proliferation, among others. We conclude that the combination of depletion of LRH-1 and SF-1 in mouse granulosa cells has profound negative effects on follicle assembly, follicle activation, further follicle development, and ovulation. Supported by PJC 166020 from the Canadian Institutes of Health Research to BDM and NG.

P-312 - Female Offspring Ovarian Chemical Metabolism Protein Abundance is Altered by In Utero Atrazine Exposure

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Atrazine (ATZ) is an endocrine-disrupting chemical, and low-dose ATZ exposure during perinatal development is linked to reproductive dysfunction and behavioral abnormalities in adulthood. Also, gestational ATZ exposure can lead to adverse outcomes, including low birth weight, delayed fetal growth, and delayed onset of puberty. To investigate the effects of ATZ exposure on female offspring, timed pregnant nulliparous sows were provided ad libitum access to water that contained an environmentally relevant concentration of ATZ (20 µg/L) or vehicle control [0.002% (v/v) ethanol]. Based on their estimated water intake and average body weight, treated sows were exposed to ATZ doses of ~0.0014 mg/kg BW. Piglets were exposed to control or ATZ in utero from gestation day 28 through farrowing and lactation (~127 days total). Sows were farrowed and allowed to nurse their piglets ad libitum. The abundance of chemical metabolism proteins in postnatal day 10 offspring ovaries (n = 3 per treatment) was measured using western blotting. Statistical analyses using unpaired t-test with no adjustment were performed using GraphPad Prism 8.4.1 software. Piglets exposed to ATZ had reduced levels of ovarian phase I biotransformation proteins CYP1A1, CYP1B1, and CYP2E1, and reduced phase III xenobiotic transporter, ABCB1 compared to the

control group ($P < 0.05$). There was no effect ($P > 0.05$) of ATZ exposure on EPHX1 or GSTP1. The ovarian steroidogenic protein CYP11A1 was also decreased due to ATZ exposure ($P < 0.05$), with no impact observed in the level of CYP19A1 between the groups ($P > 0.05$). Overall, the study suggests that ATZ exposure during gestation and lactation can impair the ovarian abundance of chemical biotransformation proteins including those involved in steroidogenesis in the female offspring of in utero exposed pigs.

P-313 - The effects of Heat Stress on the Abundance of Proteins Involved in Chemical Biotransformation in the Corpora Lutea of Post-Pubertal Gilts.

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Heat stress (HS) is an environmental stressor that modulates physiological changes that impact target organs. Seasonal infertility in pigs is associated with HS, manifested as smaller litters, delayed puberty onset, increased abortion, reduced conception rate, and a longer wean-to-estrus interval. Chemical biotransformation enzymes transform parent products into metabolites with lesser or greater toxicity. Since HS causes hyperinsulinemia, and insulin regulates chemical biotransformation, this study investigated the abundance of proteins involved in chemical biotransformation in the corpus luteum (CL). Crossbred post-pubertal gilts were exposed to thermal neutral (TN, $20 \pm 1^\circ\text{C}$, $n=7$) or cyclical HS ($35 \pm 1^\circ\text{C}$ for 12 h and 31.6°C for 12 h, $n=7$) conditions from 2 to 12 d post-estrus. The ovarian abundance of proteins involved in chemical metabolism, glutathione S-transferase Pi (GSTP1), microsomal epoxide hydrolase 1 (EPHX1), cytochrome P450 2E1 (CYP2E1), and ATP binding cassette subfamily B member 1 (ABCB1), were quantified via western blotting. Statistical analyses were performed using GraphPad Prism 8.4.1 software using unpaired t-tests without adjustment. The CL abundance of EPHX1, CYP2E1, and ABCB1 were not altered ($P > 0.05$) by HS, however, the abundance of GSTP1 was increased ($P < 0.05$) by HS. GSTP1 functions in several mechanisms including chemical biotransformation, oxidative stress response, and apoptosis regulation, thus increased luteal GSTP1 due to HS may be a protective mechanism to preserve CL function. This project was supported by the Iowa Pork Producers Association.

P-314 - Characterizing the G-protein activities of the membrane Androgen Receptor, ZIP9

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Androgens mediate their physiological functions via the classical nuclear receptor, AR. However, recent studies have identified a new plasma membrane (and non-genomic) androgen receptor- the Zn(II) channel, ZIP9. ZIP9 is ubiquitously expressed and localised to gonadal tissue. Recent studies have suggested the role of zinc transporters in ovarian follicle growth, with several ZRT-and Irt-like Protein (ZIP) family members shown to be expressed in a stage-specific manner during follicle

development, highlighted potential physiological importance of ZIP9 in modulating ovarian functions. The binding of androgens to ZIP9 has been proposed to activate “non-classical” testosterone signalling pathways, via coupling to G proteins. However, how androgens and zinc interplay to modulate G protein activation remains unclear. This study therefore aimed to determine how androgens and zinc regulate ZIP9-dependent G protein coupling. To determine the effect of ZIP9 and its interaction with ligands and G proteins, HEK293 cells over-expressing ZIP9 were utilised to assess ZIP9-dependent Gs and Gi coupling via assessment of cAMP production using a live kinetic cAMP reporter- GloSensor. To assess Gi coupling, HEK293 cells expressing ZIP9 were pre-treated with 10 μ M forskolin for 10 minutes to elevate cAMP prior to ligand treatment, whilst Gs coupling was detected with the addition of ligand alone. Cells were stimulated with 100 nM testosterone alone, 20 μ M zinc alone or the combination treatment of testosterone and zinc and cAMP production was monitored for up to 60 minutes. Experiments were conducted with a minimum of n=3 in triplicate measurements. Treatment of HEK293 cells expressing ZIP9 with either testosterone or zinc alone, or in combination had no effect on cAMP production, suggesting that ZIP9 ligands didn’t include coupling of ZIP9 to Gs. To rule out that transiently transfected ZIP9 was expressed at a protein level with the plasmid concentrations transfected, Western blot analysis of transfected cell lysates was carried out and showed that increasing Zip9 plasmid concentration increased the protein expression of ZIP9, supporting that ZIP9 was expressed and didn’t couple of Gs. Next, we investigated if ZIP9 was coupling to Gi. Interestingly, in forskolin pre-treated conditions basal cAMP was modulated by the amount of ZIP9 plasmid expressed, with increasing inhibition of forskolin-induced cAMP production corresponding with increasing ZIP9 expression. This suggests that ZIP9 constitutively couples to Gi. Next, ligand treatment was assessed. At a high Zip9 expression, treatment with either testosterone, zinc or a combination of both showed no significant change in cAMP production. Interestingly, at low ZIP9 expression, single treatments with zinc or testosterone abrogated basal ZIP9-Gi coupling. However, combined testosterone and zinc treatment enhanced cAMP production, suggesting a potential switch to Gs coupling, dependent on Zip9 expression level and ligand. To understand the potential physiological implications of this dual ZIP9-Gs/Gi coupling, isolated mouse granulosa cells were treated with either 20 μ M of zinc and 100 nM of testosterone, or co-treatment 24-hour exposure on Zip9 expression. Whilst single treatments of either testosterone or zinc alone did not affect Zip9 expression, co-treatment downregulated Zip9 expression. The G protein coupling of ZIP9 in granulosa cells is currently underway to explore the mechanism underpinning this. These data indicate that ZIP9 displays dual Gs/Gi coupling that is dependent on its expression level and ligand concentrations. This may have important implications in the regulation of reproductive disorders with high androgens including PCOS, with the ovarian roles important next steps to explore.

P-315 - TRPV3 and Cav3.2 ion Channels Functionally Interacts in Mouse eggs

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Oocyte maturation or the acquisition of meiotic competence requires a controlled expression of proteins that supports this process in preparation for fertilization. Both, oocyte maturation and fertilization are determined by a highly regulated ion homeostasis. Several ion channels, regulating diverse cellular processes, have been reported to be expressed in eggs from different species, including mammals. Fertilization starts with the release of the sperm-specific phospholipase ζ (PLC ζ) in the mature oocyte, egg. Ca^{2+} influx is required to accumulate Ca^{2+} in the oocyte in preparation for fertilization, as well as to refill its intracellular stores during fertilization, supporting Ca^{2+} oscillations and egg activation. The egg activation includes the formation of the pronucleus, cortical granule exocytosis, polyspermy blockage, and the completion of meiosis II, between other processes to support the transition to early embryonic development. The voltage-gated activated calcium channel Cav3.2 has been reported to be expressed and contributes to the refilling of the Ca^{2+} store in preparation for fertilization in mouse eggs. In addition, the transient receptor channel from the subfamily vanilloid, TRPV3, a cationic non-selective channel, has been shown to be expressed in mouse eggs, however, its physiological function is currently unknown. Here, using eggs lacking TRPV3 and Cav3.2 proteins, we evaluate their role in Ca^{2+} influx and cortical granule distribution.

Methods: Using KO animal models, confocal microscopy, bioinformatics and patch-clamp electrophysiology, we tested the expression and function of TRPV3 and Cav3.2 ion channels in mouse eggs, and evaluated their role in cortical granule dynamics.

Results: Cav3.2 currents at -20 mV are significantly decreased in TRPV3KO eggs (~8 pA/pF WT eggs vs. 3,75 pA/pF in TRPV3KO eggs). TRPV3 currents in response to the agonist 2- APB are decreased in Cav3.2KO eggs (41 pA/pF in WT vs. 30,5 pA/pF in Cav3.2KO eggs). TRPV3KO and TKO eggs show an impaired cortical granule distribution, measured as fluorescence intensity of labeled lens culinaris agglutinin, in comparison to WT eggs. Bioinformatics approaches reveal potential sites/residues for physical interaction between Cav3.2 and TRPV3 proteins.

Our results suggest a functional and/or physical interaction of Cav3.2 and TRPV3 that might modulate critical cellular processes as cortical granule distribution, underlying egg-to-embryo transition in mammals.

Acknowledgments: FONDECYT 1221308; FONDEQUIP ANID EQM200122

P-316 - A Disabled-1 (DAB1) Isoform is Differentially Expressed in Granulosa Cells of Healthy Antral Follicles and Contributes to Cytoplasmic Vesicular Transport

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The cytosolic adaptor protein Disabled-1 (DAB1) plays a role in the Reelin signaling pathway and is important in the proper development of the brain and cerebellum cortices in vertebrates. Reelin, an extracellular protein, binds to its membrane receptor, the apolipoprotein E receptor 2 (ApoER2), to induce tyrosine phosphorylation of DAB1. We have previously shown that both Reelin and its receptor ApoER2 are differentially expressed in dominant or preovulatory follicles, with Reelin being expressed in theca cells (TC) while ApoER2 is expressed in granulosa cells (GC), suggesting that Reelin is involved in a paracrine signalling pathway during follicular development. Therefore, the objective of this study was to characterize the expression and function of DAB1 in GC of bovine follicles. For this study, an in vivo model composed of GC obtained at different follicular stages was used: small follicles (SF: 2-4 mm), growing dominant follicles (DF) at day 5 of a synchronized estrous cycle and ovulatory follicles (OF) obtained at 0, 6, 12, 18 and 24 hours post-hCG injection. We found that the DAB1 isoform expressed in GC is alternatively spliced, lacking exons 7, 8 and 16, and thus missing three phosphorylated tyrosine residues (Y200, Y220 and Y232) out of the five normally found compared to the canonical brain isoform. In addition the DAB1 isoform expressed in GC included exons 10, 11 and 18 which are not found in the brain isoform. RT-qPCR results show that this DAB1 GC isoform was significantly upregulated in DF compared to OF and decreased significantly 6 hr following hCG injection. Specific antibody raised against DAB1 exon 11 showed by western blotting a single protein band from GC extract, and the signal decreased significantly 6 hr following hCG in OF compared to DF. Immunohistochemistry observations localized DAB1 isoform in GC of healthy follicles, and in cumulus cells, with a stronger signal in the corona radiata. Moreover, the DAB1 signal was associated with endosomal and perinuclear vesicles. Vesicles were also observed under the zona pellucida in association with the cumulus cells transzonal projections. Post-translational modification analyses by immunoprecipitation followed by mass spectrometry of DAB1 has to date identified 7 phosphorylated sites (S64, S90, S114, S468, T88, T101, Y115) and 4 glycosylated sites (S237, S241, S461, T121). Yeast two-hybrid screening identified potential DAB1 binding partners in GC that include: CLTA, CCT8, LAPTM4B and SRGN. Taken together, these results provide for the first time the characterization of a DAB1 isoform in bovine GC, and support a role in vesicular-transport in healthy growing follicles and a possible interaction between cumulus cells of the corona radiata and the oocyte.

P-317 - In Vitro Embryo Production can be Achieved After Delayed Harvest of Bovine Oocytes from Abattoir-derived Ovaries

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Oocytes from abattoir-derived ovaries are widely utilized for in vitro production (IVP) of bovine embryos. Most current protocols require that cumulus-oocyte-complexes (COC) be harvested from the ovary within a few hours of collection. Opportunities exist for additional research and flexibility in IVP embryo protocols if abattoir ovaries can be maintained for longer periods of time before COC collection. We hypothesized that blastocysts can be produced from ovaries maintained at 15°C for 17 or 24 hours (h) beyond normal COC harvesting time. Ovaries were obtained from an abattoir and transported 3.5 h to the laboratory at 20 – 25°C in saline containing antibiotics and antimycotics. Ovaries were then either used immediately (control) or maintained at 15°C for an additional 17 h or

24 h. All COCs were matured (25 COCs/drop; 4 drops/treatment/replicate; 8 replicates) and fertilized using pooled Holstein semen. Presumptive zygotes were cultured in SOF-BE1 drops with cleavage visually evaluated on d 2 post-fertilization. Blastocyst development was assessed on d 7.5. A portion of blastocysts (25 embryos/treatment) were assessed by immunofluorescent detection of CDX2, a trophectoderm (TE) marker, and DAPI counterstaining for total nuclei. Cleavage rate was reduced ($P < 0.003$) for the 17h group ($61.1\% \pm 3.5\%$) and the 24h group ($59.4\% \pm 3.7\%$) compared to the control group ($78.0\% \pm 2.1\%$). The percentage of blastocysts generated from total zygotes was greatest in the control group ($29.2\% \pm 2.5\%$), tended to be reduced ($P = 0.06$) in the 17 h group ($24.0\% \pm 2.3\%$), and was reduced ($P = 0.002$) in the 24 h group ($10.6\% \pm 1.1\%$). Blastocyst formation as a percentage of cleaved embryos demonstrated no differences between the control and 17 h group ($48.2\% \pm 5.9\%$ vs $41.7\% \pm 3.2\%$, respectively), but the percentage of blastocysts formed was reduced ($P = 0.02$) in the 24 h group ($20.1\% \pm 4.1\%$). Total cell number, TE cell number and ICM:TE ratio did not differ for blastocysts in each treatment, but ICM cell number was reduced ($P = 0.04$) in the 24h group when compared to the control group. No differences in ICM cell number were detected between the control and 17 h treatment groups. In summary, abattoir ovaries maintained in saline at 15°C for 17 h prior to COC collection contained a slight reduction in cleavage rates but otherwise produced blastocysts at the same rate with the same TE and ICM composition as COCs harvested at the conventional time. Delaying harvest by 24 h was harmful to blastocyst yields and quality. This project was supported by the Agriculture and Food Research Initiative Competitive Grant no. 2021-67015-34485 from the USDA National Institute of Food and Agriculture.

P-318 - Somatic Cell Fate Specification and Separation in the Fetal Ovary

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Proper differentiation of somatic cell types in the fetal ovary lays the foundation for future ovarian function in the adult. Defects in cell type differentiation lead to diseases including polycystic ovarian syndrome (PCOS), primary ovarian insufficiency (POI), and infertility in women. Understanding how each cell type is established is crucial in developing methods to intervene in ovarian diseases caused by cellular dysfunction. The two major somatic cell lineages in the ovary include supporting cells that secrete hormones and provide nutrients to the germ cell, and interstitial cells that produce hormones and maintain ovarian structural integrity. While it is known that supporting cells and interstitial cells originate from a common somatic progenitor within the fetal ovary, a critically unanswered question is how interstitial cells are specified apart from the supporting cells during ovarian development. To address this question, we performed single-cell mRNA sequencing of mouse ovarian cells at the beginning of ovary formation. We discovered two major somatic populations, one positive for the nuclear receptor Nr2f2, and the other positive for

the nuclear receptor Nr5a1. To investigate the differences between these two populations, we performed differential gene expression analysis and identified that the two cell populations express different components of an important signaling pathway, the Notch pathway. Specifically, the Nr2f2+ population expresses Notch receptor Notch3 and the downstream transcription co-activators, whereas the Nr5a1+ population expresses high level of Notch antagonist Numb. This suggests that Notch pathway is active in the Nr2f2+ population, and conversely, is repressed in the Nr5a1+ population. To test whether Notch signaling specifies somatic fate, we performed lineage tracing experiments by labeling early Notch-active cells in the fetal ovary. We found that these Notch-active cells later become interstitial cells in the adult ovaries. Single-cell mRNA sequencing of lineage-traced ovaries identified that the early Notch-active cells co-express Nr2f2 and were predicted to be endothelial cells and pericytes upon cell type analysis. Finally, to explore whether Notch signaling directs interstitial fate determination, we ectopically activated Notch pathway in the Nr5a1+ population and observed significant increase of interstitial-to-supporting cell ratio and a disrupted morphology in the fetal ovary. This suggests that a balanced Notch pathway is critical in determining the somatic composition during ovarian development. In conclusion, our results support a model in which active Notch signaling in Nr2f2+ cells specifies the interstitial cell lineage and separates them from the Nr5a1+ supporting cell lineage at the beginning of ovary formation.

P-319 - Expression levels of FSHR, IGF-1R, AMH, AMHR II , EGFR, Bax and Caspase-3 in Cumulus cells are correlated Developmental Competence of Bovine Oocytes.

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The generation of animal embryos by in vitro production (IVP) methods and of human embryos by artificial reproductive techniques (ART), is hampered by the failure of many of the embryos to develop to the blastocyst stage. This problem occurs even when cumulus oocyte-complexes (COCs) with good morphology are visually selected and used for culture. Thus, although variation in quality of COCs cannot be identified morphologically, it can have a significant effect on the success or failure of embryo development. Cumulus cells (CCs) are important for oocyte maturation and subsequent embryo development. The relationship between CCs status and the developmental competence of the oocyte is unknown; here, we hypothesized that the condition of CCs must reflect oocyte competence. Therefore, we investigated the relationship between the expression of genes in bovine CCs that are related to follicle development and to competence for blastocyst development. COCs were aspirated from bovine ovaries obtained in a slaughterhouse. The CCs located closest to the oocyte were collected with a glass pipette (about 100 cells). The collected CCs were washed in PBS and stored at -80°C until used for real-time PCR analysis. The COCs from which the CCs were collected were used for in vitro maturation, in vitro fertilization, and in vitro embryo development. Oocyte developmental competence was evaluated by screening for cleavage and development to the blastocyst stage under a stereomicroscope at 7 days after insemination. CCs collected before in vitro maturation were used for real-time PCR after RNA extraction to quantify the mRNA levels of FSHR, IGF-1R, AMH, AMHR II , EGFR, ER β , Bax, and Caspase-3; the relative levels of expression were measured using the $\Delta\Delta C_t$ method. The CCs of COCs that

developed into blastocysts were assigned to the blastocysts (BL) group, and those that failed to reach the blastocyst stage were assigned to the 2cell-Morula (2c-M) group. Gene expression levels were compared between the two groups of CCs. We found that the expression in the BL group of FSHR, IGF-1R, AMH, and EGFR were higher; and that of Bax and Caspase-3 were lower than in the 2c-M group ($P < 0.05$). Positive correlations were found between FSHR and IGF-1R expression levels ($r = 0.59$) and between ER β and EGFR expression levels ($r = 0.43$) in the BL group ($P < 0.05$). These results indicate that the state of the CCs are correlated developmental competence of bovine oocytes. The expression levels of FSHR, IGF-1R, AMH, EGFR, Bax, and Caspase-3 in CCs offer a non-invasive means of predicting oocyte developmental competence.

P-320 - Effect of Novel Plasticizers, Bisphenol S and F, on The Meiotic Maturation and Spindle Structure of Mouse Oocytes

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According to the WHO, infertility is a growing health problem across the world. One of the potential reasons for this situation is increasing exposure to endocrine-disrupting chemicals (EDCs), such as a widespread plasticizer bisphenol A (BPA). Due to its xenoestrogenic properties and harmful health effects, it has been often substituted with analogs, such as bisphenol S (BPS) and bisphenol F (BPF). BPS/BPF were detected in human body fluids, including (for BPS) follicular fluid. Their impact on organismal functions, including the reproductive system and fertility remains still largely unknown, although some studies suggest that they may influence the quality of mammalian oocytes. In our preliminary study, we checked whether BPS and BPF affect in vitro meiotic maturation of mouse oocytes and structure of their metaphase II spindles. Briefly, we cultured prophase I oocytes for 16 hrs in a medium supplemented with increasing concentrations of BPS or BPF, corresponding to their reported or expected concentration in human follicular fluid. Oocytes cultured in pure medium or medium with BPS/BPF solvent DMSO (in the respective concentration) served as controls. Afterwards, oocytes were fixed and stained for β -tubulin and DNA to visualize the spindle structure. Metaphase II spindles were classified as abnormal if did not display a typical bipolar shape and/or had misaligned chromosomes. Additionally, spindle volume and ratio of length to width were measured. Our data showed that meiotic maturation was not disturbed by any of BPS/BPF analyzed concentrations (2, 20, 200 ng/ml for BPS and 0.2 ng/ml for BPF). On the other hand, we found that 20ng/ml BPS ($n=53$; 32.08%) exposure significantly elevated the percentage of abnormal meiotic spindle formation compared with oocytes cultured in pure medium ($n=117$; 16.24%) and medium with DMSO ($n=40$; 12.50%) ($p < 0.05$). Furthermore, we showed that 20ng/ml BPS during in vitro maturation modifies spindle morphology (classified previously as normal): significantly decreased spindle volume ($n=10$; $1385 \pm 161 \mu\text{m}^3$) compared to pure medium ($n=8$; $1732 \pm 222 \mu\text{m}^3$; $p < 0.01$) or DMSO ($n=10$; $1624 \pm 251 \mu\text{m}^3$; $p < 0.05$) groups. Additionally, 20ng/ml BPS significantly elevated the ratio of spindle length to width ($n=10$; 2.44 ± 0.21) compared to pure medium ($n=8$; 2.12 ± 0.21 ; $p < 0.05$) or DMSO ($n=10$; 2.09 ± 0.11 ; $p < 0.01$) groups. To sum up, our preliminary data suggests that at least BPS may not be neutral for oocyte quality, which is why further research on their action is needed. This research was funded by the National Science Centre (Poland) grant no. 2022/45/B/NZ5/02039 to AA.

P-321 - Application of Transient Gene Expression Using Lipid Nanoparticle During Early Embryo Development.

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Zygotes and preimplantation embryos have unique features compared to other cell types. For example, they have zona pellucida (ZP), which protects zygotes/embryos against physical damage and microbial infections. Due to these features, microinjection has been a first choice for gene delivery into zygotes. Alternative methods are electroporation or use of viral vectors. In addition, it has been reported that lentivirus vector can be used for gene introduction in blastocyst embryos and this showed gene expression only in trophectoderm (TE) which will become placenta in the future. However, it requires to remove ZP for lentivirus vector infection to blastocyst. Since each method have advantages and disadvantages, we aim to find new methods to introduce gene into zygotes or embryos. We focused on lipid nanoparticle (LNP) which has been used for mRNA delivery. We have showed that LNP can be used for transient gene expression in zygotes in the previous meeting. In this study we examined whether LNP can be used for gene introduction in blastocyst embryos. To observe mRNA delivery and subsequent expression, we used mTmG reporter mice, which express tdTomato but switch to EGFP after Cre recombinase-mediated excision of the tdTomato cassette. We treated mTmG blastocyst with cre mRNA encapsulated LNP overnight and checked their fluorescence. We observed that there were embryos that showed green fluorescence without removing ZP. Interestingly we observed green fluorescence only in TE but not in inner cell mass (ICM) suggesting that LNP can use for placenta specific gene transduction. This may help us to study the function of placenta and embryo developments.

P-322 - Expression of Estrogen-Related Receptor GPR30 in Mouse Metaphase II Oocytes and Its Effect on Fertilization-Induced Ca²⁺Oscillations

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Estrogen receptors such as classical receptors alpha (ERa) and beta (ERb), and estrogen-related receptor GPR30 play a key role in the hormonal regulation of female reproduction. They may be also targeted by numerous exogenous agents, such as endocrine disrupting compounds (e.g., plasticizers such as bisphenol A) or substances used in cancer treatment (e.g., tamoxifen), which may modify their activity and, in consequence, disrupt female fertility. To address this issue, we wish to investigate whether these receptors participate in the regulation of calcium homeostasis in oocytes, a process crucial for early embryo development. Fertilization-induced oscillations of the cytoplasmic concentration of free calcium ions ([Ca²⁺]_i) serve as a mechanism responsible for activation of the embryo development. Moreover, the pattern of [Ca²⁺]_i oscillations influences implantation and postimplantation development of the embryo. In our study, we used mouse oocytes as a model. Our results indicate that mRNAs for all three receptors are present in mouse metaphase II (MII) oocytes with mRNA encoding ERb being the most highly expressed.

Subsequently, using immunofluorescence staining, we found that all three receptors were present in the oocyte cytoplasm, but ER β and GPR30 were also localized in the cell membrane and thus may affect oocyte physiology in a more direct way, which do not involve their migration into the nucleus and regulation of gene expression. Finally, we focused on GPR30, a potential target for xenoestrogens such as bisphenol A, and assessed if its activity influenced the fertilization-induced [Ca²⁺]_i oscillations. We matured prophase I (GV) oocytes in vitro in the presence of either GPR30 agonist (G1) or antagonist (G15). Subsequently, we followed [Ca²⁺]_i in in vitro fertilized MII oocytes. Our preliminary data suggest that the number and total duration of [Ca²⁺]_i transients were decreased in MII oocytes if GPR30 was activated during in vitro maturation. Interestingly, inhibition of GPR30 also shortened the duration of [Ca²⁺]_i oscillations but did not affect the number of [Ca²⁺]_i transients. Taken together, our initial data indicate that estrogen-related receptor GPR30 might influence fertilization-induced [Ca²⁺]_i oscillations. Although we still need to determine whether ER β can affect fertilization-induced Ca²⁺ oscillations, our results strongly suggest that deregulation of estrogen receptors may affect oocyte ability to respond to fertilization. This research was funded by the National Science Centre (Poland) grant no. UMO-2022/45/B/NZ5/02039 to AA.

P-323 - Maturation of Bovine Cumulus-Oocytes Complex in Microfluidic Device: Effects on Lipid Accumulation and Blastocyst Rates

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The study of oocyte in vitro maturation (IVM) is significant as it can still improve oocyte quality, which is the ability to be fertilized and to develop a healthy embryo. Based on that, new techniques applied to the in vitro maturation can help to increase the in vitro production rates. The conventional IVM method consists of culturing COCs in plates and in restricted media volume. One of the limitations of this type of culture can be the access to nutrients, hormones, and other molecules as well as the accumulation of metabolic waste, which can impact the maturation process. Compared with the in vivo situation we believe that the dynamic exchange of the microenvironment within the follicle could impact the maturation success. In this case, we constructed a reversible and reusable microfluidics device that enables cumulus-oocyte complex (COC) culture while media exchange in real-time. We used a reversible sealing and reusable microdevice to perform IVM and evaluated lipid accumulation at the end of the maturation period. Initially, we developed and validated a reusable and reversible sealing microfluidic device in laminated polydimethylsiloxane (PDMSLAM), glass, and Polymethyl methacrylate (PMMA-acrylic). The microdevice validation steps included flow rate, media recovery, media evaporation, and pH changes. After that cumulus-oocyte complex (COC) from bovine ovaries were aspirated and COCs with one or two layers of cumulus cells and homogeneous cytoplasm were selected to continue in the experiment. The COCs were matured in TCM119 B supplemented with 10 μ g/ml sodium

pyruvate, 25µg/ml gentamicin, 0.4% BSA, 5 ng/ml 10–2 IU/ml human recombinant FSH and 50 ng/ml human recombinant luteinizing hormone (LH). The COCs cultured in plates were cultured with 30µL of medium, and the oocytes cultured in microfluidic were cultured with 6µL of medium with a constant flow rate of 1µL. min⁻¹ for 22 h. We performed 5 routines with a pool of 60 COCs per routine (30 in plates-control and 30 in microfluidic device). After 21h in maturation media COCs were stripped and only the matured COCs were stained with BODIPY, and the lipid droplet profile was evaluated. The matured COCs were also parthenogenetic activated to verify whether microfluidics affects the blastocyst rate. The data were expressed as means ±SEM, and $p < 0.05$ was considered a significant difference. We observed that lipid droplets in COCs matured on microdevices were two folds smaller than in COCs matured on plates. Furthermore, we did not observe statistical differences in maturation and blastocyst rates. However, the blastocysts from COC that were subjected to in vitro maturation in microdevices showed larger diameter and an increased number of cells than oocyte matured in culture plates ($P < 0,0002$). The results suggest that microdevice does not interfere with the COC maturation process but improves lipid accumulation and blastocyst cell numbers. The microfluidic device can be a good alternative for improving in vitro embryo production.

P-324 - The Alpha 7 Nicotinic Acetylcholine Receptor (CHRNA7) May Limit Inflammatory Responses in Cultured Human Granulosa Cells

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Previous studies suggested that the neurotransmitter acetylcholine (ACh) may play a role in the regulation of the ovary. One of its target receptors is the alpha 7 nicotinic ACh receptor (CHRNA7; encoded by CHRNA7). This member of the nicotinic ACh receptor superfamily of ligand-gated ion channels is best known for fast signal transmission in neurons. It is, however, also expressed in various non-neuronal cells, where its roles are associated with different cellular functions, including anti-inflammatory responses. We recently found this receptor in the mouse female gonad in stromal cells, macrophages and oocytes. Examination of adult female Chrna7-knockout mouse ovaries indicated involvement in gonadal functions, including follicle development. According to the Human Protein Atlas, CHRNA7 is expressed in human ovarian cells, e.g. stromal cells and oocytes and, in contrast to the mouse ovary, also in granulosa cells (GCs). Therefore, the objectives of this study were to verify expression in the human ovary and to examine functions in cultured in vitro fertilization-derived GCs. By performing immunohistochemistry, we confirmed expression in follicular GCs and the cells of the Corpus Luteum. Expression was retained in cultured GCs, as indicated by qPCR, Western blotting and immunocytochemistry. Preliminary results reveal that its activation by the selective CHRNA7-agonist PNU 282987 can evoke elevations of intracellular calcium in GCs, indicating channel functionality. While GCs and luteal cells are regulated by gonadotropins, CHRNA7 transcript levels were not affected by human chorionic gonadotropin

(qPCR: n = 7) or forskolin (qPCR: n = 12), a potent cAMP activator. As in non-neuronal cells, the activation of CHRNA7 is associated, among others, with anti-inflammatory responses, we investigated this possibility in human GCs. We used Lipopolysaccharide (LPS) to induce an acute inflammatory response. We found that PNU 282987 reduced the LPS-induced IL-6/IL-8 release (qPCR: n = 7, ELISA: n = 6), implicating a role of CHRNA7 in immunological events. Hypoxia also leads to increased production of cytokines by GCs and preliminary data suggest that the selective activation of CHRNA7 by PNU 282987 can inhibit pro-inflammatory responses evoked by hypoxia (qPCR: n = 4). Proteomic studies employing PNU 282987-stimulated GCs are currently being conducted to examine further roles of this channel. Of note, the expression of the human-specific variant of CHRNA7, namely CHRFA7A, was also detected in GCs. Its role as a possible negative regulator needs to be investigated. In conclusion, the results obtained so far demonstrate expression of CHRNA7 in the human ovary and GCs and identify a role of CHRNA7 in limiting inflammatory responses. (Supported by Deutsche Forschungsgemeinschaft (DFG), project number 4324342)

P-325 - Preantral Follicles of Over 12-Month-Old Mice Have Developmental Potential for Matured Egg Production

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Mammalian ovaries contain numerous numbers of preantral follicles, which decrease with aging throughout lifespan. To utilize these follicles for efficient egg production, the previous studies succeeded in embryo production by in vitro culture of preantral follicles derived from juvenile mice, while there are few reports with successful embryo production in case of aged mice, especially at over 12-month-old. This study aimed to investigate the developmental ability of preantral follicles in aged mice by using our successful protocol in juvenile mice. Preantral follicles between 80 and 120 μm in diameter were obtained from the ovaries of juvenile (0.5-month-old) as a control, and adult (2, 7, 12, and 15-month-old, respectively) mice of BDF1(C57BL/6 $\times$ DBA/2). Based on the previous report (Kohama et al., JRD, 2022), the collected specimens were cultured by two steps; as a 1st step, isolated follicles were placed on low cell adhesion membrane inserts under 20% O_2 for 6 days followed by 0.1% collagenase treatment to remove theca cells and basement membranes, and then as a 2nd step culture, formed granulosa cells-oocyte complexes (GOCs) were cultured on high adhesion membrane inserts under 5% O_2 for 6 days. As a result, the survival rates of the preantral follicles in the adult groups were lower than that in the control: 63.2, 54.3, 63.9, and 66.7% in 2, 7, 12, and 15-month-old, respectively, vs. 76.8% in the juvenile. In vitro maturation of cumulus cells-oocyte complexes (COCs) obtained after culture produced matured eggs with lower rates in the adult groups (40.5, 51.0, 34.8, and 48.3% in 2, 7, 12, and 15-month-old, respectively), compared to that in the control (75.2%). Finally, by in vitro fertilization and embryo culture, we found that these matured eggs have potential to produce blastocysts (the blastocyst rates from 2-cell embryos in each group were 75.0, 64.7, 58.3, 100, and 15.4% in 0.5, 2, 7, 12, and 15-month-old, respectively). As far as we know, the present study shows the first evidence that the preantral follicles derived

from the ovaries in aged mice over 12-month-old have developmental ability for egg and embryo production.

P-326 - Signaling Between the LH Receptor and the NPR2 Guanylyl Cyclase in Mouse and Rat Preovulatory Follicles

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Activation of the luteinizing hormone (LH) receptor in the granulosa cells of mouse and rat preovulatory follicles causes dephosphorylation of regulatory sites on the membrane guanylyl cyclase, natriuretic peptide receptor 2 (NPR2), decreasing production of cGMP and promoting resumption of meiosis in the oocyte. However, it is unknown how LH regulates dephosphorylation of NPR2. Since NPR2 dephosphorylation occurs rapidly, within 10 minutes, we hypothesize that it is triggered by LH-induced changes in protein phosphorylation that either increase the activity of a phosphatase or decrease the activity of a kinase in the presence of constant phosphatase activity. LH stimulation rapidly increases the phosphorylation of the phosphatase subunits PPP1R12A and PPP2R5D on serines whose phosphorylation is known to increase phosphatase activity. However, LH-induced dephosphorylation of NPR2 was not prevented in follicles from mice in which serine 507 of PPP1R12A and serines 53, 81, 82, and 566 of PPP2R5D were all changed to alanines, thus opposing activation of these phosphatases. These findings indicate that LH does not reduce NPR2 phosphorylation by way of these PPP family phosphatase regulatory subunits. Alternatively, LH signaling could reduce NPR2 phosphorylation by inhibiting an active kinase. Although the kinases responsible for maintaining the basal phosphorylation of the regulatory serines and threonines of NPR2 are unknown, one candidate that stands out is glycogen synthase kinase-3 (GSK3), which is encoded by 2 similar genes, Gsk3a and Gsk3b. Phosphorylation of GSK3A and GSK3B, on serines 21 and 9 respectively, inhibits their kinase activity. Previous research has shown that mouse and rat preovulatory follicles express both GSK3A and GSK3B, and both GSK3A-serine 21 and GSK3B-serine 9 are rapidly phosphorylated in response to LH signaling, by way of protein kinase A (PKA). If GSK3 phosphorylates NPR2, it could be responsible for maintaining NPR2 activity and meiotic arrest prior to LH signaling and could link LH-induced PKA activation to NPR2 dephosphorylation, in the pathway by which LH reinitiates meiosis. In support of this hypothesis, incubation of mouse follicles with a selective GSK3 inhibitor (CHIR-99021, 10 μ M) decreased NPR2 phosphorylation. Consistent with a reduction in NPR2 dephosphorylation, this inhibitor also caused nuclear envelope breakdown in follicle-enclosed oocytes. CHIR-99021 did not cause nuclear envelope breakdown in isolated oocytes that were maintained in meiotic arrest with the PDE3 inhibitor milrinone, indicating that the GSK3 inhibitor caused meiotic resumption by acting on the granulosa cells, rather than directly on the oocyte. Mice with genetic modifications of Gsk3a and Gsk3b will facilitate future studies of the possible function of GSK3 kinases in regulating meiotic progression in preovulatory follicles. This research was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (R37 HD014939).

P-327 - Transcriptional Modifications in Cumulus Cells during Human Oocyte In vitro Maturation

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It is widely acknowledged that the communication between the oocyte and the surrounding cumulus cells (CCs) is essential for oocyte maturation. However, the intricate details of this process in humans are not yet fully understood. Attempts have been made to define oocyte quality markers based on transcriptional changes in CCs from GV and MII oocytes after ovarian stimulation during IVF cycles or after in vitro maturation (IVM) with limited success. Nevertheless, the transcriptome of CCs from fresh unstimulated GV oocytes (before IVM) has never been compared with CCs from GV and MII oocytes after IVM to unravel transcriptional modifications in human CCs that may contribute to oocyte maturation in vitro. In the present study, 25 CC samples were collected ex vivo from cumulus-oocyte complexes (COCs) located in the surplus medulla tissue of 8 patients (mean age 29 years; range 19-36) who underwent unilateral ovariectomy and ovarian tissue cryopreservation, including CCs from fresh GV COCs (before IVM, n=5) and CCs from MII and GV COCs after IVM (GV-IVM, n=8; and MII-IVM, n=12). Overall, 1763 differently expressed genes (DEGs) were significantly regulated between CCs from fresh GV (before IVM) and MII-IVM, being 1057 protein-coding genes, including upregulated DEGs such as DHCR24, RUNX2, ANGPT2, WISP2, LHCGR, and IGF2, and downregulated DEGs, such as VIT, CRHBP, ACAN, AMH, FST, and VCAN, among others. The top upregulated pathways included, VEGF-VEGR signaling, MAPK signaling, PPAR signaling, Wnt signaling, cholesterol metabolism, and fatty-acid beta-oxidation, while downregulated pathways included the mesodermal commitment pathway, TGF-beta signaling, PI3K-AKT signaling, endoderm differentiation, RANKL/RANK signaling, androgen receptor signaling and regulation of actin cytoskeleton, among others. When comparing CC from COCs that failed after IVM (GV-IVM) vs. MII-IVM, only 50 significant DEGs were detected, with 27 of them being protein-coding genes, 12 of which were upregulated and 15 downregulated. PBX1 was the only unique upregulated DEG, whereas unique downregulated DEGs included SORL1, TANC1, ERG, CCDC3, IQGAP1, and SUN1. All the remaining DEGs were also differently expressed between fresh GV and MII-IVM. These findings may improve the current knowledge of key human oocyte maturation regulators and help uncover the mechanisms of ovarian function and dysfunction. This can potentially lead to better IVM outcomes and make more competent oocytes available for fertility treatment.

P-328 - Saturated Fatty Acids are Crucial Components in Follicular Fluid for Ensuring Endocrine Regulation of Estradiol Production in Bovine Ovary

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Ovarian granulosa cells (GCs) have evolutionarily conserved steroidogenic functions mainly controlled by the consecutive actions of pituitary follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Estradiol (E2) synthesized by GCs is essential for the feedback regulation of gonadotropin production, uterine extracellular modeling, and the manifestation of estrous symptoms in dairy animals such as cows and buffaloes. Metabolic conditions like postpartum

negative energy balance in animals and diseases like diabetes and obesity in humans are characterized by increased concentrations of long-chain non-esterified fatty acids (NEFA) in follicular fluid and are associated with female subfertility. Oleic acid (OA; C18:1), palmitic acid (PA; C16:0), and stearic acid (SA; C18:0) are the predominant fatty acids in follicular fluid and constitute up to 70% of the NEFA fraction under healthy conditions. However, their concentrations are further elevated in postpartum animals due to negative energy balance-induced fat mobilization. Earlier studies in GCs indicate that unsaturated fatty acids (UFAs) downregulate and saturated fatty acids (SFAs) upregulate estradiol production. However, it was unclear whether pituitary gonadotropins induce accumulation of NEFA in the follicular fluid since FSH induces and LH inhibits estradiol production in the ovary. To address this, we have performed gas chromatography analysis of follicular fluid, which revealed no differential accumulation of NEFAs between pre- and post-luteinizing hormone surge follicles. We, therefore, wondered how estradiol production is regulated in the physiological context, as UFAs and SFAs are mutually present in the follicular fluid despite their opposite effects on E2 production. We thus performed in vitro primary GC cultures with palmitate, palmitoleate, stearate, oleate, linoleate, and alpha-linolenate (ALA), representing >80% of the NEFA fraction in the follicular fluid, and analyzed 62 different cell culture conditions to understand the regulation of estradiol biosynthesis. Our analyses show that UFAs (OA and ALA) downregulate GC's E2 production by strongly downregulating the expression of aromatase and gonadotropin receptors FSHR and LHCGR. This suggests that UFAs could hamper the function and also the gonadotropin signaling in GCs. Surprisingly, cosupplementation of SFAs (PA and/or SA) with UFAs (OA or ALA) alleviated the inhibitory effects of UFAs by rescuing E2 production, expression of gonadotropin receptors and aromatase. Next we analyzed the underpinning signaling pathways responsible for the regulation of E2 production upon NEFA treatments. Transcriptome data of oleic acid-treated GCs indicated that UFAs induce the ERK1/2 and AKT signaling pathways. Western blot validation showed that UFAs activate and SFAs inhibit the ERK1/2 and AKT pathways in GCs. Interestingly, cosupplementation of SFAs and UFAs did not induce phosphorylation of ERK1/2 and AKT proteins, suggesting a possible role of these pathways in UFA-dependent downregulation of E2 production. Next, we inhibited these pathways using chemical inhibitors PD98059 and LY294002. Attenuation of ERK1/2 phosphorylation resulted in elevation of E2 levels along with the expression of genes downregulated by UFAs, indicating SFAs-induced rescue of E2 production is mediated via inhibition of ERK1/2 signaling. ERK1/2 mRNA knockdown assay further confirmed that ERK1/2 is indeed a negative regulator of estradiol production in GCs. Overall, our results demonstrate for the first time that SFAs are vital components of the follicular fluid, without which gonadotropin signaling and the ovarian cycle would probably be shattered by UFAs in GCs. Funding: This work is funded by Deutsche Forschungsgemeinschaft (DFG; grant No. BA 6909/1-1)

P-329 - The Dummerstorf High-Fertility Mouse Line 1 – a Worldwide Unique Model for Increased Female Reproductive Performance

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The development and maturation of ovarian follicles is a complex and highly regulated process. During recent decades, several mouse models provided insights into the regulation of folliculogenesis. In contrast to the commonly used transgenic or knockout mouse models, the Dummerstorf high-fertility mouse line 1 (FL1) is a worldwide unique selection experiment for increased female reproductive performance and high fertility. FL1 mice almost doubled the number of ovulated oocytes compared to the unselected control mouse line. To gain insights into the cellular mechanisms leading to the high fertility phenotype, granulosa cells were isolated from antral follicles and used for mRNA sequencing. To obtain a global picture of how the phenotype is achieved gonadotropins, growth factors and other hormones associated with follicular development were analyzed. While FSH and IGF1 levels are significantly decreased in FL1 females, LH levels are elevated. No differences were found in insulin, prolactin and oxytocin levels in FL1 mice compared to the control mouse line. FL1 females are characterized by various alterations on endocrine and molecular levels, which have the potential to improve the follicular development. Our results indicate that the crosstalk between different intracellular signaling pathways in granulosa cells is improved, follicular atresia in FL1 is decreased due to enhanced granulosa cell survival and by improving the efficiency of intracellular signaling, glucose metabolism and signal transduction, FL1 mice have advantages in reproductive performance. The understanding of these mechanisms and their interplay might be of fundamental interest for the understanding of proper fertility in human.

P-330 - Obesity Alters Sumoylation-Dependent Proteomic Response in Ovaries of Mice Exposed to 7,12-Dimethylbenz[A]Anthracene

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Small ubiquitin-like modifiers (SUMOs) are present in all eukaryotes, and protein sumoylation is a post-translational modification that functions in chromatin-associated processes including DNA repair. Additionally, SUMOs and SUMO ligase enzymes (PIAS) localize at DNA double strand breaks. Further, ovarian roles for SUMOs have been recently described. We have previously discovered that exposure to the genotoxicant 7,12-dimethylbenz[a]anthracene (DMBA; 1 mg/kg for 7 d), in adult lean and obese mice altered the abundance of ovarian SUMO proteins thus, the hypothesis tested was that exposure to DMBA would increase sumoylation of proteins as a mode of ovotoxicity and that obesity would alter this response. Lean and obese mice (KK.Cg-a/a and KK.Cg-Ay/J) were exposed to either corn oil (CT) or DMBA (1 mg/kg) for 7d via intraperitoneal injection (n = 4/treatment) and ovaries were flash-frozen on day 2 of diestrus and stored in -80°C. Protein isolation was performed followed by immunoprecipitation of SUMO2/3 substrates using a SUMO2/3 polyclonal antibody. Precipitated proteins were digested with trypsin and labeled with tandem mass tags for liquid chromatography tandem mass spectrometry (LC-MS/MS). A total of 114 SUMO protein targets were identified and quantified between treatment groups. Obesity alone altered the abundance of 67 sumoylated proteins ($P < 0.1$). Exposure to DMBA differentially affected the level of 54 sumoylated proteins in obese compared to lean females ($P < 0.1$). Approximately two-thirds (44 proteins) of differentially abundant sumoylated proteins were

decreased in obese ovaries, but in DMBA-exposed mice, the reverse trend was observed with ~one-third (12 proteins) being reduced in obese mice, suggesting an alteration of the sumoylation-dependent proteomic response. In lean females, exposure to DMBA altered 13 sumoylation-dependent proteins ($P < 0.1$) while 33 sumoylation-dependent proteins were affected in obese mice ($P < 0.1$), supporting that the physiological status effect is greater than the DMBA exposure effect. The large proline-rich protein BAG6, which functions in DNA damage-induced apoptosis, and bromodomain-containing protein 1, which is a scaffold subunit of histone acetyltransferases, were decreased in DMBA-exposed lean mice by 1.7-fold and 2.5-fold, respectively ($P < 0.01$); increased in obese DMBA-treated mice by 1.9-fold and 2.4-fold, respectively ($P < 0.01$) and increased in obese relative to lean control mice by 0.8-fold and 1.2-fold, respectively ($P < 0.05$). These findings suggest differential epigenetic control of the DNA damage response due to metabolic status, identify ovarian SUMO target proteins, and elucidate the dynamic interplay of sumoylation as a response to ovotoxicity induced by DMBA exposure. Supported by 1R01ES030341 from NIEHS.

P-331 - Obesity Prone Mice Present Impaired Steroidogenesis Associated with SOCS3-Mediated Nodal Downregulation in Ovarian Theca-Stromal Compartment

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Obesity is a major health issue and presents multiple comorbidities, amongst them infertility. Obesity leads to systemic hormonal imbalance and diabetes, as well as ovarian failure comprising lipid accumulation, cellular stress and inflammation which culminates with decreased ovulation and infertility. Furthermore, we have previously shown that diet-induced obesity (DIO) in mice leads to the establishment of ovarian leptin resistance. Presently, we hypothesise that ovarian performance during obesity is linked to leptin and Nodal signalling disturbances. Using mouse models with contrasting responses to DIO, we unravelled the cross talk between leptin signalling modulator SOCS3 and Nodal signalling underpinning impaired steroidogenesis in obese mothers. We treated either obesity prone C56BL/6J (B6) mice or obesity resistant 129S1 (129) mice with chow diet (CD) and high fat diet (HFD) for 16 weeks (wk), and characterised the animal phenotype, profiling body weight (BW) and food intake (FI). Additionally, after culling the animals we collected blood, gonadal fat, ovaries, ovarian granulosa cells (GC) and theca/stroma cell fraction (TC) after puncturing the ovaries. Furthermore we used pharmacologically hyperleptinemic (LEPT) mouse model, in which animals were intraperitoneally injected with saline (C) or 100mg of leptin/ day (L), as well as model of genetic obesity with leptin deficiency (ob/ob) and collected ovaries from wild type (+/+) or mutants (-/-) mice. Despite presenting greater FI than the lean counterparts ($p < 0.0001$), the 129 HFD mice did not gain BW, in contrast with B6 HFD mice, which doubled their BW after DIO (38g) regarding B6 CD (20g). Both strains had increased plasma levels of leptin, but to a greater extent in B6 HFD ($p < 0.0001$). Moreover, increased BW gain in B6 HFD after DIO was associated with the upregulation in mRNA levels of white adipose tissue expansion markers Polymerase I and transcript release factor (Cavin), Secreted frizzled-related protein 5 (Sfrp5),

Mesoderm specific transcript (Mest, $p < 0.01$) and the proinflammatory genes NLR Family Pyrin Domain Containing 1 (Nlrp1) and C-C motif chemokine ligand 5 (Ccl5, $p < 0.01$). Furthermore, obesity prone B6 mice presented decreased expression of Luteinizing hormone (Lh) receptor ($p < 0.05$) in ovarian extracts and Prolactin receptor (Prlr) mRNA in TC ($p < 0.01$). Moreover mRNA expression of Steroidogenic acute regulatory protein (Star), 3 beta- hydroxysteroid dehydrogenase (3bhsd) and 17bhsd ($p < 0.05$) was decreased in TC in B6 HFD mice compared to CD, as well as progesterone receptor (PR, $p < 0.01$) and estradiol receptor a (Era, $p < 0.05$) mRNA. No significant differences were noted in 129 mice. Finally, we confirmed the upregulation of suppressor of cytokine signalling 3 (Socs3) from B6 HFD comparing to CD ($p < 0.001$) and associated downregulation of Nodal ($p < 0.01$) and its receptors Activin receptor type-2B (Acvr2b) and Activin A receptor (Alk7, $p < 0.05$). Moreover, LEPT model demonstrated increased Socs3 expression in ovarian extracts but decreased Nodal and Alk4 expression comparing to controls, while the extremely obese -/- ob/ob mice demonstrated no changes in Nodal or its receptors. Overall, impaired steroidogenesis in obesity prone mice is associated with dysregulation of Nodal signalling in TC, which is mediated by the overexpression of SOCS3. Hence, the cross talk between leptin and Nodal signalling may suppress steroidogenesis and subsequently impact oocyte development and quality in obese mothers. Further studies are needed to explore the underlying interactions. Research supported by the Polish National Science Centre (2019/34/E/NZ4/00349).

P-333 - A Phenotypic Screening Assay of Mouse Egg Activation Identifies Novel Inhibitors Targeting WEE2 for Non-Hormonal Contraceptive Applications

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WEE2 is an oocyte-specific kinase that mediates the resumption of meiosis from the metaphase II arrest at fertilization and is essential for the egg-to-embryo transition. Thus, developing compounds that selectively inhibit WEE2 without affecting kinases such as WEE1 and MYT1 represents a promising strategy for non-hormonal contraceptive development. We established a phenotypic assay for mouse egg activation to assess potential WEE2 inhibitors using a closed time-lapse imaging system (EmbryoScope+TM) whereby we tracked second polar body extrusion (PB), pronuclear (PN) formation, and two-cell cleavage. Strontium chloride (SrCl₂), a known inducer of calcium transients, was utilized to trigger synchronous egg activation. Mouse oocytes were treated for 2-hour with SrCl₂ followed by 24-hour culture. In the absence of selective commercially available WEE2 inhibitors, we used Adavosertib during egg activation as a reference compound to validate the platform. Adavosertib potently inhibits both WEE1 (K_i: 0.6 nM) and WEE2 (K_i: 10 nM), but it lacks WEE2 specificity. 24-hours of Adavosertib treatment (500 nM) markedly inhibited egg activation, with significant reductions in developmental progression (2nd PB extrusion: 87.5%, PN-formation: 3%, 2-cell cleavage: 3%) compared to controls (2nd PB extrusion: 100%, PN-formation: 100%, 2-cell cleavage: 94%). Additionally, just a brief 2-hour Adavosertib exposure during egg activation was sufficient to disrupt development. We then tested a novel series of highly selective Wee2 inhibitors developed by Schrodinger (B-1: WEE2 K_i < 1.5 nM, B-2 WEE2 K_i = 9 nM) and an

inactive enantiomer (B-3: WEE2 KI > 650 nM). These compounds are not selective for WEE1 (KI > 10,000 nM). WEE2 functions to phosphorylate the kinase CDC2 to drive egg activation. Therefore, we assessed target engagement by evaluating expression of pCDC2 and CDC2 by increasing WEE2 activity in activated eggs treated with 10 μ M B-1 and B-2 via Western blotting. pCDC2 expression increased in eggs following egg activation, but exposure to B-1 and B-2 inhibited this activity. Total CDC2 and WEE2 levels were similar across groups. Thus, B-1 and B-2 effectively inhibit WEE2 activity. When testing these inhibitors using our phenotypic egg activation assay (in 24-hour culture with compound), 2nd PB extrusion was similar across groups (Control: 100%, B-1: 100%, B-2: 100%, B-3: 100%), but PN-formation (Control: 100%, B-1: 0%, B-2: 0%, B-3: 81%) and cleavage (Control: 100%, B-1: 0%, B-2: 0%, B-3: 59%) were reduced compared to controls. Additionally, these phenotypes were dose dependent. Overall, these findings demonstrate promising novel WEE2 inhibitors, and studies are ongoing to examine their safety and efficacy in vivo as novel female contraceptive agents. This study was part of the Ovarian Contraceptive Discovery Initiative (INV-003385) funded by the Bill & Melinda Gates Foundation.

P-334 - Proteomic Approaches to Identify Age-Specific Biomarkers in Oocyte

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The increase in pregnancy at advanced maternal age (AMA, age ≥ 35) can be attributed to various factors such as socioeconomic changes and advancements in medical technology. Furthermore, despite the decline in the birth rate, the birth rate among AMA mothers has shown an increasing trend for a few decades. However, pregnancy at AMA has gradually been recognized to increase several risks, including miscarriage, stillbirth, and obstetric complications. Additionally, pregnancy at AMA can act as a risk factor for the structure and functions of oocytes, subsequently contributing to subfertility. Therefore, this study was designed to profile correlated changes during aging that can be used as age-specific biomarkers in mouse oocytes. The age-specific mouse groups were categorized into three in accordance with the reproductive age of mice equivalent to humans. The age of human sexual maturity, AMA, and menopause were used as criteria. MII stage oocytes were collected from each age-specific group and used to evaluate the differences in age-related physiological and proteomic biomarkers. Initially, nonlethal factors (e.g. tail length and body weight) and the number of ovulated oocytes per mouse showed age-dependent changes. Moreover, increased morphological abnormality of the uterus, ovary, and oocyte in the older mice group was confirmed. Additionally, the evaluation of comprehensive oocyte function (e.g. mitochondrial activity and ROS level) revealed abnormal changes in older oocytes. Proteomic research identified 14 differentially up- and down-regulated (> 3-fold) proteins between each group. These proteins are closely related to component and function of oocytes, including kinetochore, nucleus, macromolecular complex, ubiquitin-dependent process, and nucleotide binding. Consequently, in vitro fertilization assay confirmed the significantly decreased cleavage and blastocyst formation

rates. Taken together, our results implicate the cellular and molecular changes between younger and older oocytes. Therefore, continued efforts are needed to evaluate how aging affects oocytes and the intermolecular process of fertilization. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (Ministry of Science and ICT) (NRF- 2022R1F1A1066103).

P-335 - Extracellular Vesicles Isolation Method can Influence its Contents and the Bovine In Vitro Oocyte Maturation Upon Supplementation

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The quality of oocytes derived from in vitro maturation (IVM) culture systems is inferior to those matured in vivo, which can result in a low conversion to transferable embryos. This can be attributed to the fact that, during IVM, the oocyte obtained from small antral follicles loses crucial contact with theca and granulosa cells and is stimulated to undergo the early resumption of meiosis. Extracellular vesicles present in follicular fluid (ffEVs) function as carriers of molecular messages and play a crucial role in cell-to-cell communication, participating in both follicular development and oocyte maturation. However, there is limited understanding regarding the impact of the isolation methods on ffEVs contents and its use as supplement during IVM. Therefore, we initially collected follicular fluid from 3-6 mm follicles harvested from bovine ovaries from a slaughterhouse and used the same sample and volume to isolate ffEVs using ultracentrifugation (UC) or size exclusion chromatography (SEC). Next, the ffEVs isolated by UC or SEC were characterized by their miRNA profile (n= 5 pools of follicular fluid from 6 ovaries). MiRNAs were considered present when they presented an appropriate melting curve, cycle threshold (CT) lower than 35, and were expressed in all replicates. The CT data generated by amplification were normalized using an endogenous gene (bta-miR-99b) in technical duplicate. After the characterization, cumulus-oocytes complex (COCs) retrieved from 3-6 mm follicles from abattoir ovaries were submitted to IVM under different medium conditions: (i) C (Control), consisting of base medium TCM 199; (ii) UC (base medium supplemented with ffEVs separated by UC) and (iii) SEC (base medium supplemented with ffEVs separated by SEC). The COCs were matured for 9 hours to evaluate the number of transzonal projections (TZPs) and 22 hours to evaluate the maturation rate. The ffEVs miRNA profile data was analyzed using t-test analysis, while the supplementation data was analyzed using ANOVA followed by the Tukey test. Statistical significance was determined at a p-value < 0.05. We identified a total of 44 miRNAs differently expressed, being 16 up-regulated in SEC and 28 up-regulated in UC. The bioinformatic analysis indicates that these miRNAs play a role in modulating pathways associated with to the regulation of TZPs, such as the regulation of actin cytoskeleton, as well as pathways related to the oocyte maturation, such as PI3K-Akt signaling pathway, respectively. Next we supplemented COCs during IVM with ffEVs separated by the different methods, and observed lower number of TZPs (p= 0.04) and an increased maturation rate (78,9% ± 7,19) in the SEC group compared to the C group (59,34% ± 7,53; p= 0.003). In conclusion, our results demonstrate that the isolation method influence the contents of ffEVs, and that SEC isolated ffEVs affect the cellular responses. Funding: FAPESP 2021/06645-0 and 2021/12560-7

P-336 - Role of Ankyrin-repeat and SOCS-Box Protein 9 (ASB9) in PAR1 Inhibition and MAPK Signaling Pathway in the Bovine Ovary

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The Ankyrin-repeat and SOCS-box protein (ASB) family, part of the E3 ubiquitin ligases, regulates proliferation, differentiation, carcinogenesis, and cell cycle in granulosa cells. We previously identified ASB9 as an hCG-induced gene in granulosa cells (GC) of bovine ovulatory follicles. Although we reported ASB9 interactions with target binding partners including protease-activated receptor 1 (PAR1), the exact roles of ASB9 in GC activity and in various signaling pathways need to be fully investigated. In this project, we aimed to study the mechanisms of action of ASB9 in GC and determine whether ASB9 induction by LH/hCG is necessary for the regulation of target genes such as PAR1, and signaling pathways involved in GC function and activity. Cultured GC were treated with different doses of FSH, LH and Thrombin. RT-qPCR analyzes showed that thrombin increased PAR1 expression in GC ($P < 0.05$) while FSH had no effect on PAR1. Treatment with LH reduced significantly PAR1 ($P < 0.05$), even in the presence of thrombin, demonstrating downregulation of PAR1 by LH, possibly via induction of ASB9. Western blot results show that ASB9 expression in GC in vitro is increased 12 hours after treatment with LH ($P < 0.05$). Furthermore, the phosphorylation profile of MAPK3/1 (ERK1/2) in thrombin-treated GC suggests a PAR1-mediated control while the presence of ASB9 in GC appears to have a negative effect on the MAPK pathway. Finally, thrombin could block the negative effect of ASB9 on PAR1 very early (within 1 hour treatment) while this protease does not seem to affect ASB9 at 6 and 12 hours of treatment. Proliferation assays showed that ASB9 negatively regulates GC number as well as proliferation marker CCND2 while apoptosis marker BAX2 was increased with LH treatment. Together, these data show functional effects of ASB9 on GC activity through PAR1 and potential consequences on the ovulatory process in bovine species.

P-337 - Follicular fluid from overweight women displays altered energy metabolism and oocytes with smaller sizes

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Overweight is a condition associated with obesity, impacting half of the population in Brazil. Moreover, there is a concerning trend suggesting that more than half of the global population could be obese by the year 2035. On the other hand, infertility is a prevalent condition, with recent research estimating that one in six individuals will experience infertility during their lifetime. Given the increasing prevalence of these two conditions, it is becoming more common to encounter

overweight women seeking assisted reproduction treatment. It is well-established that obesity adversely affects the retrieval of oocytes, leading to a low fertilization rate, a high concentration of lipids in follicular fluid (FF), and the production of poor-quality embryos. However, identifying the underlying causes of these effects remains challenging. In this study, we explore the metabolic aspects of the follicular environment in overweight infertile women and its impact on oocytes and embryos. Recognizing the limited number of studies focusing on individual follicles and their follicular fluid, we conducted a single-center prospective analysis from March 2022 to August 2022, involving 20 patients. The recruitment was based on their Body Mass Index (BMI), leading to the formation of two groups: Normal BMI (NBMI) ranging from 18.5 to 24.9 kg/m² and High BMI (HBMI) ranging from 25 to 29.9 kg/m². Among the participants, 13 fell into the NBMI category, while 7 belonged to the HBMI category. Exclusion criteria encompassed patients with Polycystic Ovary Syndrome (PCOS), ovarian insufficiency, Endometriosis in stages III and IV, and those who had undergone prior treatments at the clinic. During the oocyte retrieval day, we conducted a meticulous puncture of follicles individually, followed by system washing. Only follicular fluid without visible blood contamination and with the presence of oocytes was utilized, and no pooling of fluids was performed. FF were analyzed for glucose, lactate, insulin, and MDA (an important oxidative stress marker). Morphokinetic assessments were conducted for all embryos (total of 81) and the diameter of matured oocytes were collected (117 oocytes). Women with High BMI (HBMI) exhibited higher levels of insulin and a twofold increase in lactate ($p=0.017$, $p=0.01$, respectively). Unexpectedly, Normal BMI (NBMI) women showed a higher amount of MDA ($p=0.004$), while glucose levels did not differ significantly ($p=0.64$). Regarding morphokinetic parameters, the Time of pronuclear fading was longer in embryos from HBMI women ($p=0.003$). Mature oocytes from HBMI individuals were found to be smaller compared to those from NBMI individuals ($p=0.03$). The observed increase in insulin in the follicular fluid (FF) was expected to result in higher glucose consumption. Consequently, cumulus cells likely reduced the glycolysis rate, transitioning to the Warburg effect, which may explain the elevated levels of lactate found in the FF. In individuals with Normal BMI (NBMI), the standard glycolysis and tricarboxylic acid (TCA) cycle process generates reactive oxidative species, resulting in increased Malondialdehyde (MDA) in follicular fluid (FF). The insufficient energy consumption likely contributed to smaller oocytes and delayed pronuclear fading. This study is the first to identify, through the analysis of individual follicular fluids, a shift in energy metabolism, revealing the Warburg effect in cumulus cells. Notably, the Warburg effect is typically associated with cancer cells and embryos. However, more studies involving a larger patient cohort are essential to validate this hypothesis, explore its impact on clinical outcomes, and delve into the metabolic dynamics of embryos.

P-338 - Analysis of the Expression of Tight Junction Proteins in Corpora Lutea of the Domestic Cat

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Corpora lutea (CLs) are usually transient endocrine glands that pass through different life cycles. In the domestic cat, formation, development/maintenance, early and late involution have been described; at the end of the cycle, they transform into corpora albicantia, which disappear completely. To characterize the CL life cycle, its regulation and functionality in more detail, we performed a transcriptome analysis using RNA sequencing (RNA-Seq). In the present sub-study, we have analyzed the data set with regard to the expression of tight junction (TJ) proteins, as these are important for paracellular structure and transport. We hypothesized that they change during the life cycle of CL, especially between the active development/maintenance stage and the subsequent regression phase. The group of TJ proteins consist of transmembrane proteins like occludin (OCLN) and claudins (CLDN), and cytoplasmic scaffolding proteins like cingulin (CGN) and the tight junction proteins 1, 2 and 3 (TJP1/ZO-1, TJP2/ZO-2, TJP3/ZO-3). They regulate the paracellular pathway for the movement of ions and solutes in-between cells. Claudins are responsible for the paracellular barrier function of TJs, and in some cases confer paracellular channel functions to the paracellular barriers of TJs. But components of tight junctions are also expressed in cell types not forming tight junctions. For RNA-Seq-analysis, we have used CLs from domestic cat of development/maintenance (dm, n = 8) and regression stages (re, n = 12). As one of the highest upregulated genes in dm compared to re ($\log_2\text{FoldChange} = -9.24$), the gene coding for claudin 4 (CLDN4) was identified. Looking at the expression of other claudins in the data set, we detected transcripts for 15 different CLDNs. Nine of them were very low expressed in both stages; of the other six, five genes were differently expressed between the stages. In addition to CLDN4, also CLDN1 and CLDN18 ($\log_2\text{FoldChanges}$: -3.49, -4.01) were upregulated in dm, whereas CLDN5 and CLDN11 ($\log_2\text{FoldChanges}$: 0.85, 3.39) were upregulated in re. Of the other TJs, OCLN, CGN, TJP1 and TJP3 were higher expressed in regression stage ($\log_2\text{FoldChanges}$: 1.37, 1.10, 0.72, 1.63), TJP2 was not differentially expressed. The protein expression of CLDN1 was analyzed by immunohistochemistry, but the staining did not reflect the gene expression. Thus, clear staining was only visible in a few, but not in all dm samples examined, as well as in some of the regression samples. We have observed staining of large and small luteal cells. Further studies would be required to characterize the protein staining of the other TJs. Interestingly, with regard to the expression of claudins in corpora lutea, only the expression of CLDN1 and CLDN5 was described before. It has been discussed that they play a role as junctional proteins and act as regulators of vascular permeability. The cytoplasm staining of steroidogenic cells for CLDN1 in our study hints at further functions. Based on our gene expression data we hypothesize that the composition of the tight junctions changes during the transition from the dm to the re stage, which may influence the type of exchange of substances and/or the para- and intracellular structures at the different stages.

P-339 - Rescue of Preantral Follicles from Ovaries of Endangered Cattle Breeds Undergoing Genetic Salvage

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The intense selection of a few highly productive breeds over the past 50 years has led to a dramatic decline in the variety of cattle biodiversity. The loss of several local breeds and the limitation in genetic diversity across many bovine species has garnered urgent attention. Italy has a wide variety of local breeds, favored by the richness of territorial environmental diversity. Specifically, the Varzese cattle breed, originating from Lombardy, is characterized by rusticity, resistance to disease,

longevity, and high milk quality. However, since the widespread of cosmopolitan breeds in the 1960s, Varzese cattle declined to the point where they are currently at risk of extinction. Strategic policies and action plans for animal genetic conservation are operative and allowed to partially rescue the breed. Indeed, the headcount of cattle, which stood at 39 in 2001 as per the Cattle Breeder Association Herd book, increased to 827 in 2023, thanks to EU policies and National plans. However, a further consistent increase is not expected due to the lack of interest of most breeders in these cattle. Therefore, intensive ex-situ preservation programs must be pursued to rescue the limited genetic material available through advanced assisted reproductive technologies. Although more challenging and less efficient than bovine sperm cryobanking, technologies that allow the preservation of oocytes will safeguard the only inheritable source of mitochondrial DNA. In this sense, the bovine ovary is a considerable source of oocytes established during fetal life and organized into follicles at various stages of folliculogenesis. However, preantral follicles, the most abundant quota of the ovarian reserve, remain unexploited. As such, our study aims to provide strategies for rescuing preantral follicles from the ovarian cortex of Varzese cattle. A pilot study was performed using Holstein-Friesian bovine ovaries as a model to histologically evaluate the follicle density. Ovarian cortical fragments collected from slaughtered heifers (12-24 months old) and cows (40-87 months old) were fixed and submitted to morphometric analyses to quantify the preantral follicle population contained within 1 mm³ of the ovarian cortex. In parallel, a mechanical isolation protocol was applied to liberate preantral follicles from the same ovary. For this purpose, fragments of the cortex of 0.16 cm³ were minced, homogenized, and filtered through sieves of decreasing pore sizes to obtain a homogenous population of preantral follicles. Using this procedure the yield of follicles per fragment in the Holstein-Friesian model was 653.25±279.71 and 105.91±89.85 (mean±SD, $p < 0.05$), with an efficiency rate per mm³ of 10.54 and 4.69% in heifers (N=8) and cows (N=11), respectively. Preliminary ovarian reserve quantification was then performed on Varzese heifers (N=2) and cows (N=2) on ovarian fragments, representing the by-products of the dissection of antral follicles used for in vitro embryo production. Our results showed a decrease in ovarian reserve with age. Comparable fragments from the same animals were then cryopreserved to construct a biobank with an estimated number of gametes that may be used in the future for biodiversity restoration, either by grafting, in vitro culture of the ovarian cortex, or follicle isolation and subsequent in vitro folliculogenesis. Beyond the expected decline of the ovarian reserve with age, our approach proposes a paradigm to evaluate the timing for the rescue interventions of female germplasm in biodiversity preservation plans for species at risk of extinction. Funded by MUR PRIN2020, No.20209L8BN4 (InfinitEGG) and RLPSR2014-2020 No.202102146691 (R-INNOVA).

P-340 - Towards the Optimization of Targeted Systems for the In Vitro Culture of Isolated Bovine Primordial Follicles

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To date, the production of live offspring through in vitro follicle culture from the ovarian reserve has only been achieved in mice, providing the proof-of-principle of the potential value of primordial follicles (PMF) as a source of fully grown oocytes. The ability to grow undifferentiated oocytes in vitro from PMF would increase the supply of fully grown oocytes destined for downstream applications in the livestock industry and fertility preservation programs. However, in large mammals, such as bovine, in vitro follicle culture systems to produce mature oocytes from PMF are still experimental due to high follicle mortality following isolation from the surrounding tissue. As evidenced in humans, isolated PMF undergo cell death after a short period in culture. While understanding the cause of follicle death is necessary to inhibit the induction of programmed cell death (PCD), it is also essential to elucidate the mechanisms responsible for activating PMF and steering them towards the next stage of development, i.e., primary follicles (PF). Mechanistically resolving both sides of the story - PMF death and development - would then provide insights into key factors responsible for deciding the fate of the PMF in culture. Condensation of this genetic repertoire would then optimize the current base culture system and improve the development of isolated bovine PMF to PF entirely in vitro. Heifer ovaries were collected from the abattoir and transported on ice to the laboratory in saline. PMF and PF were mechanically isolated, and PMF were cultured in a defined system. Follicle viability of freshly isolated (PMF T0) and PMF cultured for 16 (PMF T16) and 24 hours (PMF T24) were assessed. PMF T0, PMF T16, and PF T0 were subjected to bulk RNA sequencing, generating 50bp paired-end reads. Raw data were trimmed with TrimGalore to remove artificial constructs and low-quality bases. Trimmed data were mapped to the *Bos taurus* ARS-UCD1.3 transcriptome, and reads were quantified with Salmon. Differentially expressed genes (DEGs) between PMF T0 and PMF T16 were then obtained with DESeq2 (Wald's Test). A chi-square test was performed to determine an association of the DEGs with PCD genes. DESeq2 (Likelihood Ratio Test) was further employed to perform an inter-follicle (PMF T0-PMF T16-PF T0) time course gene expression analysis followed by hierarchical clustering of significant genes (IFGEA) with the DEGREport package in R. We observed a significant reduction in PMF viability after 16 hours of culture, while no significant differences were observed between 16 and 24 hours ($p < 0.0001$ and $p = 0.9753$, respectively, two-way ANOVA followed by Tukey's test). Furthermore, we report the transcriptome profiles of freshly isolated bovine PMF and PF and 16-hour cultured PMF for the first time. PCA showed clear clustering of all the samples. 1949 DEGs ($\text{padj} < 0.1$) were identified, which were determined to be associated with genes involved in ferroptosis and autophagy (chi-square test, $p\text{-value} < 0.05$). IFGEA yielded 4 clusters of 1173, 1570, 162, and 22 genes ($\text{padj} < 0.05$) with similar expression patterns in clusters 1-4, respectively. Our transcriptome analyses indicate that ferroptosis and autophagy are the elected PCD mechanisms bringing about PMF death in vitro and can be inhibited to nurture current culture systems. Based on comparison with PF, systems may be further optimized to allow the holistic development of isolated PMF entirely in vitro in the future. Work funded by H2020 MSCA-ITN-ETN n.860960 (EUROVA), RL PSR2014-2020 No.202102146691 (R-INNOVA).

P-341 - The Positive Role of AMH Regulation in Oocyte Number and Embryonic Development in Aged Mice

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Anti-Müllerian hormone (AMH) is known to play an important role in preserving the pool of primordial follicles by inhibiting awakening from dormancy to the growth phase. The number of follicle is decreasing with age, so maintaining the number of follicles is very important in reproductive function of aged mice. Regulation of AMH expression has a critical role in folliculogenesis and female reproductive function. We previously reported that PPAR γ agonist can modulate the level of AMH along with the foxl2/sf-1/STAT3-related network, and showed that ovaries treated with PPAR γ agonists have an increased proportion of primordial follicles in 2-day-old female ICR mice. Therefore, we studied the effects of PPAR γ agonist on the folliculogenesis of aged mice and its impact on oocyte quality and developmental competency. 15-22-week-old (Young) mice and 56-62-week-old (Aged) C57BL/DBA F1 hybrid female mice were administered with a PPAR γ agonist by intraperitoneal injection every 3 days for 21 days. To evaluate the developmental competence of these oocytes, three days after last PPAR γ agonist IP injection, mice were induced superovulation. The retrieved oocytes were subjected to in vitro fertilization (IVF) with epididymal sperm obtained from 12-week-old fertile male mice and cultured to the blastocyst stage in KSOM. In line with our previous studies, results in aged mouse groups showed that PPAR γ agonist increased the number of primordial follicles. The proportion of ovulated oocyte that reached good-quality blastocysts was significantly higher in the PPAR γ agonist-treated group compared to the control group. Also, the count of zona pellucida remnant (ZPR), an apoptotic marker associated with follicle atresia, exhibited a decrease in PPAR γ agonist-treated group. In young mice, the number of good-quality oocytes was significantly increased compared to the control group. The number of primordial follicles and the rate of high-quality blastocyst formation showed an increasing trend, but it was not statistically significant. These results demonstrated that increasing AMH expression by PPAR γ agonists has a positive effect on oocyte competence and good embryonic development, especially in aged mice. Here we report that upregulation of AMH in mice not only increases the number of ovulated oocytes but also has a positive effect on oocyte maturation and embryonic development. This study may provide new insights into the overall impact of AMH regulation on female fertility and highlight therapeutic possibility to the aged patients. This work was supported by a grant from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family affairs, Republic of Korea (HI21C1350) and NRF - 2021R111A1A01042374

P-342 - Hypoxia-Related Regulation of Luteal Function: New Insights from Gene Expression and LPS Challenge Studies in Cattle

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Considering the role of the corpus luteum (CL) in the reproductive performance of cattle, comprehending its formation, maintenance and regression remains of utmost clinical importance. Reduced oxygen content has emerged as a significant factor influencing these processes. In this study, we explored the dynamics of CL functionality, investigating gene expression patterns of hypoxia-mediating and regulating factors during the luteal phase, and in response to *Escherichia coli* lipopolysaccharide (LPS) challenge, as well as inflammation related factors. LPS, a gram-negative bacterial endotoxin, frequently causes infertility and subfertility in livestock. When

administered at 0.5µg/kg i.v., LPS affects CL function, decreases its steroidogenic capacities, and triggers systemic responses, without causing a life-threatening risk. Here: (1) CL samples were collected during different luteal stages (early, mid, and late); (2) CL biopsies from synchronized cows were obtained on day 10 of the first estrous cycle 12h after being treated i.v. with LPS or saline, and on day 10 of the subsequent cycle. Semi-quantitative real-time TaqMan PCR was performed to assess gene expression. Non-radioactive in situ hybridization (ISH) localized hypoxia inducible factor (HIF) 1α and its regulators. The transcript levels of HIF1A and its oxygen-sensitive regulators: PHD1 (EGLN2), PHD2 (EGLN1), PHD3 (EGLN3), FIH (HIF1AN), and VHL were assessed. The results demonstrated dynamic changes in gene expression patterns during the luteal lifespan, indicating active regulation of HIF1α transcriptional and functional availability. The luteal development was accompanied by increasing HIF1A transcription while it decreased in the late luteal phase. Its elevated levels at mid-luteal stage were accompanied by time-dependent expression of EGLN2, HIF1AN and VHL. In regressing CL, VHL and EGLN3 were significantly downregulated. While the LPS challenge affected only the expression of EGLN2 and EGLN3 in opposite directions, with EGLN2 being downregulated and EGLN3 upregulated, we observed a significant increase in the response of endothelial cell-specific pro-inflammatory factors ICAM1 (ICAM1) and NFκB (NFKB2), suggesting that LPS-induced vascular inflammation occurs, with concomitant hypoxia-related effects, possibly modulated through EGLN2 and -3 expression. ISH revealed compartmentalization of signals regarding the distribution of HIF1α and its regulators within the bovine CL, emphasizing the cell-specific regulation of hypoxia-related factors. Large luteal cells presented predominant signals for HIF1A. These cells were shown to exhibit higher basal progesterone (P4) production, than small luteal cells and less responsiveness to LH stimulation, implying that the basal production of P4 in the CL and the constant provision of P4 remain under the control of HIF1α in large luteal cells. HIF1A also showed signals in capillary vessels, mostly in endothelial cells, as well as in the media of smaller vessels, where it was colocalized with the other regulatory factors. EGLN2, prevailed in vessels, whereas in luteal cells it was mostly targeted to large cells, thereby colocalized with HIF1A. EGLN1 was abundantly present in larger vessels and ubiquitously distributed in small and large luteal cells, although it appeared weaker than in vessels. EGLN3 and VHL followed the localization pattern of EGLN1, but appeared to prevail in the vessels. In conclusion, this study offers new insights into hypoxia-dependent regulation of luteal function, including the spatiotemporal distribution of signals encoding for HIF1α regulatory factors. It marks the first demonstration of HIF1α expression in the context of its regulators and reveals their dynamic cellular compartmentalization. Additionally, it broadens our understanding of the LPS-mediated inflammatory responses in the CL, implicating possible hypoxia-mediated effects.

P-343 - Relationship between bone morphogenetic protein-15 (BMP-15) and formation of the hyaluronan-rich cumulus-oophorus extracellular matrix (ECM).

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It is well known that bidirectional communication between cumulus cells and oocyte is necessary for oocyte cytoplasmic maturation and subsequent developmental competence. Factors secreted by the oocyte, including BMP-15, have been shown to be implicated in the regulation of follicular cell functions. However, the complex role of BMP15 within the oocyte-cumulus complexes (OCCs) is not fully understood. To elucidate the molecular mechanisms of BMP-15 action on the regulation

of the FSH/LH signaling pathway in porcine OCCs cultured in vitro, we investigated the effect of BMP-15 on FSH/LH -induced 1/ cumulus expansion by stereomicroscopy; 2/ hyaluronan (HA) synthesis by OCCs by radioimmunoassay (RIA); 3/ expression of oocyte maturation-related transcripts (AREG, CCND2, CD44, CYP11A1, HAS2, PCNA, PTGS2, STAR and TNFAIP6) at different time points of the cultivation by real-time RT-PCR; and 4/ progesterone (P4) production either by OCCs or granulosa cells (GCs) by RIA. Results: We found that gonadotropin-stimulated OCCs in the presence of BMP-15 were 1/ full-expanded and formed elastic cumulus extracellular matrix (ECM); 2/ amount of HA retained within the ECM was significantly increased compared to FSH/LH alone (65% versus 35%, respectively). 3/ Moreover, BMP-15 enhanced FSH/LH-induced expression of the cumulus-related transcripts such as TNFAIP6 and AREG (both after 16 h) and CYP11A1 (after 24 h). 4/ Surprisingly, BMP-15 markedly increased (~69%) FSH/LH-stimulated P4 secretion by OCCs compared to FSH/LH alone, while BMP-15 caused a significant decrease (about 35%) in FSH/LH-induced P4 release by GCs. Conclusions: We have shown that addition of BMP-15 as well as differences in culture conditions, such as the absence or presence of serum, strongly affect the structural organization of oocyte-cumulus ECM and consequently the functionality of cumulus cells surrounding the oocyte. To our knowledge, this is the first study demonstrating the relationship between BMP-15 and formation of the HA-rich cumulus-oophorus ECM.

P-344 - Propranolol Treatment Increased Murine Follicular Activation After Ovarian Autotransplantation

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Ovarian follicles are classified as a functional unit of oogenesis, and it is well reported in the literature that ovarian transplantation is an alternative for maintaining female fertility. Furthermore, the mTOR signaling pathway has an effect on follicular activation and the drug propranolol has also been described as related to its activation. Thus, the objective of this study was to evaluate the effect of propranolol administration on follicular activation after ovarian auto transplantation in mice. 6-week-old female C57BL/6 mice were used (n=33), they were divided into six groups: control and propranolol-treated for 5 days; 14 days and 23 days. The body weight and estrous cycle of the animals were evaluated daily, and when in proestrus, they were subjected to anesthesia and bilateral ovariectomy. In the dorsal region of the forelimbs, two incisions were made for the ovarian grafts, followed by suturing and application of meloxicam for 3 days (5mg/kg). The control groups received daily i.p. injection according to the experimental period. of 150µl per animal, with PBS+DMSO, while the propranolol-treated received i.p. injection under the same conditions, of 150µl of propranolol (40mg/kg) + PBS. After treatment, the animals were euthanized, the ovaries removed and subjected to processing and staining in H&E (for quantification and percentage of each class of follicles: primordial, transitional, primary, secondary, antral and atretic; and morphometry of the diameter of the graft, ovary and capsule and subcapsular space area);

immunohistochemistry for FOXO3a (D19A7 Rabbit mAb, Cell Signaling TECHNOLOGY) in the 14-day experimental groups (to evaluate follicular activation through the percentage of oocytes with FOXO3a translocated from the nucleus to the cytoplasm) and the TUNEL technique (DeadEnd™ Colorimetric TUNEL System, Promega, G7132) at all experimental times (to analyze cells positive for apoptosis). For statistical analysis, GraphPad Prism 8.0.2 was used, with D'Agostino & Pearson and Shapiro-Wilk normality tests, followed by the t or Mann-Whitney test between groups, considering $p < 0.05$. Body weight did not show significant differences between the groups. However, ovarian weight was lower in the 14-day propranolol group compared to the control group ($p=0.0077$), and also lower in the 23-day control group compared to propranolol ($p=0.0289$). Regarding histological evaluation, at 5 days, there was a significant increase ($p=0.0480$) in the atretic class of the propranolol treated animals vs control. Regarding the immunohistochemical analysis for FOXO3a, no significant differences were observed between the groups. Capsular morphometry showed, at 5 days, a significant decrease in ovarian diameter between the control group ($p=0.0289$) and propranolol, and during this treatment period the majority of animals did not yet have a capsule around the ovary. At 14 days there were no significant differences and at 23 days there was a decrease in capsule diameter ($p=0.0197$) between the groups. The TUNEL method qualitatively demonstrated the presence of atresia in all groups, especially at 5 days, demonstrating that the initial days post-transplant lead to greater follicular apoptosis. Murine ovarian autotransplantation, added to the administration of propranolol daily at different experimental times, possibly helped with ovarian follicular activation, demonstrating the efficiency of the protocol and presenting itself as a possible viable alternative for preserving fertility. Keywords: Ovary; transplant, propranolol, follicular activation; Fertility. Financial Support: CAPES, CNPq, FAPEMIG and UFSJ.

P-345 - Association of Vitamin D and Its Receptor and the Severity of Ovarian Cancer

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High-grade serous ovarian carcinoma (HGSOC) and low-grade serous ovarian carcinoma (LGSOC) are the most common subhistotypes of epithelial ovarian cancer. HGSOC grows and spreads faster than LGSOC which is considered as slow-growing cancer. The 5-year survival rate of HGSOC (32.1%) is significantly lower than that of LGSOC (54.2%). Thus, efforts have been made to develop strategies for primary and adjuvant therapy relevant to HGSOC and LGSOC. Vitamin D (VD), a secosteroid hormone initially identified for its role in maintaining calcium homeostasis, exhibits pleiotropic functions. VD₃ is produced in the skin upon UV-B light exposure, which is converted to 25-hydroxyvitamin D₃ (25(OH)D₃, the major circulating form of VD) in the liver, and then 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃, the biologically active form of VD) in the kidneys. The active 1,25(OH)₂D₃ acts on VD receptor (VDR) to regulate downstream gene expression critical for cell survival, growth, proliferation, differentiation, and apoptosis. In recent years, VD has been studied as a preventive or adjuvant agent for highly prevalent cancer types. Epidemiological studies showed that VD deficiency is associated with increased mortality in breast, colon, and rectum cancer patients. VD supplementation increased 5-year survival rate of ovarian cancer patients. Therefore, experiments were designed to determine the direction action of VD on the proliferation (viability) of HGSOC and LGSOC cells. Three ovarian cancer cell lines, OVSAHO, SKOV3, and VOA6406, were selected for in vitro study. OVSAHO is an established human ovarian carcinoma cell line typically

used as a reproducible in vitro source for HGSOC, whereas VOA6406 is a typical LGSOC cell line. SKOV3 is a widely used ovarian cancer cell line. Although SKOV3 cells are not considered as HGSOC or LGSOC cells, they have a high growth rate and form spheroids in culture which are similar to OVSAHO cell behaviors. All three cell lines were cultured in a mixture of RPMI1640/MCDB105/Medium199/DMEM (1:1:1:1) containing 10% (v/v) FBS, 10,000 IU/ml penicillin, 10,000 IU/ml streptomycin, 1 × MEM non-essential amino acids, and 2 mM L-glutamine at 37 °C and 5% CO₂. Treatment groups included 1,25(OH)₂D₃ supplementation at 0 (control), 1, 5, 10, 50 nM for 72 hours. Immunofluorescent staining was performed to detect VDR expression in cultured cells. VDR protein levels were measured by western blot. Cell proliferation (viability) was assessed using CellTiter-Glo 2.0 Cell Viability Assay. VDR expression was observed in all three ovarian cancer cell lines with intensive staining located in the nucleus. VDR levels were significantly higher in VOA6406 cells than those of SKOV3 and OVSAHO cells. The 1,25(OH)₂D₃ supplementation significantly decreased cell proliferation (viability) of OVSAHO, SKOV3, and VOA6406 at 50, 5, and 1 nM, respectively. The results suggest that VD can elicit direct action on ovarian cancer cells via VDR. VD supplementation inhibits ovarian cancer cell proliferation in vitro in a dose-dependent manner. Cells with low levels of VDR expression require a high dose of VD to induce inhibitory effects. For the first time, it is revealed that HGSOC and LGSOC cells exhibit different dose-response patterns to VD treatment due to differences in VDR levels. Our data support the potential of VD as adjuvant therapy for HGSOC and LGSOC. High-dose VD treatment may be required to effectively inhibit HGSOC development. Further studies are needed to delineate the underlying mechanism of VD in regulating ovarian cancer cell proliferation.

P-346 - Co-Exposure to Mono(2-ethylhexyl) Phthalate and Elevated Temperature Inhibits Mouse Antral Follicle Growth and Steroidogenesis In Vitro

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Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer found in common consumer goods, such as household items, food packaging, and medical devices. DEHP is rapidly metabolized upon ingestion to mono(2-ethylhexyl) phthalate (MEHP), which is bioactive and known to exert endocrine-disrupting toxicities. Due to the extensive use of DEHP in consumer goods, humans are ubiquitously exposed and are vulnerable to endocrine and reproductive dysfunction by MEHP. Studies have shown that MEHP exposure disrupts the essential ovarian processes of antral follicle growth and steroidogenesis. Simultaneously, humans are ubiquitously exposed to non-chemical stressors, such as rising global temperatures, and studies have shown that exposure to elevated temperatures can impair female reproductive health. Given the progression of climate change and the unavoidable exposure to both increasing heat and DEHP, this study used an in vitro follicle culture system to test the hypothesis that exposure to high temperature (HT) will exacerbate the negative effects of MEHP exposure on antral follicle growth and steroidogenesis. Antral follicles were isolated from 4-5-week-old CD-1 mice and cultured for 96 hours in media containing follicle-stimulating hormone at a control temperature (CT; 37°C) or high temperature (HT; 42°C; 8hr/day).

Follicles were treated with vehicle control (dimethyl sulfoxide; DMSO) or MEHP (0.2, 2, and 20µg/ml) in both temperature groups for the 96-hour culture period. Separate incubators were used for the CT and HT treatment groups, in which all HT treatments occurred for 8 hours per 24-hour period to model daytime, occupational exposure to high temperatures. Follicular growth was measured every 24 hours via inverted microscopy. Follicles and media were collected at 96 hours for steroidogenic gene expression analysis and sex steroid hormone measurements, respectively (n=3-7 with 5-10 follicles/group/replicate, $p \leq 0.05$). Antral follicle growth was decreased by HT+MEHP 2µg/ml and HT+MEHP 20µg/ml (72hr, 96hr) treatment compared to the CT+DMSO control group. Further, antral follicle growth was decreased by HT+DMSO (72hr, 96hr) and HT+MEHP 20µg/ml (96hr) treatment compared to their CT treatment equivalents. Estradiol levels were decreased by CT+MEHP 0.2µg/ml treatment and in HT groups (DMSO, MEHP 0.2, 2, and 20µg/ml) compared to CT+DMSO and in HT groups (DMSO, MEHP 0.2, 20µg/ml) compared to their CT treatment equivalents. Testosterone levels were increased by CT+MEHP 20µg/ml treatment compared to CT+DMSO and decreased by HT+MEHP 20µg/ml treatment compared to its CT treatment equivalent. The mRNA levels of Hsd3b1 were decreased in CT groups (MEHP 0.2, 2, and 20µg/ml) and by HT+MEHP 20µg/ml treatment compared to CT+DMSO. The mRNA levels of Cyp17a1 and Hsd17b1 were decreased by HT+MEHP 20µg/ml treatment compared to CT+DMSO and by HT+DMSO and HT+MEHP 0.2µg/ml treatment compared to their CT equivalents. The mRNA levels of Cyp19a1 were decreased in CT groups (MEHP 2, 20µg/ml) and HT groups (DMSO, MEHP 0.2, 2, and 20µg/ml) compared to CT+DMSO and decreased in HT groups (DMSO, MEHP 0.2, 2, and 20µg/ml) compared to their CT treatment equivalents. These findings suggest that exposures to HT and MEHP inhibit antral follicle growth and disrupt steroidogenesis. The observed decreases in steroidogenic mRNA levels correlate to the decreases in estradiol levels, with HT exposure exacerbating the MEHP 2µg/ml and 20µg/ml-mediated decreases in Cyp19a1 levels. Thus, combined exposures to these ubiquitous chemical and non-chemical stressors may potentiate ovarian dysfunction. Supported by R01ES033767 and pilot funding from UL1TR001998 and P30ES026529.

P-347 - Ovulatory Angiogenic Factors and Receptors are Altered by an Environmentally Relevant Phthalate Mixture in Mouse Granulosa and Endothelial Cells In Vitro

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Phthalates are known endocrine-disrupting chemicals that people are exposed to daily. Previous work by our group has shown that phthalates decrease mouse ovarian follicular production of prostaglandins, which are key regulators of ovulatory angiogenesis. Angiogenesis is critical for successful ovulation and involves granulosa cell secretion of pro-angiogenic factors that bind to receptors on ovarian endothelial cells. Specifically, the ovulatory cascade induces granulosa cells to produce prostaglandin E2 (PGE2), prostaglandin F2α (PGF2α), and members of the vascular endothelial growth factor (VEGF) family, while endothelial cells express multiple prostaglandin E

receptors (PTGER1-4), prostaglandin F receptor (PTGFR), and vascular endothelial growth factor receptors (FLT1, KDR, FLT4). We hypothesized that phthalates alter both the production of angiogenic factors by granulosa cells and the expression of angiogenic receptors on ovarian endothelial cells. Mouse granulosa cells were isolated from immature mice 48 hours after pregnant mare serum gonadotropin (PMSG) injection. The cells were treated with vehicle control (dimethylsulfoxide, DMSO) or an environmentally relevant phthalate mixture (MPTmix, 1-500µg/mL) that was derived using urinary phthalate metabolite levels in pregnant women. This is considered environmentally relevant considering humans are exposed to a mixture of phthalates daily that are rapidly converted into active metabolites that reach the ovary. Following one hour of MPTmix exposure, the cells received human chorionic gonadotropin (hCG) treatment to induce the ovulatory cascade. The cells and media were collected at 11 hours post-hCG treatment and subjected to gene expression analysis and enzyme-linked immunosorbent assays (n=5, p≤0.05). In complementary experiments, mouse ovarian microvascular endothelial cells were treated with DMSO or MPTmix (1-500µg/mL) with or without VEGFA supplementation. The cells were collected at 24 and 48 hours for gene expression analysis (n=4, p≤0.05). Exposure to hCG+MPTmix decreased the granulosa cell production of PGE2 at doses of 1, 10, 100, and 500µg/mL and PGF2α at doses of 10, 100, and 500µg/mL compared to hCG ovulatory controls. Of the prostaglandin receptors on endothelial cells, treatment with MPTmix increased Ptger4 at 500µg/mL (24hr); and decreased Ptgfr expression at 10µg/mL (24hr) and 100µg/mL (48hr) doses when compared to DMSO. For the VEGFs in granulosa cells, exposure to hCG+MPTmix decreased the mRNA levels of Vegfd at doses of 100 and 500µg/mL and increased protein levels of VEGFA at a dose of 500µg/mL, while gene expression of Vegfa remained unchanged. Of the VEGF receptors, MPTmix decreased endothelial cell expression of Flt1 at doses of 1µg/mL and 100µg/mL (24hr); decreased Kdr expression at doses of 1µg/mL and 10µg/mL (24hr), and at 500µg/mL (48hr); and decreased expression of Flt4 at doses of 10µg/mL and 100µg/mL (24hr) when compared to DMSO controls. Supplementation with VEGFA restored the MPTmix-induced changes in the expression of all angiogenic receptors to DMSO control levels. These data demonstrate that exposure to an environmentally relevant phthalate mixture altered the production of granulosa cell-derived angiogenic factors. Additionally, phthalate exposure altered the expression of key angiogenic receptors on endothelial cells, and these toxic effects were restored with VEGFA supplementation. Thus, phthalate exposure may induce dysregulation of the communication network between granulosa cells and endothelial cells necessary for ovulatory angiogenesis. Supported by R01ES033767.

P-348 - Exposure to Phthalates Disrupts Ovulatory Luteinizing Hormone/Choriogonadotropin Receptor Signaling and Decreases Downstream Prostaglandin Production in Human Granulosa Cells In Vitro

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Humans are ubiquitously exposed to phthalates, which are widely used chemicals that disrupt female reproductive health. Phthalates are a family of solvents and plasticizers found in commonly used products including cosmetics, food/beverage containers, medical tubing, and car upholstery, among other sources. Phthalates undergo rapid metabolism in the body, and these metabolites can target the ovary. Due to their classification as endocrine-disrupting chemicals, exposure to phthalates and their active metabolites could impair ovulation. To best mimic human exposure, this study used an environmentally relevant phthalate metabolite mixture (MPTmix), which was derived from urinary phthalate measurements in pregnant women. The ovulatory process is initiated by an endogenous surge of luteinizing hormone (LH) or clinical treatment with human chorionic gonadotropin (hCG, a potent LH analogue). LH/hCG binding to its receptor (LHCGR) on ovarian granulosa cells promptly activates various intracellular signaling cascades, including pathways mediated by protein kinase A (PKA), protein kinase B (AKT), and extracellular signal-regulated kinase 1/2 (ERK1/2). Additionally, it is postulated that ERK1/2 signaling drives the production of prostaglandin E2 (PGE2) via transcription of PTGS2, a prostaglandin synthase. Increases in PGE2 are vital for the inflammatory changes that occur during ovulation, including cumulus-oocyte complex expansion, follicle rupture, and angiogenesis. We hypothesized that MPTmix exposure would disrupt essential signaling cascades involved in ovulation (PKA, AKT, ERK1/2), alter mRNA levels of PTGS2 and enzymatic activity of PTGS2 protein, and decrease PGE2 levels. Granulosa-lutein cells from follicular aspirates of women undergoing in vitro fertilization were acclimated in culture to regain LH/hCG responsiveness. Prior to hCG treatment, cells were exposed for 48hr to vehicle control (dimethylsulfoxide, DMSO) or varying doses of the MPTmix (1-500µg/ml). Following treatment ± hCG and ± MPTmix, cells and media were collected at 0, 0.5, 6, 12, 24, or 36hr to measure the levels of cAMP (upstream of PKA), p-PKA, p-AKT, p-ERK1/2, PTGS2 mRNA, PTGS2 activity, and PGE2 levels (n=3-11; p<0.05). Treatment with hCG alone rapidly (0.5hr post-treatment) increased cAMP, p-PKA, p-AKT, and p-ERK1/2 levels when compared to DMSO. However, treatment with hCG+MPTmix 500µg/ml decreased cAMP, p-PKA, and p-ERK1/2 levels relative to the hCG ovulatory control group at 0.5hr. Similarly, hCG treatment increased PTGS2 mRNA levels and PTGS2 activity at all timepoints when compared to DMSO alone. Treatment with hCG+MPTmix decreased PTGS2 mRNA levels at 6hr (500µg/ml), 12hr (1, 10, 500µg/ml), 24hr (all doses), and 36hr (100, 500µg/ml) when compared to hCG. Additionally, hCG+MPTmix treatment decreased PTGS2 activity at 24hr (all doses) and 36hr (1, 100, 500µg/ml) relative to hCG. The decreases in PTGS2 activity correlate with the 24hr and 36hr MPTmix-induced decreases (all doses) in PGE2 levels compared to hCG. These data demonstrate that phthalate exposure alters requisite ovulatory signaling cascades, and has further downstream inhibitory effects on mediators that drive functional changes leading to oocyte release. These findings in human granulosa cells exposed to an environmentally relevant mixture of phthalates suggest that phthalates may impair the ovulatory process in women. Supported by R01ES033767, R00ES028748, P30ES026529.

P-349 - Semaphorin 3E-Plexin-D1 pathway regulates ovulation, luteinization, and ovarian angiogenesis in mice

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Mammalian ovulation, triggered by the preovulatory surge of luteinizing hormone (LH), is accompanied by granulosa cell (GC) luteinization and ovarian angiogenesis. Various regulators of ovarian angiogenesis play crucial roles in ovulation and corpus luteum (CL) formation, but the picture of key regulators and the mechanisms governing their activities is far from complete. Semaphorin 3E (Sema3E), a secreted protein of the semaphorin family, regulates the guidance and migration of neurons and endothelial cells via binding to its receptor Plexin-D1; however, its role in the ovary has not been investigated. Elevated transcript levels for Sema3e in the early luteal phase were reported in mice with GC-specific deletion of CCAAT/enhancer-binding proteins alpha and beta (*Cebpa/bgc*^{-/-}), which had blocked ovulation and impaired CL angiogenesis. We hypothesize that SEMA3E-PlexinD-1 pathway is active downstream of the preovulatory LH surge and regulates ovarian angiogenesis around the time of ovulation. We first examined the expression patterns for Sema3e and *Plxnd1* in the mouse ovary following superovulation (time post-hCG is the biological equivalent of time post-LH surge). Utilizing real-time-qPCR on isolated GCs and ovarian stromal tissues, immunostaining, high-resolution spatial transcriptomics, and single-nucleus assay for transposase-accessible chromatin sequencing (snATAC-seq), we found that Sema3E and Plexin-D1 are induced by the LH surge in preovulatory follicles in dynamic spatial and temporal patterns. Sema3E is predominantly expressed in GCs. The expression of Sema3e mRNA is induced shortly after LH surge, peaks at 6 h post-hCG, and decreases to a lower level immediately before ovulation (12h post-hCG). Plexin-D1 is expressed in a sub-population of GCs and ovarian stromal tissues (primarily in ovarian vascular endothelial cells) with cell type-specific temporal dynamics. Fluorescence in situ hybridization revealed a dynamic shift of *Plxnd1*⁺ vascular endothelial cells from stromal tissues to the CL after ovulation. We then asked whether C/EBP α and C/EBP β regulate the expression of Sema3e and *Plxnd1* during the preovulatory stage. By immunostaining, snATAC-seq, and comparing Sema3e and *Plxnd1* mRNA levels in GCs and stromal tissues isolated from *Cebpa/bgc*^{+/+} control and *Cebpa/bgc*^{-/-} mutant mice, we found that mRNA levels of these two genes are dysregulated in the mutants in a cell type-specific manner. SnATAC-seq further indicated that Sema3e and *Plxnd1* are expressed in sub-populations of preovulatory granulosa cells, and C/EBP α and C/EBP β regulate their expression during the preovulatory stage by modulating chromatin accessibility. Specifically, deletion of *Cebpa/b* increased the expression of Sema3e during the late preovulatory stage. To explore the functional impact of evaluated Sema3E in the mouse ovary, we conducted intraovarian injection of mouse recombinant Sema3E to one ovary and IgG control to the contralateral ovary shortly before hCG stimulation. The injection of Sema3E resulted in reduced ovulation, disrupted CL formation, and aberrant ovarian angiogenesis both in stromal tissues and in CL. Bulk RNA-seq analysis on isolated GCs and stromal tissues revealed altered transcripts of genes regulating GC luteinization, ovarian stromal angiogenesis, and

inflammation in Sema3E-treated ovaries compared to controls. Furthermore, in vitro culturing of GCs and stromal tissues treated with Sema3E demonstrated a direct effect of Sema3E on GC luteinization and angiogenesis, with an indirect effect on stromal angiogenesis and inflammation primarily through its effects on GCs. Taken together, our findings demonstrate the role of Sema3E-Plexin-D1 pathway in regulating ovulation, GC luteinization, and ovarian angiogenesis in mice. Sema3E-Plexin-D1 pathway may be a potential therapeutic target for infertility associated with impaired ovulation and ovarian angiogenesis.

P-350 - Optimizing the Luteinization Timeline: A Comparative Study of Human Mature and Immature Granulosa Cells In Vitro

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During luteinization, the granulosa cells (GC) undergo extensive expressional changes. This transition, prompted by an increase in gonadotrophins, transforms GC into granulosa-lutein cells of the corpus luteum. However, the timeline of the transition has currently not been adequately explored. In this study, human GC (mature GC) from preovulatory follicles collected through in vitro fertilization (IVF) and GC (immature GC) from antral follicles, collected through our ovarian tissue cryopreservation (OTC) program, are compared to establish the in vitro timeline for luteinization of early and late-stage GC. During cell culture both media and GC were collected every 12 hours, to investigate changes in gene expression and hormone levels. GC was donated by six women (n=57) aged 27-40 years undergoing fertility treatment or preservation. The expression of STAR, CYP11A1, CYP19A1, and 17 β HSD genes, along with hormone assay for estradiol and progesterone, was utilized to analyze luteinization. The mature GCs displayed minor differences in gene expression and estradiol production between the control and androstenedione (100 nm) groups. There was observed a significant difference ($p \leq 0.05$), between the treatment groups in the expression of CYP11A1 after 72 hours ($p = 0.03$) and STAR after 84 hours ($p = 0.02$). Furthermore, within the androstenedione treatment group, a significant variation was noted in the expression of the gene 17 β HSD between the time points 72 hours and 96 hours ($p = 0.04$). Estradiol levels demonstrated no time-dependent difference within or between the treatment groups. The collection of immature GCs is ongoing; preliminary data indicate a gene-level trend wherein the expression of luteal cell markers, STAR and CYP11A1, increases 8-10-fold over 48 hours, while the expression of GC-specific gene markers, CYP19A1 and 17 β HSD, exhibits a decrease ranging from 1.2- to 2.6-fold over the same period. Additionally, estradiol levels increase 2-fold after 24 hours post-initiation of in vitro culture, parallel with a marked increase in progesterone for each time point resulting in a 9-fold increase after 48 hours. Preliminary observations suggest that the majority of mature GCs harvested from IVF procedures exhibit characteristics of luteinization, with a peak in luteal gene expression observed approximately 48 hours following the onset of in vitro culture, and appears unaffected by the presence of androgen supplementation. This pattern raises concerns regarding their suitability as a model for in vitro studies of luteinization. Conversely, immature GCs demonstrate an upregulation of luteal gene expression approximately 24 hours post-culture.

initiation. This is accompanied by an increase in estradiol levels up to the 24-hour timepoint, followed by a pronounced surge in progesterone. These findings may imply that GCs from antral follicles undergo luteinization between 24-48 hours after the onset of in vitro culture. Additional time points and patients will be included over the following months to specify and confirm these preliminary conclusions.

P-351 - Differential Expression of microRNAs Predicted Against Connexins 37 and 43 mRNA Between Oocytes and Cumulus Cells in Canines.

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Connexins 37 (Cx37) and 43 (Cx43) participate in the bi-directional communication between the oocyte and its companion cumulus cells (CCs). Several post-translational factors, including miRNA, control connexins; therefore, the aim of this study was to determine the expression levels of miRNAs predicted against canine Cx37 and Cx43 mRNA in CCs and oocytes. Cumulus oocyte complexes were retrieved from small (~0.25–0.39 mm) and medium (~0.4–3.9 mm) antral follicles from more than 100 dog ovaries following ovariectomies. CCs were mechanically removed from the oocytes. Specific miRNAs regulating Cx37 and Cx43 genes were selected from miRNA databases. The best miRNA for each target gene (Cx37 and Cx43) was selected considering qPCR performance (low ct value and specific melt curve) and target prediction probability with a binding p-value of 1.0, choosing for the study cfa-miR-1838 against Cx37 mRNA and cfa-miR-130b against Cx43 mRNA. The expression level of each miRNA in the oocytes and CCs from both follicle sizes was evaluated using ANOVA. The relative abundance of cfa-miR-1838 was the highest ($P < 0.05$) in oocytes from the small follicles, whereas the lowest ($P < 0.05$) value was observed in CCs from medium antral follicles. Cfa-miR-130b abundance was higher ($P < 0.05$) in oocytes compared to CCs in both follicle sizes. Therefore, the expression of cfa-miR-1838 and cfa-miR-130b differed ($P < 0.05$) between the oocytes and CCs from small and medium antral follicles. These miRNAs against canine Cx37 and Cx43 mRNA could play a role in regulating oocyte and CC cross-talk. Grant FONDCYT 1211285

P-352 - SLIT1 and -2 Act Redundantly Through an AKT/FOXO1 Pathway in Mouse Follicular Granulosa Cells

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Previous studies conducted in our laboratory have demonstrated the involvement of SLIT1 in female fertility in mice. SLIT1-deficient mice display increased fertility, characterized by higher rates of ovulation and a greater abundance of healthy antral follicles compared to wild-type (WT) mice. Our findings identified SLIT1 as an inhibitor of AKT, a crucial effector known for its roles in gonadotropin signaling. One of the well-known downstream targets of AKT is FOXO1, a transcription

factor involved in regulating various cellular processes, including cell survival and apoptosis. The Slit/Robo pathway comprises three ligands: SLIT1/2/3 and their four receptors ROBO1/2/3/4. While SLIT1 and SLIT2 share similar structures and properties, SLIT3 diverges structurally and seems more associated with angiogenesis. The current study seeks to clarify the potential redundancies among SLIT ligands and their mechanisms of action in mouse ovarian follicles. To investigate the ovarian expression of Slit/Robo genes, granulosa cells from WT mice were isolated. mRNA levels of Slit/Robo genes were assessed using RT-qPCR, which revealed the expression of all effectors in mouse granulosa cells. Western blotting analyses were conducted on cultured granulosa cells treated with exogenous SLIT1, -2, or -3 alone or in combination with FSH to investigate the potential redundant effects of SLIT ligands on the AKT pathway. The results showed that SLIT1 and -2 significantly inhibit the induction of AKT and FOXO1 phosphorylation by FSH. In line with expectations, cells treated with SLIT3 showed no alteration in AKT and FOXO1 phosphorylation levels. To explain the increased number of healthy follicles observed in SLIT1-deficient mice, apoptosis was evaluated in cultures granulosa cells treated with SLIT1. Our analysis revealed that SLIT1 treatment induces an elevation in cleaved-caspase 3 levels, as well as the proportion of TUNEL-positive cells. Furthermore, granulosa cells derived from Slit1-/- mice exhibited elevated mRNA levels of Cyp11a1, Cyp19a1, Inha, Nr5a2, and Star compared to wild-type mice. These genes have been previously identified in the literature as targets of FOXO1. To elucidate the genes regulated by SLIT1, we conducted RNA sequencing on granulosa cells from both SLIT1-deficient and WT mice. Our analysis revealed a marked impact of SLIT1 on the Renin/Angiotensin system (RAS) pathway. Subsequent qPCR analysis confirmed significant downregulation of Agt, Agtr2, Clca3a, and Mme in SLIT1-deficient mice compared to WT counterparts. Notably, RAS is also known to be regulated by FOXO1 in other cell types. To identify the receptor responsible for SLIT1 action in mouse ovarian granulosa cells, we utilized a Robo1-/-;Robo2f/f mutant model, in which the Robo2 allele is highly hypomorphic. Granulosa cells isolated from these mutants and WT mice were cultured with SLIT1 and/or FSH, followed by Western blot analyses. Interestingly, SLIT1 treatment led to a comparable decrease in AKT phosphorylation in the granulosa cells from WT or mutant mice, indicating that ROBO1 is not the exclusive receptor for SLIT1 in granulosa cells, and that neither ROBO1 nor ROBO2 may be required for SLIT1 to exert its effects. Together, these data suggest that SLIT1 induces an increase in nuclear FOXO1 levels by inhibiting AKT phosphorylation, resulting in suppression of the transcription of its target genes and the induction of apoptosis. To validate this hypothesis, ChIP-sequencing experiments will be conducted. Further investigations will be required to elucidate the mechanisms of action of SLIT2 and SLIT3 in mouse ovarian granulosa cells.

P-353 - Neurotensin Mediates Ovulation in Mouse Ovarian Follicles In Vitro

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Neurotensin (NTS) is a small peptide produced in the ovarian follicle. The ovulatory gonadotropin surge rapidly increases follicle expression of NTS mRNA in mice, rats, monkeys, and women. Inactivation of NTS within the follicle via immunoabsorption reduces ovulation in monkeys,

implicating NTS as a paracrine mediator of ovulation. NTSR1, NTSR2 and SORT1 have been identified as functional NTS receptors. We hypothesize that NTS stimulates ovulation through one or more NTS receptor(s), and that blockade of NTS action at one or more NTS receptors will decrease ovulation. A model of in vitro mouse follicle culture was used in which ovaries were removed from female 32-39-day-old C57BL/6 mice. Secondary follicles were dissected from the ovary and cultured individually in follicle stimulating hormone-containing media. After four days, follicles that increased in diameter by at least 40% and remained circular and intact were treated with or without human chorionic gonadotropin (hCG) to stimulate ovulatory changes. Total RNA was collected from follicles at 0, 4, 8, and 12 hours treated with or without hCG, and mRNA for NTS and NTS receptors was quantified via qPCR (n=4 per treatment/time). hCG rapidly and transiently increased follicle expression of NTS mRNA, with levels peaking quickly at 4 hours and then decreasing at 8 and 12 hours ($p < 0.05$). Follicular cells expressed mRNA for NTS receptors NTSR1 and SORT1 at all time points with and without hCG. SORT1 mRNA was decreased at 4, 8, and 12 hours post-hCG ($p < 0.05$) while NTSR1 mRNA remained constant. NTSR2 mRNA was not detected. In a separate experiment, follicles were treated with or without hCG for 18 hours to induce ovulation and then assessed via microscopy for follicle rupture, oocyte release, cumulus expansion, and luteinization. Media was assayed for progesterone and prostaglandin E2 accumulation by ELISA. Without hCG, ovulation was low (0-10% of follicles). hCG effectively stimulated ovulatory events after 18 hours (74-80% of follicles), including follicle rupture, release of a cumulus-oocyte-complex, luteinization of follicle cells, and increased media levels of progesterone ($p < 0.05$ at 12, 18 hours; n=10) and prostaglandin E2 ($p < 0.05$ at 18 hours; n=10). To determine if NTS receptor activation is important for ovulation, day 4 follicles were pre-treated for 1 hour before hCG administration with or without the general NTS receptor antagonist SR142948. In the presence of hCG, SR142948 resulted in a reduced rate of ovulation (45%, $p < 0.05$; n=5), indicating that NTS action at one or more NTS receptors is important for ovulation. To determine which NTS receptors are involved in ovulation, follicles were pre-treated with either the NTSR1-selective antagonist SR48692 or the SORT1-selective antagonist Spadin for 1 hour before hCG administration. Blockade of either NTSR1 or SORT1 significantly reduced the rate of ovulation (SR48692 48%, n=3 and Spadin 58%, n=4; $p < 0.05$) compared to hCG alone. Media progesterone and prostaglandin E2 in the presence of hCG was not affected by any of the antagonists. These data support the concept that locally-produced NTS is a paracrine factor which promotes ovulation in multiple mammalian species. The present findings are the first demonstration that NTSR1 and SORT1 are key receptors which mediate NTS-stimulated ovulatory events. Ultimately, NTS receptors may be useful therapeutic targets to promote ovulation in infertile women or reduce ovulation as a non-hormonal contraceptive. This work was supported by EVMS and NIH HD097675 to DMD and TEC.

P-354 - Luteinizing Hormone Stimulation of Germ-Free and Conventionalized Female Mice Reveals Microbiome-Dependent Effect on Ovarian Steroidogenesis and Ovulation

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Germ-free female mice have decreased litter sizes and increased pregnancy intervals compared to conventionally raised mice, suggesting that the microbiome affects their ovulation and/or oocyte quality. However, little is known about how the absence of a microbiome affects ovulatory stimulation and oocyte maturation. The hypothesis of this study was that female mice lacking a

microbiome have reduced fertility due to alterations in the ovarian response to LH stimulation. To test this hypothesis, we utilized two strains of mice, C57BL6/J (B6) and C3H/HeN (C3H). Mice from each strain were devoid of all microbiota (GF; B6-GF n=10, C3H-GF n=8) or conventionalized via oral gavage with fecal microbiota from conventionally reared mice upon reaching mature body weight (CVZ; B6-CVZ n=9, C3H-CVZ n=8). All mice were fed normal rodent chow until 14-16 weeks of age. Females were stimulated to ovulate with 5 IU PMSG and 5 IU hCG. Following hCG injection, only 44% of B6-GF females ovulated, compared to 100% of B6-CVZ females ($P < 0.01$). There was no difference in ovulation between C3H-CVZ (87.5%) and C3H-GF (100%). Additionally, the number of oocytes ovulated was significantly lower ($P < 0.05$) in B6-GF (11 ± 2.2) compared to B6-CVZ (17 ± 1.2), while there was no difference between C3H-CVZ (13.7 ± 2.5) and C3H-GF (11.0 ± 1.3). We then assessed steroidogenesis in response to ovulatory stimulation. Blood serum was obtained from all females to determine post-ovulatory circulating estradiol (E2) and progesterone (P4) concentrations. We detected a 5-fold increase ($P < 0.01$) in E2 concentration in C3H-GF compared to C3H-CVZ. However, E2 was not different between B6-CVZ and B6-GF. Conversely, progesterone (P4) tended to be decreased ($P = 0.09$) in B6-GF compared to B6-CVZ, while there was no difference between C3H groups. The E2/P4 ratio was significantly increased ($P < 0.05$) in B6-GF compared to B6-CVZ and tended to be increased ($P = 0.06$) in C3H-GF compared to C3H-CVZ females. Consistent with these changes in circulating steroids, the mRNA abundance of steroidogenic enzymes within whole ovaries was also altered. The abundance of Star, which moves cholesterol into the mitochondria, was increased ($P < 0.01$) in B6-GF compared to B6-CVZ, but not in C3H ovaries. Conversely, Hsd3b, which converts pregnenolone to P4, tended to be increased ($P = 0.09$) in C3H-GF compared to C3H-CVZ, but not between B6-GF and B6-CVZ females. Cyp17a1, which converts progesterone to androgens, was significantly increased ($P < 0.01$) in B6-GF compared to B6 CVZ, while there was a tendency ($P = 0.08$) for decreased Cyp17a1 in C3H-GF compared to C3H-CVZ. Lastly, Cyp19a1, which aromatizes androgens to estrogens, was significantly decreased ($P < 0.05$) in C3H-GF compared to C3H-CVZ, but not in B6. To determine whether the lack of a microbiome affected oocyte maturation and developmental competence, we assessed spindle structure and performed qPCR to quantify markers of cumulus expansion. There were no differences in the percentage of normal spindles between B6 (CVZ, 83%; GF, 71%) or C3H (CVZ, 73%; GF, 78%) experimental groups. There was also no difference in the expression of Has2, Tnfrsf6, or Ptx3 in cumulus cells from GF and CVZ mice from either strain. However, there was a significant decrease ($p < 0.01$) in the abundance of Ptgs2 in cumulus cells from B6-GF compared to B6-CVZ, but not C3H-GF and C3H-CVZ. Taken together, these data suggest the microbiome does not affect nuclear maturation. Rather, it may be essential for LH responsiveness of somatic cells and the physiological process of ovulation.

P-355 - Increased Plasma Progesterone Levels During the Estrous Cycle and Altered Follicular Fluid IL-13 May Determine if High A4 Cows Ovulate or Develop Persistent Follicles.

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Our research identified a subpopulation of cows with naturally elevated androgen levels (specifically androstenedione, or A4; High A4 cows; A4>40ng/ml) in follicular fluid compared to controls (A4<.20ng/ml). Interestingly, these High A4 cows have molecular characteristics similar to those observed in women diagnosed with polycystic ovary syndrome (PCOS). High A4 cows also are sub-fertile with a 17% reduction in calving rate, increased ovarian fibrosis, and oxidative stress. This study aimed to investigate whether elevated ovarian cortex A4 levels in cows with ovulatory (HA4O) or irregular (HA4I) cycles cause hormonal, metabolic, and immune changes compared to controls. We hypothesized that excess androgen alters hepatic liver enzymes, and endocrine hormones to allow for ovulation to occur in HA4O compared to anovulatory HA4I cows. Interestingly, HA4O cows had the highest A4 levels in ovarian cortex media, followed by HA4I and then control cows. The HA4O cows had greater circulating concentrations of progesterone (P4) and reduced estradiol (E2) during the estrous cycle when compared to the HA4I and control groups. Circulating Leptin secretion was greater in control animals compared to HA4O and HA4I groups indicating there may be differences in metabolism in High A4 cows. There were no differences in circulating non-esterified fatty acids (NEFA's) or triglycerides between the HA4O and HA4I groups. Hepatic tissue had increased cytochrome 2C (CYP2C; $p < 0.05$) and a tendency ($p < 0.06$) for increased aldo-keto reductase 1C (AKR1C) activity in High A4 groups compared to controls suggesting that steroid hormones would be metabolized at a faster rate in excess androgen females. Interleukin-13 (IL-13) concentrations were increased in follicular fluid of dominant follicles of HA4I ($p < 0.03$) compared to HA4O and Control cows. Elevated IL-13 has been demonstrated to cause inflammation, suggesting that increased inflammation may be a factor contributing to anovulation in the HA4I cows. In summary, while both High A4 groups had increased liver enzyme activity and reduced circulating Leptin, HA4O had increased circulating P4, reduced E2, and increased dominant follicle follicular fluid IL-13. The cytokine, IL-13, is an anti-inflammatory cytokine that causes inflammation and may contribute to development of persistent follicles resulting in anovulation in HA4I cows. Furthermore, elevated plasma progesterone with reduced follicular fluid inflammation may allow for HA4O cows to ovulate despite the high levels of A4 in the ovarian microenvironment.

P-356 - Folliculogenesis in Polycystic Ovaries in the Domestic Cat

Alisia Nolasco, Za'Chari Bey and Rajeev Chandra

Polycystic ovary syndrome (PCOS) is the most common cause of anovulatory infertility, but the mechanism of anovulation remains uncertain. The typical gross morphology of anovulatory

polycystic ovaries is the presence of multiple antral follicles 2–10 mm in diameter, which signifies the arrest of follicle development prior to the preovulatory phase. Anovulation in PCOS is characterized by arrested growth of antral follicles. Although arrested antral follicle growth reflects the abnormal endocrine environment, there is increasing evidence of abnormalities of follicle development from the very earliest, gonadotropin-independent stages. The underlying molecular basis of this fundamental ovarian abnormality remains to be determined. Folliculogenesis is the cycle of maturation of a follicle within the ovary of the adult human female. A follicle is a membranous sac of cells that contains an immature egg cell, called an oocyte. The primary investigation of this research includes a study identifying various stages of follicle development with particular reference to antral follicle development and their respective sizes during the estrous cycle of the domestic cat, *Felis catus*. This understanding will help enhance the ability to assess risk and develop preventative strategies of ovarian dysfunction such as Polycystic Ovarian Syndrome, as highlighted. Ovaries from adult female domestic cats were obtained from routine spaying procedures conducted at a local veterinary clinic. The primary methods utilized in the study include histology of the ovarian tissue. Briefly, ovaries were surgically removed during a spaying procedure at a veterinary clinic and washed in PBS. Ovarian tissue was fixed in formalin solution, followed by rinsing in a graded ethanol series (70%, 95%, 100%). Tissues were then embedded in paraffin, serially sectioned (5 µm), mounted onto microscope slides, and stained with hematoxylin and eosin by a standard histological procedure. Gross follicular morphology, including their respective sizes, was evaluated for various developmental stages of the ovarian follicles. Key words: Ovary, Follicles, Ovulation, Folliculogenesis, Antrum

P-357 - Sex-specific Consequences of Poor Early-life Parental Nutrition on the Reproductive Performance Across Two Generations of Mice

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Nutrition significantly influences various aspects of reproductive outcomes, including gamete quality, hormonal balance, and the progression of pregnancy. Emerging research explores gender-specific mechanisms through which dietary habits may shape individual reproductive health and leave a lasting impact on the fertility of subsequent generations. In this study, we investigated the impact of malnutrition during the early postnatal period on the reproductive performance and fertility of female and male mice, spanning two successive generations. Newborn pups (F1) were assigned to either the control group (CON), where mothers were fed ad libitum, or the lactation undernutrition group (LUN), where mothers were provided with 50% of the daily portion of chow consumed by control dams. After weaning (postnatal day 21 = PND21), F1 offspring was fed ad libitum. Sexually mature F1 mice were bred in three different combinations: CON female x CON male (CC), LUN female x CON male (LC) and LUN female x LUN male (LL), to generate F2 offspring not directly exposed to undernutrition. A morphometric analysis of ovaries and testes was performed at PND21, while follicle count and daily sperm production were assessed in adults. Upon reaching sexual maturity, estrus cycles were monitored in F1 and F2 females. The hormonal status of both sexes was evaluated by measuring androstenedione, progesterone, luteinizing hormone (LH), and testosterone (males only) in blood plasma. To assess reproductive performance

over time, breeding pairs from the F1 and F2 generations were monitored for the number of pups and litters over a 20-week period. LUN females displayed irregular initial estrus cycles compared to CON. At PND21, their ovaries exhibited smaller sizes with fewer antral follicles, a trend that persisted into adulthood with decreased follicle counts. Furthermore, LUN females showed lower androstenedione levels and elevated LH levels at PND21, with lower progesterone levels observed in sexually mature animals. Notably, these effects were not detected in the second generation of female mice. The scenario differed significantly for males. Early-life malnutrition affected the morphological appearance of the testes. Both LUN and LL males exhibited decreased daily sperm production compared to the CON and CC groups, respectively. Additionally, LUN and LL males displayed lower levels of androstenedione and testosterone at PND21, along with elevated levels of LH. LUN females had limited reproductive capacity after 15 weeks of breeding, whereas LUN males had a decreased total number of delivered offspring over time compared to control pairs. LL females paired with CC males appeared to have slightly better reproductive performance over time than LL males paired with CC females. In summary, early-life malnutrition in the F1 generation impairs their reproductive performance. Notably, adverse effects on gamete production and hormonal balance in the next generation (F2) were observed only in males. This study highlights the crucial role of parental nutritional history in determining the reproductive performance in a sex-specific manner. Research supported by Polish National Science Centre [2018/31/B/NZ4/03527].

P-358 - Cumulus-Oocyte-Complexes of Juvenescence Mice Expand into Smaller Areas Compared to Their Adult Counterparts During In Vitro Maturation

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It is well known that with advancing reproductive age, egg quality declines with associated deterioration in fertility outcomes. However, the quality of eggs during reproductive juvenescence is less well understood. Currently, eggs are cryopreserved for fertility preservation in children with cancer and other diseases or treatments that jeopardize their ovarian function. Therefore, it is important to understand the quality of these eggs. Emerging research demonstrates that egg quality in children is inferior to adults with increased aneuploidy rate in the younger age group. A study from our lab demonstrated that mitosis, cell proliferation and cell cycle pathways are down-regulated in cumulus cells (CCs) of adolescents undergoing fertility preservation compared to oocyte donors which represent a group with optimal gamete quality. Cumulus cells are intimately linked with the oocyte, support its metabolism and growth, and undergo expansion prior to ovulation. Cumulus expansion is necessary for successful ovulation and fertilization, and is impaired with advanced reproductive age, which may be indicative of underlying poor gamete quality in the older age group. Our objective in this study was to assess the cumulus expansion in peri- and early post-pubertal mice compared to adults, given that reproductive juvenescence in mice is also associated with increased chromosome abnormalities in the egg. We assessed cumulus expansion in vitro in 3 age cohorts: peri-pubertal (Day 16-21), early post-pubertal (Day 22-28), and control adult mice (9 weeks). Cumulus-oocyte-complexes (COCs) were retrieved from the ovaries of Pregnant Mare Serum Gonadotropin (PMSG) injected mice. Individual COCs were imaged pre-expansion, cultured in individual wells in the presence of 10ng/ml EGF and 5% FBS for 12-14

hours, and then imaged post-expansion. Expanded COCs were then treated with hyaluronidase, CCs were removed, and metaphase II oocytes were stained for spindles and chromosomes. Peripubertal mice exhibited significantly smaller COC areas prior to maturation compared to adults ($377.6 \pm 5.6 \mu\text{m}^2$ (peri-pubertal), $448.6 \pm 26.5 \mu\text{m}^2$ (early post-pubertal), $466.8 \pm 16.5 \mu\text{m}^2$ (adult) $p=0.03$). These COCs also expanded into smaller areas compared to early post-pubertal and adult mice ($683.3 \pm 8.4 \mu\text{m}^2$ (peri-pubertal), $776.6 \pm 24.6 \mu\text{m}^2$ (early post-pubertal) $p=0.0005$, $772.2 \pm 17.5 \mu\text{m}^2$ (adult) $p=0.0008$). In metaphase II eggs, chromosome alignment analyses trended more favorably for the adult cohort with a mean of 94.6% appropriately aligned chromosomes on meiotic spindles in this age group, compared to 78.1% in early post-pubertal and 77.4% in peri-pubertal mice. COC area of reproductively juvenescent mice is smaller pre- and post-expansion in vitro compared to adults. Additionally, chromosomes tend to be misaligned on metaphase II spindles of juvenescent mice compared to adults. This may be reflective of the suboptimal gamete quality in the younger end of the age spectrum and future research will focus on investigating the implications of this finding for determining the reproductive potential during reproductive juvenescence. This work is supported by the Department of Obstetrics and Gynecology Start Up funds (EB).

P-359 - Exploring the Therapeutic Potential of Salmon Egg and Plant Callus-Derived Polydeoxyribonucleotide (PDRN) for Wound Healing and Tissue Regeneration

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Polydeoxyribonucleotide (PDRN) is a mixture of polynucleotides primarily extracted from the sperm cells and eggs of chum salmon or salmon trout for clinical and therapeutic applications. It activates the adenosine A_{2A} receptor and exhibits several pharmacological properties, including promoting wound healing, angiogenesis, anti-inflammatory, anti-apoptotic, and tissue repair. Although PDRN is already used for clinical and cosmetic purposes, additional research is needed to further characterize its bioactivities and applications. The aim of this study was to evaluate the application of PDRN derived from salmon eggs and cultured plant tissues. We investigated the degradability of salmon eggs PDRN-coated bioresorbable sutures and the histological changes that occur in response to implantation with the suture. After implantation of threads coated with salmon eggs-derived PDRN, there were no significant signs of inflammation in the thread-loaded regions of rat skin during the experimental period. As the duration of the thread transplantation period increased, the thread remaining at the experimental site gradually became transparent and fragmented. There were no significant differences in tensile strength between non-coated and PDRN-coated threads throughout the loading period. The fibrous bridging effect was evident near the thread insertion site, and newly formed dense collagen fibers stained were observed near the PDRN-coated thread insertion site. Furthermore, there was an increase in the expression of genes related to collagen synthesis. In addition, salmon-derived PDRN induced the proliferation of human skin fibroblast cells (CCD-986sk) and stimulated MAPK signaling proteins. We also cultured *Brassica oleracea* var. plants. Various plant tissues (such as *Brassica oleracea* var. *italica*) were cultured, and PDRN was

extracted from stable callus. We investigated the potential of plant callus-derived PDRN to substitute animal-derived PDRN. The plant callus-derived PDRN did not induce cell cytotoxicity and reduced inflammatory responses in lipopolysaccharide (LPS)-induced Raw 264.7 cells. These results suggest the possibility that PDRN derived from eggs and plants can be utilized in clinical and pharmacological products for wound healing, tissue regeneration post-surgery, and repair.

P-360 - Luteal Fibroblasts Produce Prostaglandin F2 α in Response to IL1 β in a MAPK-mediated Manner

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The corpus luteum is a temporary endocrine gland that is crucial for pregnancy, as it produces the progesterone needed to maintain optimal uterine conditions for embryo implantation. In the absence of a conceptus, the corpus luteum becomes non-functional and undergoes rapid tissue remodeling to regress into a fibrotic corpus albicans, in a process initiated by prostaglandin F2 α (PGF2 α) in ruminants. Early luteal regression is characterized by elevated levels of luteal cytokines. Because the luteal tissue micro-environment is poorly understood. Further, the contribution of luteal fibroblasts to luteal regression remains to be elucidated. The aim of this study was to determine the response of bovine luteal fibroblasts to inflammatory cytokines. Because such cytokines are known to induce prostaglandin production, we hypothesized that bovine luteal fibroblasts produce PGF2 α in response to tumor necrosis factor α (TNF α) and interleukin 1 β (IL1 β). To test our hypothesis, we established primary cultures of bovine luteal fibroblasts and treated them with TNF α (10 ng/mL) and IL1 β (10 ng/mL). Protein and mRNA were collected for western blot and RT-PCR analysis, and culture medium was collected for PGF2 α ELISA. Secreted arachidonate metabolites were also analyzed by LC/MS. Statistical analysis was performed by two-way ANOVA with a Tukey's post hoc multiple comparison test, or one-way ANOVA as appropriate. Short-term treatment (2-120 min) revealed that both cytokines induced canonical mitogen activated protein kinase (MAPK) signaling in luteal fibroblasts by stimulating the phosphorylation of ERK1/2, p38 MAPK, JNK. However, IL1 β induced more robust activation of these kinases than TNF α ($p < 0.05$). Treatment for 24 h revealed that TNF α increased PGF2 α , cytosolic phospholipase A2 (cPLA2) protein, and prostaglandin-endoperoxide synthase 2 (PTGS2) protein nearly 2-fold, but not at a statistically significant level. IL1 β elevated PGF2 α levels in the culture medium over 20-fold ($p < 0.0001$), as well as mRNA and protein for enzymes involved in prostaglandin synthesis, cPLA2 (4-fold, $p < 0.0001$) and PTGS2 (22-fold, $p < 0.01$). Pre-treatment of luteal fibroblasts with Vehicle (DMSO), MEK inhibitor, U0126 (10 μ M), p38 inhibitor, SB203580 (20 μ M), or JNK inhibitor, SP600125 (10 μ M) for 1 h prior to 24 h treatment with IL1 β (10 μ g/mL) abrogated the stimulatory effects of IL1 β . IL1 β -induced PGF2 α secretion was diminished in U0126 ($p < 0.001$), SB203580 ($p < 0.001$) and SP600125-treated ($p < 0.05$) fibroblasts. Inhibition of ERK and p38 MAPKs also decreased IL1 β -induced cPLA2 protein ($p < 0.001$). However, inhibition of JNK induced an increase in both cPLA2

and PTGS2 protein. IL1 β still elevated PTGS2 protein in response to U0126; however, that response was blunted compared to the vehicle control (4-fold in U0126-treated fibroblasts vs 20-fold in vehicle control, $p < 0.01$), and p38 MAPK inhibition diminished the IL1 β -induced PTGS2 response ($p = 0.148$ vs inhibitor control without IL1 β). Mass spectrometry analysis of arachidonate metabolites following treatment with IL1 β revealed significant increases in other prostaglandins (PGE2, PGD2, 6-Keto-PGF1). All together, we have identified luteal fibroblasts as potential inflammatory mediators operating in the luteal microenvironment during luteal regression. This study was supported by funding from the American Heart Association (AHA 23PRE1018741, to CFM), as well as funding from USDA NIFA Grant 2023-67015-40795 (JSD).

P-361 - Eggs Derived from Cultured Follicles In Vitro Generate Embryos with Altered ZGA Gene Expression

Edward J. Grow

In vitro culture has enormous promise as a tractable experimental system to study follicle development and also as a translational approach to produce eggs for fertility treatments. Although well-established in the mouse system, most oocytes from in vitro follicle culture (IVFC) generate developmentally compromised embryos. To investigate the molecular mechanism underlying this phenotype, we have performed single-cell RNA-seq in oocytes from IVFC and resultant embryos. We find that a subset of oocytes in culture transcriptionally drift and precociously activate markers of ZGA. Additionally, we find that only ~50% of oocytes establish nuclear competency characterized by appropriate chromatin condensation. After egg activation, many embryos derived from IVFC oocytes arrest at the early ZGA stage and express high levels of early 2-cell stage transcripts such as Obox4. Importantly, these effects are not seen in oocytes developed in vivo and subjected to in vitro maturation. In summary our results show that IVFC might impart long-lasting potentially transgenerational effects on oocytes and embryos.

P-362 - Effects of Maternal Antral Follicle Count in Bos Taurus Indicus Cattle on The Genetic Merit of Male Offspring and Antral Follicle Count of Female Offspring

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This study evaluated the effects of antral follicle count (AFC) in female cattle on offspring characteristics. Recently calved multiparous Bos indicus cows (Nelore; n=222) were evaluated using ultrasonography on random days of their estrous cycle to determine the AFC and were classified into “low” (≤ 15 follicles), “intermediate” (≥ 16 and ≤ 29 follicles), and “high” (≥ 30 follicles) AFC groups. Weight and scrotal circumference (SC) of male offspring from these cows (n=127) were determined from 20 to 27 months, and the data were added to a genetic evaluation program (economic total genetic merit, MGT_e and TOP value) that uses the kinship matrix to evaluate the genetic relationship between animals. The AFC of female offspring from these cows (n=95) was

evaluated to analyze the relationship between the AFC of mothers and daughters. The effects of maternal AFC on the genetic merit of male and female offspring were analyzed using GLIMMIX and GLM, respectively. Correlations were assessed using the Pearson's coefficient. Male offspring of cows with high AFC had superior MGTe ($P=0.005$) and TOP values ($P=0.01$) than those from cows with low AFC. Additionally, the AFC of mothers was positively correlated with MGTe ($R=0.33$; $P<0.0001$) and negatively correlated with TOP values ($R=-0.32$; $P<0.0001$). The SC ($P=0.01$), but not body weight of the offspring ($P=0.46$) was affected by maternal AFC. The daughters' AFC were correlated ($R=0.29$; $P=0.004$) with mothers' AFC and were influenced by maternal ($P=0.05$) but not paternal ($P=0.77$) effect. In conclusion, cows with high AFC produced males with greater MGTe, superior TOP values and higher SC. Maternal AFC did not influence the weight of male offspring but was correlated with the AFC of daughters.

P-363 - Emerging Roles of Filamin A in the Human Ovary and Granulosa Cells

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Filamin A (FLNA) was identified as a cytoskeleton associated protein that crosslinks actin filaments. By now, it is clear that it is a multifunctional protein, which serves as an intracellular signaling scaffold, coordinating pathways linked to cell differentiation, development, and morphogenesis. FLNA is widely expressed in various human cell types and roles in certain tumors have been discussed in recent years. Yet, surprisingly FLNA and its roles have not been addressed in the human reproductive system, in which morphogenic events and cell differentiation are of great importance. We recently initiated such studies. In the human male gonad, we described FLNA in germ cells and peritubular cells, and shed light on its role in seminoma. We now address the role of FLNA in the female gonad. Data mining, including transcriptomic and proteomic data, suggested the presence of FLNA in various human ovarian cell types, including granulosa cells and oocytes. Immunohistochemical examinations of sections from human and nonhuman primate ovaries demonstrated robust expression of FLNA in follicular granulosa cells, luteinized granulosa cells in the corpus luteum, as well as oocytes. Further investigations using fluorescence microscopy and Western blotting (WB) uncovered the sustained expression of FLNA in cellular models, specifically in cultured in vitro fertilization-derived granulosa cells (IVF-GCs) and the human granulosa-like tumor cell line KGN. Moreover, our observations indicated a downregulation of FLNA in response to forskolin and/or hCG treatment in both cell types (IVF-GCs: qPCR $n=13$, WB $n=8$; KGNs: qPCR $n=6$, WB $n=6$; statistical significance determined by Student's t-test $\alpha=0.05$). To gain insights into the functions of FLNA in the human ovary, we conducted immunoprecipitation experiments followed by mass spectrometry analysis in IVF-GCs ($n=4$) and KGN cells ($n=3$). Among the top 20 proteins identified for each cell type, there was an overlap of 8 interaction partners, including stress-70 protein (HSPA9) amongst others, in addition to cell-type specific interactors. Notably, in GCs proteins with important cell-specific roles, namely the enzyme cholesterol side-chain cleavage

(CYP11A1), stress-70 protein (HSPA9) and gap junction alpha-1 (GJA1) were identified. Currently, further studies are under way to examine their intracellular co-localization. In summary, our results reveal the presence of FLNA in the ovary, in IVF-derived GCs as well as KGNs, its regulation in response to FSK/hCG, and its interaction partners with physiological significance relating to steroidogenesis, mitochondrial import and cell-cell communication. (Supported by DFG project number 491030536).

P-364 - Effects of Inhibiting CXCL12-CXCR4 During Implantation on Pregnancy-Associated Glycoproteins (PAGs) in the Sheep Placenta at Midgestation.

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Failure of trophoblast cells to properly attach and invade uterine endometrium leads to placental insufficiency, a root cause of preeclampsia, fetal growth restriction, and fetal morbidity and mortality. Further, placental insufficiency also predisposes offspring to cardiovascular disease, type 2 diabetes, insulin resistance, obesity, hypertension, and stroke during adulthood. Currently, no therapy exists to treat placental insufficiency and due to ethical standards study of this disorder in humans is difficult, necessitating the need for animal models. Research using livestock animal are proving to be effective models to address placental insufficiency pathogenesis. Using a sheep model, we previously demonstrated that suppressing the signaling of a chemokine, CXCL12, and its receptor, CXCR4 at the fetal-maternal interface during implantation with a pharmacological inhibitor (AMD3100, 1X dose) results in placental insufficiency by d90 of gestation. Interestingly, investigation of the placenta on d20 of gestation after suppressing CXCL12 revealed fewer trophoblast multinucleated giant cells expressing pregnancy-associated glycoproteins (PAGs) in the placenta compared to control. Trophoblast giant cells, similar to human syncytiotrophoblast cells, form a syncytium and invade the ovine endometrium, albeit to a lesser degree than in humans. PAGs are essential for successful pregnancy in ruminant animals, playing multiple roles in placental development, maternal-fetal communication, and pregnancy maintenance. Based on our results with fewer PAGs on d20 we hypothesized that ewes with placental insufficiency at d90 of gestation would present with fewer PAGs in the placenta compared to control. To test, on d12 post-breeding, osmotic pumps were surgically installed in ewes to deliver three different amounts (1X, 1.5X or 3X) of AMD3100 or saline (control) into uterine lumen ipsilateral to corpus luteum for 14 days. On d90 placentomes were collected and immediately immersed in 4% paraformaldehyde and later embedded in paraffin according to standard histological procedures. Immunofluorescence staining and analysis was conducted on placentomes to quantify PAG presence. Compared to control, ewes treated with 1X AMD3100 at the fetal-maternal interface during implantation had fewer ($P < 0.05$) PAG expressing cells on d90. However, similar levels of immunoreactive PAGs in placentomes were observed between control ewes and those subjected to higher doses of AMD3100 (1.5X and 3X) during implantation. Our data underscore the importance of CXCL12-CXCR4 axis during placentation and provide evidence that altering CXCL12-mediated signaling during implantation induces enduring placental effects manifesting later in gestation as placental insufficiency. As serious inadequacies in placental formation, such as trophoblast invasion, underpin many cases of implantation failure and contribute to pregnancy complications, further

characterization of our sheep model may prove useful for studying pregnancy complications stemming from compromised implantation. Research supported by National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health (NIH). Grant number 1SC1GM139712-01.

P-365 - Placenta-Specific RNA Interference of Transthyretin in Sheep

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During the first half of gestation, both the fetus and placenta are reliant on maternally derived thyroid hormone (TH). Maternal hypothyroidism is associated with miscarriage, fetal growth restriction, and preeclampsia; even mild hypothyroidism may negatively impact development. The predominant TH in both fetal and maternal circulation is thyroxine (T4) which can be converted to triiodothyronine (T3) or reverse triiodothyronine (rT3) by the iodothyronine deiodinase enzymes 2 (DIO2) and 3 (DIO3), respectively. DIO2 and DIO3 are abundantly present in the placenta. Transplacental T4 delivery to the fetus is critical, but how T4 evades placental metabolism is not well understood. Interestingly, the placenta also produces transthyretin (TTR) which binds T4. Evidence exists for TTR-T4 trophoblast uptake, and TTR has been hypothesized to protect and shuttle maternal T4 to fetal circulation; however, this shuttling mechanism has not been directly tested in vivo. We have detected TTR, DIO2, and DIO3 mRNA within cotyledons in sheep and have localized their proteins in placentomes. The purpose of this study was to use lentiviral-mediated RNA interference (RNAi) to directly investigate the role of TTR in TH transport across the placenta at mid-gestation (day 75 of gestation) in sheep. At this timepoint, the fetal thyroid has not yet begun producing substantial amounts of T4. Blastocysts were collected from Dorper ewes on day 9 and infected with lentivirus targeting either TTR (TTR RNAi) or a non-targeting sequence (NTS) before being surgically transferred into recipient ewes. At gestational day (GD) 50 and 70, ultrasound doppler velocimetry was utilized to assess pregnancy status and umbilical artery blood flow. At GD 75, ewes carrying TTR RNAi (n=4) or NTS (n=4) singleton pregnancies were weighed and anesthetized for collections. Uterine and umbilical blood was collected for T3 and T4 quantification by ELISA. TTR, DIO2, and DIO3, mRNA quantification by qPCR was conducted in cotyledon samples. Fetal measurements, qPCR, and ELISA results were compared by two-tailed t tests in GraphPad Prism 10 with a p-value of ≤ 0.05 denoting statistical significance. Lentiviral-mediated RNAi of TTR in ovine placental tissues resulted in a significant decrease in TTR mRNA in cotyledons ($p=0.01$) and resulted in an approximate 30% reduction in umbilical vein total T4 at GD 75 (0.658 $\mu\text{g/dL}$ vs. 0.915 $\mu\text{g/dL}$; $p=0.021$). No significant differences were detected in umbilical artery, uterine vein, or uterine artery T4, and no significant differences in T3 were observed. T3 and T4 were significantly lower in fetal plasma compared to maternal plasma, regardless of treatment. TTR RNAi fetuses (TTR RNAi vs. NTS) did not have significantly different fetal weights (209g vs. 199g; $p=0.57$) or liver weights (14.2g vs. 12.3g; $p=0.15$), but they tended towards shorter femur lengths (4.25cm vs. 4.6cm; $p=0.086$) at GD75. Major gaps exist in our understanding of how transplacental TH transport occurs, but these results suggest that our in vivo approach provides a model to study the role of TTR in placental TH transport. Overall, these results indicate that placental inhibition of TTR leads to significantly less T4 availability for fetal use, supporting the hypothesized shuttling mechanism of TTR, and while the TTR RNAi fetuses may experience hypothyroidism, significant fetal phenotypes may not be detected until later in gestation. This work was supported by NIH Grant HD093701 and

Agriculture and Food Research Initiative Competitive Grant no. 2019-67015-29000 from the USDA National Institute of Food and Agriculture.

P-366 - Maternal Myostatin Deficiency Modulates Placental Gene Expression and Signaling Pathways

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Introduction: Intrauterine growth restriction (IUGR) affects approximately 9% of pregnancies, and is associated with adverse outcomes like preterm birth and stillbirth. IUGR also poses risks for long-term health effects, including musculoskeletal deficiencies, obesity, and insulin resistance in adulthood. Placental insufficiency is a major contributor to IUGR. Myostatin acts as a negative regulator of muscle development. Our previous research revealed that Wildtype (WT) fetuses carried by myostatin deficient dams (Mstn+/-) exhibited 25% higher weight than WT offspring from WT dams, alongside increased muscle mass and bone strength in adulthood. Notably fetal myostatin concentrations were unaffected. Metabolomics analysis highlighted differences in amino acids and lipopolysaccharides in serum of fetuses from Mstn+/- dams. Moreover, WT placentas from myostatin deficient dams displayed larger labyrinth and junctional zone areas. This study aims to investigate the impact of maternal myostatin reduction on gene expression. **Experimental Design:** We performed reciprocal crosses of WT and Mstn+/- parents with WT dams serving as controls for the Mstn+/- dams. Placentas were collected at E17.5 day of pregnancy. Total RNA was extracted from five male and five female WT placentas from each cross and submitted for sequencing. Read counts were aligned and DEseq2 was used to identify differentially expressed genes. **Results:** There were 297 differentially expressed genes between the two maternal genotypes (adj. p-value < 0.05). There were 44 transcripts upregulated at least 15-fold in the placentas from a myostatin-deficient mother compared to those from a WT mother. These include Dsg3, Marco, Acod1, Il1b, Retnlg and Hp which all part of the inflammatory response pathway, the most upregulated pathway overall. There also significant upregulation of transcripts for Gpr132, Gpr84, Rnd1, Plcd1, all members of signal transduction pathways. **Conclusion:** These results suggest that maternal myostatin reduction may affect offspring growth in part by altering placental signaling through both inflammatory and growth factor signaling pathways.

P-367 - In Vitro Fertilization Significantly Impacts the Female Placental Proteome in a Mouse Model

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There is significant evidence that placental development is impacted in pregnancies conceived by assisted reproductive technologies (ART). Specifically, ART pregnancies have increased risk of preeclampsia, placental abruption, and morbidly adherent placentas. Abnormal placental morphology, specifically the developmental delay of placental vasculature and overgrowth of the endocrine compartment, impaired placental transport, reduced fetal weight, altered gene expression, and loss of DNA methylation have been robustly observed in a mouse model of in vitro fertilization (IVF). Identifying placental protein differences with IVF, pathological and compensatory, would help inform potential interventions for placental insufficiency as well as identify proteins that have a yet uncovered, but important role in placental development. We determined the placental protein abundance differences in IVF and spontaneously-conceived male and female mouse placentas by data-independent acquisition mass spectrometry at five timepoints (E11.5, 12.5, 14.5, 16.5, and 18.5). Proteome analyses were conducted on placentas (one male and one female closest to litter mean) from each litter (n=4-5 concepti/group/timepoint). Peptides below 1% FDR were used and statistical analysis was performed using Two-way ANOVA and Tukey HSD post hoc tests. All p-values reported were corrected for multiple hypothesis testing. Our results demonstrate placental proteins exhibit normal, dynamic change over placental development and that IVF induces an overall reduction in the abundance of several placental proteins at the earliest timepoint, E11.5. Several of the identified affected proteins are known to be important for placenta development. We further determined that placental protein levels are more affected in females. To our knowledge, this is the first proteomic analysis of mouse placentas at multiple timepoints along prenatal development. Thus, in addition to the insights into changes with IVF, this study provides a rich resource to understand protein abundance of proteins of interest over placental development for the research community.

P-368 - B-Raf and Epigenetic Regulation of Placentation

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Defective placentation has been associated with the “great obstetrical syndromes”. Placentation is a complex process and relies on the coordinated regulation of various pathways. Others and we observed that B-Raf expression increases from 9.5 days post coitum (dpc) to 11.5 dpc during placentation. B-Raf^{-/-} mice are not viable and die at 10.5 to 11.5 dpc. Necropsy demonstrates vascular malformation with thin and dilated fetal vessels with apoptotic cells in mice's placentas with disorganized trophoblast layers. Of note, conditional knockout of B-Raf only in the epiblast (not in trophoblast) did not lead to fetal demise. This evidence supports that placental vascular defect secondary to trophoblast dysregulation in B-Raf^{-/-} mice is the chief cause of mid-gestation fetal death and not vasculature in fetus per se. Thus, we tested the hypothesis that B-Raf kinase regulates trophoblast apoptosis through the B-Raf-STAT-Bcl2 pathway. Results demonstrate that B-Raf- Signal Transducer and Activator of Transcription 3 (STAT3)-Bcl2 is critical for the survival of these cells. Furthermore, B-Raf kinase regulates promoter methylation and histone phosphorylation to mediate downstream effects. The findings indicate that B-Raf epigenetically regulated trophoblasts' survival and placentation.

P-369 - Placental Cell Type Specific Transcriptional Responses to Maternal Obesity: Implications for Fetal Growth

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Maternal obesity is associated with increased infant birth weight, elevating the risks of long-term diseases in offspring. Despite this, the specific strategies employed by placental cells in response to maternal obesity for modulating placental or fetal growth remain elusive. Our study delves into the distinctive responses of placental cell types to maternal obesity and their correlation with infant birth weight. Using single-nuclei RNA sequencing, we analyzed human placental samples from a Chilean cohort, stratified into three groups: normal maternal BMI and appropriate baby birthweight for gestational age (N-AGA, n=4); obese AGA (O-AGA, n=4) with maternal BMI $30 \leq \text{BMI} < 35$; and obese with large baby birthweight for gestational age (O-LAGA, n=4). Thirteen major cell types were identified and all cell types presented differentially expressed genes (DEGs). Notably, in syncytiotrophoblast, gene expression increased in both obese groups, with a more pronounced effect in the O-AGA group. Conversely, in cytotrophoblast cells, the gene expression decreased in obese groups, with a more significant decrease in the O-AGA group. Fibroblasts from the O-LAGA group exhibited 87 DEGs compared to N-AGA, while the O-AGA group had 43 DEGs. Pathway analysis revealed perturbations in hormonal response and secretion in syncytiotrophoblasts, modulation of nutrient sensing and tissue growth regulating pathways in cytotrophoblasts, and altered responses to oxygen-containing molecules in fibroblasts from placentas of obese mothers. These results help advance our understanding of the placenta's role in fetal development and shed light on the potential adverse effects of maternal obesity on both maternal and fetal health.

P-370 - Appropriate Glycemic Management Protects The Germline But Not The Uterine Environment in Hyperglycemia

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Emerging evidence indicates that parental diseases can impact the health of subsequent generations through epigenetic inheritance. Recently, it was shown that maternal diabetes alters the metaphase II oocyte transcriptome, causing metabolic dysfunction in offspring. However, type 1 diabetes (T1D) mouse models frequently utilized in previous studies may be subject to several confounding factors due to severe hyperglycemia. This limits clinical translatability given improvements in glycemic control for T1D subjects. Here, we optimize a T1D mouse model to investigate the effects of appropriately managed maternal glycemic levels on oocytes and intrauterine development. We show that diabetic mice with appropriate glycemic control exhibit

better long-term health, including maintenance of the oocyte transcriptome and chromatin accessibility. We further show that human oocytes undergoing in vitro maturation challenged with mildly increased levels of glucose, reflecting appropriate glycemic management, also retain their transcriptome. However, fetal growth and placental function are affected in mice despite appropriate glycemic control, suggesting the uterine environment rather than the germline as a pathological factor in developmental reprogramming in appropriately managed diabetes.

P-371 - Gestational Age-Specific Expression of Placental Endoglin in Normal Human Pregnancy

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The study examined the expression of Endoglin (Eng) in normal human pregnancy throughout gestation. Placentas were collected from normotensive women from first, second and third trimester of normal pregnancy, without medical complications, and analyzed by ELISA using monoclonal antibody (mAb) to human Eng as capture antibody. Independent T Test and Spearman's correlation were performed. 280 placentas were analyzed. Age was comparable between the groups. The mean + SD Eng, expressed as nano gram per 100 milligram tissue were: 58.82 + 26.82; 45.33+ 26.96 and 28.95 + 11.89 for first (n=115), second (n=79), and third trimester (n=86), respectively; showing a decline in protein expression with an increase in gestational age. Spearman's correlation also reflected the decline showing a significant negative correlation ($r = -.506$, $p = .000$) between Eng protein and gestational age in days. The findings show higher Eng expression in the first trimester of normal pregnancy when a hypoxia state prevails in the placenta but as placental environment transitions to normoxic state, a steady decline in Eng was observed. There are reports that Eng expression is up-regulated in preeclamptic placentas. Increased Eng results in overexpression of its soluble form (sEng) in maternal circulation. sEng by coupling with angiogenic proteins impairs placentation, deranges angiogenesis and promotes vasoconstriction. Currently the use of mAb to sEng, administered during early pregnancy to women at risk for preeclampsia seems promising, with the hope that by inhibiting the action of sEng, the production of several angiogenic molecules can be increased; giving clinicians a potential way to postpone delivery. It therefore follows that identifying the dose at which the mAb to sEng could be administered is informative and could largely depends on the expression of Eng in the first and second trimester of pregnancy. Our findings provide preliminary information which is currently unavailable in the literature.

P-372 - Leukemia Inhibitory Factor Signaling in Human Trophoblast Cell Development

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In the early stages of human pregnancy, cells from the developing placenta, termed extravillous trophoblast (EVT) cells, enter the uterus. The entry of EVT cells into the uterus results in remodeling of uterine arteries to meet the increased demand of blood flow and oxygen during pregnancy. Dysregulation of the molecular controllers responsible for this structural uterine remodeling can lead to pregnancy complications such as early pregnancy loss, preeclampsia, intrauterine growth restriction, pre-term birth, or placenta accreta. Human trophoblast stem (TS) cells provide a powerful in vitro model to dissect regulatory processes controlling trophoblast cell lineage development. Previously, a uterine-derived cytokine called leukemia inhibitory factor (LIF) was implicated as a candidate regulator of early placentation in the mouse. In the present study, we performed in-situ hybridization, RT-qPCR, and bulk RNA-sequencing to help discern LIF-signaling in human first trimester placental tissue and in human TS cells. In situ hybridization revealed EVT cell columns were positive for LIF receptor (LIFR), and its downstream signaling partners, janus kinase 1 (JAK1) and signal transducer and activator of transcription 3 (STAT3). LIFR and JAK1, but not STAT3, transcript levels increased as TS cells transitioned from the stem to EVT cell state. We also examined the effects of LIF on human TS cells. Acute exposure to LIF resulted in an increase in phosphorylated STAT3. The effects of chronic LIF on EVT cell differentiation was examined morphologically followed by RT-qPCR and bulk RNA-sequencing. At the end of differentiation into EVT cells, LIF-treated TS cells failed to fully differentiate and instead exhibited features similar to TS cell stem state. We then used RT-qPCR to quantify stem and EVT cell state markers within control or LIF-treated cells following differentiation. LIF treatment corresponded to an increase in selected stem markers (NPPB and PEG10) and a decrease in EVT cell markers (FLT4 and FSTL3). Bulk RNA-sequencing assisted in expanding the list of additional stem and EVT cell markers. In summary, LIF-signaling has prominent restraining effects on development of the EVT cell phenotype. This may reflect a pathway utilized by the uterine compartment to restrain excessive intrauterine EVT cell invasion and a regulatory mechanism susceptible to dysregulation in disease states characterized by abnormalities in intrauterine EVT cell invasion. This research was supported by K-INBRE P20 GM103418 (S.L.S), Lalor Foundation (S.L.S., A.M.), and NIH grants (HD020676, HD099638, HD105734).

P-374 - Epigenetic Profiling in Late Gestation Porcine Placenta Reveals Sex-Dependent Adaptations to Fetal Endocrine Status

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The placenta has crucial roles in supporting fetal development by facilitating nutrient transport and gas exchange. In pigs, as gestation progresses, structural changes in the placenta occur to accommodate the growth and increasing nutrient requirements of the developing fetus. However, the epitheliochorial porcine placenta forms a restrictive barrier that greatly limits the transplacental passage of specific molecules, including immunoglobulins and thyroid hormones. Thyroid hormones are vital for prenatal development, influencing organ differentiation and fetal growth. Beginning in early gestation, fetal pigs largely rely on autonomous thyroid hormone production rather than maternal thyroid hormone supplementation. As a result, disruptions to fetal thyroid

status can negatively impact fetal growth and viability, particularly during late gestation when fetal growth is especially rapid. In swine, both pathogenic and induced hypothyroidism models have been shown to alter placental thyroid metabolism, suggesting fetal endocrine status may influence placental physiology. To better understand this relationship, placental tissues were isolated at gestation day 86 following 21-day maternal treatment with methimazole (MMI) to induce fetal hypothyroidism, and tissues were also obtained from gestational age-matched controls. We used CUT&RUN to evaluate the enrichment of histone marks (H3K4me3, H3K27ac) and the SWI/SNF central ATPase BRG1 in placental tissue, which, to our knowledge, represents a novel avenue of investigation in this area. Analysis of n=12 samples per treatment group (n=6 samples each from male and female fetuses per group) demonstrated differential enrichment of all three epigenetic features in placental tissue obtained from MMI-treated fetuses. Importantly, we saw that existing sex-specific differences in placental epigenetic features were exacerbated by fetal hypothyroidism, indicating that the porcine placenta is impacted by fetal endocrine status during late gestation. Taken together, these findings provide novel insights into the existence of sex-specific differences in placental chromatin state and shed light on the intricate interplay between fetal thyroid hormone levels and placental epigenome regulation.

P-375 - Effects of Dietary Supplementation of Creatine on Fetal Muscle and Placental Development in Mid-and-Late Gestating Gilts

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Effects of Dietary Supplementation of Creatine on Fetal Muscle and Placental Development in Mid-and-Late Gestating Gilts Maddison A. Olivarez¹, Arianna N. Lopez¹, Claire Stenhouse¹, Robyn M. Moses¹, Makenzie Newton¹, Nirvay Sah¹, Heewon Seo², Joe W. Cain², Carli Lefevre², Alexandria Ross², Jeffery Wiegert¹, Guoyao Wu¹, Gregory A. Johnson², Fuller W. Bazer¹ Departments of Animal Sciences¹ and Veterinary Integrative Biosciences², Texas A&M University, College Station, TX, USA 77843 The creatine-creatine kinase-phosphocreatine (Cr-CK-PCr) system plays a role in interorgan metabolism among the kidneys, liver, and muscle to yield adenosine triphosphate (ATP) for cellular bioenergetics. The Cr-CK-PCr system has been hypothesized to have a role in fetal-placental development in pigs as energy demands increase during gestation. This study tested the hypothesis that dietary supplementation of creatine from Day 10 to either Day 60 or 90 of gestation would enhance Cr-PCr metabolism in the placenta to improve placental and fetal muscle development. Gilts were synchronized to estrus during the spring using synthetic progesterone, Matrix, and observed for estrus (Day 0). Gilts were then artificially inseminated at 12 h and 24 h after first insemination following observed estrus. Gilts were assigned randomly to one of 4 groups: Day 60 CON, Day 60 Cr, Day 90 CON, or Day 90 Cr (n=10 gilts/treatment). All gilts were fed a diet of 2lbs containing 14% crude protein twice daily and gilts assigned to the Cr treatment groups were supplemented with 30 g/day Cr from Day 10 to either Day 60 or 90 of gestation when tissues were collected by hysterectomy. This study had one replicate where gilts were synchronized in spring and

tissues collected in the summer and another where gilts were synchronized in fall and tissues collected in the winter. Following euthanasia, individual placentas were separated from the endometrium and their lengths and weights were measured. Samples from the endometrium, chorioallantois, and amnion and were snap frozen in liquid nitrogen for analysis of gene and protein expression and fixed in 4% paraformaldehyde for histological analysis. Additionally, biceps femoris muscle from 3 male and 3 female fetuses closest to the mean weight were snap frozen in liquid nitrogen, frozen in OCT gel or fixed in 4% paraformaldehyde. Statistical analyses revealed that placental weights from gilts supplemented with Cr were lighter than those from CON gilts at Day 60 ($P=0.02$) of gestation. However, there were no significant treatment effects on placental weights due to dietary supplementation at Day 90 of gestation. Additionally, no significant effects on placental weights were detected between seasonal replicates. For placental length, there were no treatment effects; however, there were statistically significant differences between replicates from both Day 60 ($P=0.01$) and Day 90 ($P=0.02$). D60 and D90 tissues collected in the summer had greater mean placental weights than tissues collected in the winter. Results of this study suggest that dietary supplementation of Cr had limited effects on placental weight and length. Currently, mRNAs for key enzymes in the pathway for synthesis of PCr and ATP and transporters for Cr and guanidinoacetate are being quantified. Further, placental expression of creatine, phosphocreatine, and guanidinoacetate proteins is being determined. This research was supported by Agriculture and Food Research Initiative Competitive Grant no. 2022-67015-36376 from the USDA National Institute of Food and Agriculture.

P-376 - Endometrial Expression and Secretion of Insulin-like Growth Factor 2 Facilitates Uterine Angiogenesis and Trophoblast Differentiation during Placenta Development

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During early pregnancy, the uterine stromal cells, under the influence of the ovarian steroid hormones, undergo a dramatic transformation to form the decidua, a secretory tissue that surrounds the growing fetus. The decidual cells control diverse functions within pregnant uterus, including the formation of an extensive maternal vascular network that supports embryo development. Proper differentiation and migration of the trophoblast cells, critical for forming functional placenta, are also influenced by yet unknown factors secreted by the decidual cells. A major challenge is to identify the signaling molecules emanating from the decidual cells that communicate with the endothelial and trophoblast cells within the implantation chamber to ensure successful pregnancy establishment and maintenance. We have recently developed a unique mouse model in which conditional knockout (cKO) of the gene encoding the transcription factor Runx1 in the uterus led to severe subfertility due to deficient decidual angiogenesis and an impairment in trophoblast cell lineage development and subsequent invasion into decidua during placentation. Interestingly, we found that expression of insulin-like growth factor 2 (IGF2), a protein known to influence both angiogenesis and trophoblast differentiation, was significantly reduced in decidual cells in the absence of Runx1. In this study, we investigated the role Runx1 plays in

coordinating IGF2 expression and secretion in human endometrial stromal cells (HESC) undergoing decidualization. We found that Runx1 controls the expression of the *Igf2* gene by directly interacting with a distinct upstream regulatory region. Notably, decidualizing HESC secrete extracellular vesicles (EVs) which are taken up by various cell types within the uterus to influence critical functional events during pregnancy. Mass spectrometry analysis revealed that EVs secreted by HESC harbor IGF2 as a protein cargo. Upon treatment of these cells with Runx1-specific siRNA, IGF2 was absent from the EV cargo. Functional studies showed that, while delivery of control HESC-derived EVs into human endothelial cells HUVEC promoted vascular network formation, similar treatment with EVs obtained from Runx1- or IGF2-depleted HESC failed to do so. When Runx1 or IGF2-depleted EVs were added to human trophoblasts stem cells, their differentiation into extravillous trophoblast lineage was also reduced compared to the addition of control EVs. To examine the role of decidual IGF2 in vivo, we have recently created an IGF2 cKO mouse model in which *Igf2* gene is conditionally deleted in the uterus. Our initial results indicate that female IGF2 cKO mice exhibit marked subfertility, presumably due to post-implantation uterine defects. Collectively, these findings suggest that Runx1-driven expression of IGF2 in decidual cells plays a critical role in mediating cell-cell communication during uterine angiogenesis and trophoblast differentiation to ensure proper placenta formation. This work was supported by the Eunice Kennedy Shriver NICHD/NIH R01 HD090066 and R21 HD109726 (to ICB and MKB). JRB is supported by the NIEHS training grant T32 ES007326.

P-377 - Single Cell RNA Sequencing Identifies CXADR as a Fate Determinant of the Placental Exchange Surface

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Early placentation is a crucial period in fetal development. Abnormalities in the formation of the placenta underly major pregnancy complications. The differentiation of the trophoctoderm into the different trophoblast lineages is critical for the remodeling of the maternal spiral arteries and the

establishment of placental blood supply and to generate the syncytial surface which is essential for gaseous and nutrient exchange between the mother and the fetus. Studying the early differentiation events in-vivo is difficult and mouse trophoblast stem (TS) cells are one useful in-vitro model to achieve this goal. Here we used single cell RNA sequencing analysis of differentiating mouse trophoblast stem cells to uncover the gene expression patterns that define the entry into the trophoblast lineages. We induced mouse trophoblast stem cell differentiation by using a Mitogen-activated protein kinase kinase inhibitor (PD0325901) or by withdrawing conditioned medium and sampled cells at 0h, 1h, 4h, 24h, 36h and 48h (n=3-4). We fabricated polydimethylsiloxane microfluidic devices and encapsulated single cells with barcoded beads in microdroplets to construct single cell transcriptomic Drop-seq libraries. Our dataset comprised a total of 136,291 cells with an average of 1,740 reads and 1,059 genes per cell. We explored the heterogeneity of mouse trophoblast stem cells before differentiation through clustering with Seurat. We identified a sub-population of mouse TS cells with a gene expression profile similar to differentiating cells suggesting they were primed for differentiation. Differential gene expression analysis identified NELL2 Interacting Cell Ontogeny Regulator 1 (Nicol1) as a potential marker for the predominant non-primed stem cell population. Further bioinformatic analysis of the full timecourse in Seurat identified candidate clusters for the junctional zone and placental labyrinth progenitor cells. Gene regulatory network analysis in SCENIC revealed that the labyrinth progenitor cell cluster, characterized by activity of the glial cells missing transcription factor 1 (Gcm1) regulon, also exhibited increased activity of the PHD Finger Protein 8 (Phf8) and E2F Transcription Factor 8 (E2f8) regulons. Pseudotime analysis in Monocle and marker gene expression revealed that the junctional zone and syncytiotrophoblast layer I lineage precursor cells initially shared a similar differentiation trajectory, while the syncytiotrophoblast layer II lineage was already transcriptionally distinct by 48h. Pseudotime analysis showed that the Coxsackievirus and adenovirus receptor (Cxadr) transcript is retained in the syncytiotrophoblast layer I and junctional zone trajectory but is not expressed in the early syncytiotrophoblast layer II progenitors. Immunofluorescent staining of mouse trophoblast stem cells revealed that loss of CXADR preceded syncytialisation. We then determined the effect of CRISPR-Cas9 mediated knockout of Cxadr in TS cells. Upon differentiation this lead to increased cell fusion but with upregulation of syncytiotrophoblast layer I markers and reduction of syncytiotrophoblast layer II markers as measured by RT-qPCR. This suggests that this gap junction protein is involved in maintaining the correct balance between the two layers of syncytiotrophoblast in the mouse labyrinth by slowing or suppressing the syncytiotrophoblast layer I differentiation. Correct trophoblast differentiation is essential to the establishment of the placental exchange interface. These findings enhance our understanding of the early events during mouse trophoblast differentiation and if a similar mechanism is present in human trophoblast this may effect human pregnancy outcome. This work was supported by BBSRC and Sphere Fluidics Ltd.

P-378 - Effect of visfatin on human placental endocrinology. In vitro studies on BeWo cell line and villous explants from third-trimester human pregnancy

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Visfatin, an adipocytokine primarily known for its role in energy metabolism, has been recently implicated in pregnancy with evidence suggesting that its levels fluctuate across individual trimesters. Our previous data indicated visfatin expression in the placental cell lines and different compartments of the human placenta. In this study, we aimed to investigate the *in vitro* effect of visfatin on placental cell endocrinology with underlying molecular mechanisms. Using placental BeWo cell line and villi obtained from both normal and pathological pregnancies, including intrauterine growth restriction (IUGR), preeclampsia (PE), and gestational diabetes mellitus (GDM), we examined the dose (1-100 ng/mL) and time (24-72 h) dependent impact of visfatin on the secretion of key steroids (progesterone and estradiol) and protein hormones (human chorionic gonadotropin, human placental lactogen, and placental growth hormone (ELISA). We also investigated the effect of visfatin on the protein expression of steroid enzymes, including 3- β -hydroxysteroid dehydrogenase and aromatase, as well as the aforementioned protein hormones (Western blot). Moreover, we studied the visfatin effect on phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and protein kinase A (PKA). In contrast, pharmacological inhibition of the above kinases was used to further explain visfatin-mediated effects on selected hormones secretion. Experiments were carried out at least three times, and statistical analysis was performed in GraphPad Prism 8 using Student t-test as well as ANOVA ($p < 0.05$). Our results provide comprehensive insights into the modulatory role of visfatin in the human placenta and identify key endocrine molecular players involved in this process. The findings underscore the complex interplay between adipocytokines and placental endocrinology, shedding light on potential therapeutic targets for pregnancy-related complications. This research contributes to a deeper understanding of the intricate mechanisms governing maternal-fetal interactions and may pave the way for developing novel interventions to improve pregnancy outcomes. Funding: Diamond Grant IX 0110/DIA/2020/49

P-379 - Down Syndrome Trophoblasts from Induced Pluripotent Stem Cells Display Differentiation Defects

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Down Syndrome (T21) pregnancies are at an increased risk of adverse outcomes resulting from placental insufficiency, yet the etiology behind these defects is unknown. Additionally, T21 placental explant cultures display defects in fusion and differentiation into multinucleated syncytiotrophoblasts (STB). However, placental explants are derived from mid- to late- term placental tissue and offer limited potential to functionally explore mechanistic differences in T21 trophoblast differentiation potential. We hypothesize that aberrant development of T21 placental trophoblast cells occurs early in gestation during peri-implantation and that differentiation of induced pluripotent stem (iPS) cells may serve as an appropriate model system to study peri-implantation T21 trophoblasts. Here, we report that T21 trophoblasts can be modeled *in vitro* by T21 iPS cells following exposure to BMP4, a TGF β inhibitor (A83-01), and a fibroblast growth factor receptor inhibitor (PD173074). Directed differentiation of a T21 and an isogenic euploid control

(D21) iPS cell line from the same donor results in expression of putative trophoblast lineage markers, GATA3 and TFAP2C, by immunofluorescence (IF), and cells secrete human chorionic gonadotropin beta, a hallmark of STB. Differentiated iPS cells can be further separated into mononucleated cytotrophoblast-like cells and STB-like cells based on qPCR analysis of each population. Following differentiation (n=3), T21 cells exhibit reduced cell fusion compared to D21. Furthermore, expression of putative STB markers, CGB, ERVW-1, and GCM1 are reduced in T21 compared to D21. Future studies characterizing the global transcriptome of 6 age- and sex-matched iPS cell lines will aim to identify a mechanism by which STB differentiation is disrupted in T21 placentas. This work is supported by a Sie fellowship from the Linda Crnic Institute for Down Syndrome.

P-380 - Placental vascular development throughout gestation in beef heifers

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Placental vascular development is essential for transplacental exchange of nutrients, respiratory gases and metabolic wastes between the maternal and fetal systems to ensure optimal fetal growth and development. While research has quantified placental vascular development in several mammalian species, studies in cattle are limited. This study sought to enhance our comprehension of placental vascular development in beef heifers by analyzing the expression of CD31 and CD34 (endothelial cell markers). A total of twenty-six primiparous, pregnant, crossbred, Angus beef heifers were included in the study, with placental samples collected at four gestational time points: days 63 (n=6), 83 (n=6), 161 (n=7), and 250 (n=7) (length of gestation approx. 280 days). Placentome samples were processed by fixation, embedding, and sectioning at 5 mm. Immunofluorescence staining for CD31 and CD34 was conducted, followed by analysis with Image-Pro Premier software. Vascularity was assessed through analyzing capillary area density (CAD) and capillary number density (CND) in fetal cotyledon (COT) and maternal caruncle (CAR) regions. Data were analyzed with PROC GLM and PROC REG of SAS, with heifer as the experimental unit. Significance was determined at $P \leq 0.05$ and tendency at $P = 0.10$. The CAD serves as a determinant of resistance and thus placental blood flow capacity. Differences in CAD were observed on days 83 and 161 of gestation, with CAR greater than COT (23.61 ± 1.43 vs. 15.25 ± 0.47 at day 83, $P = 0.05$; and 34.39 ± 0.97 vs. 23.87 ± 1.05 at day 161, $P = 0.02$). However, on day 250, CAD of COT tended to be greater than that of CAR (53.50 ± 1.09 vs. 44.75 ± 1.53 at day 250, $P = 0.11$). Similarly, CND, which reflects capillary branching, was greater for CAR vs. COT early in gestation (0.89 ± 0.01 vs. 0.62 ± 0.01 at day 63, $P = 0.001$; and 1.05 ± 0.04 vs. 0.70 ± 0.03 at day 83, $P = 0.03$). In late gestation, the CND of COT was slightly greater than in the CAR, but again this difference was not significant (1.81 ± 0.02 vs. 1.63 ± 0.03 at day 250, $P = 0.15$). Moreover, CAD exhibited cubic increases for both CAR and COT throughout gestation ($R^2 = 0.687$ and 0.898 , respectively; $P < 0.01$), with COT showing a greater proportional rate of increase (3.9-fold) compared to CAR (2.7-fold). The CND of CAR displayed a cubic increase ($R^2 = 0.720$; $P < 0.01$) with a smaller increase (1.8-fold) compared with COT (2.9-fold), which also demonstrated a cubic increase throughout gestation ($R^2 = 0.872$; $P < 0.01$). This study establishes the patterns of placental vascular development throughout gestation in primiparous beef heifers. For CAR, both CAD and CND exhibited notable increases in early pregnancy, followed by a slower rise in late pregnancy. Conversely, in the COT, there was a

pronounced increase in CAD and CND in late pregnancy. These findings deepen our understanding of placental vascular development in beef heifers. Supported by USDA-NIFA-AFRI grant numbers: 2021-67015-34277 to LPR, 2021-67016-33389 (d 83), and 2022-67016-36479 (d 250).

P-381 - Epigenome Profiling Reveals an Essential Role for Jun-N-Terminal Kinase Signaling During Human Trophoblast Stem Cell Specification and Differentiation into Terminal Lineages

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Due to ethical and practical constraints on access to first-trimester placental tissues, the study of human trophoblast specification and differentiation into specialized lineages has remained limited. The recent derivation of human trophoblast stem cells (hTSCs) from naive human pluripotent stem cells (hPSCs) allows for mechanistic investigation of developmental time points and cell fate decisions that we lack access to in vivo. By mapping genome-wide chromatin accessibility and active and repressive histone marks in the naïve hPSC and hTSC states, we have produced an epigenetic atlas of cis-regulatory regions driving hTSC specification in vitro. This analysis revealed an enrichment of Jun-N-Terminal Kinase (JNK) binding motifs in regions associated with hTSC-specific active histone marks. Chemical inhibition of JNK signaling in hTSCs and trophoblast organoids caused spontaneous differentiation into terminal trophoblast lineages as assessed by morphology and gene expression analysis. When attempting to differentiate hTSCs from the naïve state in the presence of this inhibitor, the cells displayed growth restriction and abrogated hTSC specification. To investigate how JNK-associated factors contribute to hTSC specification and maintenance, we have identified specific JNK targets via differential phosphorylation status upon inhibitor treatment. We are currently exploring these transcription factors in greater detail to assess their individual and combinatorial impact on hTSC specification and maintenance using a deactivated Cas9 system. By integrating these genetic studies of JNK-associated factors with genome-wide location analyses to identify DNA binding regions, we will gain a comprehensive understanding of the role of the JNK signaling pathway during hTSC specification and differentiation into terminal trophoblast lineages.

P-382 - Pro-Inflammatory Interleukin 1 β Affects the Function of Decidualized Uterine Stromal Cells – Clues from the Canine Model

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Well-timed uterine immune dynamics are crucial for the establishment and outcome of pregnancy. For instance, both implantation and parturition are events marked by increased immune activity, whereas the maintenance of pregnancy is associated with decreased intrauterine immune activity in several species, including the dog. A disproportionate immune response is frequently associated with loss of pregnancy. Indeed, in dogs, inflammatory and infectious uterine diseases rank among the main causes of abortion, but underlying mechanisms still remain unexplored. The transcriptional availability of interleukin 1 β (IL1 β), key mediator of inflammatory responses, is

increased in the canine uterus during placentation, but not in the matured placenta. Conversely, this cytokine is recurrently described as being upregulated in the canine uterus during inflammatory and infectious events. The outcome of pregnancy in dogs is tightly linked to a proper function of maternally-derived decidual cells, mainly due to their unique expression of the progesterone receptor (PGR) in the canine placenta. Indeed, interference with the activity of decidual cells, e.g., by blocking PGR function, results in preterm pregnancy termination. Previous works using an in vitro model of canine decidualization, the immortalized dog uterine stromal (DUS) cells, identified possible immunoactive mechanisms in decidual cells, including an increased transcriptional availability of the IL1 β receptor 1 (IL1R1). However, functional studies are still missing. Following this, the present work aimed to investigate the modulatory effects of IL1 β in the canine placenta, mainly focusing in decidual cells function. To further support the proposed responsiveness of decidual cells to IL1 β , the expression and localization of IL1R1 were investigated in samples of pregnant dogs throughout gestation (n = 3-5 animals/pregnancy stage), spanning from pre-implantation to parturition luteolysis. The intrauterine transcriptional availability of IL1R1 was increased in the mature placenta, namely during mid-gestation (days 35-40 of pregnancy) and parturition luteolysis, when compared with the time of implantation (day 17) or early placentation (days 18-25) (P < 0.05). Furthermore, IL1R1 could be localized with immunohistochemical staining in decidual cells, in addition to endothelial cells and trophoblast, in placentae collected during mid-gestation. The functional effects of IL1 β were explored by exposing DUS cells decidualized in vitro with cAMP to different concentrations of IL1 β (0.1, 1 or 10 ng/ml) for 24h. All tested concentrations of IL1 β significantly decreased the transcriptional availability of factors linked to decidual function, such as the decidualization markers IGF1, PTGES and PTGS2/COX2 (P < 0.001), but also the extra-cellular matrix components ECM1 and TIMP2 (P < 0.01). Finally, transcriptional availability of the gap junction component CX43, but also its protein amounts detected with immunofluorescence, were decreased by IL1 β in decidualized DUS cells (P < 0.001). These findings suggest that IL1 β can disturb placental tissue homeostasis, at least in part, by disrupting the function of decidual cells. This may comprise one of the underlying mechanisms leading to pregnancy loss (abortion) in bitches, but also in other species with decidual placenta, during inflammatory/infectious events.

P-383 - Disrupting the CXCL12-CXCR4 Chemokine Axis at the Fetal-Maternal Interface During Implantation in Sheep Results in Less Placental VEGFA at Midgestation

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Failure of proper placental vascular remodeling and development results in placental insufficiency which is a root cause of preeclampsia, fetal growth restriction, and fetal morbidity and mortality. The causes of placental insufficiency are poorly understood but origin of disease is thought to occur during placental development (placentation). Specifically, dysregulation of placental angiogenesis has emerged as one of the main pathophysiological features in the development of placental insufficiency and its clinical consequences. The chemokine, CXCL12 working through its receptor CXCR4 plays pivotal roles during implantation and placentation by stimulating synthesis of vascular endothelial growth factor (VEGFA), considered the most potent inducer of angiogenesis

and chief player in placental growth and vascularization. Interestingly, VEGF in turn drives expression of both CXCL12 and CXCR4 creating a powerful feed forward angiogenesis loop. Dysregulation of the CXCL12-CXCR4 axis is implicated in pregnancy complications including miscarriage and preeclampsia but precise roles of this axis during placentation are unclear, especially at the fetal-maternal interface. Using a sheep model, we previously demonstrated suppressing CXCL12-CXCR4 signaling with a pharmacological inhibitor (AMD3100, 1X dose) at the fetal-maternal interface during implantation diminishes placental vascularization with drastic reduction in VEGFA by d20 of gestation. Ewes also present with reduced trophoblast invasion and compromised uterine remodeling. If allowed to continue gestating, at midgestation, dams exhibit placental insufficiency. In the current study, a more in depth investigation into the effects of CXCL12-CXCR4 on placental VEGFA production was conducted by delivering increasing concentrations of AMD3100 at the fetal-maternal interface during implantation and investigating placental VEGFA on d90. We hypothesized increasing the amounts of AMD3100 (1X, 1.5X, and 3X doses) during implantation would cause dose-response impacts to placental vascularization evidenced by altered VEGFA production. On d12 post-breeding, osmotic pumps were surgically installed in ewes to deliver three different amounts (1X, 1.5X or 3X) of AMD3100 or saline (control) into uterine lumen ipsilateral to corpus luteum for 14 days. On d90, placentomes were collected and immediately immersed in 4% paraformaldehyde for 24h and embedded in paraffin according to standard histological procedures. Immunofluorescence staining and analysis was conducted on placentomes to quantify VEGFA presence and subsequent vascularization. Images obtained were analyzed via Image J and integrated densities were analyzed in a mixed procedure via SAS. Differences in VEGFA expression between male and female fetuses was observed only in control placentomes with females displaying more ($P < 0.05$) VEGFA than male fetuses. Ewes exposed to 3X AMD3100 exhibited less ($P = 0.008$) VEGF compared to control. Immunoreactive levels of VEGFA tended to decrease in the 1X and 1.5X AMD3100 treated ewes compared to control. Our data underscore the importance of CXCL12-CXCR4 axis during placentation and provide evidence that altering CXCL12-mediated signaling during implantation induces enduring placental vascular effects manifesting later in gestation. As serious inadequacies in placental formation and vascularization underpin many cases of implantation failure and contribute to pregnancy complications, further characterization of our sheep model may prove useful for studying pregnancy complications stemming from compromised implantation. Research supported by National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health (NIH). Grant number 1SC1GM139712-01.

P-384 - Developing a 3D Long-Term Trophoblast Culture System to Study Fetal-Maternal Interactions in Cattle

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The greatest limitation of in vitro 2-dimensional trophoblast cell culture systems used to study placental physiology is the inability to accurately mimic the complexity of tissue structure and physiology of in vivo placentation. Three-dimensional (3D) long-term cultures have the potential to represent the in vivo microenvironment of placental tissue more accurately, with cell-cell, and cell-extracellular matrix interactions. Our goal is to establish and characterize a 3D long-term bovine trophoblast cell culture system that accurately recapitulates bovine placental physiology and can

be used to study placental function and embryonic loss in cattle. We hypothesize that the 3D culture will resemble the morphology, gene expression, and protein expression of placental tissue. Eight Angus cows were estrus synchronized using OvSynch + CIDR (controlled internal drug-releasing dispenser), superovulated, and artificially inseminated. Embryos were flushed transcervically at sixteen and twenty-one days after artificial insemination. Concepti retrieved from two day-16 pregnancies and one day-21 pregnancy were identified under a dissecting microscope, mechanically separated from mucus and uterine secretions, the embryonic disk was removed, and incubated for 2 minutes in 0.25% of trypsin in Dulbecco's Modified Eagle Medium (DMEM). The tissue was placed in T25 flasks with Trophoblast Organoid Medium containing DMEM/F12, N2 supplement, B27 supplement minus vitamin A, primocin, N-acetyl-L-cysteine, L-glutamine, recombinant EGF, CHIR99021, recombinant R-spondin-1, recombinant FGF-2, recombinant HGF, A83-01, prostaglandin E2, and Y-27632. Tissue was cultured in an incubator at 37°C, 5% CO₂. Trophoblast tissue was collected at 0, 3, 6, and 12 months in culture. Day 40 and 80 fresh placental tissue and cultured fibroblast cells were used as controls for gene expression analysis. Cellular viability and proliferation rates were assessed using the CyQuant XTT cell viability kit over time. Expression of genes related to trophoblast development, function, and differentiation was assessed. The presence of placental alkaline phosphatase (PLAP) was assessed using a sandwich ELISA. Cell morphology was evaluated by a pathologist. Collagen and extracellular matrix deposition were evaluated using picosirius red and Masson trichrome stains. Cell viability and proliferation results showed proliferation in all culture groups throughout the 48-hour assay. Histological analyses demonstrated that some cells differentiate into binucleate cells and that these cells form spheroid structures that resemble trophoblast organoids. Gene expression analyses have demonstrated that these cells maintain the expression of trophoblast-specific genes such as PD-L1, PRP-VII, PAG11, PAG 12 and PTSG2 up to 12 months after the start of culture. In addition, placental alkaline phosphatase (PLAP) was secreted in the supernatant of cultured trophoblast cells. There was no evidence of extracellular matrix deposition. These results indicate that this 3D long-term trophoblast cell culture model is morphologically and molecularly similar to placental tissue. Further characterization of this 3D model is needed to better understand if it could be a useful tool to study fetal-maternal interactions in cattle. This project was supported by the Utah Agriculture Experiment Station Project no. 1459.

P-385 - Evaluation of Anti-Müllerian Hormone Plasma Levels as a Predictor of Pregnancy Outcome in the Rhesus Macaque

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Due to their close evolutionary relationship to humans and aptness of the species for creating disease and developmental models, the rhesus macaque is one of the most utilized non-human primates in biomedical research. To maintain an appropriate population for research progress, and

provide developmental models for study, the success of time-mated breeding programs is critical. Key factors in time-mated breeding include menstrual and hormone tracking, ovulatory phase assessment, timed-pairing, and early pregnancy confirmation. Here, we focused our efforts on evaluating if ovarian cycle day 3 plasma levels of anti-Müllerian hormone (AMH), a known biomarker for ovarian reserve, can predict pregnancy outcome in naturally cycling rhesus macaques. Previously, our laboratory demonstrated that day 3 plasma levels of AMH could be used as a predictor of oocyte yield following a controlled ovarian stimulation; therefore, we hypothesized that a correlation between AMH collected on day 3 could also be used to predict pregnancy outcome in a naturally cycling population. A total of $n=83$ plasma samples from $n=44$ animals were collected and AMH levels were quantified by ELISA (AnshLabs, AL-105). Data were interrogated by Pearson's coefficient and Point-biserial correlation coefficient; our results indicated no significant correlations between AMH levels and pregnancy outcomes for a given cycle ($r_{pb} = -.110$, $p = .322$). There was also no relationship between AMH and number of previous pregnancies ($r_{pb} = -.183$, $p = .097$), nor AMH and total number of pregnancies ($r_{pb} = -.179$, $p = .105$). As expected, we observed a negative correlation between AMH levels and age of the animal ($r = -.231$, $p = .035$), in that the older the animal, the lower the AMH value. Interestingly, we observed a positive correlation between AMH levels and estradiol surge day ($r = .306$, $p = .005$), but not peak estradiol levels ($r = .034$, $p = .762$). We conclude that AMH does not serve as a reliable metric for determining pregnancy in a time-mated breeding program. These data indicate that the connection between AMH and age may aid in establishing a screening metric for reproductive potential when selecting new animals, and in assessing animals currently within the time-mated breeding program. Additionally, taking the relationship between estradiol peak day and AMH into consideration may allow for more accurate assessments of individual animals' ovulation cycles. This is an essential component in time-mated breeding in advising appropriate day of pairing, in addition to tailoring total number of days paired to match predicted ovulation days. The correlation between AMH and estradiol surge day indicates a relationship to ovulation; investigation of AMH and ovulatory factors, such as luteinizing hormone, should be assessed. Further research on prematurely diminishing or non-cycling levels, as well as supplementation, of AMH in the rhesus macaque is needed to identify possibilities of promoting or extending reproductive fecundity.

P-386 - Estradiol-Induced Labor Initiation: Changes on Gene Expression of Maternal Uterine and Cervical Tissues in Periparturient Ewes.

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The onset of labor is a complex process involving various hormonal and physiological pathways that remain to be completely understood. Herein, we investigated the influence of estradiol (E2) in the initiation of parturition of periparturient Rambouillet ewes. To this end, two experiments evaluated the relationship between E2 circulating levels, birth timing (Exp.1), and gene expression (Exp.2) on the myometrium, endometrium, cervix, and caruncle tissues. Both experiments utilized

the same approach but with varying E2 dosages. Multiparous ewes between 139 to 142 days of gestation were randomly assigned to either the E2 group, which received six Silastic implants of 50 mg of E2 in Exp.1 (300 mg/ewe; n=12) and four in Exp.2 (200 mg/ewe; n=6); or the control (C) group, which received 6 Silastic empty implants in Exp.1 (n=12) and 4 in Exp.2 (n=6). The implants were inserted subcutaneously in the axillary region of the ewe and removed two days after parturition (Exp.1) or at the time of slaughter, 26h after treatment (Exp.2). Maternal blood samples were drawn one day before treatment initiation in Exp.2 and 8h (Exp.1) or 26h (Exp.2) from the beginning of treatment to measure circulating levels of E2 and progesterone (P4) using immunoassay. Cross sections of the uterus, caruncles, and cervix were collected for RNA-Seq analysis. After RNA-Seq data quality control and mapping, differentially expressed genes (DEGs) were identified using DESeq2. Results indicated that before treatment, E2 levels between groups were similar. However, at 8h (Exp.1) and 26h (Exp.2) after treatment, E2 concentration was significantly higher in the E2 group (192.91 ± 17.32 vs. 10.97 ± 20.47 pg/ml at 8h $p=0.01$, and 149.21 ± 55.93 vs. 30.61 ± 11.73 at 26h $p=0.01$). No differences on the P4 level between groups were found at any point. Exp.1 showed a significant difference in days to deliver after treatment: 2.84 ± 1.02 in the E2 group vs 7.18 ± 1.11 in the C group ($p=0.01$). Furthermore, the E2 treatment significantly impacted gene expression in all the tissues tested ($p\text{-value} \leq 0.1$ and $\log_2$ fold change $|\geq 0.5|$). The cervix exhibited the strongest effect, with 8,073 DEGs, including E2 upregulation of PTGS-2, PTGES2, ESR2, and IL-6, and downregulation of IL-10, ACTA2, MYH11, GJA1, and TIMPs. The endometrium also showed significant changes, with 722 DEGs, including E2 upregulation of OXTR and ESR1 and downregulation of SULT1E1 and TIMPs. The caruncles had a more modest response, with 109 DEGs, while the myometrium was less responsive to the treatment, with 90 DEGs, including E2 upregulation of PTGS-2. Our findings suggest that E2 causes significant changes in gene expression in the maternal reproductive tissues, leading to parturition without a decline in circulating levels of P4. These effects appear to be primarily driven by the alterations in the cervix, where E2 upregulated genes associated with inflammation and cervical ripening and downregulated genes associated with smooth muscle contraction and tissue integrity. Supported by USDA-NIFA-AFRI grant 2021-67015-34277 to LPR and AJC

P-387 - Concentrations of Amino Acids and Expression of Their Transporter in the Pig Uterus Throughout Pregnancy

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Progesterone (P4) is an essential hormone for the establishment and maintenance of pregnancy. The porcine conceptus (embryo/fetus and placental membranes), requires histotroph, a mixture of hormones, cytokines, amino acids, and other nutrients, to undergo elongation, implantation/attachment, and placentation essential for development of the embryo/fetus. Solute Carrier Transporters (SLC) transport amino acids (small neutral, large neutral, anionic, cationic) across membranes. To characterize SLC expression during gestation, gilts were assigned to cyclic or pregnant groups, with hysterectomies performed on either Day 9, 12, or 15 of the estrous cycle or Day 10, 12, 15, 20, 25, 30, 35, 40, or 60 of gestation. qPCR was used to measure relative changes in

the endometrial expression of select SLC mRNAs. Expression of SLC1A4 mRNA was elevated on Day 15 of both the estrous cycle and pregnancy, and further increased between Days 35 and 60 of pregnancy ($p < 0.05$). SLC1A5 mRNA expression was also elevated on Day 15 of the estrous cycle and pregnancy, and increased significantly ($p < 0.05$) from Day 25 through Day 60 of gestation. Expression of SLC7A10 mRNA was slightly elevated on Day 10 of pregnancy, decreased until Day 25 and then increased significantly ($p < 0.05$) to Day 60 of gestation. No significant changes in expression between days were detected in cyclic gilts. Expression of SLC6A9 mRNA was greatest on Day 12 of the estrous cycle and Days 10 and 12 of pregnancy, but decreased significantly by Day 15 of both the estrous cycle and pregnancy ($p < 0.05$). SLC7A8 mRNA expression increased between Days 10 and 15 of gestation, and slowly decreased through Day 30. Expression of SLC7A8 mRNA increased between Days 9 and 12 of the estrous cycle, and decreased by Day 15. The secretion of histotroph is regulated by P4, estrogen (E2), and other paracrine signals. To study the contribution of P4 to amino acid transport across the endometrial luminal epithelium (LE) during pregnancy, ovariectomized gilts received daily injections of either P4 or corn oil (CO) from Day 12 through Day 39 post-estrus. HPLC was used to determine concentrations of amino acids in the uterine lumen on Day 40 in P4- and CO-treated gilts. Aspartate, serine, glycine, threonine, citrulline, arginine, taurine, alanine, methionine, valine, phenylalanine, isoleucine, leucine, and lysine were significantly more abundant in uterine flushings of P4-treated than CO-treated gilts. Next, qPCR was used to analyze the endometrial mRNA expression of the facilitated solute carrier transporters, SLC1A4, SLC1A5, SLC6A9, SLC7A8, SLC7A10, and SLC38A1, to determine effects of P4 on their expression. SLC6A9 mRNA expression increased ($p < 0.05$) in response to P4 whereas P4 treatment decreased ($p < 0.05$) expression of SLC7A10 mRNA. There was no significant difference in the relative expression of mRNAs for the other amino acid transporters. The results from these studies indicate a dynamic role of P4 in the regulation of solute carrier expression in the endometrium for transport of amino acids across the endometrial LE to support elongation, implantation and placentation of pig conceptuses. This research was supported by Agriculture and Food Research Initiative Competitive Grants no. 2022-67015-37229 from the USDA National Institute of Food and Agriculture.

P-388 - Modification of the expression profiles of Prnp gene and marker genes in early stage embryo by DNMT1 modulators

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The products of Prnp gene are the functional molecule in development and disease. One of the possible reasons of prion disease is the amount of PrPC in a tissue, and controlling the expression levels of PrPC is suspected as a way of protection the prion. DNA methylation is a major epigenetic mechanism and plays critical roles in the silencing of retrotransposons, genomic imprinting, and cell differentiation. So far, the epigenic modification is happened during early embryonic stage, but it is not fully unmasked the epigenic regulation during cleavage. In this study the expression of the Prnp was examined in the cleavage stage and the evaluation the changes of developmental genes was did by DNMT1 modifying molecules, S-adenosylhomocysteine (SAH), N-phthalyl-L-tryptophan (RG108), and maybridge (Myb1). Development to the blastocyst stage was significantly higher in 10

mM Myb but lower in 1 mM SAH than the control. The expression profiles of Prnp were modified by the inhibitors and the patterns were different between them. The expression of the potency related genes and lineage related genes were also modified with the inhibitor specific patterns. RNAseq results showed the high expression of carbohydrate metabolic genes in Myb1 treated embryos, immune related genes in SAH, and cell proliferation related genes in RG108. Myb1 treated embryos were transferred into the uteri of pseudopregnant mice and got the normal pups. Put together it is suggested that the gene expression is modified with the DNMT1 inhibitor specific manner and Myb1 is a useful DNMT1 inhibitor for the early stage embryo development along with the regulation of Prnp expression. Grants: This study was supported by the NRF-2021K1A4A7A02098794 and NRF-2023R1A2C3007223

P-389 - Physiologic and Physical Interactions between the TRPV4 Channel and Oxytocin Receptor Modulate Human Myometrial Contractility

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Preterm birth and preterm labor remain significant public health concerns, affecting millions of infants worldwide. Previously, we demonstrated that transient receptor potential vanilloid-4 (TRPV4) channel activity modulates myometrial contractility in murine models. The present work addressed the hypothesis that direct interaction between the TRPV4 channel and the oxytocin receptor (OXTR) regulates calcium signaling and contractility in the human myometrium. After obtaining informed consent, myometrial tissue was collected from pregnant non-laboring women at term gestation (≥ 37 weeks) undergoing cesarean section ($n=7$). Myometrial biopsies were obtained from the upper margin of the lower uterine segment incision following delivery of the placenta. Myometrial tissue was used for histological analyses or enzymatically dispersed for the isolation and culture of human uterine smooth muscle cells (HuSMCs). Spatial protein expression and co-localization of TRPV4 and OXTR were assessed by immunofluorescence. Proximity ligation assays (PLA) were performed to visualize protein-protein interactions in situ by detecting the formation of fluorescent signals when the TRPV4 and OXTR were < 40 nm apart. To determine whether TRPV4 channel activation mediated contraction via OXTR, dynamic calcium (Ca^{2+}) imaging was performed to measure changes in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$). HuSMCs were loaded with the Ca^{2+} -sensitive fluorophore, fura-2, and then stimulated with GSK1016790A ($1\mu\text{M}$), a TRPV4 agonist, or oxytocin ($1\mu\text{M}$) in the presence or absence of extracellular Ca^{2+} . Contractility was evaluated by collagen gel contraction assay, HuSMCs were treated with oxytocin ($1\mu\text{M}$) in the presence or absence of GSK1016790A, and the cross-sectional diameter of each gel was measured. Specific pharmacological antagonists of TRPV4, RN-9893 (500nM) and GSK2728745 (1nM), were used in the presence or absence of oxytocin, with $[\text{Ca}^{2+}]_i$ and myometrial contractility as outcome measures. Co-localization of TRPV4 and OXTR was demonstrated via immunofluorescence. PLA showed proximity between TRPV4 and OXTR, supporting the notion of their physical association. In HuSMC treatment with either GSK1016790A or oxytocin increased

[Ca²⁺]_i ($p < 0.0001$ vs baseline). The absence of extracellular calcium attenuated the GSK1016790A and oxytocin-induced increases in [Ca²⁺]_i. In contractility assays, oxytocin treatment decreased the cross-sectional diameter of collagen gels loaded with HuSMCs, by 27±5% ($p < 0.05$ vs control), consistent with contraction. Co-treatment with GSK1016790A potentiated the oxytocin-induced contraction by 10±6% ($p < 0.05$ vs oxytocin). Moreover, pharmacological antagonists of TRPV4, RN-9893 and GSK2728745, markedly attenuated oxytocin-induced increases in [Ca²⁺]_i by 81% ($p < 0.05$ vs. oxytocin) and 75% ($p < 0.05$ vs. oxytocin), respectively. We conclude that activation of either the TRPV4 channel or OXTR increases HuSMCs [Ca²⁺]_i and contractility, effects contingent on entry of extracellular calcium. Moreover, the physiologic effects of activation of either the TRPV4 or the OXTR were potentiated by activation, and attenuated by blockade, of the other. The physiologic interaction between TRPV4 and OXTR was supported by evidence of a physical interaction between the channels. These findings underscore the importance of the TRPV4 channel and interactions with the OXTR in regulating myometrial contractility. Targeting TRPV4-OXTR interactions presents a promising avenue for therapeutic interventions aimed at managing preterm labor and reducing the incidence of preterm birth.

P-390 - Mastitis Infection During Pregnancy Alters the Liver Proteome of Calves Postnatally

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Neonatal dairy calves are particularly vulnerable to respiratory and gastrointestinal illnesses, potentially due to developmental programming. We hypothesize that high milk yields and/or mastitis infections during pregnancy will impact hepatic protein expression of those factors involved in cellular function and metabolism. Holstein bull calves (N = 39) were brought to the University of Rhode Island Peckham Farm within 24 hours of birth. Based on their dam's milk production and somatic cell counts (SCC) during pregnancy, calves were categorized into three groups: HI (top 25% of herd M305, n = 7), MOD (lower 60% of herd M305, n = 16), HIMAST (HI with SCC ≥ 200,000 cells/mL; n = 15). At ten weeks of age, calves were slaughtered and tissue samples from the liver were immediately collected and snap-frozen in liquid nitrogen. Proteomic analyses of the liver tissue was conducted on a subset of these calves (n = 4 per treatment) using ultra-performance liquid chromatography-tandem mass spectrometry on a Q Exactive HF mass spectrometer (ThermoFisher Scientific, Waltham, MA; University of Connecticut Proteomics and Metabolomics Facility, Storrs, CT). Proteins that were differentially expressed were quantified and identified utilizing the average precursor intensity in Scaffold v5.3.2 ($P \leq 0.05$; Proteome Software, Inc; Portland OR). Panther (Panther v18.0; Mi et al., 2021) was used for protein ontology analyses. A total of 3,673 proteins were identified across the treatment comparisons. A subset of 665 differentially expressed (DE) proteins were identified from this total number (MOD vs HI: 263; HI vs HIMAST: 195; MOD vs HIMAST: 207; $P \leq 0.05$). Two differentially expressed proteins Tensin (TNS)-2 and UDP-glucuronosyltransferase (LOC1001389) were identified as common between the three treatment groups. TNS2 and LOC1001389 were reduced in HIMAST when compared to HI and MOD ($P \leq .05$). Conversely, HI calves exhibited greater protein expression of TNS2 and LOC1001389 when compared to MOD calves ($P \leq 0.04$). Proteins were classified into the following groups: molecular function, biological processes, and protein class with subclassifications within each. Data

presented are the classifications and subclassifications with the greatest number of proteins. A total of 104, 76, and 83 proteins were classified into the molecular function: catalytic activity category for MOD vs HI, MOD vs HICAST, and HI vs HICAST respectively. 129, 99, and 95 proteins were classified within biological processes: cellular processes category for MOD vs HI, MOD vs HICAST, and HI vs HICAST. A total of 79, 61, and 58 proteins were classified within protein class: metabolite interconversion classification for MOD vs HI, MOD vs HICAST, and HI vs HICAST. These data support the hypotheses that the level of milk production and/or mastitis infection during pregnancy can impact the expression of a wide variety of proteins that are involved in cellular function as well as metabolism.

P-391 - ADAMTS4 is a Crucial Proteolytic Enzyme for Versican Cleavage in the Amnion at Parturition

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Hyalactan cleavage may play an important role in extracellular matrix remodeling. However, the proteolytic enzyme responsible for hyalactan degradation for fetal membrane rupture at parturition remains unknown. Here, we revealed that versican (VCAN) was the major hyalactan in the amnion, where its cleavage increased at parturition with spontaneous rupture of membrane. We further revealed that ADAMTS4 was a crucial proteolytic enzyme for VCAN cleavage in the amnion. Inflammatory factors may enhance VCAN cleavage by inducing ADAMTS4 expression and inhibiting ADAMTS4 endocytosis in amnion fibroblasts. In turn, versikine, the VCAN cleavage product, induced inflammatory factors in amnion fibroblasts, thereby forming a feedforward loop between inflammation and VCAN degradation. Mouse studies showed that intra-amniotic injection of ADAMTS4 induced preterm birth along with increased VCAN degradation and proinflammatory factor abundance in the fetal membranes. Conclusively, there is enhanced VCAN cleavage by ADAMTS4 in the amnion at parturition, which can be reenforced by inflammation.

P-392 - Mammalian Implantation and Subsequent Pregnancy – A Story of MiRNA Evolution and Co-option

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MiRNAs have long been associated with animal innovation. Specifically, it is known that bursts of miRNA diversity coincide with functional and morphological innovation. Indeed, placental mammals have over 13 miRNA families identified thus far that are unique to this clade. However, we know from gene regulatory network biology that “newer” miRNAs have fewer targets and major transitions such as the shift in reproductive strategy in mammals from oviparity to viviparity are most likely supported by a combination of new miRNAs and older miRNAs. Here we consider the role of “older” miRNAs (i.e., miRNAs that predate the origin of mammals) in the evolution and diversification of mammal reproductive strategies. Using a set of >400 mammal genomes, we have applied machine learning approaches to identify miRNAs and their homologs across all species in our set. From the large presence absence matrix, we extracted all miRNAs that are “older”, we mapped their evolutionary trajectories (e.g., expansions and contractions of miRNA gene families) and using independent methods have identified their targets, pre- and post-mammal origin, to

explore shifts in the regulatory network through time and to identify key shifts that coincide with the origin of mammals. The likely impacts of these “older” miRNAs and their target profiles on mammal implantation strategies and subsequent embryonic development are explored.

P-393 - Loss of Function Mutations in ALPL Alter Nutrient Abundance in Fetal Plasma, and Allantoic and Amniotic Fluids in Sheep

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Tissue-nonspecific alkaline phosphatase (TNSALP; encoded by the ALPL gene) has an important role in the regulation of phosphate homeostasis postnatally. We recently reported spatio-temporal alterations in expression of TNSALP and its enzymatic activity in ovine utero-placental tissues during gestation, yet the role of TNSALP in the regulation of placental development and function is unclear. To determine the role of TNSALP during pregnancy in ruminants, wildtype (WT) ewes and ewes with heterozygous (Het) or homozygous (Hom) loss-of-function mutations in ALPL (c.1077 C >G; generated by CRISPR/Cas9) were bred with fertile rams. Ewes were euthanized and hysterectomized on Day 97 of gestation. Placentae from Het ewes were longer, and had abnormally large and irregularly shaped placentomes, while placentomes from Hom ewes were smaller and more numerous. These results suggest that loss of function mutations in ALPL affect placental morphology and surface area, both of which are critical for placental transport of nutrients. We hypothesized that these alterations in placental morphology and surface area in response to loss of function mutations in ALPL altered placental nutrient transport and thus the abundance of nutrients in fetal plasma and fetal fluids that are important nutrient reservoirs for the fetus. To test this hypothesis, the abundance of key nutrients required for fetal development were quantified in fetal plasma, and amniotic and allantoic fluids from WT, Het, and Hom fetuses on Day 97 of gestation. Het fetuses had more urea nitrogen ($P=0.07$) and less 25(OH)D, aspartate aminotransferase (AAT) and gamma-glutamyl transferase (GGT) activity ($P<0.05$) in plasma compared to WT fetuses. Het fetuses had less creatine phosphokinase (CPK) activity compared to Hom fetuses ($P<0.05$) in plasma. Hom fetuses had more glucose ($P<0.001$) and potassium ($P<0.01$), but a lower sodium: potassium ratio ($P<0.01$) and AAT activity in plasma compared to WT and Het fetuses. TNSALP activity in fetal plasma was positively correlated with amounts of bilirubin ($P<0.05$), magnesium ($P<0.05$), AAT ($P<0.01$), and CPK ($P=0.058$) in fetal plasma. Fetal fluid analyses demonstrated that Het fetuses had greater concentrations of glucose in amniotic fluid compared to WT ($P<0.01$) and Hom ($P<0.05$) fetuses. Similarly, Het fetuses had greater

concentrations of glucose in allantoic fluid compared to WT fetuses ($P < 0.05$). Allantoic fluid from Hom fetuses contained more calcium than for WT ($P < 0.05$) and Het ($P = 0.10$) fetuses, which is consistent with decreased fetal skeletal mineralization. Allantoic and amniotic fluids from Het fetuses had greater concentrations of urea nitrogen than WT fetuses ($P < 0.05$). Amniotic fluid from Het fetuses contained more bilirubin ($P = 0.05$) and creatinine ($P < 0.01$) compared to WT fetuses. In contrast, amniotic fluid of Het ($P < 0.05$) and Hom ($P = 0.10$) fetuses contained less chloride than WT fetuses. The significant correlations between fetal ALPL genotype and fetal TNSALP activity with specific nutrient abundance in plasma and fetal fluids suggest significant alterations in fetal development in response to loss of function mutations in TNSALP by mid-gestation. Collectively, these findings suggest loss of function mutations in TNSALP alters the nutritional composition of fetal plasma, and allantoic and amniotic fluids, and positions TNSALP as an important regulator of placental development and function, far beyond what is widely accepted as simply regulating phosphate homeostasis.

P-394 - Effect of Bacitracin Methylenedisalicylate Supplementation on Pelvic Organ Prolapse Incidence in Late Gestation Sows

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Throughout the last decade, rising sow mortality rates have afflicted U.S. pork producers, as sow mortality has increased from an average of 9.0% in 2014 to a record high of 15.8% in 2023. Much of this increase is the result of an elevated incidence of pelvic organ prolapse (POP), as POP accounted for 22.6% of sow mortality in 2023 alone. Despite being widely recognized as not only an economic issue but also a sow welfare and production issue, mitigation strategies are lacking. Antibiotics represent a potential avenue for prevention of this condition, as some work indicates vaginal microbiota differences in sows at elevated risk for POP. Therefore, we conducted a study to evaluate the effect of bacitracin methylenedisalicylate (BMD) supplementation in late gestation sows on POP incidence. This study utilized 2,289 sows housed on two commercial sow farms from the same production system. Administration of BMD differed between farms, with farm A utilizing administration of BMD soluble through the water and farm B utilizing administration of BMD as a feed additive. A total of 1,014 sows were on trial at farm A, with 522 in the control group and 492 in the BMD group. A total of 1,275 sows were on trial at farm B, with 709 in the control group and 566 in the BMD group. Sows were administered BMD for two weeks prior to farrowing and perineal score (PS) evaluations were conducted during the last week of gestation to determine presumed POP risk. In brief, a PS of 1 (PS1) presumes low risk of prolapse and has little to no vulva swelling, protrusion, or swelling of the perineal region. By comparison, a PS of 3 (PS3) presumes high risk of prolapse and has moderate to severe vulva swelling, protrusion, and swelling of the perineal region. Outcomes analyzed included the effect of BMD treatment on PS, POP incidence, and litter characteristics. Analysis of PS by treatment revealed that BMD administration did not affect PS at either farm ($P \geq 0.13$). At farm A, 60.0% of control sows and 60.6% of BMD treated sows were scored a PS1 while 2.9% of control sows and 5.4% of BMD treated sows were scored a PS3. At farm B, 78.3% of control sows and 76.7% of BMD treated sows were scored a PS1 while 2.8% of control sows and 3.1% of BMD treated sows were scored a PS3. Further, BMD treatment did not affect POP incidence at either farm ($P \geq 0.71$), with 2.7% of control sows and 2.8% of BMD treated sows experiencing POP at farm A, and 2.8% of control sows and 3.2% of BMD treated sows experiencing POP at farm B. When evaluating litter characteristics, BMD treated sows had a significantly lower

number of stillborn piglets compared to control sows at both farms ($P \leq 0.05$). Although BMD administration did not reduce POP incidence in the current study, further investigation into the effect of BMD treatment on incidence of stillborn piglets is warranted. This project was supported by the National Pork Board and the Foundation for Food and Agriculture Research.

P-395 - Pig Conceptuses Utilize Extracellular Vesicles for Interferon Gamma-mediated Paracrine Communication with the Endometrium

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Interferon-gamma (IFNG) is a pro-inflammatory cytokine secreted by the trophectoderm cells of porcine conceptuses (embryo and associated extra-embryonic membranes) during the peri-implantation period of pregnancy. IFNG is a type 2 interferon that, unlike the type 1 IFN-tau of ruminants, does not act as a signal for pregnancy recognition. Instead, IFNG modifies the inflammatory immune response within the endometrium and, when deleted from conceptuses through gene editing, disrupts implantation and survival of conceptuses. The mechanism by which IFNG is secreted or released by conceptus trophectoderm and transported into the endometrium for communication with cells in the endometrial stroma has not been determined. In the present study, we performed immunofluorescence staining to localize IFNG to specific cells at implantation sites on Day 13 (n=4) and Day 15 (n=4) of pregnancy. Immunoreactive IFNG protein was detected in both trophectoderm and endometrial luminal epithelium (LE) on Day 15 of pregnancy, although we previously reported expression of IFNG mRNA only by conceptus trophectoderm suggesting IFNG protein in endometrial LE is of conceptus origin. We next cultured endometrial explants (n=3) from Day 11 of pregnancy with increasing doses of IFNG (0, 10, 50, 100 ng/mL). The explant culture procedure removed the endometrial LE barrier present in vivo, allowing IFNG to directly contact stromal cells. Real-time PCR analyses revealed that IFNG increased ($P < 0.001$) expression of mRNAs CXCL9, CXCL10, CXCL11, B2M. These effects were not detected when IFNG was infused into the uterine lumen in vivo by McLendon et al. (Biology of Reproduction. 2020;103(5):1018-1029). These observations suggest a delivery mechanism that permits IFNG to be taken up by endometrial LE during pregnancy. Extracellular vesicles (EVs) are released from cells, contain molecular signals for intercellular communication, and can cross significant biological barriers including the plasma membranes of cells. We hypothesized that the trophectoderm of pigs releases EVs that contain IFNG for paracrine communication with the endometrium. We isolated EVs from the uterine fluid of gilts on Day 11 (n=4), 13 (n=4), and 15 (n=6) of pregnancy and Day 15 of the estrous cycle (n=5). Using Western blotting, IFNG was detected in EVs from Day 15 of pregnancy, but not in EVs from Day 15 of the estrous cycle. Mean size and total numbers of EVs increased from Day 11 to Day 15 of pregnancy. Real-time PCR demonstrated increased expression of IFNG-stimulated genes in endometrial explants treated with EVs from Day 15 of pregnancy (n=4) including CXCL9 ($p < 0.0001$), CXCL10 ($p < 0.0001$), CXCL11 ($p = 0.001$), IRF1 ($p = 0.0002$), B2M ($p = 0.0150$), CTLA4 ($p = 0.0071$), PDL1 ($p < 0.0001$), and IDO1 ($p = 0.003$). EV-mediated transport of IFNG was confirmed by the presence of

immunoreactive IFNG protein in the endometrial LE of explant cultures of whole uterine sections cultured with EVs from Day 15 of pregnancy. Results of this study suggest that EVs are involved in IFNG transport into the endometrial LE which enables paracrine communication between the conceptus and cells within the endometrial stroma, during the peri-implantation period of pregnancy. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2016-67015-24955 from the USDA National Institute of Food and Agriculture.

P-396 - Endometrial TGF β Signaling via TGFBR2 Coordinates Estrogen Response during the Peri-Implantation Window and is Critical for Pregnancy

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Establishment of pregnancy is a complex process that requires bidirectional communication between the maternal endometrium and the implanting embryo. Given that 1 in 4 pregnancies experience complications or result in pregnancy loss, defining the signals that coordinate endometrial receptivity is critical for improving pregnancy outcomes. Our previous work has demonstrated that the transforming growth factor β (TGF β) pathway is critical for preparing the endometrium for implantation and for optimal pregnancy outcomes. Specifically, we showed that TGF β signaling controls the endometrial response to estrogen and progesterone signaling. To identify the mechanisms controlling the dialog between TGF β /estrogen/progesterone signaling during early pregnancy, we generated a new mouse model with conditional inactivation of the TGF β type 2 receptor, TGFBR2, using progesterone receptor cre (PRcre) ("Tgfbr2 cKO"). Compared to controls, mice with conditional TGFBR2 deletion are infertile and generated only one pup over the course of a six-month fertility trial (controls, 584 pups; Tgfbr2 cKO, 1 pup). Mice with conditional TGFBR2 deletion also experienced high rates of mortality during the breeding trial (7/12 perished). To determine a potential role of Tgfbr2 cKO on ovarian function, we monitored estrous cycling of control and Tgfbr2 cKO mice for 30 days and found no statistical difference in the number of days spent in each phase or in the total number of cycles in the 30-day period between groups. Additionally, superovulation studies showed no statistically significant difference in ovulated oocytes between control and Tgfbr2 cKO mice. Given the lack of significant ovarian defects in Tgfbr2 cKO mice, we focused on the effect of conditional TGFBR2 deletion on endometrial function to explain the infertility phenotype. Timed mating analyses demonstrated that defects in pregnancy occurred during the peri- and post-implantation stages, where compared to controls, Tgfbr2 cKO mice displayed fewer implantation sites at 4.5 days post coitum (dpc), 5.5 dpc, 6.5 dpc, and 8.5 dpc. Almost no implantation sites were recovered at 12.5 dpc in the Tgfbr2 cKO mice, indicating that embryo resorption was likely completed by this point. Molecular analyses of control and Tgfbr2 cKO implantation sites indicate a dysregulation of early pregnancy starting at implantation, that worsens throughout pregnancy and results in abnormalities in decidualization, uterine natural killer cell (uNK) invasion, and angiogenesis. At 4.5 dpc, implantation sites of Tgfbr2 cKO mice demonstrate an increase in estrogen-response genes such as Lifr, Lif, Lcn2, and Muc1, suggesting

unopposed estrogen response during early pregnancy. To further characterize the differences in hormone response between control and *Tgfr2* cKO mice, we utilized murine endometrial epithelial organoids to define differences at the first place of maternal contact with the implanting blastocyst – the endometrial epithelium. Following estrogen and progesterone treatment, *Tgfr2* cKO organoids displayed an upregulation of *Esr1*, as well as estrogen and progesterone-response genes, while no significant difference in *Pgr* expression was observed. These data indicate both increased endometrial epithelial estrogen response and impaired progesterone response in the epithelium of *Tgfr2* cKO mice. Ultimately, our studies indicate that TGF β coordinates the endometrial response to estrogen and progesterone, controls uNK cell recruitment, and that defective TGF β signaling leads to impaired implantation and pregnancy defects during late gestation. Studies were supported by Eunice Kennedy Shriver National Institute of Child Health and Human Development grants R00-HD096057, R01-HD105800. Diana Monsivais, Ph.D. holds a Next Gen Pregnancy Award (NGP10125) from the Burroughs Wellcome Fund.

P-397 - Neuregulin-1 signaling in corpus luteum

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The corpus luteum (CL) is an absolute requirement for reproductive success to regulate the estrous/menstrual cycle and pregnancy by producing progesterone (P4). CL is a transient ovarian endocrine gland that develops from a dominant ovulated follicle in the ovary by the mid-cycle surge of luteinizing hormone (LH). The lifespan of CL is tightly regulated by survival and cell death signals, including endocrine (LH), intra-ovarian regulators, and cell-cell interactions. Neuregulin 1 (NRG1) is a member of the epidermal growth factor-like factor family that mediates its effect through the erythroblastoma (ErbB) family. However, the detailed mechanisms associated with the interplay of NRG1 in CL function are unknown. In this study, we investigated the potential impact of NRG1 on rat CL and luteal cell (LC) function. Our experimental results suggest that LH has differential effects on the expression of NRG1 and ErbB receptors in CL during pregnancy. On day 12 in pregnant rats, NRG1 and ErbB2/3 receptors are highly expressed in LCs with a positive correlation with StAR expression and serum LH and P4 concentrations. In contrast, on day 22, pregnant rats had significantly decreased serum LH and P4, suggesting luteolysis had occurred with low expression of NRG1 and ErbB receptors. To define the role of NRG1 in LCs, monolayer LCs were cultured and treated with different doses of cytokines in the presence of exogenous recombinant NRG1 or absence of NRG1 (knockdown, siNRG1) since various cytokines are produced within the CL for vascularization. Under these experimental conditions, siNRG1 alone or cytokines-treated LCs showed significantly higher cell death in a time and dose-dependent manner when compared with parallel controls. In contrast, exogenous NRG1 co-treated LCs with cytokines showed delayed apoptosis. These results are further corroborated by pro-survival and anti-inflammatory protein expression. Furthermore, these experimental studies also provided direct evidence that the gain of NRG1 maintains the phosphorylation of ErbB2 and ErbB3, along with PI3K, Akt, and Erk. In contrast, a loss of NRG1 directly inhibited the phosphorylation of ErbB2/3, Akt, and ERK-1/2 in LC.

Collectively, these results provide new evidence of the supporting role of NRG1 in maintaining the function of CL. Acknowledgments: This study was partly supported by National Institutes of Health Grants 1 SC1 GM130544-01A1, 1SC3GM113751, and G12RR03034. This research was conducted in a facility constructed with support from the Research Facilities Improvement Grant C06RR018386 from the National Institutes of Health National Center for Research Resources.

P-398 - Age-Related Changes in the Uterine Environment during the Peri-Implantation Period of Pregnancy.

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Decidualization and placentation defects are a major cause of age-related reproductive decline. This study tested the hypothesis that age-related changes in the uterine environment begin during the window of receptivity (WOR) in which suboptimal priming of endometrial stromal cells for decidualization may create a cascading effect, leading to compromised decidualization and placentation. To excluding ovarian aging factors, we used an artificial decidualization protocol in this study. After ovariectomy and two weeks of rest to eliminate endogenous ovarian steroids, aged (45-48 weeks of age) and young (6-8 weeks of age) female mice (n=21-24) were administered daily E2 (100 ng per mouse) injections for three days. After two days of rest, mice were treated with E2 (6.7 ng per mouse) and P4 (1 mg per mouse) for three days. On the second day [DD-1; mimicking gestational day (GD) 3.5], a subset of both aged and young mice (n=7) was euthanized 6 hours after E2 and P4 administration. On the third day (DD0; mimicking GD 4.5), the remaining mice were administered a single injection of 0.05 ml of sesame oil to the right uterine horn, and the uterine tissues (n=8) were collected 6 hours later. The rest of the aged and young mice (n=7) continued receiving E2 and P4 for one additional day and were then euthanized 6 hours after the injection (DD1; mimicking GD 5.5). Initial qPCR analyses showed that both the P4- and E2-mediated pathways were downregulated ($P < 0.01$) as well as methylcytosine dioxygenase family (Tet1, Tet2 and Tet3) in DD-1 aged uteri. Immunohistochemical analyses revealed decreases of PGR expression in the uterine stromal cells as well as increases of proliferation marker Ki67 in the uterine luminal epithelium cells of aged uteri at DD-1. Furthermore, there were increases in 5-methylcytosine (5mC) and decreases in 5-hydroxymethylcytosine (5-hmC) in aged uteri as compared to young uteri at DD-1. At DD0, qPCR analyses showed that aged female mice exhibited decreases ($P < 0.01$) in mRNA expression of Ptg2 and Foxo1 when compared to the young controls. At DD1, aged mice exhibited decreases ($P < 0.01$) in the ratio of decidual to control horn weight compared to young female mice. There were also decreases ($P < 0.01$) in decidualization markers Igfbp1, Bmp2, and Prl8a2 in aged decidual uteri as compared to young controls at DD1. DD-1 uteri was further subjected to RNA-seq analyses. A total of 1,432 differentially expressed genes (DEG; 913 up and 519 down) were identified between young and aged uteri at DD-1. Canonical pathway analyses identified the top enriched pathways in the aged uteri, including activation of extracellular matrix organization, cellular stress and injury, and inhibition of cell cycle. We further identified 599

upstream regulators enriched by aging, including activation of TP53, IL6, IL4, AR, MUC1 and BCL2; and inhibition of MYC, SIRT1, SIX1, HAND2, PGR and ESR1. This study confirms that alterations in uterine pathways during WOR impact implantation and decidualization and provides insights into understanding how aging affects the uterine function independently of the ovary.

P-399 - Does Lipopolysaccharide Influence Bovine Endometrial Response to Interferon Tau?

Anup K. Talukder; John A. Browne; P. Lonergan

The uterine environment during the peri-implantation period is crucial for conceptus growth, elongation and attachment leading to a successful pregnancy establishment in cattle. Postpartum uterine disease is a major factor affecting fertility in high-producing dairy cows through disruption of ovarian and uterine function. Lipopolysaccharide (LPS), a well-known endotoxin and the major component of the outer membrane of Gram-negative bacteria, causes poor uterine receptivity by inducing excessive inflammation at the maternal-fetal interface. We aimed to investigate the effect of interferon tau (IFNT), the conceptus-derived maternal recognition signal, on bovine endometrial gene expression in the absence or presence of LPS. Endometrial explants were collected at a local abattoir from Holstein Friesian cows (n=4) during the mid-luteal stage of the oestrous cycle, and cultured in RPMI medium supplemented with 5% fetal calf serum without (control), or with IFNT (100 ng/mL), LPS (1 mg/mL), or both IFNT and LPS for 24 h in 5% CO₂ in humidified air. Gene expression was analysed by RT-qPCR. Treatment effects were considered significant at $P < 0.05$. Incubation with IFNT upregulated ($P < 0.05$) the well-known classical interferon-stimulated genes (ISGs: ISG15, OAS1, MX1 and MX2) as well as selected ISGs (CMPK2, IFI35, TRIM38, TNFSF10) from our previous study (Talukder et al., 2023 doi: 10.1016/j.theriogenology.2023.07.033) and downregulated expression of IL1B in endometrial explants. Incubation with LPS increased ($P < 0.05$) expression of inflammation-related genes (TNFA, IL6, and IL1B) as well as ISG15 and MX1 in endometrial explants but did not alter endometrial response to IFNT in terms of the studied ISGs. These results suggest that the expression of ISGs, upregulated by conceptus-derived IFNT, is not altered in the endometrium in the presence of LPS; however, the increased expression of inflammation-related genes induced by LPS indicate an altered endometrial immune response that may be associated with compromised pregnancy establishment. The work was funded by the Irish Research Council (GOIPD/2021/46).

P-400 - Gestational Intermittent Hypoxia Impairs AT2R-Mediated Cardiovascular Protection in Female Offspring on a High-Fat, High-Sucrose Diet

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Gestational intermittent hypoxia (GIH), a hallmark of maternal obstructive sleep apnea, exhibits sex-specific consequences, leading to hypertension and endothelial dysfunction in adult male offspring but not in females. This study investigated whether GIH-exposed female offspring, a "protected" group against the hypertensive effects of maternal GIH exposure, exhibit increased susceptibility to hypertension and cardiovascular dysfunction when fed a high-fat, high-sucrose (HFHS) diet, and whether this effect could be reversed by pharmacological intervention activating the angiotensin II type 2 receptor (AT2R). Female offspring of GIH-exposed (10.5% O₂, 8 h/day, E10-21) and control dams were assigned either an HFHS diet or a control diet from 12 weeks of age. Blood pressure was monitored weekly using the CODA tail-cuff system. At 28 weeks of age, the

control and GIH offspring with and without HFHS diet received a systemic infusion of either CGP42112 (AT2R agonist) or saline for 2 weeks through mini osmotic pump, and blood pressure was recorded. At 30 weeks, the animals were euthanized, and mesenteric arteries were collected for assessment of vascular reactivity and protein analysis. Plasma was collected for measurement of bradykinin levels using ELISA. The HFHS diet induced similar increases in body weight gain and blood pressure in both control and GIH female offspring. Vascular contractile responses were unaltered, but endothelial-dependent vascular relaxation was impaired, accompanied by decreased AT2R and eNOS expression and reduced plasma bradykinin levels in both control and GIH female offspring on the HFHS diet. CGP42112 administration effectively mitigated HFHS-induced hypertension and endothelial dysfunction only in control female offspring, accompanied by restoration of AT2R, eNOS, and bradykinin levels, but not in GIH counterparts. These findings suggest that GIH induces endothelial dysfunction and AT2R insensitivity in female offspring exposed to an HFHS diet.

P-401 - Uterine injury during diestrus leads to placental and embryonic defects in future pregnancies in mice

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The uterus is central to mammalian reproduction and gynecological health, and yet its capacities to regenerate itself and to interact with the embryo remain poorly understood. While the uterine is capable of scarless regeneration each menstrual or estrous cycle, C-sections and other uterine injuries contribute to scarring, resulting in either infertility or disorders like placenta previa and placenta accreta in subsequent pregnancies. With rates of C-section at approximately 30% of deliveries in the US and projected to continue to climb, a deeper understanding of the mechanisms by which these pregnancy disorders arise and opportunities for intervention are needed. Here we describe a rodent model of uterine injury on subsequent in utero outcomes. Using this mouse model, we recapitulated several features of human disorders, including implantation failure, previa-like embryo misspacing, and accreta-like overinvasive placentas. Strikingly, only uteri injured during the diestrus phase of the estrous cycle displayed subsequent embryo misspacing via persistent cyclooxygenase (COX) pathway perturbations following poor wound healing. Using RNA-seq, we identified perturbations in the expression of components of the COX/prostaglandin pathway after recovery from injury, a pathway that has previously been demonstrated to play an important role in embryo spacing. Therefore, we demonstrate that uterine injury engenders a long-term molecular

“memory” of damage that ultimately leads to numerous placental and embryonic developmental defects.

P-402 - Prolonged Gestation in Cows Carrying IVP Calves is Not Predicted by Bovine Pregnancy-Specific Proteins B

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Prolonged gestation is well recognized in cattle carrying in vitro produced (IVP) pregnancies, but to date has been largely uninvestigated. Increasing gestational length beyond the typical 285-day average is associated with larger birthweight and poor outcomes in the resultant neonate. Increased incidence of prolonged gestation and large offspring syndrome (LOS) have been reported with the use of IVP and in vitro fertilization (IVF). In vitro-produced embryos often display inferior binucleate trophoblast cell development and activity compared to in vivo-derived embryos, resulting in lower levels of hormones like progesterone and pregnancy-associated glycoproteins (PAGs) in maternal circulation. It has previously been reported that cows carrying conventional pregnancies with prolonged gestation have lower concentrations of PAGs at term. This work aimed to assess maternal serum concentrations of the PAG, Bovine Pregnancy-Specific Proteins B (PSPB), in cows with prolonged gestation and subsequently delivering a LOS affected calf. Blood samples were collected from nine prolonged gestation IVF recipient heifers (GL 290 ± 2 days) and seven control pregnant heifers (GL 275 ± 5 days) with pregnancies conceived by artificial insemination. PSPB was measured using a Sandwich ELISA. Concentrations of PSPB were compared using a Welch t-test. PSPB concentrations in prolonged gestation IVF females (Median: 1.4551 ng/ml, Range: 0.8211 – 2.1345 ng/ml) were not different ($P=0.9459$) from those of control pregnancies (Median: 1.3024 ng/ml, Range: 0.9025 – 2.0434 ng/ml). Our findings suggest that serum PSPB concentration may not be a reliable indicator of prolonged gestation in cattle carrying LOS pregnancies as it is in prolonged gestation of conventional pregnancies.

P-403 - Loss of Inhibin Negative Feedback in the Pituitary Leads to Enhanced Ovulation but Pregnancy Failure in Mice

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Follicle-stimulating hormone (FSH) is an essential regulator of female gonadal function. Inhibins are TGF β family ligands that suppress FSH synthesis by pituitary gonadotrope cells. Inhibins require a co-receptor, betaglycan or TGFBR3L, to mediate their actions. Female mice with a gonadotrope-specific deletion of betaglycan or global deletion of Tgfbr3l exhibit enhanced follicle growth and produce larger litters compared to controls. Females with both co-receptors knocked out (hereafter dKO) show marked increases in serum FSH levels, ovulate 3-4 times as many eggs in natural cycles

compared to controls, but are infertile. dKO females become pregnant and show an increased number of implanted embryos at 7.5 days post coitum (dpc). Nevertheless, by 10.5 dpc, their embryos are dead or dying. Wild-type surrogates give birth to live young following transplantation of embryos from dKO females. Conversely, pseudopregnant control but not dKO females carry transferred wild-type embryos to term. Thus, the maternal environment in dKO mice cannot support full-term pregnancies. We suspected a deleterious effect of elevated estrogens. Consistent with this idea, treatment with the aromatase inhibitor anastrozole increased embryo survival in dKO mice. We are currently characterizing the maternal hormonal environment throughout gestation and investigating placental structure/function to gain more insight into the precise nature and cause of pregnancy failure in dKO mice. Collectively, the data indicate that ovarian overstimulation, due to the loss of inhibin negative feedback, is compatible with enhanced ovulation but not fertility.

P-404 - Maternal Tamoxifen Effects on Mouse Pregnancy and Placental Development

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A significant reduction in pregnancy rates is observed among reproductive age female cancer survivors treated with tamoxifen (TAM). Tamoxifen is a selective estrogen receptor modulator (SERM) used to treat estrogen-sensitive (ER+) breast cancer. While it has protective estrogen receptor inhibitory effects in breast tissue, it is an agonist in uterine tissue. We have used a mouse model to examine the effects of TAM on implantation and the placenta after exposure. There were two primary cohorts of CF1 female mice – age-matched control (n=27) and a TAM-treated group (n=54). Mature female mice received a diet of regular chow (control) or chow supplemented with 250 mg of TAM per Kg of chow for 3 weeks, then TAM treatment ended, all mice were fed control diet, and mice were housed for 1-4 weeks washout prior to being mated with fertile B6D2F1 males. The pregnancy rate of the control group was 67% and 26.3% for the treated group. The average number of pups was 13.2 per litter and 4.9 per litter for the control and treated group, respectively. Tamoxifen treated females had significantly lower pregnancy rates, even after extended wash-out: 7.7%, 13.3%, 26.3%, 28.6% (0, 1, 2, 3wks wash-out) compared to controls 67%. Time to conception was longer for TAM females, 4, 2, 3, 4wks vs 0wks for controls. Placentas were collected at E16 of development, fixed, and embedded in paraffin, sectioned using a microtome, scanned, and analyzed after conducting immunohistochemistry (IHC) or Hematoxylin and Eosin (H&E) staining. Images were scanned for positive staining of the antibodies CD31 (endothelial cells), TPBPA, GLUT1 (glucose transporter), and P57. Uteri with implantation resorption sites were found only in the treated group of mice. GLUT1 was ubiquitously stained throughout the entire placenta. Using the antibody TPBPA as a marker for the junctional zone spongiotrophoblasts, and H&E staining, we were able to obtain the average surface area measurements of the primary layers of the fetal placenta; the labyrinth and junctional zone (JZ). In the control group, the fetal placenta was made up of 59.4% labyrinth and 40.6% JZ. In the TAM treated group, the fetal placenta was made up of 45.3% labyrinth and 54.7% JZ, with spongiotrophoblast cells of the JZ growing in a disordered fashion into the labyrinth zone. Quantification of P57 expression in the labyrinth zone of the placenta revealed a significant decrease in p57 expressing trophoblast cells in the labyrinth zone of TAM treated mice. In addition, CD31 quantification showed a significant increase in CD31 immunoreactivity in the labyrinth zone of the TAM treated mice. Along with the reduced P57 in the trophoblast cells in the labyrinth zone, this suggests reduced numbers of trophoblast cells in the labyrinth zone of TAM treated mouse placentas. These results suggest potential long-term effects of

tamoxifen on the placenta and pregnancy, even after an extended wash-out period. Keywords: Tamoxifen, pregnancy rates, placenta and implantation Financial Support: Wright Foundation Grant, University of Southern California; Future Interdisciplinary Research Exploration (FIRE) Grant, College of ACES, University of Illinois Urbana-Champaign

P-405 - Serum Metabolites Differ in Late Gestation Sows at Various Risk for Pelvic Organ Prolapse

Zoë E Kiefer, Jamie M. Studer, and Jason W. Ross

Sow mortality is an economic and welfare concern for U.S. pork producers. Specifically, pelvic organ prolapse (POP) is estimated to account for approximately 21% of all sow mortality, and a direct cause of POP has yet to be identified. Previous work has begun to characterize physiological contributors to this phenomenon. Specifically, differences in inflammatory biomarkers and analytes in circulation have been identified in sows at varying POP risk. The objective of the current study was to utilize metabolomics to further evaluate differences in circulating metabolites between late gestation sows with presumed high or low risk for POP as well as between sows that did or did not subsequently experience POP. We hypothesized that metabolites would differ between sows at varying risk for POP. During late gestation (days 110-115), sows (n = 106) from two farms were evaluated for POP risk using a 3-point perineal scoring system. Briefly, a perineal score (PS) of 1 (PS1) presumes little to no risk of POP, a PS of 2 (PS2) presumes moderate risk of POP, and a PS of 3 (PS3) presumes high risk of POP. Blood samples were collected from PS3 sows (n = 56) and parity matched PS1 sows (n = 50). Out of these 106 sows, 29 subsequently experienced POP and 77 did not, all sows that prolapsed were assigned PS3. Serum metabolites were evaluated via gas chromatography mass-spectrometry (GC-MS) with a non-targeted approach. Metabolite comparisons were evaluated between PS3 sows and PS1 sows, sows that subsequently experienced POP versus sows that did not, and PS3 sows that subsequently did or did not experience POP. When comparing PS3 and PS1 sows, 14 metabolites were observed to be significantly different ($P \leq 0.05$), and five metabolites tended to be different ($0.05 < P \leq 0.10$). Of these 19 metabolites, 15 are known and four are unknown. When comparing sows that did or did not subsequently experience POP, six metabolites were significantly different ($P \leq 0.05$), and seven metabolites tended to be different ($0.05 < P \leq 0.10$). Of these 13 metabolites, 10 are known and three are unknown. Differences in 2-hydroxybutyric acid, erythritol, lysine, ornithine, phenylalanine, proline, tyrosine, and valine were observed ($P \leq 0.10$) in both the PS and POP comparisons. When evaluating the PS3 assigned sows that did or did not experience POP, three metabolites were significantly different ($P \leq 0.05$) and two metabolites tended to be different ($0.05 < P \leq 0.10$). Of these metabolites, four are known and one is unknown. The comparison of POP outcome demonstrated differences in 5-Oxoproline. 9-Octadecenoic acid was observed to be different in the PS3 POP outcome comparison and different based on PS. No metabolites were observed to be different across all three comparisons. Collectively, these data indicate sows at high risk for POP have differences in serum metabolites compared to sows at low risk, and differences exist between sows that did or did not subsequently experience POP. Focusing on metabolites that differ between high and low risk sows as well as between sows that did or did not experience POP can lead to a better understanding of what contributes to POP risk. This project was supported by the National Pork Board and the Foundation for Food and Agriculture Research.

P-406 - Maternal Adaptation to Nutrient Restriction in Sheep Herds from Chilean Patagonia: A Random Response or a Repeatable Trait?

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Maternal nutrient restriction (NR) occurs in Patagonian sheep herds due to low quality of pastures, leading to small for gestational age (SGA) offspring. However, there are NR ewes producing lambs whose birth-weight is unaffected (Non-SGA), likely due to metabolic and placental adaptations. It is unknown whether these adaptations are repeatable across gestational seasons (GS), and if so, which traits are associated. Our objective was to determine the repeatability of the maternal programming leading to SGA or Non-SGA offspring, and evaluate associated traits. Experiments were ethically approved (CICUA 01/22), and conducted during two consecutive GS (GS1 and GS2). During GS1, 171 single-bearing ewes of similar weight and age were assigned to NR (n=136) or Control (CN, n=35) groups. Both groups grazed on natural pasture, representative of Patagonia, throughout the pregnancy (Crude Protein (CP) 6.1%, Metabolizable Energy 1.6 Mcal/Kg, stocking rate 0.9 ewes/hectare; dry matter 525 kg/hectare). The CN group additionally received concentrate supplementation (Suralim®, CP 22%, EM 2.5 Mcal/Kg) to meet their CP requirements according to NRC, from gestational day (GD) 70 to term. Offspring birth weight (BW, Kg) was registered and animals born to NR ewes were segregated into quartiles based on BW. Lambs within the upper and lower quartiles formed the Non-SGA (n=34, 5.08±0.08 kg) and SGA (n=34, 3.61±0.08 kg) groups respectively. Lambs in the CN group (n=35, 4.72±0.07 kg) were not separated into quartiles. Mothers of Non-SGA, SGA, and CN lambs formed the mNon-SGA (n=34), mSGA (n=34), and mCN (n=35) groups respectively, and were evaluated during GS2. Within those groups, single-pregnant ewes were selected, and received the same nutritional treatment described for GS1. Maternal weights (MW, Kg) were registered on GD 70, 90, 110, 125, and 140. Plasma glucose and β -hydroxybutyrate (BHB) were analyzed on GD 70, 90, and 140. Lamb BW, placental weight, and placentome weight and number were registered at delivery. NR ewes were segregated into mNon-SGA and mSGA groups based on lamb BW as described for GS1. Data was analyzed using repeated measures analysis or ANOVA. For GS2, offspring BW differed among Non-SGA, SGA, and CN groups (5.31±0.10a, 3.79±0.10b, 4.95±0.09c; $P < 0.0001$). In GS2, a 66.7% of mNon-SGA ewes and 80% of mSGA ewes were also identified as such in GS1. These ewes that repeated the mNon-SGA (n=10) or mSGA (n=12) condition, plus the mCN group (n=18 for GS2) were considered for statistical analysis. There were no differences ($P > 0.05$) in MW at GD 70 and 90, while mSGA group showed lower ($P < 0.05$) MW than mNon-SGA and mCN at GD 110 and 125, and all groups differed at GD 140 (66.10±1.70a mCN; 59.20±1.99b mNon-SGA; 50.70±2.20c mSGA, $P < 0.005$). Glucose and BHB differed only at GD 140 ($P < 0.05$). Glucose (mmol/L) was lower in mNonSGA (2.03±0.15a) than mCN (2.56±0.11b) and mSGA (2.77±0.14b), while BHB (mmol/L) was reduced ($P < 0.05$) in mSGA (0.33±0.08a) compared to mNon-SGA (0.69±0.08b), and mCN was intermediate (0.56±0.06ab). Placental weight (g) was reduced ($P < 0.05$) in mSGA (375.69±27.84a) compared to mCN

(507.62±35.49b) while mNon-SGA was intermediate (472.00±35.50ab). Placentome weight (g) was reduced ($P < 0.05$) in mSGA (105.38±10.01a) compared to mCN (159.50±12.77b) and mNon-SGA (161.50±12.80b). There were no differences in placentome number ($P > 0.05$). These results indicate that the maternal adaptation to NR is repeatable across GS, and it is mediated by a differential whole-body metabolic response and placental adaptations. FONDECYT 11220188.

P-407 - Identification of ISG15 Conjugated Proteins in Murine Reproductive Tissues During Early Pregnancy

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Interferon stimulated gene 15 (ISG15) is an evolutionarily conserved ubiquitin homolog. It becomes up-regulated in the uterus of most mammalian species in response to the implanting embryo despite significant species differences in the processes of implantation and placentation. Based on studies conducted primarily in virally-infected cells, ISG15 functions intracellularly by becoming covalently tethered to targeted conjugate proteins to modulate their stability and/or activity. ISG15 is also secreted through an unknown mechanism where it functions as a cytokine by binding and activating LFA-1 integrin to promote natural killer cell and T-cell emigration and interferon-gamma secretion. We previously demonstrated that female mice carrying the *Isg15* null mutation delivered 50% fewer pups and had abnormally low numbers of uterine natural killer cells on d7-d10 of pregnancy. Tight regulation of ISGylation through specific ubiquitin-like enzymes was also shown to be fundamentally important for normal pregnancy in that female mice deficient in the ISG15 deconjugating enzyme USP18 also had compromised pregnancies with a dramatic increase in mortality at the embryo-fetal transition. Despite these efforts to establish a fundamental role for ISGylation during early pregnancy, and despite its conservation across diverse mammalian species, the functions of this enigmatic pathway remain largely unknown in female reproductive tissues. The objectives of this study were to: 1) fully evaluate the expression of the ISG15 pathway during early murine pregnancy and human endometrial stromal cell (HESC) decidualization; and 2) begin establishing a mechanism of action through the identification of proteins that become covalently linked to ISG15 and track their fate in murine reproductive tissues. Uterine expression of ISG15, its conjugation to target proteins, and the entire ISG15 conjugation/deconjugation enzymatic pathway increased on the mesometrial side of the implantation site starting on d7 of pregnancy, with a sustained increase through d11. In parallel, ISG15 and its conjugates were elevated in the ovary on d9 of pregnancy compared to the diestrus ovary. Human ISG15 and its conjugating/deconjugating enzymes were similarly up-regulated at the mRNA level in HESCs induced to undergo decidualization in vitro. Magnetic beads coated with anti-ISG15 antibody were used to immunoprecipitate ISG15 conjugates from murine mesometrial decidual tissue lysates on d9 of pregnancy, with non-pregnant (diestrus) uterine tissue used as a negative control. Isolated ISG15 conjugates were then identified using tandem mass tag-based proteomic profiling. Peptide sequences were identified from a total of 602 proteins with high z-scores based on established

adjusted p-values (≤ 0.05). Chief among the most highly enriched decidual ISG15 conjugated proteins included those associated with interferon signaling (e.g., STAT1, STAT3, JAK1, IFIT1, IFIT4, OAS3), immune cytokine signaling, signal transduction (e.g., PKA and CAMK2 pathways), transcriptional regulation (e.g., WNT pathway), steroidogenesis, the ubiquitin system, and cell stress. Reciprocal immunoprecipitation studies coupled with the In Situ Proximity Ligation Assay are being used to validate ISG15 conjugates in anticipation of future experiments that will evaluate the functional consequences of ISGylation in the uterus and corpus luteum of early pregnancy. This study was supported in part by the Curtis and Marian Rochelle Endowment in Animal Science.

P-408 - Cervical ISG15 and PGR mRNA expression during early pregnancy in Holstein heifers

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We aimed to determine the effect of pregnancy on gene expression of Interferon-stimulated gene 15 (ISG15) and progesterone receptor (PGR) in the cervix of Holstein heifers. Sixteen heifers were synchronized with two injections of prostaglandin (Estrumate®, MSD, Argentina) separated by eleven days. Animals were sham-inseminated with semen extender only (open, n=6), or inseminated with frozen semen from one bull and were diagnosed pregnant by ultrasound at day thirty post insemination (pregnant, n=10). Blood by coccygeal venipuncture and cervical cells samples using a cytological brush were obtained at 14, 16, and 18 after insemination. Serum progesterone (P4) was determined by a solid-phase radioimmunoassay using a commercial kit. Total RNA was obtained with Trizol reagent (Life Technologies) and the concentration and purity of the RNA was determined using a spectrophotometer (nanodrop ND 1000). Total RNA was treated with DNase using a DNA-free™ kit (Ambion, Austin, TX, USA). For each sample, copy DNA (cDNA) was synthesized by reverse transcription using a SuperScript III transcriptase (Invitrogen) with random primers and total RNA as a template. ISG15 and PGR gene expression was determined by real-time PCR (qPCR) using the Rotor-Gene™ 6000 kit (Corbett Life Sciences, Sydney, Australia). Gene expression was calculated by relative quantification to the exogenous control (β -actin) and normalized to the geometric mean of the endogenous control, taking into account the respective amplification efficiencies. Serum progesterone concentrations and the transcripts determined by real-time PCR were analyzed by a Glimmix procedure (Proc Glimmix; SAS Studio®) including group, day and their interaction as a fixed effect. Significance was considered with $\alpha \leq 0.05$, and tendency between 0.05 and 0.10. Serum progesterone concentrations were higher in pregnant than open heifers (6.5 ± 0.48 vs 4.2 ± 0.57 ng/mL, $P=0.0077$). There was no effect of day or the interaction between group and day on progesterone concentrations. The cervical expression of ISG15 mRNA was greater in pregnant than open heifers (1.76 ± 0.46 vs 0.13 ± 0.04 , respectively, $P < 0.0001$), being different in all days (Fold-Change: 12.0, 21.5, and 9.4, on days 14, 16, and 18, respectively). There was an interaction between group and day ($P=0.033$), as pregnant heifers presented greater ISG15 mRNA expression on day 16 than on day 14 and 18 ($P < 0.05$), but no differences according to the day were found in open heifers. The PGR mRNA expression tended to be affected by the

interaction between group and day ($P=0.0779$), while open heifers increased during the estrous cycle, pregnant heifers maintained PGR expression. We concluded that the cervical expression of ISG15 mRNA could be used as a pregnancy biomarker as early as day 14 post insemination.

P-409 - Differential Regulation of In Vitro Decidualization by Circulating Exosomes Isolated from Recurrent Pregnant Loss (RPL) Women and Normal Pregnant Women

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Proper implantation is a major determinant of successful pregnancy which requires an extensive cross-talk between the trophoblast cells of the blastocyst and the endometrial cells of the receptive uterus. Recently, shedding of exosomes and microvesicles has been identified as an important tool for intercellular communication among several different cell types. However, their importance in mediating embryo-maternal interactions during pregnancy is just beginning to be recognized. Decidualization is one of the important processes required for implantation and further sustenance of pregnancy. Hence, it can be hypothesized that exosomes mediating the maternal-fetal cross-talk influence the decidualization process also. Previous literature suggests that dysregulation of the decidualization process can lead to recurrent pregnancy loss (RPL). Therefore, the aim of this study was to investigate whether circulating exosomes isolated from pregnant women with RPL history modulate decidualization differently than those isolated from normal pregnant women. Blood samples were collected from pregnant women with RPL history ($n=25$), normal pregnant women (gestational age 8-12 weeks; $n=20$) and healthy non-pregnant women with regular menstrual cycle ($n=20$). Exosomes were isolated and their size distribution was determined using Nanoparticle Tracking Analysis. The isolated exosomes were characterized by western blotting. Decidualization was induced in human endometrial stromal cell line, THESC cells, by addition of medroxy progesterone acetate (MPA), cAMP and estrogen (EPA mix). THESC cells were incubated with exosomes isolated from different sources. The effect of exosomes on decidualization was evaluated by observing the morphology and measuring the decidual markers such as prolactin and IGFBP-1. Exosomes isolated from the serum samples had size in the range of 50 – 150 nm and concentration ranging from $0.5 - 4.0 \times 10^{11}/\text{ml}$. Presence of exosomes was confirmed by western blotting with CD9, CD81 and TSG101 antibody. THESCs treated with EPA mix showed increased levels of the decidualization marker prolactin in a time-dependent manner. THESCs incubated with exosomes isolated from RPL women showed reduced decidualization compared to those incubated with exosomes from normal pregnant women as indicated by lower levels of prolactin and IGFBP-1. Morphological parameters such as area, roundness of cells and f-actin structure were also affected differently by exosomes from the two groups. In conclusion, our data showed that exosomes isolated from normal pregnant and RPL pregnant women had different effect on regulating the decidualization process.

P-411 - The Zinc-finger Domains of Wilms' Tumor 1 Protein are Critical for Uterine Receptivity During Pregnancy

Wilms' Tumor 1 (WT1) is a zinc-finger transcription factor required for cell survival, growth and differentiation. We and others have shown that WT1 is abundantly expressed in the stromal cells of the secretory endometrium and the decidua in the first and second trimesters in humans. However, the functional roles of WT1 in the uterus during pregnancy is unknown. Mice that carry the *Wt1f/f* allele flanking exons 8 and 9 of the *Wt1* homolog gene were bred to *Pgrcre/+* mice to generate *Pgrcre/+Wt1f/f* (*Wt1D/D*) mice. The *Wt1D/D* mice produced a shortened WT1 protein lacking the second and third of the four DNA-binding zinc-finger domains, validated via genotyping and western blot analyses. After 6-month breeding trial, *Wt1D/D* female mice were infertile ($P < 0.001$) as compared to the *Wt1f/f* control mice (6.0 ± 0.4 pups per litter). Also, haploinsufficiency was spotted in which significant reductions ($P < 0.01$) in pups born per litter (3.4 ± 0.5 versus 6.0 ± 0.4), as well as survival pups per litter at weaning (1.0 ± 0.5 versus 5.6 ± 0.4) were observed in *Wt1+/D* mothers as compared to the *Wt1f/f* controls. The rates of stillbirth and second-day neonatal death were also high ($P < 0.05$) in *Wt1+/D* females. Among the stillborn pups from *Wt1+/D* females were the underdeveloped fetuses with congenital limb defects. Ovarian functions were not affected by *Wt1* mutation in which no differences in the number of blastocysts at gestational day (GD) 3.5 and serum P4 levels at GD 4.5 were observed between *Wt1D/D* females and the *Wt1f/f* controls. Blue dye injection analyses of implantation sites showed that uteri of *Wt1D/D* females exhibited no implantation responses at GD 4.5 and 5.5. Histological analyses further revealed that blastocysts can only attach to the luminal epithelium but failed to invade it and initiate decidualization at GD 4.5. However, at GD 6.5 and 9.5, smaller decidual bulb with reabsorbing blastocysts was observed in *Wt1D/D* uteri as compared to the control, suggesting delayed and defective implantation, decidualization and placentation. *Wt1+/D* females also exhibited no implantation responses at GD 4.5 and 5.5 and embryos were found attached to the luminal epithelium whereas at GD 6.5 and 9.5 decidual bulb were observed on *Wt1+/D* mice as compared to *Wt1f/+* control mice. Immunohistochemical analyses further showed that ESR1 decreased in glandular epithelium (GE) but increased in the stroma of *Wt1D/D* uteri at GD 3.5 as compared to the *Wt1f/f* control. PGR was downregulated in the stromal cells of *Wt1D/D* uteri between pre-decidual (GD 3.5) and post-decidual stage (GD 9.5). In addition, PTGS2 was failed to express in stromal cells of implantation sites at GD 4.5 *Wt1D/D* uteri as compared to the *Wt1f/f* control. At decidual day -1 (DD-1; one day before artificial decidualization to mimic GD 3.5), *Areg* mRNA was decreased ($P < 0.01$) but *Wnt4*, *Ltf*, and *Bmp2* were increased in *Wt1D/D* uteri as compared to *Wt1f/f*. Moreover, the ability of the endometrial stromal cells to undergo an artificially induced decidualization was severely compromised ($P < 0.01$) in *Wt1D/D* uteri, with failure of induction in *PrL*, *Igfbp1*, *Pgr* and *Areg* at DD2 and DD5. These results suggest that *Wt1*, via its second and third DNA-binding zinc-finger domains, plays critical roles in implantation and decidualization, thereby governing placentation and fetal development during pregnancy.

P-413 - Targeting Matrix Metalloproteinase 9 to Modulate Uterine Contraction

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Matrix metalloproteinase 9 (MMP9) expression is elevated in maternal tissues and fluids during both term and preterm labor. We performed experiments to test the hypothesis that MMP9 contributes

to labor by enhancing uterine contractions and that targeting this enzyme could serve as a mechanism to relax the uterus. Ex-vivo experiments were performed with human uterine tissues donated by women undergoing Cesarean-sections at term. Uterine tissue was immediately transported to the laboratory in physiological buffer and myometrial samples dissected into 10x1x1 mm strips which were suspended in tissue organ baths filled with Krebs solution at 95% O₂, 5% CO₂ at 37 °C. One end of each strip was connected to a force transducer and changes in isometric tension were recorded. Tissues were maintained under 2-3 g basal tension for at least 30 min and then primed with 60 mM KCl for 3 min. Baseline spontaneous or oxytocin-induced (0.6 IU/mL) contractions were recorded for 30 min and served as the baseline for comparison of treatments with recombinant human MMP9 or the specific inhibitor AG-L-66085. Statistical analyses were performed using Prism version 10.0.1. Addition of MMP9 to physiologically relevant levels (10 nM) enhanced the oxytocin-mediated contractile response over time (n=6 patients, p=0.0003), primarily by increasing contraction frequency compared to vehicle (PBS) (p< 0.0001). Specific inhibition of MMP9 with 1 micromolar AG-L-66085 reduced the oxytocin-mediated contractile response over time (n=8 patients, p=0.0075) by dampening both contraction amplitude (p< 0.0001) and frequency (p=0.0498) compared to vehicle (DMSO). Effects of AG-L-66085 on spontaneous contractions (in the absence of peptide agonist) were highly variable, with contractions completely abolished in two patient samples. AG-L-66085 had an inhibitory effect in six additional uterine samples, reducing the contractile response over time (p=0.0415) and contraction amplitude (p=0.038) compared to vehicle (DMSO). These data suggest MMP9 contributes to the physiology of labor and could serve as a molecular target to regulate this process.

P-414 - Direct Proteomic Profiling VEGF Responsive Sulfhydromes in Human Uterine Artery Endothelial Cells

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Angiogenesis is a key mechanism that causes uterine blood flow to rise that supplies nutrients and carries out maternal-fetal exchanges of respiratory gases to support fetal growth and survival. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that stimulates human uterine artery (UA) endothelial cell (hUAEC) hydrogen sulfide (H₂S) production; yet, how VEGF stimulates uterine angiogenesis is unknown. Formations of persulfides (-SSH) from free thiols (-SH) on cysteines in proteins, referred to as S-sulfhydration, have been recently identified as the main mechanism that H₂S exerts its biological function. This study using our recently developed low-pH quantitative thiol reactivity profiling (LP-QTRP) proteomics platform hypothesized that VEGF stimulates hUAEC sulfhydrated proteins (SSH-Ps) that are involved in angiogenesis regulation. Primary hUAEC were treated with or without VEGF (10 ng/ml) for 30 min. Total hUAEC proteins were labeled by tag-switch using CN-biotin and biotinylated SSH-Ps were then quantified by immunoblotting. Total SSH-Ps in the hUAEC proteome, i.e., sulfhydrome, were labeled and trypsinized; SSH-peptides (SSH-PPs) were enriched from the tryptic peptides and then sequenced by LP-QTRP proteomics platform that can identify and quantify SSH-PPs aligned on respective SSH-

Ps using LC-MS/MS. Bioinformatics tools were used to determine the biological functions of VEGF-responsive SSH-Ps and their interactomes. Levels of total SSH-Ps were significantly greater in VEGF-treated vs. untreated hUAEC. In the untreated and VEGF-treated hUAEC sulfhydromes, there were hundreds of differentially regulated sulfhydrated proteins (DRSPs) characterized by SSH-PPs that map reactive cysteine(s) in each SSH-P. Bioinformatics analyses revealed that the VEGF-responsive DRSPs are involved in regulating many key biological processes and interactomes including extracellular matrix constitution (thrombospondin, integrin and pregnancy-specific glycoprotein), cytoskeleton remodeling (vimentin), antioxidation (peroxiredoxin) and cell-cell interaction (endosialin), important for regulating uterine angiogenesis. Altogether, we concluded that VEGF stimulates protein sulfhydration in hUAEC; the unique VEGF-responsive DRSPs demonstrate that VEGF differentially regulates sulfhydration of specific proteins involved in angiogenesis regulation, informing novel roles of protein sulfhydration in uterine angiogenesis regulation by augmented endogenous H₂S production. [AHA Postdoc Fellowship 903757 (to JB) and RO1 HL70562 and RO1 HD105699 (to DBC)].

P-415 - SPACE SPERM – The Importance of Gravity in Sperm Navigation, Fertilisation and Early Embryo Eormation.

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Space and space exploration attracts curiosity, excitement, and innovation nationally and internationally, with growing interest in Mars settlement, deep space exploration and Moon mining. However, effective long-term human settlement of celestial bodies and exploration of deep space poses many challenges, including but not limited to the requirement for mammalian reproduction in micro-gravity environments. It is crucial to understand the impact of low gravity on the earliest stages of space-born life, including the viability of conception and healthy fetal development in mammals, processes of reproductive success which are normally dependant on Earth's gravity. Utilising a gamete and embryo culture suitable dual-axis 3D clinostat to simulate microgravity we aimed to determine if exposure of mammalian sperm and embryos to simulated micro-gravity altered functional and developmental capacities. In this study non-capacitated mouse (N&#3f10) and human (N&#3f6) sperm were challenged to swim through an Ibidi 6-channel μ -slide for 1-6 h at either normal gravity (NG) or stimulated micro-gravity (OG), in conditions that mimic the female reproductive tract (media – G-IVF PLUS, 37oC, 6% CO₂, 5% O₂). Sperm recovery and motility kinetics were assessed by computer-assisted semen analyser (CASA). Additionally, non-capacitated mouse sperm (N&#3f7) underwent in vitro fertilisation (IVF) for either a 4 or 24 h fertilisation window at NG or OG and zygotes subsequently cultured in GT-L at NG for 5-days. 2-cell cleavage rates were assessed at 24 h and on-time blastocyst development and cell

numbers/allocation (Oct4 – inner cell mass and Nanog – epiblast fetal cells) at 96 h post-insemination. A subset of embryos underwent time-lapse culture to assess timing of PN fading, PN to 2-cell cleavage, and PN to blastocyst development. Additionally, porcine sperm (N&#3f3) underwent in vitro fertilisation for a 6 h fertilisation window at NG or 0G, and the zygotes subsequently cultured in NCSU23-plg and NCSU-G at NG for 7-days at 38.5°C, 6% CO₂, 5% O₂. Porcine 2-cell cleavage rates were assessed at 96 h and on-time blastocyst development at 144 h post-insemination. Exposure of both human and mouse sperm to 0G reduced its ability to navigate a channel slide, with a 40% reduction in sperm recovery after 1 hour (both $P < 0.05$) and a reduction in both amplitude lateral head movement and curve linear velocities in the mouse (-33%, $P < 0.05$), although total motility was unchanged in both species. Fertilisation rates in the mouse were reduced by 33% following 4 h fertilisation at 0G ($P=0.003$), although unchanged during a 24 h fertilisation window. On-time blastocyst rates were reduced by 22% (all $P < 0.05$) following any fertilisation period at 0G. Interestingly, in those embryos that did make it to blastocyst, exposure to 0G increased epiblast fetal cells (+30%, $P < 0.05$), although, blastocyst total cell numbers were unaffected. Similar effects were observed in the pig, where there was a 20% reduction in fertilisation rate ($P=0.067$) and a 23% reduction in on-time blastocyst rate ($P=0.046$). This study indicates that microgravity can negatively influence sperm's ability to navigate and fertilise an egg. Even after returning to normal gravity, embryos exposed to microgravity during fertilisation have reduced on-time blastocyst rates, and an increased fetal cell cellular allocation. The ability of mammals to reproduce in micro-gravity environments currently seems limited.

P-416 - Transcriptional Regulation of Ribosomal RNA by a Germ Granule Component, Meioc, for Differentiation of Germline

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Recent studies in both embryonic and adult stem cells demonstrated that stem cells require low translation rates to maintain the undifferentiated status. However, mechanisms used by stem cells to control translation are still unclear. Here we describe a novel ENU-generated mutant zebrafish in which germline stem cells (GSCs) fail to differentiate, and novel properties of the responsible gene *meioc* as a germ granule component that controls upregulation of rRNA transcripts. Upregulation of 5.8S, 18S, 28S rRNAs was observed in 1-4-cell cyst spermatogonia in wild-type zebrafish, while the mutant maintained spermatogonia with low levels of those rRNAs. The encoded Meioc protein is observed in cytoplasmic granules from a portion of 1-2-cell cyst germ cells, and interacts with Piwil1 (Ziwi). Depletion of Meioc resulted in accumulation of Piwil1 in nucleoli. The nucleolar localization of Piwil1 was diminished by inhibition of RNA polymerase I activity, and Piwil1 associated with unprocessed pre-rRNA. Although the *piwil1*^{-/-} mutants have a germ cell-depleted testis, we found that heterozygous *piwil1*^{+/-}-spermatogonia exhibited reduction of Piwil1. The *piwil1*^{+/-} spermatogonia upregulated rRNA transcripts, and the *piwil1*^{+/-};*meioc*^{-/-} spermatogonia then recovered differentiation later than those in *meioc*^{-/-}. Furthermore, Piwil1 interacted with HP1a in nucleoli, and *meioc*^{-/-} spermatogonia exhibited high levels of H3K9me3 and methylated CpG in the 45S-rDNA region. These results indicate that zebrafish GSCs maintain low rRNA transcriptional activity with high H3K9me3 and methylated CpG, and that Meioc has a function of

upregulating rRNA transcripts and promoting differentiation by preventing localization of Piwil1 and HP1a in nucleoli. All animal experiments were approved by the Animal Care and Ethics Committees of the National Institute of Genetics and carried out according to the guidelines.

P-417 - Exploring the Impact of Hypoxia on Gonadal Steroidogenic Cells: New Insights from Proteomics and Spheroid Development in Leydig Cells

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The modulatory role of reduced oxygen (O₂) tension (hypoxia) is an essential part of physiological steroidogenic processes. Hypoxia inducible factor (HIF) 1a, a master regulator in biological responses to hypoxia, has been implicated as a direct transcriptional regulator of Steroidogenic Acute Regulatory (STAR)-mediated steroidogenesis. Studies have shown that the degree of hypoxia, duration of exposure to reduced O₂ tension, as well as the tissue/cell type and the stability of HIF1a, impact steroidogenesis to varying degrees. Here, we aimed to identify the largescale changes that occur in the proteome of Leydig cells mediated by cAMP, HIF1a and O₂ levels using a quantitative LC-MS/MS approach. Two Leydig cell lines (MLTC1 and MA10) were used, treated with (db)cAMP or without (control) for 6h in serum-free medium under different O₂ tensions (20%, 10% and 1%). Echinomycin, a functional blocker of HIF1a was applied to evaluate the HIF1a effects. In contrast to previous reports utilizing steroidogenic models, the cells were subjected to continuous hypoxia within a controlled hypoxia workstation, ensuring no oxygen bursts occurred during manipulation of cells. The stabilization of cells in hypoxia was initiated 24 hours before treatments. Various contrasts were set to assess the impact of cAMP, the influence of O₂ on cAMP-mediated responses, and the effects of HIF1a. Differentially expressed proteins (DEPs) were used for downstream functional enrichment analysis ($P < 0.05$, FDR < 0.01). While, compared with their controls, cAMP at both 20% and 10% O₂ upregulated proteins involved in cholesterol metabolism, the cAMP treated cells at 1% O₂ downregulated proteins involved in mitochondrion organization, mitochondrial transport and NADH dehydrogenase complex assembly, biological processes essential for steroidogenesis. The contrast 10% vs 20% O₂ after cAMP treatment showed DEPs involved in cytokine secretion and transmembrane transporter activity, indicating highly responsive oxygen sensors. On the other hand, the contrast 1% vs 20% O₂ after cAMP treatment resulted in 166 DEPs, upregulating cytokine secretion-related factors, and downregulating, i.a., Hippo signaling-related factors and sialylation, as well as modulating activity of kinases. Interestingly, echinomycin decreased essential proteins involved in ribonucleoprotein complex biogenesis, indicating a possible role of HIF1a in global protein synthesis within steroidogenic cells. In a parallel study, the functional involvement of HIF1a in STAR-mediated steroidogenesis was confirmed by applying water-soluble (22R)-22-hydroxycholesterol to echinomycin-treated cells. This rescued progesterone production despite suppressed STAR expression. To optimize the verification of the newly discovered target molecules from our proteomics data, we designed a new 3D culture model for steroidogenic cells. The MA10 Leydig cells were plated in non-adherent 6 well plates for 48h, and resulting spheroids were assessed for viability, morphology and steroidogenic capacity. Histology revealed closely packed spheroids with high cell-density. The PCNA staining confirmed

proliferation without signs of central necrosis, accompanied by low cleaved-caspase-3 expression, altogether indicating low apoptotic index. The spheroids responded to cAMP with increased STAR gene and protein expression and showed increased progesterone levels after treatment. In conclusion, our preliminary data offer new insights into hypoxia and HIF1a-dependent regulation of steroidogenesis, indicating functional targets of translational value. The successful production of steroidogenic spheroids from the MA10 cell line could provide an alternative 3D in-vitro model for steroidogenic studies, one that might be more relevant for in vivo comparisons, and that will ultimately help broaden our understanding of the hypoxia-mediated effects in steroidogenic cells. Supported by SNSF project nr. 310030_207895

P-418 - Detection Of Perivitelline Membrane Bound Sperm gDNA Profiles Using Quail-Specific Microsatellites

Kendra Roberts; Thomas Jensen

Microsatellites are small tandem repeat DNA sequences interspersed throughout an organism's genome. The number of microsatellite repeats can differ, allowing for genetic identification of individuals. This technique has been used for human identification in crime investigations and paternity determination. Likewise, microsatellite analysis can also be utilized for animal identification. For example, dog and horse parentage confirmation is critical to breed management, captive breeding facilities can use microsatellites to establish pedigrees for optimal genetic diversity pairings, and wildlife studies identification of individuals can help estimate emigration and immigration, and in zoos, microsatellites have been utilized to determine paternity in animals managed in colonies. Microsatellites have previously been used in studies to determine paternity, estimate extrapair copulations (EPCs), and cryptic mate choice/sperm storage gland function in birds. However, these studies are based on the paternity of the offspring. However, if an EPC event does not result in offspring, the EPC is not "counted," and the actual rate of EPC events (as opposed to extrapair paternity) estimate is skewed. We previously presented a method to isolate gDNA from the perivitelline membrane to confirm mating, in addition to fluorescent microscopy identification of sperm heads embedded in the PVM). However, no one, to our knowledge, has explored the possibility of using PVM-bound sperm to genetically identify sperm donors. Such information would be invaluable to basic avian reproductive physiology through more accurate assessments of female cryptic choice, sperm-storage gland sperm selection, or EPC events. We identified four quail-specific microsatellite primers exhibiting allele diversity in our quail research population, allowing us to differentiate between two male and two female quail from our research colony. One male was allowed to mate with the females, and eggs were collected for PVM removal and gDNA isolation, followed by microsatellite PCR amplification. We successfully isolated gDNA (5-10 ng/mL, 250-500 ng) at a high enough concentration to attain microsatellite profiles for each egg. All the eggs exhibited microsatellite amplification, allowing us to build a DNA profile for each egg. However, due to too many common alleles, only one microsatellite was able to confirm male-only gDNA by excluding both females in a third of the eggs and excluding the non-breeding male in all the eggs. This project could significantly impact basic avian reproductive research and captive management, allowing zoo and conservation managers to estimate EPC rates within a species or population. This is important because studbooks are based on actual pairings, which, depending on the EPC, may not accurately reflect true paternity. Knowing the estimated EPC will allow facilities to decide if

chick paternity testing costs are warranted for studbook accuracy. We will use this method to investigate cryptic female choice, including sperm-storage gland sperm selection.

P-419 - Epigenetic Effects of Adverse Lifestyle Choices in Males

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Obesity is an epidemic in the US, with 1 in 3 adults and 1 in 5 children being obese. Additionally, obesity is a major risk factor for metabolic disorders. Hispanic communities in particular have a high prevalence of metabolic disorders and obesity associated with low socioeconomic status and low health literacy. While genomic impacts of offspring health are well-established, there is now increasing evidence that epigenetic determinants, such as genomic patterns of DNA methylation or specific histone modifications, also impact offspring health. Research into paternal origins of health and disease has identified epigenetic changes in sperm that propagate effects of a father's lifestyle and environmental exposures to his offspring, potentially predisposing adverse intergenerational health outcomes. However, the mechanisms responsible for the transmission of information from a male's soma to his germ line, and then from his germ line to his offspring, remain unknown. The primary goal of our study is to understand how obesity and lack of exercise impact epigenetic programming and gene expression profiles in both somatic cells and germ cells in young Hispanic men. Nucleic acids were extracted from peripheral blood mononuclear cells (PBMCs) and sperm from obese, inactive (OI; n=46), and lean, active (LA; n=15) Hispanic men aged 18 to 40 years. Genomic DNA was bisulfite converted and processed for detection of DNA methylation at individual CpG sites using the Infinium MethylationEPIC v2.0 BeadChip. The resulting raw data is being analyzed using the R package, Sesame, to identify genome-wide differences in DNA methylation patterns between samples from OI and LA groups. Results will be presented describing the DNA methylation status at individual CpGs in both somatic and germ cells from each group. In addition, gene expression is being assessed in the PBMCs by RNA-seq and analyzed using the DESeq2 analysis package. Using Sesame's built-in regulatory feature enrichment analysis, KnowYourCG, we will determine the association of differentially methylated DNA sites with PBMC gene expression. We will also determine the association of differentially methylated DNA sites in both sperm and PBMCs in OI group with annotated genomic regions including enhancers, promoters, and transcription factor binding sites. Finally, we will use Gene Set Enrichment Analysis (GSEA) to identify gene pathways that show significant up- or down-regulation of gene expression data in PBMCs from the OI men compared with LA men.

P-420 - Systems Investigation Reveals an Unexpected LH-mediated Signal Transducer of Steroidogenesis in Leydig Cells

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Trophic hormone regulation of gonadal and adrenal steroidogenesis elicits acute responses, initiating the production and release of steroid hormones within minutes. The discovery of the steroidogenic acute regulatory protein (STAR) as an essential mediator of this process has focused subsequent studies on signaling and transcriptional responses around the established understanding of STAR. However, recent findings from our laboratory demonstrate that STAR is not a protein kinase A (PKA)-regulated phosphoprotein, and that it functions alone within the mitochondrial intermembrane space as a cholesterol shuttle, challenging the basis of several key interpretations made in this field. Consequently, we decided to reconstruct *de novo* the cellular impacts of luteinizing hormone (LH) exposure using precision nuclear run-on sequencing (PRO-seq) to uncover the identity of transcriptional regulators and predict upstream signaling mediators that form the basis of a steroidogenic response. We performed PRO-seq using MA-10Slip5 cells at baseline and after a 15-minute LH exposure, a timepoint that results in a 2.3-fold increase in STAR mRNA and a 6.9-fold increase in progesterone production. From this dataset, we generated a high-resolution map capturing all active transcriptional regulatory sites (enhancers and promoters) associated with steroidogenesis across the mouse genome by using the dREG and tfTarget pipelines. This analysis highlighted differences in expressed transcriptional regulatory elements (dTREs), identifying 508 dTREs responsible for LH-mediated effects, corresponding to 336 protein coding genes. We employed a Hidden Markov model within RSAT for *de novo* motif discovery, identifying putative transcription factors associated with these dTREs. The analysis revealed three motif families with a normalized correlation greater than 0.85, responsive to LH: the ETS-domain family (including Erf, Elk, and Gapba), the NF-Y family (Nyfa and Nfyc), and the SP/KLF family (Sp3, Sp6, Sp8, Sp9, Klf2, Klf6, Klf13, Klf14). Intriguingly, despite the frequent implication of CREB in prior signaling studies, it was not identified in our current analysis. By analyzing the target genes identified by dTREs using Ingenuity Pathway Analysis®, we unraveled the primary mechanisms activated by LH. These include activation of ER stress, Rho signaling, MAPK signaling, mTOR signaling, and EGF signaling. Additionally, our findings highlight pathways that support mitochondrial protein import, COPI-mediated anterograde transport, endocytosis, cholesterol biosynthesis, and transcriptional activation of gene expression by Srebp (sterol regulatory element-binding protein). Contrary to the current understanding, we found that cAMP-mediated signaling was downregulated following LH exposure. Given its proximity to LH receptor responses at the plasma membrane interface, we investigated RhoA as a potential signal transducer in LH-induced steroidogenesis. We utilized an inducible, constitutively active form of RhoA (RhoA.Q63L) in MA-10Slip5 cells and observed a full steroidogenic response without LH stimulation, confirming RhoA as a central mediator of LH signaling. Induction of RhoA.Q63L resulted in progesterone production levels that were 4-fold higher than those induced by LH alone. This finding challenges the prevailing model of LH signal transduction, which is thought to occur via LH-GPCR activation of adenylyl cyclase and subsequent protein kinase A (PKA) activity, and suggests that a reevaluation of this model is warranted. In summary, this study provides a detailed analysis of LH signaling impact in Leydig cells. The original mechanisms of transcriptional regulation and signaling uncovered by our

research, while unexpected, are unprecedented and critically important for advancing our understanding of steroidogenesis.

P-421 - Glucose Metabolism as a Driver for Embryonic Sertoli Cell Differentiation

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The gonadal primordium, unlike many other tissues in the body, has the unique ability to differentiate into two completely different organs, testes in the male and ovaries in the female. During gonadal sex differentiation, every cell in the gonad must commit to an ovarian or testicular fate. This process is orchestrated by the supporting cell lineage. The supporting cells are the first ones to differentiate into either Sertoli cells in the testes or pre-granulosa cells in the ovary. Once they differentiate, they control the fate of the rest of the gonadal cells. Sex determination of the supporting cells is therefore critical for proper establishment of sex-specific reproductive organs. In the fetal testis, sex determination of the supporting lineage into Sertoli cells involves the upregulation of thousands of genes over a short period of time, suggesting a genome-wide chromatin remodeling process. One uncharted mechanism for such regulation is cell metabolism, which has gained interests as a novel regulator of cell fate and differentiation. Glucose metabolites play important roles in chromatin modification, linking cellular metabolism to epigenetic control of gene expression. We hypothesized that a change in the cell metabolic state is required to achieve genome-wide modification during sex determination of gonadal supporting cells. We first performed single-cell RNA-sequencing analysis of the supporting cell lineage in the mouse embryos to identify sex specific differences in genes involved in glucose metabolism. Sertoli cells exhibit a higher expression of enzymes associated with glucose metabolism, including glycolysis, the hexosamine biosynthetic pathway and mitochondrial oxidative phosphorylation, than the pre-granulosa cells. We used a combination of specific chemical inhibitors and ex-vivo gonadal cultures to evaluate the role of each pathway in supporting cell differentiation. When gonads were cultured with 2-Deoxy-D-glucose, an inhibitor of the second step of glycolysis, Sertoli cells failed to differentiate. However, perturbation of the last step of glycolysis (Shikonin), and mitochondrial glucose oxidation (UK-5099) had no impacts on Sertoli cell differentiation. This suggests that glucose is used a non-ATP-generating pathway that branches from glycolysis. With this in mind, we examined the hexosamine biosynthetic pathway, a side-branch of glycolysis that results in post-translational modification (O-GlcNAcylation) of histone or non-histone proteins. Notably, Sertoli cells showed higher cytoplasmic and nuclear protein O-GlcNAcylation compared to pre-granulosa cells. The Inhibition of nuclear O-GlcNAcylation (OSMI-1) disrupted Sertoli cell differentiation, mirroring the perturbations observed in the 2-Deoxy-D-glucose inhibition. Moreover, Sertoli cells expressed higher levels of Gfat1, the rate-limiting enzyme of the hexosamine biosynthetic pathway, than the pre-granulosa cells. Based on these results, we propose that male supporting cells shuttle glucose into the hexosamine biosynthetic pathway, which then facilitate nuclear O-GlcNAcylation to induce Sertoli cell differentiation. These findings shed new light on the pivotal role of the hexosamine biosynthetic pathway in the intricate process of gonadal sex differentiation.

P-423 - TEX15 Regulates Spermiogenesis Through Interaction with TASOR2

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In mouse spermiogenesis, round spermatids undergo extensive morphological changes to transform into elongated spermatids with a sickle head and a tail. Both head and tail are essential for fertilization. Manchette, a transient microtubular platform, appears in step 8 round spermatids and disappears in step 13-14 elongating spermatids, plays an important role in sperm head morphogenesis and tail formation. Meanwhile, canonical histones are replaced by transition proteins and then by protamines which compact nucleus by six-fold. Only 1% of histones are retained in mouse epididymal sperm. Many chromatin factors contribute to this differentiation process. We previously reported that TEX15, a testis-specific protein, is essential for retrotransposon silencing and meiotic progression in male germ cells. Here we report that the DUF3715 domain in TEX15 is required for retrotransposon silencing. Importantly, we found that TEX15 plays a novel role in spermiogenesis. Postnatal inactivation of TEX15 leads to abnormal mushroom head morphology in sperm and male subfertility but not de-repression of retrotransposons. TEX15 is associated with TASOR2 and MPP8 but not TASOR in postnatal testis. TASOR2, sharing the DUF3715 domain with TASOR and TEX15, gradually forms large aggregates in the nuclei from step 2 to step 8 round spermatids right before spermatid nuclear condensation. Both TEX15 and MPP8 localize to TASOR2 foci in the nuclei of round spermatids. While the formation of TASOR2 and MPP8 foci is independent of TEX15, the formation of TEX15 and MPP8 foci is dependent on TASOR2. TASOR2 knockout sperm also displayed abnormal head shape, resulting in subfertility. However, manchette and tail are formed normally and histones are not significantly retained in sperm from either mutant. In the future, we plan to carry out RNA-seq and ChIP-seq analyses of mutant round spermatids and characterize new TEX15-interacting proteins to elucidate the function of TEX15 and TASOR2 in spermiogenesis. In summary, TEX15 plays an important role in spermiogenesis through interaction with a distinct HUSH2 complex (TASOR2-MPP8-PPHLN1).

P-424 - Glutamine Protects Mouse Spermatogonial Stem Cells Against NOX1-Derived ROS for Sustaining Self-Renewal Division

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Reactive oxygen species (ROS) are generated from NADPH oxidases and mitochondria; they are generally harmful for stem cells. Spermatogonial stem cells (SSCs) are unique among tissue-stem cells because they undergo ROS-dependent self-renewal via NOX1 activation. However, the

mechanism by which SSCs are protected from ROS remains unknown. Here, we demonstrate a crucial role for Gln in ROS protection using cultured SSCs derived from immature testes. Measurements of amino acids required for SSC cultures revealed the indispensable role of Gln in SSC survival. Gln induced Myc expression to drive SSC self-renewal in vitro, whereas Gln deprivation triggered Trp53-dependent apoptosis and impaired SSC activity. However, apoptosis was attenuated in cultured SSCs that lacked NOX1. In contrast, cultured SSCs lacking Top1mt mitochondria-specific topoisomerase exhibited poor mitochondrial ROS production and underwent apoptosis. Gln deprivation reduced glutathione production; supra-molar Asn supplementation allowed offspring production from SSCs cultured without Gln. Therefore, Gln ensures ROS-dependent SSC-self-renewal by providing protection against NOX1 and inducing Myc.

P-425 - Intracytoplasmic Sperm Injection Induces Transgenerational Abnormalities in Mice.

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In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are 2 major assisted reproductive techniques (ARTs) used widely to treat infertility. Recently, spermatogonial transplantation emerged as a new ART to restore fertility to young patients with cancer after cancer therapy. To examine the influence of germ cell manipulation on behavior of offspring, we produced F1 offspring by a combination of two ARTs, spermatogonial transplantation and ICSI. When these animals were compared with F1 offspring produced by ICSI using fresh wild-type sperm, not only spermatogonial transplantation-ICSI mice but also ICSI-only control mice exhibited behavioral abnormalities, which persisted in the F2 generation. Furthermore, although these F1 offspring appeared normal, F2 offspring produced by IVF using F1 sperm and wild-type oocytes showed various types of congenital abnormalities, including anophthalmia, hydrocephalus, and missing limbs. Therefore, ARTs can induce morphological and functional defects in mice, some of which become evident only after germline transmission.

P-426 - COVID-19 Compromises Spermatogenesis Even After Recovery

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The SARS-CoV-2 virus has infected over 760 million people worldwide in the last three years and caused almost 7 million deaths. SARS-CoV-2 uses the TMPRSS2 protease and ACE2 receptor to infect host cells. Even though it is mainly a respiratory disease, both proteins are expressed in many tissues, including several testicular cell types. Abnormal levels of sex hormones and a decrease in sperm quality have been observed in patients during and after recovery from COVID-19. Furthermore, severe damage caused by inflammation has been detected in the testis of infected men. In addition, SARS-CoV-2 has been found in the testis. Thus, our objective was to explore the potential impact of COVID-19 on the male reproductive system. First, we analyzed the morphology of testis sections from patients deceased by COVID-19 and compared them to control samples of similar ages. Overall, COVID-19 samples displayed various anomalies commonly associated with compromised spermatogenesis, such as vacuolization of Sertoli cells, detachment of the germinal epithelium, or thickening of the basal lamina. Next, we studied the presence of different relevant biomarkers of spermatogenic cells, DNA damage, and leukocytes in these samples. A higher fraction of T lymphocytes and macrophages were detected in the peritubular spaces of COVID-19 samples compared to controls, thus confirming the infiltration of immune cells in the peritubular tissue of the testis. In addition, the seminiferous tubules of COVID-19 samples showed fewer UTF1-positive spermatogonia, which represents the spermatogonial stem cell population from which all sperm cells derive. Moreover, UTF1-positive spermatogonia presented more DNA damage than control cells, suggesting that COVID-19 could compromise spermatogenesis even after recovery. So, we conducted a study to examine the impact of SARS-CoV-2 infection on the testes of a small group of male individuals who had recovered from the virus infection. These patients also showed a decrease in the number of UTF1-positive spermatogonia, which presented more DNA damage, compared to controls. Finally, viral RNA was found in a fraction of COVID-19 necropsies. Nonetheless, more studies are needed to understand the impact of COVID-19 in spermatogenesis, especially in those patients that have recovered from the infection. Financial Support Research was supported by the Eunice Kennedy Shriver National Institute of Child Health & Human Development of the National Institutes of Health (R00HD090289), Ferring COVID-19 Investigational Grant, MWRI Research and Education Funding and the Magee-Auxiliary Woman Scholar endowment (MARS), Ferring COVID-19 investigational grants in Reproductive Medicine and Maternal Health (FIN0045760) and Impact of COVID-19 on male reproductive Health - Fundació La Marató de TV3 (677/U/2021). MLP is a recipient from a fellowship from the AGAUR (2023 FISDU 00058).

P-427 - Histological Analysis of Gonadal Sex Differentiation of Gonads in *Chamaeleo Calyptratus*.

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Early embryonic development is the period when all internal structures are formed, including gonadal primordia. The gonadal primordia, or gonadal ridge, differentiate into testes or ovaries through sexual differentiation. The process of gonadal development is still poorly understood in many vertebrates, especially in reptiles, especially lizards. The study focused on describing gonadal development, with particular emphasis on gonadal ridge formation and sexual differentiation of gonads in the veiled chameleon (*Chamaeleo calyptratus*). The study used histological staining according to Dubreuil's modifications and immunohistochemistry to visualize the structure of the gonadal development. Gonadal primordia are formed on the ventro-medial surface of the mesonephros. These gonadal ridges are covered with coelomatic epithelium, in which the primordial germ cells complete their migration. In the central part of the gonadal ridge, somatic cells accumulate. This is the first stage of the formation of the gonadal cortex. In differentiating testes, the gonadal medulla grows, while in differentiating ovaries, the cortex grows. A mature ovary is characterized by a cortex that equally surrounds the gonad on all sides. The testis, after differentiation, is a gonad with a reduced cortex and an enlarged medulla. The development of gonadal ridge in the *Ch. calyptratus* showed common developmental features found in representatives of squamate reptiles. On the other hand, the process of gonadal differentiation in the *Ch. calyptratus* analysed in this study revealed a certain diversity of processes the development of gonads that is unusual in reptiles. The analysis indicated that gonadogenesis in reptiles is not a conservative process.

P-428 - A New Insight into Testis Development of the Zebra Finch (*Taeniopygia Guttata*)

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Zebra finches have emerged as a widely used avian species in research. Nevertheless, the gonadal development of these birds has remained largely unexplored. The objective of this study was to elucidate the early formation of gonadal ridges in zebra finches, the process of sexual differentiation in the gonads, and the subsequent development of testicular structure. Eggs were collected on the day they were laid and incubated at 37.5 °C and 70% humidity. Embryos dissected from eggs were staged according to the Hamburger-Hamilton stages. We employed the histological staining according to Dubreuil's modifications, and compared it with immunofluorescence. We observed a notable asymmetry in gonadal development, with the left gonadal ridge consistently larger than the right, even before the settlement of primordial germ cells (PGCs). Following the settlement of PGCs, the left gonadal ridge displayed a significantly higher number of PGCs compared to the right, further accentuating the asymmetry. The left gonadal ridge lacked a distinct division into cortex and medulla due to the abundance of germ cells. The right differentiated testis contained testis cords and a thin surface epithelium. In some individuals left testis demonstrated a hermaphroditic structure, featuring testis cords and an extensive cortex resembling the cortex found in ovaries. Additionally, a number of mature males exhibited abnormalities in the structure of the left testis and impaired spermatogenesis, including atypical spermatid clusters or a complete absence of spermatozoa. This study provides insight into the distinct development of testes in

zebra finches, characterized by a high number of germ cells and significant cortical growth in the left testis. Understanding the molecular mechanisms underlying the development and maintenance of this hermaphroditic structure could provide valuable insights into the evolutionary adaptations of zebra finches and possibly other avian species.

P-429 - A Loss-of-Function Variant in HIPK4 Causes Male Infertility with Oligoasthenoteratozoospermia

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Male infertility, a prevalent reproductive disorder, encompasses a multitude of genetic factors that remain incompletely understood. In this study, we identified HIPK4 as a novel genetic determinant that contributes to male infertility in humans. which induced decrease in the number of sperms and severe sperm morphological anomaly Genetic analysis unveiled a homozygous missense variant in the HIPK4 gene (NM_144685.5: c.1115C >T; p. Ser372Leu) in the patient, resulting in a significant reduction in HIPK4 protein expression. Immunofluorescence analysis demonstrated the localization of HIPK4 in the acrosome of normal sperm, whereas a reduced staining intensity was observed in the patient Liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) analyses and and Co-immunoprecipitation found two interactors of HIPK4 , CALM1 and IQCN. We subsequently confirmed that HIPK4 can phosphorylate IQCN in the presence of CALM1. Further investigation indicated a poor prognosis for the patient undergoing regular intracytoplasmic sperm injection (ICSI) treatment. Our study identifies a novel gene, HIPK4, which contributes to male infertility caused by sperm head defects. This discovery enhances our understanding of the gene variant spectrum and the underlying etiology of oligoasthenoteratozoospermia, thereby contributing to the advancement of genetic analysis in this field.

P-430 - The Induction of Stress via Social Isolation in a Rat Model: The Effect on Male Reproductive Parameters

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Background and Aim: The recent increase in the global prevalence of psychological stress necessitates an exploration into the potential role of psychological stress as a contributing cause to male infertility. In order to advance this, the current study aimed to assess the effect of stress, induced via social isolation in a rat model, on male reproductive parameters. **Methods:** This study consisted of 18 male Wistar rats. Nine rats were stressed via social isolation prior to sacrifice (isolated males, IM) while the remaining 9 were housed in groups, serving as controls (group-housed controls, GHC). All rats were sacrificed by decapitation. Both epididymides were harvested

for biochemical analyses (eNOS and p-eNOS expression by western blotting, Total Antioxidant Capacity (TAC) assessment by means of an ORAC assay) and sperm extraction for the analysis of sperm motility, morphology and viability parameters. Results: A significant increase in corticosterone levels was observed in the IM group, confirming stress in the animal model. Analysis of sperm kinematic parameters revealed that the curvilinear velocity (VCL) ($P = 0.0320$), VAP ($P = 0.0053$), straight line velocity (VSL) ($P = 0.0053$) and linearity (LIN) ($P = 0.0261$) were significantly decreased in the experimental (IM) group. Furthermore, the percentage normal morphology decreased significantly in the stressed rats ($P < 0.0001$). Total motility, progressive motility, viability as well as the percentage of rapid, medium and slow motile sperm, showed no statistical significance between the stressed IM rats and the GHC controls. Total antioxidant capacity (TAC), endothelial Nitric Oxide (eNOS) expression and testosterone levels remained unchanged. Discussion: The present study showed that psychological stress induced by social isolation resulted in a significant increase in corticosterone levels coupled with a reduction in normal sperm morphology and kinematic parameters. TAC, eNOS expression and Testosterone levels were explored as possible causative mechanisms but remained unchanged. Thereby alternative mechanisms for reduced sperm quality that should be explored in follow-up studies. Conclusion: Psychological stress induced by social isolation in rats negatively impacts male reproductive parameters, however, the mechanism through which this phenomenon ensues requires further elucidation beyond that of the induction of oxidative stress.

P-431 - The Time Doesn't Stop: Impact of Paternal Aging on Fertility in a Mouse Model

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This study worked with the hypothesis that aged mice present alterations in testosterone levels, sexual behavior, and sperm physiology, negatively influencing fertilization processes and early in vitro embryo development. Three experiments were conducted using male C57BL/6J mice aged 4 (young/control), 19, and 24 (senile/experimental) months. Data were analyzed using SAS software. Were performed ANOVA One-Way and the post-hoc test LSD. A level of 5% significance was considered. In the first experiment (4, n=6; 19, n=5; 24-month, n=7), behavioral tests were performed, including open field, elevated T maze, and social interaction with synchronized young females. The experimental groups exhibited a decrease in rearing ($p=0.023$), and center distance moved ($p<.0001$), as well as in the frequencies of entry into the central zone ($p=0.002$) and the peripheral zone ($p=0.002$). The time in the central area was longer in the aged groups ($p=0.001$), while the movement time ($p=0.001$) and average speed ($p<.0001$) in that area were lower. In the social interaction test, the aged groups showed a shorter social interaction time ($p=0.010$) and a lower frequency of female chasing ($p=0.003$), and there was a trend for a decrease in mating attempt frequency ($p=0.082$). In the second experiment (4, n=11; 19, n=5; 24-month, n=9), male reproductive capacity was evaluated. Serum testosterone levels, testicular weight, sperm

morphology, CASA motility, integrity of plasma and acrosomal membranes (FITC/IP), oxidative stress (CELLROX green), mitochondrial membrane potential (JC1), susceptibility of chromatin to acid challenge (modified SCSA), and respective condensation (CMA3), sperm capacitation status (CTC), and impacts on in vivo fertilization rate were assessed. The 24 months group showed lower serum testosterone levels ($p=0.018$). Aged groups exhibited a higher percentage of individual ($p=0.0001$), multi-defects ($p=0.0361$), and total defects ($p<.0001$) compared to the control group. The 19-month group demonstrated a higher percentage of tail defects ($p=0.023$), and the 24-month group had a higher percentage of cells with midpiece defects ($p=0.006$). Males at 19 months exhibited higher intermediate mitochondrial membrane potential ($p=0.032$) and damaged plasma membrane with oxidative stress ($p=0.003$). The 24-month group showed a higher percentage of deprotaminated sperm ($p=0.045$). The percentage of capacitated sperm ($p<.0001$) was decreased in aged groups compared to the control, with the 19-month group showing increased acrosome reaction ($p=0.016$). In the third experiment (4, $n=5$; 19, $n=5$; 24-month, $n=7$), in vitro embryo development rate, blastocyst stages, and the first cell differentiation (marked with CDX2 and SOX2) from young females mated with males from control and experimental groups were analyzed. It was found that aged groups had lower cleavage rates (D1.5; $p=0.0290$), blastocyst ($p=0.0070$), and embryo development rates (D4.5; $p=0.0120$). In the evaluation of specific blastocyst stages, the 19-month group showed more early blastocysts (EB; $p=0.0051$), and aged groups had less expanded (BX; $p=0.0402$) and hatched (HB; $p=0.0003$) blastocysts. There was a reduction in the total cell number ($p=0.004$) and trophectoderm (CDX2-positive; $p=0.016$) in the aged groups. The 24-month group had fewer cells for inner cell mass (SOX2-positive; $p=0.035$). Aged groups exhibited few fluorescence intensities for CDX2 ($p<.0001$). Specifically, the 24-month group showed lower fluorescence intensity for SOX2 ($p<.0001$). This study demonstrated the negative effect of paternal age on sexual behavior, and sperm quality, potentially leading to delayed first-cell division and consequently poorer early embryonic development in vitro. This work was supported by the Coordination of Improvement of Higher Education Personnel (CAPES).

P-432 - MRNIP Interacts with Sex Body Chromatin to Support Meiotic Progression, Spermatogenesis, and Male Fertility in Mice

Samina Kazi, Julio M, Castañeda, Audrey Savolainen, Yiding Xu, Ning Liu, Huanyu Qiao, Ramiro Ramirez-Solis, Kaori Nozawa, Zhifeng Yu, Martin M, Matzuk, Renata Prunskaitė-Hyyryläinen

MRNIP Interacts with Sex Body Chromatin to Support Meiotic Progression, Spermatogenesis, and Male Fertility in Mice Samina Kazi¹, Julio M, Castañeda², Audrey Savolainen¹, Yiding Xu³, Ning Liu³, Huanyu Qiao³, Ramiro Ramirez-Solis⁴, Kaori Nozawa⁵, Zhifeng Yu^{5,6}, Martin M, Matzuk^{5,6}, Renata Prunskaitė-Hyyryläinen¹ ¹Faculty of Biochemistry and Molecular Medicine, University of Oulu, Oulu, Finland ²Research Institute for Microbial Diseases, Osaka University, Suita, Japan ³Department of Comparative Biosciences, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA ⁴University of Texas Health Science Center, San Antonio, Texas, USA ⁵Department of Pathology & Immunology, Baylor College of Medicine, Houston, Texas, USA ⁶Center for Drug Discovery, Baylor College of Medicine, Houston, Texas, USA The MRN complex interacting protein (MRNIP) was initially identified as a protein involved in maintaining genome stability by playing a role in DNA double-strand break repair processes in mitosis. It has also been shown that MRNIP is an important factor for the protection of the replication fork during mitosis and is involved in promoting DNA double-strand break repair sensing by liquid-liquid phase separation. Our and other

research works have established a central MRNIP role in male meiosis using Mrnip knock-out mouse models. We show that Mrnip is ubiquitously expressed in multiple tissues both in mice and humans and that the gene expression in mice testes begins postnatally. Our analysis demonstrates that MRNIP is specific to male, not female meiosis. It is expressed during the first wave of spermatogenesis and becomes specific to meiocytes from mid-pachytene until the diplotene stage in prophase I. The deletion of Mrnip leads to a four-fold reduction in testes size and weight but normal body weight in mice. The histological and sperm motility analysis revealed seized spermatogenesis. Deeper studies showed that deletion of Mrnip leads to reduced sex body formation, impaired meiotic sex chromosome inactivation, and defective retroposition of X-linked genes to autosomes. Furthermore, we observed the formation of droplet-like accumulations of MRNIP that coalesce to create a distinct sub-nuclear compartment resembling nucleoli through meiotic progression. The analysis demonstrates that MRNIP droplets within the nucleus closely associate with the sex body during diplotene. Taken together, the loss of Mrnip causes meiocyte apoptosis at the diplotene stage leading to a failure in the formation of mature and motile sperm cells causing male mice infertility. This work was supported by grants from the Academy of Finland 285151 and Profi6 336449 and the Sigrid Jusélius Foundation to R. P.-H.; Health and Bioscience doctoral program of the University of Oulu to S.K. and A.S.; the Ministry of Education, Culture, Sports, Science and Technology (MEXT)/Japan Society for the Promotion of Science (JSPS) KAKENHI grants JP18K14715 and JP20K15804 to JMC; National Institute of Health R00 HD082375 and R01 GM135549 to H.Q.; a P30 Cancer Center Support Grant (NCI-CA125123, HTAP), the Eunice Kennedy Shriver National Institute of Child Health and Human Development R01HD088412 and P01HD087157 to M.M.M., and the Bill & Melinda Gates Foundation INV-001902 to M.M.M.

P-433 - Standardization of Sperm Analysis using Computer-Assisted Sperm Analysis (CASA) from Cynomolgus Monkey (*Macaca Fascicularis*) Semen in Primate Resources Center

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Non-human primates (NHPs) are phylogenetically close to humans (*Homo sapiens*) and are appropriate animal models because of their physiological similarities to humans. NHPs are studied in physiology, neuroscience, and reproductive biology. Cynomolgus monkeys (*Macaca fascicularis*) is a well-known NHP species widely used in various studies are reproductively similar to humans. Sperm in particular are important for successful fertilization and proper embryonic development. Therefore, the purpose of this study was to accurately evaluate fertility by evaluating the sperm quality of Cynomolgus monkeys. Like this we standardized the sperm of cynomolgus monkeys male, which can be used for developmental research. Semen from five cynomolgus monkeys was recovered using an automatic electric ejaculation system. In order to standardize cynomolgus monkey sperm, we investigated the motility, viability, morphology and acrosome reaction, which are indicators of semen analysis evaluation. Average motility (%) of sperm (n=5): progressive; 81.83 ± 5.42 and non-progressive; 18.17 ± 5.42 . Average vitality (%) of sperm (n=5): live; 79.03 ± 4.07 and dead; 20.97 ± 4.07 . Average morphology (%) of sperm (n=5): normal; 64.27 ± 2.30 , head defects; 4.86 ± 1.65 , midpiece defects; 2.02 ± 0.84 , tail defects; 28.09 ± 1.37 , and cytoplasmic droplets;

0.76 ± 0.65. Average acrosome reaction Ca⁺ non-treated group (%) of sperm (n=5): non-reaction; 84.5 ± 5.6 and reaction; 15.5 ± 5.6. Average acrosome reaction Ca⁺ treated group (%) of sperm (n=5): reaction; 56.4 ± 1.5 and non-reaction; 43.6 ± 1.5. In conclusion, we confirmed our sperm analyzed data from cynomolgus monkeys, and the results presented are considered to indicate reproductive ability.

P-434 - Molecular Interaction Between Nucleolus and Chromatoid Body of Male Germ Cells Evidenced by in Vitro and in Vivo Nucleolar Blocking

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The chromatoid body (CB) is a cytoplasmic structure of male germ cells that has a role in the regulation of mRNA transcription during spermatogenesis. It also participates in the control of gene expression through the action of small non-coding RNAs. The complete proteome analysis of CB molecular structure has identified abundant presence of their classical molecular markers, as well as the identification of many transient proteins (e.g., the nucleolar protein fibrillarin; rRNA 2'-O-methyltransferase). The nucleolus is a sub-compartment of the nucleus, which has the primary function in the biogenesis of ribosomes. It has been shown that the nucleolar cycle during gametogenesis contributes to the reestablishment of nucleolar morphology, which is an important factor for removal of induced cell damage. Here, we evaluated the effect of nucleolar blocking on the CB organization. BMH-21, an RNA polymerase I (Pol I) inhibitor, was used to block the nucleolar activity in male germ cells in vitro and in vivo. We found a change in the amount of MVH protein in germ cells of the groups treated with BMH-21 by WB analysis, as well as the failure in the structural organization of the CB in germ cells undergoing the same treatment by IF analysis. Additional data collected in vivo detected the physical interaction between fibrillarin and MVH by Co-IP. Moreover, RT-PCR analyzes in germ cells of animals treated with BMH-21 showed increased expression of Gm10486, a gene normally kept silent in the CB. Our results corroborate previous evidence of the link between the physiology of the nucleolus and the CB in male germ cells, which reinforces the role of these structures in determining male fertility.

P-435 - Post-Thaw Characteristics of Ram Spermatozoa and Activity of Antioxidant Enzymes after Supplementing Semen Extender with Mn²⁺-, Zn²⁺- or Cu²⁺ -Nanosuccinate

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The main goal of this experiment was to determine the effects of Mn²⁺-, Zn²⁺- or Cu²⁺-nanosuccinate added to semen extender on ram semen characteristics and activity of antioxidant enzymes after thawing. This study utilized six clinically healthy Texel rams aged 2-4 years during seasonal anestrus (April-May). Ejaculates (n=36; 6 per ram) collected into an artificial vagina were evaluated for volume, sperm concentration and motility, and then divided into isovolumetric control and experimental fractions (n=10 per ejaculate). Control semen was diluted with lactose-yolk-tris-citrate-glycerin medium (LYTCGM) and nanosuccinates (Mn²⁺- and Zn²⁺-nanosuccinate: 2.5, 5.0 and 7.5 µg/l; Cu²⁺-nanosuccinate: 1.25, 2.5 and 3.75 µg/l) were added to LYTCGM in experimental semen samples. Extended semen was loaded into 0.25-ml straws (64 x 10⁷ spermatozoa/ml), equilibrated for 2.5 h and then frozen in liquid nitrogen. Post-thaw sperm motility was determined with Computer Assisted Semen Analysis (CASA) and the activity of antioxidant enzymes (superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT)) was measured with a spectrophotometric technique. The addition of an intermediate dose of Mn²⁺- and Zn²⁺-nanosuccinate to LYTCGM was associated with a significant increase in ram sperm progressive motility, and the lowest and intermediate dose of both nanosuccinates improved sperm motion kinetics/velocity parameters. Moreover, Mn²⁺- and Zn²⁺-nanosuccinates at a dose of 5.0 µg/l, consistently decreased (P< 0.05) SOD activity and boosted (P< 0.05) GPX and CAT activity in ram semen samples after thawing. Alternatively, the addition of Cu²⁺-nanosuccinate to LYTCGM (highest dose) significantly reduced the progressive motility and velocity of thawed ram spermatozoa, increased the percentage of sperm with acrosomal/head defects and seminal SOD activity, and depressed CAT (highest dose) and GPX (all doses) activity. In summary, the addition of Mn²⁺- and Zn²⁺-nanosuccinate to semen extender appeared to have beneficial effects on sperm motility/motion kinetics and structural integrity, whereas Cu²⁺-nanosuccinate generally had debilitating effects on the post-thaw semen characteristics in rams.

P-436 - Transition Nuclear Protein 2 (TNP2) mRNA Cargo: a Putative Marker for Assessment of Breed Differences?

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Artificial insemination (AI) centres and farm management require fine control of the quality status of the boars. In particular, sperm quality through conventional methods fails to address the presence of sub-lethal characteristics that led to a detrimental effect not only on the sperm itself but also on the final fertility performance. Boar sperm plasma membrane presents a high proportion of polyunsaturated fatty acids, increasing its susceptibility to oxidative stress, such as the induced by in vitro handling (e.g. centrifugation, and dilution), causing chromatin alterations. In this sense, DNA integrity is an already established quality marker of sperm fertilizing ability. DNA integrity relies, among other factors, on DNA packaging, being the histones, commonly found in eukaryotic cells, mostly replaced by protamines in mammalian spermatozoa. Protamines are

acidic proteins that form very complex structures with DNA, forming disulfide bridges between the cysteine residues of protamines during sperm epididymis transit. There are two main types of protamines: protamine 1 (PRM1) and protamine 2 (PRM2), the latter present only in horses and humans with a known function. In addition, the transition nuclear proteins (TNPs) represent 90 % of the chromatin basic proteins during histone removal. This study aimed to investigate the mRNA transcripts content of the PRM1, PRM2, TNP1 and TNP2, in pig spermatozoa from two commercial breeds (Landrace (LD) vs Large White (LW)), with a field fertility record (determined based on a farrowing rate of at least 100 inseminations; high-fertility (HF) vs low-fertility (LF)). For this purpose, the relative abundance of mRNA transcripts for protamines and transition nuclear proteins was analyzed through quantitative polymerase reaction (qPCR) analysis in a total of 12 boars (LD n=6 & LW n=6; HF n=6 & LF n=6) with fertility record. Total RNA isolation from sperm samples was performed by using the miRNeasy mini kit (Qiagen, Germany) following the manufacturer's protocol. qPCR was performed by using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA), after the first-strand cDNA synthesis (High-Capacity RNA-to-cDNA™; Fisher Scientific, Sweden). The following commercial primers (all porcine-specific and from Bio-Rad, USA) were used: GAPDH (loading control), PRM1, PRM2, TNP1, TNP2. Calculations of the relative mRNA content were performed by using the Pfaffl method. After normal distribution and homoscedasticity control, parametric data were analyzed through a T-test and non-parametric data through Kolmogorov-Smirnov using GraphPad Prim 8. Our qPCR results showed a significant decrease in the TNP2 mRNA transcripts in the Large White Boars relative to Landrace (LD: 1.00 ± 0.36 vs. HF: 0.57 ± 0.33 , $P=0.048$), being the rest of the transcripts non-significant. TNP2 is closely linked to the two protamine genes, which might suggest their origin by gene duplication and, perhaps, retaining some common functions. In contrast to TNP1, abundantly expressed and conserved in various mammals, TNP2 is poorly expressed and protein abundance varies among species. Therefore, our results might suggest that the differential expression of TNP2 found among breeds could be linked to a non-described yet differential compaction status. Finally, no differences between high and low fertility boars were found in any of the mRNA transcripts analyzed. In conclusion, our preliminary results of breed-specific differences pave for further studies to elucidate whether this differential expression could have an impact on sperm in vitro handling techniques, in particular paying attention to DNA integrity preservation. Supported by RyC2020-028615-I and PID2022-136561OB-I00, funded by MCIN/AEI /10.13039/501100011033 (Spain) and FEDER funds (EU).

P-437 - Cytoplasmic Dynein 2 is Required to the Regulation of Intraflagellar Transport System in Sperm Flagellum Formation.

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Sperm flagellum formation is one of the major events in spermiogenesis. In sperm flagellum formation, many substances necessary for flagellar formation are transported along microtubules, and intraflagellar transport (IFT) system is responsible for this process. During the cilium formation, which are motile similar to sperm flagella, kinesin 2 and cytoplasmic dynein 2 have motility activity and play a fundamental role in the function of the IFT system. Kinesin 2 is known to important for sperm flagellum formation, but involvement of cytoplasmic dynein 2 is unclear. It is generally

believed that IFT system is common to both cilia and sperm flagella. However, since numerous testis-specific genes are related to sperm flagellar formation and there are differences in the structure compared to cilia (mitochondria sheath, outer dense fiber and fibrous sheath, etc.), it is significant to study IFT system with a focus on the sperm flagella. Therefore, the purpose of this study is to elucidate the molecular mechanism of IFT system focusing on cytoplasmic dynein-2. Our study leads to understand the largely unknown mechanism of IFT system of sperm flagellum formation. To explore the function of cytoplasmic dynein 2, we generated knockout mice (KO mice) in which one of the cytoplasmic dynein 2 subunits was deleted. We then evaluated the fertility of the generated male KO mice. Next, we conducted phenotypic analysis of the male KO mice using tissue staining and immunochemistry, mainly for the following three. 1): Histological analysis of seminiferous tubules and epididymal spermatozoa. 2): Dynamic analysis of cytoplasmic dynein 2 subunit. 3): Dynamic analysis of IFT proteins (IFT81,88,140) and kinesin 2 (KIF3A), which are a component protein of the IFT system. The male KO mice showed complete infertility. Periodic Acid Schiff - Hematoxylin staining in the seminiferous tubules showed that flagella defects were observed in KO mice. In epididymal cauda, most of spermatozoa flagella were severely shortened. In addition, disruption of the cytoplasmic dynein-2 subunit expression was detected in the sperm flagella of KO mice, such as loss of localization and ectopic localization, which were not seen in WT. Moreover, IFT 88 and KIF3A also showed the same abnormal localization in KO mice. These results indicate that the entire structure of IFT system is impaired in KO mice. Thus, failing to normal function of IFT system should cause the lack of transport of substances necessary for flagellar formation, and result in the severe shortened flagella. In this study, we revealed for the first time that cytoplasmic dynein-2 is important for male fertility and sperm flagellum formation. Furthermore, we showed that cytoplasmic dynein-2 regulates the structural maintenance and function of the entire IFT system.

P-438 - The Addition of a Xenobiotic Prior to Cryopreservation of Bovine Semen Increases Its Resilience Post-Thaw

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Extremolytes, a distinct class of compatible solutes, play a pivotal role in protecting macromolecules and cell structures from extreme environmental stress. The objectives of this study were to assess the biocompatibility of a bacterial derived extremolyte xenobiotic with bull sperm during the cryopreservation process and to assess the potential of the xenobiotic to shield sperm under challenging conditions such as osmotic and heat stress. Semen was collected from bulls (n=8) in a commercial stud and diluted in OptiXcell2 containing either 0 mM (control), 0.5, 5 and 50 mM of the xenobiotic. The semen was packaged into artificial insemination straws and held at 4 °C overnight following which it was cryopreserved as per routine procedures. Subsequently, the semen was thawed and the motility (computer assisted sperm analysis) as well as the viability, membrane integrity and acrosomal status (flow cytometry) were assessed. Following this, the thawed bovine sperm was exposed to heat (42 °C for 4 h) and osmotic (150 mOsm for 15 min) stress. All results are reported as mean ± s.e.m. Sperm cryopreserved in the presence of the

xenobiotic at concentrations ranging from 0.05 to 5 mM had similar motility, viability, membrane fluidity, and acrosomal integrity compared to the control post thaw ($P > 0.05$). At a concentration of 50 mM, there was a decline in total motility compared to the control ($49.5 \pm 4.11\%$ and $65.5 \pm 4.05\%$, respectively; $P < 0.05$). Incubation of the control sperm in media of low osmolarity reduced total motility to $21.4 \pm 4.16\%$ and addition of the xenobiotic did not affect this. Similarly, there was no effect of the xenobiotic on sperm viability across all treatment groups ($P > 0.05$). However, when sperm were subjected to heat stress at 42°C for 4 h, a significant interaction between time and the treatment was observed on sperm motility ($P < 0.01$). This was manifested by no difference between treatments at 0 h but after a 2-h incubation, there was a 36% decrease in motility in the control group ($P < 0.001$) while the xenobiotic treatment groups maintained their motility. These results indicate that the xenobiotic assessed is not cytotoxic to sperm in the range 0 to 5 mM. and while no protective effects were observed when the sperm was subjected to osmotic stress at 150 mOsm, promising results were observed with improvement in sperm motility under heat stress conditions. This shows the potential application of the xenobiotic to shield sperm under challenging conditions, with further studies needed to assess additional protective effects of the xenobiotic on sperm.

P-439 - Chemical Profiling of Testicular Parenchyma in Rams Using an Exploration of Echointensity Bands and a Novel Computer Algorithm (r-Algo) Increasing Specificity and Accuracy of Echotextural Analyses

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The main purpose of this study was to employ ultrasound image segmentation to increase the consistency and accuracy of determining chemical composition of rams' testicular parenchyma from its mean pixel intensity (image brightness). Ten testes obtained from sexually mature Karakul rams were scanned ex situ with an 8-MHz linear-array transducer, in the longitudinal and transverse plane, and all ultrasonograms were saved as digital images. The Kjeldahl method was used to determine the amount of crude protein, an oven-drying method was used to determine the moisture content, and a Soxhlet extraction of dried samples was used to determine the fat content of testicular tissue samples. Digital images of testicular parenchyma were normalized and subjected to computerized analysis using ImageProPlus analytical software (bitmapping). Then two different approaches, namely echo intensity (EI) banding and algorithmic sequestration of resultant pixel intensity values (bitmaps), were compared for their usefulness in detecting quantitative correlations between numerical pixel values and proximate chemical composition of testicular tissue. Using 25- or 50-pixel intensity bands for testicular ultrasonograms obtained in the longitudinal (Long) and transverse (Trans) plane, we identified two Long EI bands (26-50 and 0-50) for which mean numerical pixel values (NPV) were significantly correlated with fat content, and four Trans EI bands with mean NPV significantly correlated with testicular tissue constituents (51-75, 76-100 and 51-100 with protein content, and 0-50, 51-75, and 51-100 with moisture). For Long images, the accuracy of predicting the fat, moisture and protein content of the testicular parenchyma using r-Algo-identified pixel intensity clusters was $87.43 \pm 2.50\%$ (pixels 99-114), $99.54 \pm 0.11\%$ (84-89), and $90.66 \pm 1.18\%$ (49-55), respectively. For Trans images, the respective accuracy values were $86.37 \pm 1.49\%$ (52-58), $99.34 \pm 0.13\%$ (54-77), and $91.19 \pm 2.02\%$ (50-67). Algorithmic detection of specific pixel intensity ranges appears to be an optimal method of pixel

sequestration for determining precise correlations between first order echotextural characteristics (i.e., NPVs, pixel heterogeneity and frequency distribution) and chemical composition of the testes. Our present results highlight the importance of incorporating this method of computer-assisted image analysis for ultrasonographic monitoring of changes in testicular biochemistry/histophysiology.

P-440 - High Fertile Boars Present a Decrease in the mRNA Cargo of TRPC1, a Key Regulator of Sperm Motility

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Subfertile boars largely impact the economic status of artificial insemination (AI) centers and farms. Thus, the primary objective for AI centers is to find suitable biomarkers for early identification of subfertility individuals. Sperm activity is regulated by Calcium (Ca^{2+}), a key molecule that plays an essential role in the modulation of motility, sperm capacitation, and the acrosome reaction. However, whether the modulation of calcium trafficking from sperm cells is linked with the presence of subfertile animals remains to be elucidated. The transport of Ca^{2+} is mainly mediated not only by CatSper channels (sperm-specific) but also by transient potential canonical channels (TRPC). CatSper facilitates the entrance of cations during sperm capacitation by alterations in membrane potential and pH, via the cAMP-PKA signaling pathway, which plays a pivotal role in sperm egg penetration and its mutation are linked to infertility in humans. On the other hand, TRPCs, mainly located over the membrane of the tail and head of spermatozoa, regulate hyperactivation as well as the acrosome reaction. This study aimed to investigate the mRNA transcript cargo for the β , γ , and δ subunits of CatSper, as well as TRPC channels 1, 4, and 6 in boar spermatozoa from two distinct commercial breeds (Landrace (LD) vs Large White (LW)), with fertility record (high fertility (HF) vs low fertility (LF) (determined based on a farrowing rate of at least 100 inseminations)). For this, the relative abundance of mRNA transcripts for TRPC and CatSper subunits was determined through qPCR analysis in a total of 12 boars (LD n=6 & LW n=6) with fertility record. Total RNA extraction from sperm samples was performed with the miRNeasy mini kit (Qiagen, Germany) following the manufacturer's protocols. Then, after the synthesis of the first-strand cDNA (High-Capacity RNA-to-cDNA™; Fisher-Scientific) the quantitative polymerase chain reaction (qPCR) was performed by using the SsoAdvanced Universal SYBR-Green Supermix and commercial primers for GAPDH (loading control), TRPC1, TRPC4, TRPC6, CatSper- β , CatSper- δ , and CatSper- γ ; (Bio-Rad, USA). Relative mRNA content was calculated using the Pfaffl method. Data were assessed for normal distribution with the Shapiro-Wilk test and parametric data analyzed through a T-test, while non-parametric data through Kolmogorov-Smirnov ($p < 0.05$, GraphPad Prism8). qPCR results showed a significant decrease in the TRPC1 mRNA transcript in HF boars (LF: 1.00 ± 0.03 vs. HF: 0.61 ± 0.16 , $P = 0.026$), being the rest of transcripts non-significant. TRPC1 plays a crucial role in the acrosome reaction and sperm motility, as it encodes store-

operated Ca²⁺ channels (SOCs) associated with these functions, being observed a decrease in transcription during capacitation directly correlated with a reduction in motility. This might suggest a potential connection with interactions in the sperm reservoir for storage within the female's cells. Results from the comparison of the two included breeds (LD & LW), the relative abundance of CATSper- γ mRNA increase in the Large White breed (LD: 1.00 $\pm$ 0.17 vs. LW: 1.73 $\pm$ 0.18, P=0.027). CATSper- γ is influenced by external signals such as ligands and cell-cell interactions during sperm maturation in the epididymis and/or the female reproductive tract. In addition, an increase in the relative expression of mRNA has been described in capacitated boar spermatozoa. No significant differences were found in the remaining transcripts. Our preliminary results of breed-and fertility-specific differences pave for further studies in order to find suitable male biomarkers of fertility performance in the boar. Funded by MCIN/AEI /10.13039/501100011033 (Spain) and FEDER funds (EU) (RyC2020-028615-I and PID2022-136561OB-I00).

P-441 - Assembly and Maintenance of the Centromeric Histone CENP-A in the Male Germ Line

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The control of cell division in germ cells that give rise to gametes (eggs and sperm) is important as errors can lead to aneuploidy or infertility. The centromere is the essential chromosomal locus that directs accurate chromosome segregation at cell division. Rather than DNA sequence, it is the incorporation of the histone H3 variant CENP-A that epigenetically specifies centromere identity and function. During the cell cycle, DNA replication in S phase results in the dilution of CENP-A at centromeres. To offset this dilution, newly synthesised CENP-A must be assembled and maintained at centromeric chromatin each cell cycle to ensure continued centromere function. Compared to somatic cells, germ cells display distinct properties with respect to CENP-A assembly and maintenance. For example, in germ line stem cells or in spermatocytes undergoing meiosis, CENP-A is assembled at unique cell cycle times or in unexpected amounts. An additional conserved property of CENP-A is that it is retained on mature sperm nuclei despite the removal of most other histones and replacement by protamines. In the fruit fly *Drosophila melanogaster*, the transgenerational inheritance of paternal CENP-A on sperm is critical for early embryonic development. Here I will present our latest investigations into the molecular mechanisms controlling CENP-A assembly, maintenance and function in the male germ line, using the both the fruit fly and bovine model systems.

P-442 - Differential Expression of Oestrogen and Androgen Receptors in Spermatozoa from Different Boar Breeds and Fertility Levels

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Boar spermatozoa undergo maturation and acquire motility upon reaching the epididymis. Evidence among species suggests that this maturation is mediated by steroid hormones acting on the epididymis. Regulation of steroid hormones is crucial in livestock such as the porcine species. The epididymal function relies on androgens and estrogens, modulating the expression and secretion in the pig epididymis. Androgens (testosterone, androsterone and androstenedione) exert effects via the androgen receptor (AR), at the cellular level, influencing epididymal segmentation and luminal fluid composition. Androgen-regulated proteins are likely involved in sperm membrane remodeling, facilitating zona pellucida attachment. Estrogens play a vital role in regulating accessory sex glands and influence epididymal contractility through the RhoA/Rho-kinase pathway, potentially regulating the ejaculation process. Estrogen receptors (ER) are crucial for epididymal function and fertility maintenance. Previous studies suggest estrogen-regulated proteins contribute sperm membrane remodeling for proper sperm-oocyte interaction. Sperm storage in the epididymis cauda may also depend on estrogen-regulated proteins. Expression of AR and ER in spermatozoa varies among and within species, with limited research regarding AR and ER presence in adult boar sperm. The purpose of this study was to identify the presence of estrogen and androgen receptors mRNA in pig spermatozoa from two different commercial hybrid breeds (Landrace (LD) vs Large White (LW)). Moreover, a comparative analysis was carried out between two levels of fertility (high fertility (HF) vs low fertility (LF)). Briefly, a total of 12 boars (LD n=6 & LW n=6) with fertility record (determined based on a farrowing rate of at least 100 inseminations) were used. Total RNA extraction was isolated from sperm samples by using the miRNeasy mini kit (Qiagen, Germany) following the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was performed by using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA), after the first-strand cDNA synthesis (High-Capacity RNA-to-cDNA™; Fisher Scientific, Sweden). The following commercial primers (porcine-specific) were used: GAPDH (loading control), ER and AR (all purchased from Bio-Rad, USA). Calculations of the relative mRNA content were performed by using the Pfaffl method. After normal distribution and homoscedasticity control, a T-test (parametric) and Kolmogorov-Smirnov (non-parametric) were carried out (GraphPad Prism 8). Results in our qPCR showed a significant increase in the expression of AR mRNA transcripts in Large White vs Landrace boars (LD: 1.00 ± 0.77 vs. LW: 13.33 ± 19.23 , $P=0.0022$). However, under our experimental conditions, there was no expression of ER mRNA transcripts. Moreover, no significant differences were found between HF and LF boars in any of the mRNA transcripts analyzed. Androgens play a key role in maintaining epithelial cell morphology and preventing cell death. The expression of AR in the cells of the epididymis can determine changes in the organ's ability to carry out its important functions of maturation, transport and protection of spermatozoa. Moreover, it is known that in the low presence of androgens, spermatozoa present altered motility, lose their fertilizing capacity and even die, suggesting that the presence of androgens is essential for both the development and maintenance of epididymal functions. Further studies are needed to evaluate the potential reason behind the differences in the expression of these receptors among groups of different breeds. Supported by RyC2020-028615-I and PID2022-136561OB-I00, funded by MCIN/AEI/10.13039/501100011033 (Spain) and FEDER funds (EU).

P-443 - Histological and Metabolomic Analysis of Testis Tissues in Japanese Macaques (*Macaca fuscata*) Aiming the Establishment of In Vitro Spermatogenesis Technique in Primates

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Recent studies demonstrated successful in vitro spermatogenesis from spermatogonia using organ culture methods of mouse and rat testicular tissues. Application of this technique to other mammals including endangered species and humans would be helpful to preserve the fertility of immature males and to obtain gametes from individuals with spermatogenic failure. However, the mechanisms of spermatogenesis and the physiological environment in the testis seem to vary widely among species. In this study, the testis organ culture of Japanese macaques (*Macaca fuscata*) was carried out based on the previous studies of mice. In addition, we performed a histological and metabolomic analysis to collect useful information for the establishment of in vitro spermatogenesis techniques for primates. Testes were obtained from Japanese macaques aged 5-14 years in Tama Zoological Park (Tokyo, Japan) from November to January by skilled veterinarians for the management of population. Then tissues were transported to the laboratory on ice and used for the following experiments. Testicular fragments (1-2 mm³) were cultured for 8 weeks on agarose gels half-soaked with culture medium containing 10% knockout serum replacement (n=5). Cultured tissues were collected at 1,2,4,6,8 weeks of culture followed by a 4% PFA fixation. The states and functions of germ cells and Sertoli cells (SC) were evaluated by immunohistochemistry for DDX4 (germ cell marker), Ki67 (proliferating marker), acrosin (acrosome protein), SOX9 (SC nucleus marker), vimentin (SC marker), androgen receptor (AR) and aldehyde dehydrogenase (ALDH)1/2 which oxidizes retinal to retinoic acid. Non-target metabolome analysis in testis tissues of macaques (n=7) and C57BL/6 mice (26 weeks old, n=4) was conducted using gas chromatography-mass spectrometry to identify the difference in metabolomics profile between the two species. MetaboAnalyst (<https://www.metaboanalyst.ca>) was used for the statistical analysis. Each type of germ cell (spermatogonia, spermatocytes, and spermatids) and proliferating cells were observed in all testis tissues before culture. However, the number of germ cells and proliferating cells decreased immediately after the beginning of the culture ($p < 0.05$). The acrosin immunosignal was not detected at 6-8 weeks of culture although elongated nuclei of spermatids were observed throughout the culture period. These results indicate spermatogenesis and cell proliferation in monkey testes did not progress successfully in culture condition that works in mice. SC markers SOX9 and vimentins were detected before and after culture suggesting SCs remained on the basal membrane of seminiferous tubules. Clear immunosignals of AR and ALDH1/2 were observed before culture in nuclei and cytoplasm of SCs, respectively, but attenuated within two weeks. Since both AR and ALDH are crucial for spermatogenesis, optimal conditions to maintain the SC function need to be identified. Finally, the metabolomic analysis revealed that peak intensities of 37 metabolites such as L-cystine and L-glutamic acid were higher, and 21 metabolites such as cytidine and xanthine were lower in testes of Japanese macaques compared with mice ($p < 0.05$). It suggests that spermatogenesis in each animal proceeds in much different nutrient environments. These results provided useful information to improve the culture methods for successful in vitro spermatogenesis in primates. In the further study, culture medium and its supplements will be examined based on the metabolic profiles. This study was supported by JSPS KAKENHI 23H02232.

P-444 - ARMC2: A Multifaceted Protein Involved in both Flagella and Cilia Formation and Function.

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In humans and mice, the absence of the ARMC2 protein is linked to Multiple Morphological Abnormalities of the Flagellum (MMAF), characterized by the absence or abnormality (coiled, short, irregular shape) of spermatozoa flagella. Patients with this condition exhibit disorganization of flagellar axonemal microtubules and the absence of the axonemal central pair. Recently, knockout (KO) animals also displayed signs of ciliopathies, such as hydrocephalus and situs inversus, indicating a potential role of ARMC2 in cilia formation and function. To unravel ARMC2's involvement in cilia formation, we examined the ultrastructure and morphology of two multiciliated epithelia expressing Armc2: the trachea and the oviduct. Unlike spermatozoa, no apparent morphological abnormalities were observed at the tissue level in either tissue. However, transmission electron microscopy of the trachea revealed two major defects: disorganization of basal bodies at the plasma membrane and loss of cilia axonemal symmetry. Additionally, scanning electron microscopy showed shorter tracheal cilia in KO mice. Oviductal cilia exhibited no obvious ultrastructural defects, but KO females showed subfertility with a decrease in the number of pups compared to wild-type counterparts. Ongoing experiments analyzing beat frequency and infundibulum cilia pattern, along with cumulus-oocyte complexes collection, will further elucidate the observed female subfertility. Furthermore, histological analysis of Armc2-deleted mouse brains indicated cerebral ventricular dilatation associated with the observed hydrocephaly phenotype. In conclusion, our findings highlight that the ARMC2 protein not only plays a role in flagella formation but also contributes to cilia formation and function, shedding new light on the investigation of ciliopathies.

P-445 - Human Benign Leydig Cell Tumour - Biochemical Evaluation

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Recently we reported expressional alterations in 219 genes of Leydig cell tumours but nowadays there is still a lack of full basic biochemical characteristics of Leydig cell tumours. The discovery of potential biochemical markers for tumour management from early detection, treatments, and control of therapy results may markedly supplement genetics data. Leydig cell micronodules were obtained from patients with azoospermia who were qualified for testicular biopsy. The biochemistry of Leydig cell tumours was analyzed using histological staining and spectrophotometric measurements of total proteins, carbohydrates, lipids and nucleic acids. In addition, the levels of calcium (Ca^{2+}), copper (Cu^{2+}), zinc (Zn^{2+}) and selenium (Se^{2+}) ions were measured. When compared to healthy testis we revealed, for the first time, that in the interstitial tissue with Leydig cell tumours, great amounts of proteins, carbohydrates, lipids, and nucleic acids were dislocated from the seminiferous tubules. Measurements of organic compounds amount showed a decrease ($P < 0.001$) in their content in Leydig cell tumours that may be related to the altered biochemical structure of many of them. We found only changes in Cu^{2+} levels ($P < 0.05$) but not in concentrations of Ca^{2+} , Zn^{2+} , and Se^{2+} in Leydig cell tumours. This specific result may be promising for designing further approaches to manage this tumour based on combining morphological and molecular data.

P-446 - Exploring Oxylipid Changes in Human Sperm During Cryopreservation

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Reactive oxygen species (ROS) have been associated with decreased sperm motility and a high number of defective sperm in the ejaculate. Sperm membranes contain an abundance of polyunsaturated fatty acids (PUFAs), making them especially susceptible to lipid peroxidation. ROS can activate phospholipases, leading to the cleavage and liberation of PUFAs from membrane phospholipids, which can then be oxidized via a combination of non-enzymatic and enzymatic reactions. Signaling by oxygenated derivatives of free PUFAs, also known as bioactive lipids, regulates intercellular communications and plays pivotal roles in signal transduction, inflammation, cell death, and disease. During sperm cryopreservation, there is a burst of ROS that impacts sperm membranes. The study of the oxylipidomic profile may reflect the functional status of sperm cells. This study aimed to detect and quantify changes in the levels of oxylipids during the cryopreservation of human semen samples. To accomplish our objective, semen samples from normozoospermic sperm donors (n=15) were cryopreserved. Routine semen analysis and semen lipidomics were performed before cryopreservation and after thawing. Briefly, the fresh sample was divided into two aliquots. One aliquot was frozen with SpermFreeze™ (Fertipro, Belgium) according to the manufacturer's instructions. After 72 hours, samples were thawed at 37°C for 5 minutes. Lipids from fresh and cryopreserved-thawed samples were extracted using the hexane method followed by solid-phase extraction to obtain the free fatty acid fraction. Oxylipids present in those samples were analyzed by liquid chromatography–mass spectrometry (LC-MS/MS). T-tests were applied to compare both groups ($p < 0.05$). Enrichment pathway analysis was performed using MetaboAnalyst 6.0. The oxylipidomic analysis showed differences in the lipid profile in post-thawed samples. In particular, the enrichment analysis exhibited an activation of the arachidonic acid pathway, with the following oxylipids levels statistically increased after thawing: 12-oxo-eicosatetraenoic acid (12-oxoETE), Prostaglandin E2 (PGE2), and Isoprostanes F2α IV 5-iso PGF2). There was a decrease in the concentrations of 11-hydroxy-eicosatetraenoic acid (11-HETE), 15-hydroxy-eicosatetraenoic acid (15-HETE), and Prostaglandin F2α (PGF2 α), indicating an activation of pro-inflammatory pathways during cryopreservation. At the same time, the concentration of components of the eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) pathway increased (Resolvin D3 and (RvD3), Resolvin E1 (RvE1)) ($p < 0.05$). In addition, a small activation of the linoleic pathway was observed. The modifications of the oxylipid pattern did not correlate with the sperm parameters, as expected in a group of normozoospermic men. We conclude that LC-MS/MS is a sensitive method to detect modifications of the lipid peroxidation status during sperm cryopreservation. A change in the lipidomic phenotype was observed in cryopreserved-thawed samples. Although a prevalence of pro-inflammatory lipids was detected in the cryopreserved-thawed group, resolvins were also detected. The latter are PUFA that may represent specialized pro-resolving mediators in tissue injuries, but their function is not known in sperm physiology. The study of biolipids (oxylipidomics) in sperm under different experimental conditions may be exploited to develop lipid-targeted therapies and strategies to protect cell membranes against oxidative attack.

P-447 - Protective Effects of Monosodium Glutamate on the Preparation of Freeze-Dried Sperm in Mice

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Freeze-dried (FD) preservation of sperm allows long-term storage of genetic resources at room temperature without the use of liquid nitrogen. However, compared to fresh sperm, the success rate of offspring from those FD sperm is reduced by one-third. In this study, we focused on the use of monosodium glutamate (MSG) as a new protective agent for mouse FD sperm, which is a type of amino acid and enables yeast to survive after FD. In mammalian sperm, sugars and chelating agents are mainly used as protectants against FD treatment, but no examples have been found using amino acids. In this study, HTF medium was used as the basic medium. When mouse sperm were preincubated in HTF medium for 1 hour and then replaced with HTF medium containing 0.5-10% MSG, almost all sperm at 5% MSG and all sperm at 10% MSG lost their motility immediately after incubation. To determine the protective effect of MSG during FD treatment, those sperm were freeze-dried just after replacing MSG-HTF, and the appearance and morphology of those sperm after rehydration was observed. Then, those sperm were injected into oocytes and development rate of embryos to blastocyst and its cell number were examined. Some of embryos were transferred into recipient and the birth rate were measured. There was no difference in the appearance of the FD sperm between control and MSG treated sperm after rehydration. However, when embryos were cultured after ICSI, the developmental rate to the blastocysts was significantly increased with increased the concentration of MSG, reaching a maximum of 65% at 3% MSG compared to control (34%; $P < 0.05$). No significant differences were found in the number of cells in the blastocysts between experiments. When 2-cell embryos were transferred to the recipient, the birth rate peaked at 42% (30 pups) at the 3% MSG, more than twice as high in the control (20%; $P < 0.05$, 17 pups). Next, to determine whether the addition of MSG in sperm preincubation medium and FD treatment medium had any positive/negative effects, sperm were incubated in 1% MSG-HTF for 1 hour, followed by FD treatment in the same medium. When ICSI were performed using those sperm, both the developmental rate of embryos to the blastocysts (50%) and offspring (37%) were increased compared to controls (38% and 20%, respectively), though the difference was not statistically significant. This study shows for the first time that MSG, in which usually used for the yeast, also has a protective effect for mammalian sperm against FD treatment. It was also shown that the addition of MSG during preincubation before FD treatment had no adverse effect on FD sperm. Since MSG has been suggested to be involved in membrane protection and antioxidant effects in yeast FD, it is considered to have a similar effect on mouse sperm. Future work is needed to find a new and unique method of freeze-drying mouse sperm to improve the birth rate by referring to freeze-drying methods for more different microorganisms.

P-448 - Optimization of Rehydration Process for Freeze-Dried Mouse Sperm

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Purpose: Freeze-drying is an effective method for the long-term preservation of mouse sperm at room temperature and it plays a crucial role in the conservation of genetic resources. However, freeze-dried (FD) mouse sperm exhibit a significant reduction in birth rates after ICSI to about one-third compared to fresh sperm, and the detailed causes of this reduction remain unclear. This study

aims to elucidate the impact of the rehydration process, previously unexplored, on DNA damage in freeze-dried sperm and following development after ICSI. Methods: Sperm and oocytes from ICR strain mice were used. Freeze-dried sperm were produced using conventional methods. For the rehydration process, we tried the following three methods that affect the soaking speed of the rehydration solution into the cytoplasm of FD sperm. 1) Type and amount of rehydration solution: Distilled water or culture medium (HTF), and the volume were examined. 2) Viscosity of rehydration solution: PVP was added to the rehydration solution at concentrations of 0 to 12% to alter its viscosity. 3) Temperature of rehydration solution: The temperature of the rehydration solution was adjusted from 0.5 to 90°C. In all experiments, the soaking speed of each condition was simply measured using filter paper. DNA damage in FD sperm after rehydration was assessed using the alkaline comet assay. The quality of embryos fertilized with those sperm by ICSI was assessed using abnormal chromosome segregation (ACS) at the two-cell stage, developmental rate to the blastocysts, and birth rates after transfer of two-cell embryos into the recipient female. Results: 1) No significant effects of different rehydration solutions or volume on DNA damage of FD sperm or birth rates were observed. 2) As the viscosity of the rehydration solution increased, DNA damage of FD sperm also increased, leading to a significant decrease in both the blastocyst rate and the birth rate. 3) As the temperature of the rehydration solution increased, a significant reduction in DNA damage of FD sperm was observed, and the highest birth rate was obtained at 50°C (0.5°C: 23%; RT: 22%; 37°C: 29%; 50°C: 37%; 70°C: 31%; 90°C: 35%). Discussion: The various rehydration methods tested, from 1) to 3), suggest that the soaking rate of the rehydration solution is important to prevent/reduce the DNA damage of FD sperm during the rehydration process. While the mechanism remains unclear, a hypothesis was proposed that damage arises from the difference in expansion rates between areas of the nucleus where the soaking of solution has progressed and those where it has not. Interestingly, normal fertilization rates decreased when rehydration was performed with rehydration solutions above 70°C, but when those oocytes were artificially activated after ICSI, the birth rate of those embryos did not reduce. These results suggested that while DNA damage could be reduced with increased temperature or soaking rate, thermal denaturation damage occurred to the protein of FD sperm. This study shows for the first time that improving and optimizing the rate of soaking speed of rehydration solutions could lead to a much higher success rate of producing offspring from FD sperm. Future efforts should focus on minimizing DNA damage not only in the drying process but also rehydration process to achieve birth rates comparable to those of fresh sperm.

P-449 - Proteins of Huntington's Disease Pathway Participate in Pig Sperm Capacitation

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We have reported previously that proteins of neurological disorder-associated (NDA) pathways, such as Huntington's disease (HD) and Parkinson's disease, are highly represented in both the human and porcine sperm zincoproteomes. Therefore, it is plausible that NDA pathway proteins may play an important role in sperm physiology, especially in sperm capacitation which can be

modulated in artificial reproductive therapies/technologies (ART). With this hypothesis and aim in mind, we used small molecule inhibitors of HD pathway proteins during pig in vitro capacitation (IVC) and monitored changes in sperm motility, velocity, and movement parameters by using computer-assisted semen analysis (CASA); as well as capacitation status – monitored with our zinc signature assay; sperm viability – monitored with nuclear propidium iodide (PI) incorporation; acrosomal integrity probed by fluorescently labeled peanut agglutinin (PNA); and cholesterol efflux assessed with BODIPY-cholesterol. We used the following small molecule inhibitors: Oligomycin A (cat # 41-105, R&D Systems; inhibitor of ATP synthase F0 in mitochondria), Azoxystrobin (cat # NC1774837, Chem Service; inhibitor of cytochrome b-c1 complex in mitochondria), Dynarrestin (cat # HY-121802; Medchemexpress; inhibitor of dynein 1 and 2 in axoneme), and Pitstop 2 (cat # HY-115604; Medchemexpress; inhibitor of amphiphysin with clathrin interaction in Golgi/acrosome). We report that the addition of Oligomycin A or Azoxystrobin in IVC media had a significant inhibitory effect on sperm motility ($P < 0.05$), including total and progressive motility; curvilinear, average path, and straight line velocities; linearity, straightness, and beat cross frequency when compared to vehicle control IVC spermatozoa. Furthermore, both Oligomycin A and Azoxystrobin significantly decreased ($P < 0.05$) the capacitation-associated sperm zinc efflux and PI incorporation into the nucleus. Spermatozoa incubated with Azoxystrobin during IVC had a significantly higher ($P < 0.05$) percentage of intact acrosomes, while Oligomycin A treatment significantly decreased cholesterol efflux during IVC. Spermatozoa capacitated with Dynarrestin exhibited significantly decreased ($P < 0.05$) curvilinear velocity, amplitude of lateral head displacement, and increased cholesterol efflux when compared to IVC vehicle control. Lastly, a significant reduction ($P < 0.05$) of zinc efflux was observed in Pitstop 2 IVC spermatozoa. Our results provide the first functional evidence of the participation of NDA pathway proteins in sperm capacitation. The reversible nature of the selected inhibitors enables them to be used as modulators of semen extenders, insemination doses, or in vitro fertilization media for both human and animal ART. Supported by USDA-NIFA grant 2021-67015-33404 (PS) and 2022-67015-36298 (KK), MU Research Council grant (PS), and MU CAFNR Joy of Discovery Seed Grant program (PS).
Keywords: Sperm Capacitation, Neurological Disorder-Associated Pathways, Zincoproteome, Pig, Small Molecule Inhibitors

P-450 - CCDC183 is Essential for Axonemal Compartmentalization during Spermiogenesis

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Sperm flagellum plays a crucial role in male fertility. By using a flagellum as a driving force, spermatozoa travel long distances to reach female gametes. An axoneme, a motility apparatus composed of a “9+2” microtubule arrangement, is found throughout the sperm tail except for the endpiece. The “9+2” arrangement is composed of nine outer doublets surrounding a pair of single central microtubules. Axoneme extension from the distal centriole localized on the cell surface

causes the plasma membrane to protrude from the cytoplasm. The centriole then migrates and associates with the nuclear envelope to bridge the plasma membrane with the nucleus. The centrioles and nucleus then move toward the cell surface on the opposite side. This causes an inward folding of the centriole-associated plasma membrane called cytoplasmic invagination, which allows compartmentalization of the flagellum. Although this cytoplasmic invagination and axonemal compartmentalization in early spermatids has been observed for a long time, it has not been studied in depth until now. Here, we generated *Ccdc183* knockout mice using the CRISPR/Cas9 system to reveal the protein function of the testis-specific protein CCDC183 in spermiogenesis. We demonstrated that CCDC183 absence causes male infertility with morphological and motility defects in spermatozoa. Owing to the lack of CCDC183, centrioles after elongation of axonemal microtubules do not connect to the cell surface and nucleus during spermiogenesis, which causes subsequent loss of cytoplasmic invagination around the flagellum. This results in abnormal compartmentalization of the axoneme, and cytosol-exposed axonemal microtubules collapse during spermiogenesis. In addition, ectopic localization of accessory structures, such as the fibrous sheath and outer dense fibers, and abnormal head shape as a result of abnormal sculpting by the manchette are observed in *Ccdc183* knockout spermatids. Our results indicate that CCDC183 plays an essential role in axonemal compartmentalization to form functional spermatozoa during spermiogenesis.

P-451 - Unveiling Sperm Capacitation Dynamics: A Novel Spectral Flow Cytometry and Stochastic Modeling Approach

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Mammalian sperm undergo a maturation process known as capacitation, either in the female reproductive tract or under in vitro conditions, to become competent for fertilization. Despite advancements in understanding capacitation's molecular mechanisms, challenges persist, notably in high-precision sperm selection for assisted reproductive technologies like intracytoplasmic sperm injection. The phenotypic heterogeneity and dynamic changes exhibited by capacitating sperm pose difficulties for selection, with sperm heterogeneity and the role of sperm subpopulations in fertility remaining largely unexplored. Currently, there are no quantitative models and few experimental techniques to explore large-scale signaling kinetics or the emergent properties of subpopulations. Here, we introduce a novel 'high-dimensional' culture array system combined with spectral flow cytometry to analyze sperm phenotype heterogeneity at single-cell resolution in large cell populations and examine the dynamic interplay between intracellular calcium levels and the acrosome reaction in capacitating mouse epididymal sperm. Additionally, we employ stochastic modeling to predict individual sperm capacitation trajectories from cell population-scale data. This approach not only fills a crucial gap in sperm biology research but also

sets the stage for advanced 'next generation' male fertility diagnostics, with implications for clinical, agricultural, and basic reproductive biology research.

P-452 - Age-Associated Metabolomic Changes in Human Spermatozoa

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Advancing age is associated with a significant decline in fertility in men. Globally, aging associated reproductive health and wellbeing are assuming considerable importance as lifestyle changes have led to delayed childbearing and an increasing number of advanced age couples seeking fertility treatment. For example, the number of men between 35 and 54 years of age fathering children has increased 25% since 1980 in the United States. Understanding age-related changes in spermatozoa is crucial for addressing fertility challenges in aging men. Very limited attention has been focused on age-related functional genomics, especially metabolomic changes in human spermatozoa. The present study addressed this issue. The study involved 18 fertile Arab/Saudi men aged 21 to 51 years. The participants underwent clinical assessments at King Abdulaziz University Hospital, Jeddah, and were categorized into three age groups: young adult (21–30 years; n = 6), late adult (31–40 years; n = 7), and advanced age (41–51 years; n = 5). Semen samples were gradient purified, and purified spermatozoa were subjected to untargeted metabolomic analysis using LC-MS/MS. The spectral data were processed using XCMS and the metabolites were identified using the Human Metabolome Database (HMDB). Statistical and differential analyses were performed using MetaboAnalyst. Mean ($\pm$ SD) ages among the three groups were different (27.8 ± 2.6 years, 34.3 ± 2.9 years, and 47.2 ± 4.2 years; $P < 0.05$). Mean sperm concentrations were higher ($P < 0.05$) in late adult group (85.4 ± 22.0 M/ml) than young adult group (55.3 ± 25.5 M/ml) but comparisons for the advanced age group (78.0 ± 38.2 M/ml) were not significant. No significant differences were found in other semen analysis endpoints. Metabolomic analysis revealed 469 peak intensities on LC-MS/MS in the three groups. From these peaks, 380 metabolites were identified through HMDB, whereas 89 peak intensities could not be identified. The principal component analysis of 380 metabolites showed distinct separate clustering of metabolite expression for young adult and advanced age groups whereas the data for late adult group aligned intermediately with both groups. The ANOVA analysis showed significant differences ($P < 0.05$) in 167 metabolites among the three age groups. Using a fold-change threshold of ≥ 2 , pair-wise analysis showed differential abundance of 31 metabolites in young adult vs. late adult groups (23 upregulated, 8 down regulated), 179 metabolites in young

adult vs. advanced age groups (96 upregulated, 83 down regulated), and 39 metabolites in late adult vs. advanced age groups (16 upregulated and 23 down regulated). Comparing the young adult group with advanced age group, 179 metabolites exceeded variable importance in projection (VIP) score ≥ 1 indicating their significant contribution to group differentiation. Among these top VIP scored metabolites, L-homocysteine, L-cysteine, (S)-hydroxyoctanoyl-CoA, glutathione, and acetyl CoA were downregulated, and HMG-CoA, pentadecanoyl-CoA, CL(22:6/20:4/22:6/22:6), PIP(TXB2/22:3(10Z,13Z,16Z)), and uracil were upregulated in the advanced age group. Of note was almost complete absence of homocysteine in the spermatozoa of all the men in advanced age group. These deregulated metabolites are involved in crucial processes of sperm physiology including energy metabolism, sperm development and function, mitochondrial integrity and function, antioxidation, cholesterol metabolism, etc. In conclusion, dynamic metabolomic changes in advancing age potentially impact crucial human sperm physiological functions affecting normal sperm fertility. Acknowledgement: The authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this research work through project number 1132.

P-453 - Bio-Electrosprayed Mammalian Sperm Remain Viable

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Bio-electrospray (BES) is a very attractive technique for depositing desired biomolecules or solutions containing various cell types and other targets through the principle of applying a high voltage to a conducting needle accommodating the flow of a target containing' suspension. The research presented explores the effectiveness and safety of BES in handling one of the most specialized cell in the body, the mammalian sperm. In this work, bovine and human sperm are handled using BES technology with diagnostic sperm assessment before and after application of laminin, a major extracellular glycoprotein, with known important roles in cell differentiation, migration and adhesion. Compared to current storage methods of sperm using slow freezing or vitrification where a large proportion of motile spermatozoa become immotile, we demonstrate that BES technology results with very low loss of sperm motility and morphokinetic parameters. This preservation is controlled by the capacity for spermatozoa to be compartmentalized which would allow them to be frozen and thawed without inflicting any negative aspects on those encapsulates. Further work into functional survival of sperm cells will also be discussed in terms of fertilization and embryo development. BES therefore seems to be a promising method for encapsulating and storing spermatozoa to use later, which will be helpful in application where there is a low number of motile spermatozoa available such as in patients with oligozoospermia, or in agriculture and aquaculture industries for animal species of high genetic merits.

P-454 - Development of a Plasmid Repository Encoding Oocyte Membrane Proteins to Unveil Novel Sperm-Oocytes Interactions: A Focus on Human CRISP

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In an era marked by heightened fertility concerns, exploring the intricate interaction between sperm and oocyte proteins is a captivating pursuit. While several sperm proteins have been identified as essential for fertilization, the corresponding protein partner on the oocyte membrane (oolemma) is known only for a subset of these proteins. In this context, Cystein-Rich Secretory Proteins (CRISP) are particularly interesting as they are primarily expressed in the male genital tract of mammals and are known to play key roles in fertilization. In humans, three CRISP have been identified. CRISP1 and 3 are secreted by the epithelium of the epididymis and associate with the surface of spermatozoa during their epididymal transit, whereas CRISP2 is of testicular origin and expressed *ab initio* in spermatozoa. Both CRISP1 and CRISP2 are involved in gamete fusion, interacting with common and yet unidentified complementary sites in the oolemma. Interestingly, in rats, CRISP1 interaction with the oolemma was shown to be mediated by a small region of 12 amino acids corresponding to an evolutionarily conserved structural motif among CRISP. In this study, we analyzed published human and murine oocyte proteomes using various software tools for predicting sub-cellular localization and topology. This analysis led to the compilation of a catalog of around 300 oocyte membrane proteins, each featuring at least one ecto-domain. Using Gateway cloning, the coding sequences of these proteins were integrated into vectors allowing the expression of the proteins in fusion with GFP. These plasmids were transfected into HeLa cells, each pool of cells expressing a membrane oocyte protein fused with GFP. To identify CRISP oocyte interactor(s), these cells are screened with synthetic peptides of both human CRISP1 and CRISP2 corresponding to the 12 amino acid region involved in the interaction with the oolemma. We first validated the use of these peptides on human oocytes. Our findings demonstrated that CRISP1 and CRISP2 peptides exhibited binding affinity to the oolemma, whereas a control peptide from a different region of CRISP2 failed to bind. We believe our plasmid library and methodology will contribute to identifying egg CRISP interactor(s) as well as to uncover novel protein interactions between oocytes and spermatozoa.

P-455 - Physiological Characterization of Purinergic Signalling in Mouse Seminiferous Tubules Using an ATP Biosensor

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Purinergic signaling mechanisms mediate important physiological processes within the seminiferous tubules. However, detecting release of purinergic signals such as ATP has been challenging due to several technological limitations of available approaches. Here, we investigate purinergic signalling processes in mouse seminiferous tubules. Using the novel ATP biosensor cATP1.0, we monitor extracellular ATP release in intact whole-mount preparations of mouse testis. Live-cell recordings in a custom-designed ex vivo imaging chamber enable observation of local ATP release and ATP-dependant signals in defined areas of the seminiferous tubules. Ongoing analysis aims to identify potential correlations between ATP release and tubule contractions. Together, our results will enhance our understanding of testicular purinergic signalling and provide further insights into male reproductive physiology.

P-456 - Fine-Tuning Inbreeding Analysis Reveals Genomic Regions Associated with the Abundance of Kinetic Subpopulations in Fresh and Frozen Stallion Sperm

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Sperm quality is a major contributor to the fertility of livestock animals. This issue is even more important in stallions since this species is well-known for its moderate fertility in comparison with domestic animals. But also, stallions show a lack of sperm freezability in nearly 30% of the horses. Inbreeding depression, triggered by the mating of close relatives, was demonstrated as a cause of a decrease in sperm quality in stallions, but no studies were yet performed assessing its effect on the kinetic subpopulation patterns in stallion sperm. We determined the effect of homozygosity on sperm kinetic subpopulations in fresh and frozen/thawed samples from 18 Pura Raza Española stallions. To this, we first analyzed 148 sperm samples (8 per stallion, 72 fresh, and 72 frozen/thawed) using the motility module in a Sperm Class Analyzer (SCA™, Microptic, Spain). Three velocities (VCL, VSL, and VAP), three composite indexes (STR, LIN, and WOB), and two traits associated with sperm head movement (ALH and BCF). Thereafter, we performed a two-step clustering discriminant analysis to determine the existence of sperm kinetic subpopulations, after which each spz was allocated to one of these. All the individuals were also genotyped using an HD-SNP array (Axiom670K, Thermofisher™) to determine a runs-of-homozygosity-based inbreeding value (FROH) at the genomic and chromosomal level. Finally, we correlated the inbreeding values with the abundance of each sperm subpopulation in the fresh and frozen/thawed samples separately. The phenotypic analyses revealed the existence of three different sperm subpopulations: Sp1, including slow but progressive spz; Sp2, including fast and progressive spz and Sp3, including medium-fast but not progressive spz. Genomic analysis showed a large variability in terms of homozygosity, ranging from 1.65 to 22.21% at the genome level, but also at the chromosome level. For instance, FROH ranged between 0 and ~24% in ECA11 and between 0 and ~60% in ECA18 and ECA23. In fresh samples, most of the correlations between FROH and SP abundance were positive in Sp1 (commonly associated with moderate fertility) and negative in Sp2 (which is the sperm motility pattern most associated with fertilization success). But also, the magnitude of the association varied greatly among chromosomes, being the increase of FROH in

ECA4 and ECA15 being the most associated with a decrease in Sp2. In frozen samples, FROH increase was mostly associated with an increase in SP1, but results in SP2 were much more variable and with a lesser magnitude. On the contrary, the increase in FROH in ECA14 and ECA23 was associated with a decrease in the abundance of Sp3 (associated with an increase in fertilization failure due to a lack of progress and premature capacitation triggered by the freezing process). Overall, this preliminary study demonstrated that the increase of inbreeding in certain chromosomes is associated with changes in the sperm kinetic subpopulation patterns in stallion sperm, in particular in fresh samples, where the abundance of sperm subpopulations associated with increased fertility declined with the increase of genomic inbreeding. In addition, our results suggest that inbreeding can affect differentially the freezability of stallion sperm. Further studies on a large stallion dataset are being developed to validate these results.

P-457 - Tex46 Knockout Male Mice are Sterile Secondary to Sperm Head Malformations and Failure to Penetrate Through the Zona Pellucida

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Each year, infertility affects 15% of couples worldwide, with 50% of cases attributed to men. It is assumed that sperm head shape is important for sperm-zona pellucida penetration but research has yet to elucidate why. We generated testis expressed 46 (Tex46) knockout mice to investigate the essential roles of TEX46 in mammalian reproduction. We used RT-PCR to demonstrate that Tex46 was expressed exclusively in the male reproductive tract in mice and humans. We created Tex46^{-/-} mice using the CRISPR-Cas9 system and analyzed their fertility. Tex46 null spermatozoa underwent further evaluation using computer-assisted sperm analysis (CASA), light microscopy, and ultrastructural microscopy. We used immunoblot analysis to elucidate relationships between TEX46 and other acrosome biogenesis-related proteins. Mouse and human TEX46 are testis-enriched and encode a transmembrane protein which is conserved from amphibians to mammals. Loss of the mouse TEX46 protein causes sterility due to abnormal sperm head formation and decreased motility. Tex46 null spermatozoa morphologically lack the typical hooked sperm head appearance and fail to penetrate through the zona pellucida. Electron microscopy of the testicular germ cells reveals malformation of the acrosomal cap, with misshapen sperm head tips and the appearance of a gap between the acrosome head and the nucleus. TEX46 is essential for sperm head formation, sperm penetration through the zona pellucida, and male fertility in mice and is a putative contraceptive target in men.

P-458 - The Antioxidative Effect of MntBAP on Boar Sperm Cryopreservation

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Antioxidants protect cellular function and structure by neutralizing the oxidative stress caused by increased ROS during sperm freezing. Cryopreservation has been studied with antioxidants such as superoxide dismutase, melatonin, vitamins, polyphenols, carotenoids, and minerals, and demonstrated encouraging results. Many studies have utilized mentioned antioxidants to increase the efficiency of sperm freezing and improve the success rate of artificial insemination and pregnancy. MnTBAP (Manganese(III) meso-tetrakis(4-benzoic acid) porphyrin) is one of the newly synthesized antioxidants that has been reported to show positive effects on sperm morphology and capacitation in human, ram, and stallion. In this study, porcine semen was treated with 0, 50, 100, and 150uM of MnTBAP based on Tris-egg-yolk extender and frozen to determine whether MnTBAP can assist the status of sperm during cryopreservation. After thawing, motility, viability, acrosome integrity, mitochondrial potential (MMP), caspase activity, membrane lipid disorder, DNA fragmentation, capacitation ability, and embryo development were measured. First, motility was assessed using the CASA system (Computer-assisted sperm analysis), and the 100uM treatment group (66.8%) showed the highest motile rate compared to the control group (51.1%), so the remaining analyses were conducted comparing the two groups ($P < 0.05$). The following fluorescence staining was applied to examine the control and 100uM group by fluorescence microscopy, and it was found that the viability stained with SYBR-14 and propidium iodide (PI) was 41.7% vs 62.4% and the acrosome integrity stained with Pisum sativum agglutinin and fluorescein isothiocyanate was 77.9% vs 86.4%, which was a significant difference ($P < 0.05$). In addition, mitochondrial membrane potential (MMP) measured by rhodamine 123 and PI were 46.5% vs 51.9%, the fragmented rate estimated by the Sperm-sus-halomax kit was 63.4% vs 57.4%, and caspase activity detected by staining with sulforhodamine was 30.1% vs 22.9%, which tended to be higher in the treated group but did not show statistical significance. Finally, to investigate the effect on fertilization and embryo development in vitro, the straw was dissolved and treated with 2.5×10^7 cells/ml. As a result, the cleavage rate was 77.6% vs 84.1% and the blastocyst rate was 9.7% vs 11.4% ($P < 0.05$). In conclusion, these results suggest that antioxidant treatment has a positive effect on sperm freezing-thawing experiments, which increases fertilization capacity and leads to increased embryo development. This study was supported by the National Institute of Wildlife Disease Control and Prevention as a “Specialized Graduate Scholl Support Project for Wildlife Diseases Specialists”.

P-459 - A Molecular Mechanism of Transcriptional Activation by a Mouse Testis-specific Long Noncoding RNA, Tesra

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Many functional long noncoding RNAs (lncRNAs) are expressed in the mouse testis, but detailed molecular mechanisms of their action are poorly understood. We identified Tesra, a mouse testis-specific lncRNA, at the mouse Prss/Tessp locus and found that it is localized in both germ cells and Leydig cells. In this study, based on the knowledge that lncRNAs often regulate the expression of neighboring genes, we tested the possibility that Tesra regulates expression of the Prss/Tessp genes and investigated a molecular mechanism underlying it. We first performed the chromatin isolation by RNA purification (ChIRP) assay and found that Tesra bound to chromatin region of the Prss42/Tessp-2 promoter in germ cells from the testis. Moreover, the overexpression of Tesra increased the Prss42/Tessp-2 expression level and its promoter activity in the in vitro cell culture system. Thus, the Prss42/Tessp-2 gene, which is specifically expressed in spermatocytes, is likely to be a target of Tesra. To reveal a molecular mechanism underlying the regulation of the Prss42/Tessp-2 gene by Tesra, we searched for Tesra-binding proteins by the Ribotrap assay and the mass spectrometry analysis. One candidate protein, polypyrimidine tract binding protein 2 (PTBP2), was expressed in correlation with both Tesra and Prss42/Tessp-2 during testis development. Since both Tesra and PTBP2 are known to be localized in the nucleus of spermatocytes and our RNA immunoprecipitation (RIP) assay revealed the interaction between PTBP2 and Tesra in the nucleus of testicular germ cells, we next investigated the involvement of PTBP2 in the gene regulation by Tesra. We established the in vitro reporter gene assay system in which the expression of Tesra was induced by the Tet-on system and thereby, Prss42/Tessp-2 promoter activity was enhanced to drive the luciferase gene. The addition of doxycycline to this system induced the Tesra expression and increased luciferase activity, and the knockdown of PTBP2 significantly decreased luciferase activity. Lastly, we confirmed that PTBP2 bound to chromatin at the Prss42/Tessp-2 promoter in germ cells from the testis by the chromatin immunoprecipitation (ChIP) assay. Taken together, our results suggest that Tesra activates transcription of the Prss42/Tessp-2 gene in spermatocytes and that PTBP2 contributes to the Prss42/Tessp-2 transcriptional activation by Tesra. Because the Prss42/Tessp-2 gene is involved in the regulation of meiotic progression, Tesra may be an important regulator of spermatogenesis.

P-461 - Time-Dependent Changes in PI3K/AKT Signaling During Capacitation in a Mouse Model

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Sperm must undergo functional maturation through the process of capacitation to gain the ability to fertilize. During capacitation, numerous proteins are involved and are either phosphorylated or dephosphorylated. However, the molecular mechanism of capacitation has not been clearly identified. The present study aimed to identify changes in PI3K/AKT-related proteins over various time periods (0, 30, 60, and 90 min) in a capacitation-inducing medium. Mouse spermatozoa were cultured in media supplemented with bovine serum albumin (BSA) to induce capacitation, and various sperm functions were assessed. The results showed no significant difference in cell viability

and sperm motility; however, significant increases in several kinematic parameters were observed after 90 min of incubation. In addition, acrosome reaction and capacitated spermatozoa were observed to increase in a time-dependent manner. Moreover, the expression of protein kinase A and tyrosine phosphorylation significantly increased. Interestingly, phosphorylation of PI3K/AKT-related proteins was found to increase overall depending on the incubation time. These results suggest that PI3K/AKT-related proteins may play an early and essential role during capacitation, regulating the functions of mouse spermatozoa. Our findings will contribute to further understanding of the interactions and molecular mechanisms of the PI3K/AKT signaling pathway in the capacitation process.

P-462 - Assessing of Fertilization and Developmental Ability of Mouse Spermatozoa Head Separated from Tail by Freeze-drying Treatment

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PURPOSE: Long-term preservation of mammalian sperm is needed worldwide for the strain maintenance. However, cryopreservation using liquid nitrogen is expensive to maintain and there is a risk of sperm loss due to accidents or disasters. Freeze-drying (FD) technology can easily preserve sperm at room temperature for more than one year. However, the birth rate of FD sperm is about 20%, which is significantly lower than that of fresh sperm. It is necessary to select FD sperm which has the normal developmental ability for the improvement of the experimental efficiency, but no such method exist yet. Recently, we identified a number of spermatozoa whose heads and tails had been separated by FD treatment (Yang, Ito et al., JRD 2023), but their fertilization and developmental ability have not yet been compared between separated and intact sperm. In this study, we examined whether the sperm head separated from the tail by FD treatment had normal fertility and developmental potential after ICSI. **METHODS:** Sperm were collected from ICR and B6 strains, and FD sperm were prepared using conventional method. First, the rates of tail-separated (head-only) sperm and tail-connected (intact) sperm in fresh, freeze-thawed (FT), and FD sperm were compared. 0.5 M trehalose was added in a part of the sperm suspension to investigate whether the difference of sperm suspension affect those rates and sperm quality. ICSI was performed using head-only FD sperm or intact FD sperm. To examine the extent of DNA damage of FD sperm after fertilization, the brightness of γ -H2Ax expressed in the male pronuclei were measured by immuno-staining. To examine the quality of embryos, the frequency of abnormal chromosome segregation (ACS) was observed at the 2-cell stage, or the 2-cell stage embryos were transferred into recipient female mice and examined offspring rate. **RESULTS:** The rates of head-only sperm in FD group (ICR:29% and B6:45%) were higher than those in fresh group (ICR:2% and B6:18%). In γ -H2Ax assay and ACS frequency, there was no significant difference between head-only FD sperm and intact FD sperm. When trehalose was added, the rate of head-only FD sperm decreased but DNA damage was not improved. The head-only sperm rate was affected by the difference of sperm suspension though trehalose did not have a role as FD protectant. Next, the fertilization rate and the developmental rate to the 2-cell stage of embryos derived from head-only sperm were also similar with those of intact sperm. The offspring rates of embryos fertilized with head-only sperm were comparable to those of intact sperm (ICR:21% vs. 33%; B6 31% vs. 31%, respectively). **DISCUSSION:** The higher rate of head-only sperm in B6 than in ICR indicated that the

tolerance to physical damage was different between mouse strains. However, regardless of mice strains, this damage is likely only in the neck region since birth rates were comparable between head-only sperm and intact sperm. On the contrary, in general of mouse ICSI, the tail must be cut off and only the sperm head are injected into oocytes. Therefore, the efficiency of ICSI can be greatly improved if sperm tail has been cut beforehand. In this study, we concluded that head-only FD sperm is viable for obtaining next generation and the separation phenomena of the sperm head and tail proved to be of value for a variety of uses including the improvement of ICSI efficiency.

P-463 - Advancements in Mouse Testis Organoid Formation and Characterization: A 3D In vitro Model for Studying Spermatogenesis

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Testis is a complex organ where different cells interact to achieve healthy sperm production. Several factors can compromise this process, leading to fertility failure. However, studying these complexities in vivo can be challenging, necessitating the use of simpler systems to understand the dynamics between cells during spermatogenesis. Organoids offer a promising solution by providing an in vitro model that mimics in vivo processes. While many organs have been successfully replicated using organoids, creating functional testis organoids has remained elusive. To address this gap, we have developed a novel protocol for generating mouse testis organoids in vitro. Our protocol involves forming and maintaining organoids from a testis cell suspension. Organoid formation was achieved by aggregating 1.2×10^6 cells isolated from a mix of 10-day post-partum mouse testes, enriched with 5×10^5 germ cells from a mix of 16-day mouse testes, in AggreWell™400 plates, yielding approximately 1200 organoids per well. We utilized Dazl-EGFP transgenic mice alongside wild-type mice in our experiments. These transgenics were produced by our research group and contain the promoter of Dazl (2 kb sequence in the 5' flanking region of the mouse Deleted in Azoospermia-Like –Dazl- gene) expressing EGFP. They exhibit high expression of EGFP in spermatocyte stages, with weaker expression in spermatogonia and round and elongating spermatids. Following formation, organoids were collected and cultured for up to 35 days in small groups (approximately 15-20 organoids) in spermatogenesis culture medium at 34°C to foster the development and differentiation of testis cells. Over time, small organoids spontaneously aggregated to form larger structures. We conducted structural analysis of 5 µm waxed organoid sections using Hematoxylin/Eosin staining, identified apoptotic cells using the TUNEL assay, and determined the distribution of different testis cells through immunohistochemistry. Additionally, we analyzed the expression of specific spermatogenesis-related genes using qPCR at various points during in vitro culture and compared them with in vivo testis expression levels. Our analyses revealed a higher concentration of cells in the periphery of the organoids, surrounded by isolated or small clusters of cells in the central region. TUNEL analysis showed a small number of apoptotic cells in small organoids, with an increased presence of compromised cells as the organoids grew. Immunohistochemistry indicated an internal localization of Leydig and peritubular cells, while Sertoli and germ cells were primarily located externally, with the testis barrier (Collagen IV) serving as a reference point. Additionally, acrosome reaction was observed on the exterior of the organoids.

The use of Dazl-GFP transgenic mice facilitated monitoring the location and progression of germinal cells during the culture process through EGFP expression. These results demonstrate the successful maintenance of testis cells in vitro, with cells aggregating and localizing strategically to preserve testis structure and function. Our 3D in vitro model presents a valuable tool for studying cell-to-cell interactions and the spermatogenesis process, offering a simpler alternative to in vivo studies.

P-464 - 3D Robust Spatiotemporal Molecular Model of Acrosome Biogenesis

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Biogenesis of acrosome is a unique cellular process which taking place in the testis during the process of spermiogenesis (final stage of spermatogenesis during which round spermatid develops into the testicular spermatozoa). As a unique cellular process, it includes large amount of cell-specific proteins and their organization into the complex cytoskeletal, membranous and acrosome matrix structures. The aim of the presented project is a spatiotemporal characterization of the process in the mice model under physiological and pathological conditions with emphasis on 3D microscopic analysis of cleared seminiferous tubules. Here, utilization of the massive parallel image processing on the seminiferous tubules volumetric data enables reconstruction of the individual (sub)cellular components (e.g. acrosome, Golgi complex, nucleus) as 3D surface models. Furthermore, specific monoclonal antibodies, molecular dyes and mice transgenic models allow molecular profiling of individual structures of interest. Finally, the correlation of obtained massive parallel 3D imaging data with various standard laboratory techniques (e.g. electron/holographic microscopy, immunocytochemistry, immunohistochemistry, flow cytometry, immunoblot) enables building robust and complex models of the acrosome biogenesis process and analyze its individual stages from new perspectives. Such models would further improve our understanding of the natural acrosome biogenesis and (sub)cellular and molecular etiopathogenetic mechanisms of its disrupted development. The work was supported by Grant Agency of the Czech Republic GACR 23-06591S and by Institutional support of the Institute of Biotechnology RVO:86652036.

P-465 - Spermiogenesis in 3D: Pitfalls and Challenges

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In recent years, there has been a large breakthrough in imaging and data processing methods. One of them, volume imaging, presents a powerful tool to visualize (sub)cellular structure in its tissue context and enable us to explore its spatiotemporal changes. Moreover, using serial sections and 2D microscopy can lead to loss of information and incomplete knowledge of dynamics of (sub)cellular processes, e.g. spermiogenesis. So far, an atlas of sperm development in 3D is still

missing even though the stages of spermiogenesis are well-known and described in testicular sections. In our research, we utilized the CLARITY clearing technique which provides great optical properties and mechanical support to our samples. We utilized existing protocols to detect individual stages of spermiogenesis in individual seminiferous tubules (iSTs) under transmitting light. Next, we detected and analysed DAPI (nuclei) and PNA (acrosomes) signals in iSTs using confocal microscopy (LSM 880). Even though we obtained optically transparent and well-stained samples, we faced serious issues during the data processing. We employed commercially available Imaris software to render and statistically evaluate surface-rendered nuclei from individual stages of spermiogenesis. Leading information for the surface creation is the signal intensity. Therefore, nucleoli are more likely to be rendered than the whole nucleus as their staining is more intense. This results in an artificially increased number of nuclei in the sample. Secondly, iST contains various cells with different sizes and shapes of the nucleus (15 μm vs 5 μm , round shape vs oval). This poses a challenge for proper filter settings to capture all nuclei as individual objects. In the presented work, we focused on optimizing surface rendering in Imaris software, so it is suitable to describe spermiogenesis. This approach can lead to new insights into mechanisms behind physiological and pathological spermiogenesis and help us understand male infertility. Moreover, we compared the surface rendering abilities of Imaris with various software using diverse algorithms to visualize individual steps of spermiogenesis in 3D. The work was supported by Grant Agency of the Czech Republic GACR 23-06591S and by Institutional support of the Institute of Biotechnology RVO:86652036.

P-466 - Single Cell RNA Sequencing of Testicular Tissues from Infertile Men with Loss of Function Variants

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Cellular and molecular alterations underlying male infertility remain largely unknown. As a result, about 70% of men are not receiving a causal diagnosis for their condition. While an increasing number of genetic causes has been associated with male infertility in recent years, functional importance of identified candidate genes remains to be assessed. Prominent candidate genes include those involved in meiotic recombination. In line with this, impairment of meiotic recombination often leads to an arrest of spermatogenesis at meiosis and thus, male infertility. During meiotic recombination, heterodimers of MSH4 and MSH5 bind to the DNA and stabilize double Holliday junctions. Moreover, proteins forming part of the mammalian ZSS complex, namely SHOC1, TEX11, and SPO16, interact with M1AP and support stabilization of early recombination intermediates, enabling crossover formation. Here, we aimed to assess the cellular and molecular alterations caused by homozygous loss-of-function (LOF) variants in spermatogenesis-relevant

genes involved in meiotic recombination. To this end, we generated single cell RNA sequencing (scRNA-seq) datasets of testicular tissues obtained from men with full spermatogenesis (controls; n=3) and men with germ cell differentiation failure associated with LOF variants in MSH5, SHOC1, and M1AP (n=3). Patients underwent testicular biopsy at the Centre of Reproductive Medicine and Andrology in Münster for evaluation/treatment of azoospermia. Control group data was supplemented with two previously published datasets generated elsewhere. Following preprocessing and an established bioinformatic workflow, we obtained RNA expression data for controls (~24,000 cells) and the cases (~15,000 cells). According to respective marker gene expression, these cells were assigned to germ cell (spermatogonia, spermatocytes, and spermatids) or somatic cell types (Sertoli, Leydig, perivascular, endothelial, peritubular, and immune cells). Interestingly, in the patient with a LOF variant in MSH5 histological analyses showed that 41% tubules displayed spermatogonial arrest, 23% showed a spermatocyte arrest, and no tubules contained spermatids. In line with these histological results, majority of germ cells captured by scRNA-seq (>85%) were assigned to spermatogonial stage. In contrast, testicular tissues from patients with LOF variants in M1AP and SHOC1 showed a prominent spermatocyte arrest pattern, with 76% and 93% of seminiferous tubules displaying this phenotype, respectively. Consistently, scRNA-seq data of these patients showed decreasing cell numbers from pachytene-stage onwards, as 90% and 98% of their germ cells were assigned to spermatogonia or early spermatocytes. Furthermore, we investigated cell-specific expression of the mutated genes in all samples. Expression of MSH5 and M1AP was detected in the controls but not in the patients carrying the respective LOF variant, while the patient with SHOC1 LOF variant showed expression of the mutated gene, aligning with the prediction that this variant only leads to slight shortening of the resulting protein and thus, likely does not cause a complete loss of function. In addition, the expression of ZZS members TEX11 and SPO16 was analyzed. As expected, TEX11 was specifically expressed by spermatocytes and early spermatids whereas SPO16 expression was observed in spermatogonia and spermatocytes in all patients. In conclusion, LOF mutations in MSH5 and M1AP, but not SHOC1, seem to ablate the expression of these genes in the germline. Moreover, these datasets reveal transcriptomic changes caused by disturbance of meiotic recombination genes in human testis and elucidate their role in male infertility, providing the basis to uncover the cellular and molecular alterations caused by the LOF variants via differential gene expression and trajectory analyses.

P-467 - In Vitro Assessment of Di(2-Ethylhexyl) Phthalate Induced Alterations in Epididymal Semen Quality and Sperm-Oviduct Binding Efficiency in Dromedary Camels (*Camelus dromedarius*)

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Dromedary camels (*Camelus dromedarius*) play a vital role in the lives of countless communities across arid and semi-arid regions, providing both economic sustenance and cultural significance. Unfortunately, *Camelus dromedarius* frequently face infertility challenges due to various known and unknown factors. Among these potential threats, endocrine disruptors like di(2-ethylhexyl) phthalate (DEHP) have emerged as a growing concern. DEHP is a ubiquitous environmental contaminant known to negatively impact reproductive parameters in diverse species, including humans. This study aimed to investigate the potential reproductive toxicity of DEHP on *Camelus dromedarius*, specifically focusing on its effects on epididymal semen quality and sperm-oviduct binding efficiency. Epididymal semen samples were collected from freshly slaughtered adult camels and exposed to varying DEHP concentrations (0, 10, 25, and 50 micromolar) for 60 minutes in vitro. Subsequently, sperm from each treatment group were co-incubated with oviductal explants for an additional 60 minutes. We meticulously evaluated the impact of DEHP on various epididymal sperm parameters, including viability, morphology, and DNA integrity. Additionally, their binding affinity to the oviductal explants was assessed. Our findings revealed significant alterations in epididymal sperm viability, morphology, and DNA integrity following DEHP exposure, with statistically significant differences observed at a p-value of less than 0.05. Notably, the sperm-oviduct binding index displayed a significant reduction in groups treated with 25 and 50 micromolar DEHP concentrations compared to the other groups. Interestingly, no significant difference was observed between the 25 and 50 micromolar groups. These compelling findings demonstrate that DEHP exposure can negatively impact crucial *Camelus dromedarius* sperm parameters, including their ability to bind to the oviduct. Given the significant role of *Camelus dromedarius* in arid and semi-arid regions, further research is warranted to elucidate the underlying mechanisms of DEHP toxicity and develop mitigation strategies to safeguard *Camelus dromedarius* reproductive health and contribute to sustainable camel husbandry practices.

P-468 - In Vivo Investigation of Ca²⁺ Signaling in Seminiferous Tubules.

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The life-long process of fertile sperm production is a fundamental mechanism of reproduction. Nevertheless, the complex and precisely orchestrated mechanisms behind this phenomenon largely remain a black box. To gain insights into these mechanisms we focus on the seminiferous tubules, the primary functional units of mammalian testis, and its three main cell types, i.e., peritubular, germ, and Sertoli cells. We address cell type specific Ca²⁺ signaling due to its established role in regulation of cellular activity. Here, we use in vitro and in vivo live cell imaging approaches to study non-evoked Ca²⁺ signaling events in all three cell types. We employ Cre-loxP genetics to conditionally express the genetically encoded fluorescence Ca²⁺ reporter GCaMP6f. Our studies show that all three cell types exhibit distinct Ca²⁺ signaling patterns with unique characteristics. We demonstrate that Ca²⁺ activity is both age- and endocrine state-dependent, and it is affected by gonadotropins such as FSH and LH. Moreover, cross-correlation analysis reveals unexpected coordinated ensemble activity. Collectively, our findings provide a deeper understanding of male reproductive physiology.

P-469 - Identification and Characterization of Novel Candidate Post-Fertilization Sperm Mitophagy Determinants

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Maternal inheritance of mitochondria and mitochondrial DNA (mtDNA) is conserved across most animal species. Paternal mtDNA leakage and mitochondrial heteroplasmy may have detrimental effects on offspring health and fitness outcomes. To ensure exclusively maternal mitochondrial inheritance and normal preimplantation development, paternal mitochondria in mammals are targeted and selectively degraded shortly after fertilization through a species-specific process called post-fertilization sperm mitophagy. This phenomenon has been extensively studied in recent years and it has been discovered that sperm mitochondrial degradation is mediated by the ubiquitin-proteasome system and autophagic proteins and pathways. Previously, the ubiquitin binding pro-autophagic receptors SQSTM1 and GABARAP as well as protein dislocase VCP, which mediates the presentation of ubiquitinated sperm mitochondrial proteins to the 26S proteasome, have been reported to support sperm mitophagy. Following these findings, a unique cell-free system (CFS) recapitulating early post-fertilization events was developed to identify new potential pro-autophagic proteins. This system combines porcine oocyte extracts and primed boar spermatozoa that have been demembranated and subjected to disulfide bond reduction to mimic processes occurring in vivo during and after sperm-oocyte fusion. The porcine CFS also recapitulates the early post-fertilization sperm mitophagy events and has been used to study early fertilization proteomics. The system was used in conjunction with mass spectrometry and 185 proteins with statistically significant changes in abundance between control and cell-free-treated spermatozoa were identified. We hypothesize that in addition to SQSTM1, GABARAP, VCP, autophagy, and the ubiquitin-proteasome system (UPS), other autophagic proteins and mechanisms also play a role. Several sperm and oocyte proteins consistent with this hypothesis have been validated as candidate mitochondrial inheritance determinants. The present study focuses on new potential pro-autophagic candidates, including FUN14 domain containing 1 (FUNDC1), a mitophagy receptor involved in mitochondrial quality control following hypoxic stress, optic atrophy 1 protein (OPA1), a regulator of mitochondrial morphology and cristae maintenance, which is also known to regulate mitochondrial fission, dynamin 2 (DYN2), a protein proposed to induce membrane fission to complete mitochondrial division, and mitochondrial fission regulator 1 (MTFR1), a regulator of mitochondrial dynamics that promotes mitochondrial fission and protects against oxidative stress. These proteins are being studied in porcine model by using cell imaging, in vitro fertilization (IVF) and the aforementioned cell-free system to decipher their role in post-fertilization sperm mitophagy. To further investigate their role in the mitophagy process, the proteins will be inhibited by antibody transfection into zygotes. Identification of novel regulators of post-fertilization mitophagy may help to further unravel the mechanism of mitochondrial inheritance and provide insight into early embryonic development, as well as a better understanding of mitochondrial diseases resulting from failed sperm mitophagy. This study is funded by USDA NIFA Animal Reproduction Grant number 2020-67015-31017.

P-470 - TMC5-CIB1 Complex at the Spermatid Plasma Membrane is essential for Spermiogenesis

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Spermiogenesis is the developmental process during which haploid round spermatids transform into sperm by undergoing a series of unique morphogenetic events including nuclear condensation, flagellum formation, and excess cytoplasmic elimination. The molecular mechanisms driving these processes are poorly understood, representing a significant knowledge gap. To address this gap, we examined an understudied member of the 'transmembrane channel-like' (TMC) protein family with potential roles in mechanotransduction, TMC5. We found TMC5 resides in a complex with the spermiogenesis-essential protein 'calcium and integrin binding protein 1' (CIB1). Indeed, both TMC5 and CIB1 co-localized at the plasma membrane in round, elongating, and condensing spermatids, in TMC5-mCherry knockin mice and by immunostaining, respectively. A direct interaction between TMC5 and CIB1 was validated by co-immunoprecipitation. To define its role in spermiogenesis, we generated *Tmc5* knockout (KO) mice; these mice exhibited disrupted spermatid elongation resulting in azoospermia and male infertility. Strikingly, CIB1 was also mislocalized from the spermatid plasma membrane. Although the precise role of TMC5 is unknown, based on known roles for founding members of the TMC family (TMC1-2) in cytoskeletal regulation and plasma membrane homeostasis in other systems, we hypothesized a similar role for TMC5 in spermiogenesis. In support of this, comparative super-resolution microscopy of *Tmc5* WT and KO spermatids revealed: 1) extensive F-actin network dysregulation, including loss of F-actin at spermatid plasma membrane; 2) disassembly of the spermatid-containing acrosome-acroplaxome-manchette complex that modulates spermatid nuclear re-shaping; and 3) aberrant phosphatidyl serine externalization. In summary, we identified a novel membrane protein that is essential for spermiogenesis. Current efforts are focused on defining the molecular function of the TMC5-CIB1 complex, and results will provide new insight into the complex molecular mechanisms that regulate spermatid morphogenesis.

P-471 - Transgenerational Epigenetic Inheritance and Autism Spectrum Disorder: Linking Meiotic Dysfunction and Cognitive Impairment

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We identified the first imprinted genes on the mouse X chromosome, *Xlr3b/4b/4c*. They are imprinted in the brain in a pattern of paternal repression and are highly expressed in testis. Given these observations, we have been interested in the potential link between the events of meiosis and those of neurodevelopment. We have generated transgenic mice using siRNA to target the functional copies of *Xlr3* in germ cells. Functional analyses of our transgenic and wild-type animals have revealed that *Xlr3* protein localizes to the sex chromosomes during Meiotic prophase I. Since *Xlr3* is present on the sex chromosomes during prophase I, including the period encompassing Meiotic Sex Chromosome Inactivation (MSCI), we next assessed the expression of several X and Y

linked genes, which are normally epigenetically silenced during MSC1. We found that Xlr3 knockdown results in disruption of MSC1, leading to inappropriate expression of X and Y genes. These observations led to the hypothesis that Xlr3 knockdown begets the establishment of epimutations on the X chromosome during male meiosis and are then passed across generations via the sperm, an example of transgenerational epigenetic inheritance. The transgenerational effect of this epimutated X is manifest in daughters of transgenic knockdown males by disruption of the imprinting of Xlr3b/4b/4c in the brain. In other words, we observed reactivation of the normally silent paternal allele of these genes. To explore the persistence of this epimutation across multiple generations, we devised a novel breeding scheme using a full-length inverted X opposite the epimutated paternal progenitor X to prevent recombination in F1 females. Thus, they pass the putatively epimutated X intact to sons which allowed us to generate a cohort of 25 F2 male mice that only possessed the putatively epimutated X. These F2 males were subjected to a battery of behavioral tests for social interaction, spatial learning, and repetitive behaviors. F2 males with epimutated X chromosomes were behaviorally distinct from WT controls in the novel object recognition, Morris Water Maze, and open field tests. After behavioral testing was completed, testes and brains of the F2 males were collected and RNA sequencing will be performed to assess differential gene expression. Finally, we explored the molecular nature of the X chromosome epimutation by examining DNA methylation patterns in the sperm of knockdown males compared to controls. Transgenic knockdowns have methylation patterns that are distinct from controls, and we have identified differentially methylated regions (DMRs), with many loci of interest on the X chromosome. Intriguingly, the DMRs identified on the X chromosome are restricted to the pseudoautosomal region (PAR), and Gcna which has necessary functions in spermatogenesis. We hypothesize that knockdown of Xlr3 causes epigenetic changes that destabilize the PAR which may lead to X-Y pairing defects and aneuploidy. In summary, Xlr3 function is critical to MSC1 and the establishment of epigenetic signatures on the X chromosome during male meiosis. Disruption of Xlr3 function leads to the production of sperm carrying a broadly epimutated X with multigenerational consequences to gene regulation and neurodevelopment. This research has implications for male biased disorders such as Autism Spectrum Disorder. We hypothesize a female protective effect in Autism susceptibility may rely on proper establishment and maintenance of epigenetic programming of the X chromosome involving Meiotic Sex Chromosome Inactivation in males.

P-472 - HyperSperm, a New Sperm Preparation Method for In Vitro Fertilization (IVF), Improves Sperm Capacitation, Increasing the Number of High-Quality Blastocysts in Mice.

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In vitro fertilization (IVF) treatments often result in low numbers of high-quality embryos, leading to the need for repeated treatments. While most approaches to improve IVF focus on oocytes and embryos, there is increasing evidence that sperm plays a fundamental role in embryo

development. HyperSperm is a novel sperm preparation method based on biomimicking sperm capacitation, a process that naturally occurs inside the female reproductive tract. This prospective experimental study aimed to test whether HyperSperm could provide improved capacitation parameters in semen samples from mice. Further, fertilization and embryo development were assessed both in vitro and in vivo in a mouse model of IVF, using 3-10 animals in each set of experiments. Sperm were either incubated in control conditions (TYH-HEPES medium) or treated with HyperSperm. Sperm motility and kinematic parameters were assessed by Computer-Aided Sperm Analysis (CASA), and acrosome integrity by flow cytometry. Fertilized eggs from IVF experiments were cultured in KSOM, and resulting blastocysts were transferred to pseudo-pregnant females. The number of implanted fetuses at day 7 and of pups born were recorded. Statistical differences were determined by paired T-test, with significance set at $p < 0.05$. Hyperactivated motility, a key hallmark of capacitation, was significantly higher in HyperSperm group compared to Control ($19.1 \pm 3.2\%$ vs $8.8 \pm 0.2\%$; $p = 0.032$; $n = 3$). HyperSperm-treated sperm exhibited higher fertilization rates compared to Control, as the percentage of 2-cell embryos ($162/215$ vs $112/231$ 2-cell embryos/eggs; $71.9 \pm 6.4\%$ vs $51.9 \pm 5.5\%$; $p = 0.032$; $n = 10$), and gave rise to higher embryo development rates ($144/162$ vs $75/112$ blastocysts/fertilized eggs; $85.1 \pm 5.8\%$ vs $65.1 \pm 6.2\%$; $p = 0.029$; $n = 10$). Once transferred, blastocysts derived from HyperSperm produced more implantation sites ($31/43$ vs $39/66$ implantation sites at day 7/blastocysts transferred; $76.0 \pm 11.2\%$ vs $48.7 \pm 10.3\%$; $n = 7$), resulting in significantly higher litter sizes ($16/39$ vs $5/57$ pups born/blastocysts transferred; $41.1 \pm 4.2\%$ vs $8.3 \pm 5.4\%$; $p = 0.013$; $n = 5$). In terms of safety, HyperSperm treatment did not result in a decrease in sperm motility ($83.5 \pm 1.1\%$ vs $85.7 \pm 2.5\%$; $p > 0.05$; $n = 3$), and the percentage of sperm with intact acrosomes was similar between groups ($37.8 \pm 4.6\%$ vs $33.6 \pm 5.7\%$; $p > 0.05$; $n = 3$). Overall, HyperSperm treatment enhances sperm capacitation, fertilizing ability, and embryo development, leading to an increased yield of term pups while maintaining a high safety profile. These findings underscore its potential to influence pre and post-implantation embryo development, and to improve IVF results on a per treatment basis.

P-473 - BSA-Mediated Rapid Calcium Uptake Initiates sAC Activation and cAMP Production During Mouse Sperm Capacitation

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Mammalian sperm require capacitation to fertilize oocytes while traveling through the female reproductive tract. Capacitation can also be induced in vitro by incubating in capacitation medium (CAP) containing energy sources, albumin, Ca^{2+} , and HCO_3^- . We have previously shown that only a subpopulation of mouse sperm increases the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) during the incubation in CAP. This rise became rapidly evident, within 1 min of incubation and it remained stable for at least 90 min. Although previous reports have demonstrated that sAC requires both

HCO₃⁻ and Ca²⁺ to be fully active, addition of each component alone produces a moderate increase in soluble adenylyl cyclase (sAC) activity. While HCO₃⁻ uptake has been studied, the mechanism for Ca²⁺ influx leading to the initial sAC activation is currently unknown. In the present work, we aimed to investigate early [Ca²⁺]_i changes required for the initial activation of sAC. We hypothesize that the Ca²⁺ ions required for activation of sAC are delivered by a CatSper-independent mechanism activated by bovine serum albumin (BSA). For that purpose, we analyzed early changes in [Ca²⁺]_i during capacitation in individual live sperm. Sperm were loaded with the Ca²⁺ dye Fluo-4 AM and analyzed by flow cytometry and single cell imaging. We observed that unlike the late capacitation-associated [Ca²⁺]_i increase, the rapid rise was independent from the CatSper channel complex, as CatSper1 KO sperm showed a significant rapid increase in [Ca²⁺]_i (p=0.0001 against CatSper1 KO 0 min; n=5) similar to the one from wild-type sperm (p< 0.0001 against CatSper1 WT 0 min; n=5). This early capacitation-associated [Ca²⁺]_i increase originated in the sperm head followed by diffusion of Ca²⁺ to the flagellum. To determine which constituent of the CAP medium influences the initial Ca²⁺ uptake, [Ca²⁺]_i levels were measured in CatSper1 KO sperm after exposing them to either HCO₃⁻ or BSA. We observed that this Ca²⁺ increase is produced only in response to BSA (p >0.05 against CAP 1 min; n=5) and not to HCO₃⁻ (p=0.04 against CAP 1 min; n=5), and the direction of Ca²⁺ flow was like the one observed with CAP media. To assess the activation of sAC by the rapid Ca²⁺ uptake, we monitored the intracellular cAMP levels by ELISA, in the absence of IBMX. After 1 min of incubation in non-capacitating media, sperm displayed only low levels of cAMP. Addition of BSA induced a significant increase in cAMP (p=0.04 against NC 1 min; n >6), similar to what is observed in CAP media (p=0.01 against NC 1 min; n >6). The levels of substrates phosphorylated by PKA in CatSper1 KO sperm, analyzed by immunoblotting using a specific antibody, behaved in agreement with these results, increasing after BSA exposure. Furthermore, the rapid [Ca²⁺]_i increase and the consequent intracellular cAMP rise were dependent on the presence of extracellular Ca²⁺ in the medium. Altogether, our results suggest that a subpopulation of mouse sperm increases [Ca²⁺]_i very rapidly during capacitation due to a CatSper-independent influx of extracellular Ca²⁺, induced by BSA. This rise in [Ca²⁺]_i is involved in the initial activation of sAC and consequently in cAMP production. These data provide critical insight into the molecular mechanisms behind sAC regulation that remained unknown and reveal the first stages that trigger the capacitation signaling pathways. Funding: Male Contraceptive Initiative; Eunice Kennedy Shriver National Institute of Child Health & Human Development, NIH (R01HD106968).

P-474 - Identification of Paternal Factors Compromising Pregnancy Establishment and Reproductive Outcomes in Bovine Model

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The artificial insemination (AI) is the driver of efficiency in cattle breeding via the use of genetically superior sires through genomic selection aimed at improving production traits of their progeny. This

biotechnological approach has significant impact on economy, production and environment and thus is widely, and increasingly applied worldwide. However, optimizing the production traits involves the use of a small number of often closely related sires causing reduced fitness and fertility. Detailed field AI service records enabled us to identify 85 AI bulls with acceptable sperm quality but substandard fertility outcomes. Genome sequencing revealed that these sires carry rare, homozygous single nucleotide polymorphisms in the genes known to regulate preimplantation embryo development and/or impact sperm quality. Genome sequenced bulls were sorted into subgroups based on the prevailing sperm morphological defect (e.g., knobbed acrosome, nuclear vacuoles, midpiece defects) and the rare, deleterious gene variants shared by all bulls within each group were identified. Our interest is currently focused on phenotype characterization of five carrier bulls with sperm nuclear vacuoles (NVs) phenotype that share mutations in fertility-associated genes PEG3, MSL3, PIR, EP400 and TDRD9. We developed a state-of-the-art high throughput sperm phenotyping pipeline including cell imaging, image-based flow cytometry, proteomics and protein structure modeling. Preliminary results have mapped the localization of the above proteins of interest in the spermatozoa, spermatids and testis of wild-type, non-carrier bulls and our biological control (NV spermatozoa from bull with testicular injury). The expression of mutation-affected proteins is being determined in spermatozoa of NV sires harboring the corresponding deleterious mutations, with anticipation of revealing the changes in sperm function and morphology. Results of this research will advance the understanding of spermatogenesis and link idiopathic infertility etiologies underlying pregnancy failure due to heritable paternal factors, with implication for more efficient livestock breeding, accommodating challenges listed in The Dublin Declaration of Scientists on The Societal Role of Livestock. Moreover, study results are also likely to transfer to human reproductive medicine, particularly the area of pregnancy establishment of recurrent pregnancy loss. This study was supported by grant 2023-67015-39262 from USDA NIFA Animal Breeding, Genetics and Genomics Program. Seed funding was provided by University of Missouri College of Agriculture, Food and Natural Resources. In-kind contributions were received from Genex Cooperative and Select Sires Inc.

P-475 - Elucidating the Proteome of Seminal Plasma-Derived Extracellular Vesicles from GnRH-II Receptor Knockdown Boars with Poor Semen Quality

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The pig is the only livestock species encoding functional proteins for the second form of gonadotropin-releasing hormone (GnRH-II) and its receptor (GnRHR-II). Previously, we demonstrated that GnRHR-II localizes to the connecting piece of boar sperm, whereas GnRH-II is present within porcine seminal plasma. To examine how the interaction of GnRH-II/GnRHR-II may influence sperm function, we utilized our transgenic swine line with universal knockdown (KD) of GnRHR-II. Compared with littermate controls, GnRHR-II KD boars produce ejaculates containing 39% fewer sperm with diminished total and progressive motility (23% and 31%, respectively), a 2-fold decrease in DNA stability, and reduced energetics (10% increase in mitochondrial

depolarization). Thus, GnRHR-II KD boars have fewer, less motile sperm of poor quality. Recently, seminal plasma-derived extracellular vesicles (spEVs) and their bioactive cargos (e.g., proteins) have been implicated in the regulation of sperm function. Therefore, the objective of this study was to evaluate the proteome of spEVs from GnRHR-II KD and littermate control boars, as well as assess GnRH-II concentrations within seminal plasma. Enzyme-linked immunosorbent assays were utilized to quantify GnRH-II concentrations in seminal plasma of transgenic (n = 14) and littermate control (n = 13) boars. Data were analyzed via the MIXED procedure of SAS with a model including genotype (fixed effect) and litter (random effect). A subset of these samples (n = 5 per genotype) was subjected to differential ultracentrifugation to isolate spEVs. After validation of spEVs by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM), proteomics was performed. Proteome Discoverer (ThermoFisher; version 2.4) and Mascot software (Matrix Science) were utilized. Proteins were considered differentially expressed at $P \leq 0.05$ following Benjamini-Hochberg adjustment, and tendencies were considered $0.05 < P \leq 0.10$. Results indicate that concentrations of GnRH-II were significantly reduced ($P = 0.0476$) within seminal plasma from GnRHR-II KD boars compared with littermate controls. Proteomics of spEVs detected 556 proteins. Of these, 73 were differentially expressed proteins (DEPs), and 14 spEV proteins tended to differ between genotypes. Among these, 32 DEPs were upregulated and 55 DEPs were downregulated in spEVs from GnRHR-II KD versus littermate controls. Noteworthy upregulated proteins include: hyaluronidase, zonadhesin, zona pellucida binding protein 2, acrosin binding protein, sperm acrosome associated protein 3, acrosin, lactadherin, amine oxidase, disintegrin and metalloproteinase domain-containing protein 5-like. Downregulated proteins include: pheromaxin A, protein kinase A, glutathione peroxidase 4, inactive ribonuclease-like protein 10, epididymis-specific alpha mannosidase, and ADAM metalloproteinase domain 2. Importantly, these DEPs have been implicated in critical reproductive processes including spermatogenesis, sperm maturation, sperm quality, capacitation, the acrosome reaction, cumulus cell dispersion, hyperactivation, zona pellucida binding, cryopreservation success, oviductal binding, ovulation, farrowing rate, and pheromonal transport. Thus, DEPs within spEVs may be a cause or consequence of the poor semen quality exhibited by GnRHR-II KD boars. Ultimately, these data elucidate the biology of reproduction in the boar, revealing novel proteins and potential biomarkers or therapeutic targets to enhance boar fertility. Furthermore, these transgenic swine also ubiquitously express the reporter gene ZsGreen1, which was detected within spEVs from GnRHR-II KD boars via proteomics, confocal microscopy, TEM, and fluorescent NTA, thereby representing a novel resource to study the interaction of spEVs with sperm and the trafficking of spEVs within the female reproductive tract. Supported by USDA/NIFA predoctoral fellowship (2017-67011-26036) and AFRI (2017-67015-26508) funds.

P-476 - The Effect of Ifosfamide and Cyclophosphamide on the Pre-Pubertal Testis

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As childhood cancer survival rates improve, it is increasingly important to focus on reducing toxicities and late effects of treatment, including the adverse impact of treatment on future fertility.

Alkylating chemotherapy agents are commonly used in paediatric oncology treatment regimens, and are widely accepted to be amongst the most gonadotoxic anti-cancer drugs. Much of our understanding of the detrimental effects of alkylating agents is drawn from clinical studies, and relatively less is understood about the direct effect of individual agents on gonadal tissue. Understanding this damage may enable development of protective strategies, and this is of particular relevance to pre-pubertal males, for whom there are currently no clinically available methods of fertility preservation. Here, we examine the direct effects of two commonly used alkylating agents – cyclophosphamide and ifosfamide – on the pre-pubertal testis, using an in vitro culture model. Clinical studies support that cyclophosphamide is significantly gonadotoxic, but relatively less is known about the effect of ifosfamide on male fertility. Testes were dissected from male mice (n=6) on postnatal day 5, cut into fragments, and cultured in α MEM with 10% KSR at 34⁰ and 5% CO₂. Tissue was randomly allocated as control or to receive treatment with a metabolite of either ifosfamide (4-hydroperoxyifosfamide; 4HI) or cyclophosphamide (4-hydroperoxycyclophosphamide; 4-OOH-CP), at the following concentrations: 1 μ g/ml, 10 μ g/ml, 20 μ g/ml, 100 μ g/ml. The metabolites were used because ifosfamide and cyclophosphamide are pro-drugs which require activation through hepatic metabolism; concentrations were selected based on the limited data available about patient serum levels. After six hours of exposure, tissue was moved to drug-free media. Media was then changed every 24-48 hours until day 5, at which point tissue was fixed in NBF. Immunohistochemistry was then performed to quantify germ cells (MVH), spermatogonial stem cells (SSCs; PLZF), Sertoli cells (SOX9), and peri-tubular myoid cells (SMA). ELISA was performed on culture media to assess testosterone production. Data were analysed in GraphPad Prism using one-way ANOVA with Sidak or Dunnett's post-hoc test. Exposure to 4HI and 4-OOH-CP caused a significant loss of germ cells relative to control at all but the lowest treatment concentration ($p < 0.001$). SSC density was significantly reduced by treatment with 20 μ g/ml ($p=0.049$) and 100 μ g/ml 4-HI ($p=0.043$), and by treatment with 100 μ g/ml 4-OOH-CP ($p=0.027$). Sertoli cell density was significantly reduced only by treatment with the highest concentration of 4-OOH-CP (100 μ g/ml; $p=0.006$), and density of peri-tubular myoid cells was not affected by any concentration of either drug. Media testosterone concentration increased in a dose-dependent manner, reaching a significant increase at 20 μ g/ml of either metabolite ($p=0.001$); however, this was followed by a significant reduction in testosterone levels relative to control following treatment with 100 μ g/ml of either 4HI ($p=0.005$) or 4-OOH-CP ($p=0.002$). These findings suggest that ifosfamide and cyclophosphamide selectively damage the germ cells of the immature testis, with higher concentrations specifically targeting the subpopulation of SSCs. There is relative sparing of the somatic cells, with damage to Sertoli cells only seen at the highest concentration of the cyclophosphamide metabolite. However, testosterone production is affected in a dose-dependent manner. Given that testosterone secretion would be expected to be relatively low before puberty, further work is required to examine whether this is a clinically relevant effect of treatment or whether it may reflect the culture model.

P-477 - Successful Classification of Normal and Abnormal Ram Sperm by a Machine Learning Model

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Sperm morphology is a key in vitro predictor of ram fertility. A regular feature of semen assessment, sperm morphology assists in the identification of infertile or sub-fertile rams or semen samples, thus ensuring maximum reproductive efficiency of natural and artificial breeding programs. However, the efficacy of morphology assessment is limited by high levels of variability between assessors and labs and it's difficult to standardise. Indeed, preliminary work by our group indicated industry experts (n=4) using a simplified yes/no system of normal/abnormal agreed on only 73% (585/800) of sperm, which fell to 27% (212/800) of sperm in a comprehensive 30 category labelling system. One alternative to subjective morphological assessment of sperm is the application of machine learning. Machine learning has a multitude of applications and has already been applied to object recognition and labelling in other reproductive assessment contexts such as embryo quality. As such, this study aimed to utilise machine learning, specifically a convolutional neural network, to create an objective tool for morphological classification of ram sperm. Ram sperm obtained from 73 sires were fixed (5% formalin in PBS) and mounted on glass slides. Images of 100-200 sperm per sire were captured using differential interference contrast microscopy (400x magnification; numerical aperture = 0.75), on a Olympus DP23 camera, and cropped to 512x512 px resolution using a novel machine learning tool. Sperm were labelled as normal or abnormal by experienced morphologists. Sperm (N=2323) that had 100% classification consensus between the morphologists were randomly divided into training, validation and test data sets for the development of the machine learning model, at a ratio of 60:20:20, respectively. The model was constructed using a Resnet 50 scaffold with two dense layers of 512 then 272 units and an optimal learning rate was decided upon running multiple hyperparameters. The data points were put through the model at a 224x224 px resolution and kept as colour (RGB) images. TensorFlow was utilised to monitor for overfitting and to automatically stop the model learning before it reached that point. The model was found to have a 95% accuracy for classifying sperm as normal or abnormal on the unseen test data set and was able to achieve a 97% accuracy on the training data. This study demonstrated that machine learning can be used to successfully classify ram sperm as normal or abnormal. Future research into this area will focus on expanding the number of morphological categories the model is able to classify in order to facilitate objective assessment of sperm in clinical and research situations that require more complex morphological classification systems.

P-478 - Accelerated Mitochondrial Dynamics Promote Spermatogonial Differentiation

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Spermatogonial stem cells (SSCs) maintain a pool of undifferentiated spermatogonia and the subsequent production of progenitor spermatogonia that are poised for differentiation during spermatogenesis. At different stages of spermatogenesis, germ cell mitochondria differ remarkably in morphology, architecture, and functions. However, it remains elusive how mitochondria change these features to impact the SSC fate decision during spermatogonial development. Mitochondrial features are largely regulated by continuously go through fusion and fission cycles, the processes that are collectively called mitochondrial dynamics. In this study, we found that mitochondrial number, morphology, and DNA copies were not altered during spermatogonial differentiation. By

contrast, increased opening of the mitochondrial permeability transition pore (mPTP) along with decreased mitochondrial membrane potential (MMP) was observed during this process. Importantly, we found that regulators of mitochondrial fusion (MFN1 & MFN2) and fission (DRP1) were both upregulated in differentiating spermatogonia. Accelerated mitochondrial dynamics in turn promoted spermatogonial differentiation. Using a Drp1 conditional knockout mouse model, we demonstrated that Drp1 deficiency in germ cells led to the stage-specific block of spermatogenesis at differentiating spermatogonia. In addition, our data suggested that MFN1 promoted differentiation through the regulation of metabolism, while DRP1 impacted spermatogonial differentiation via mPTP opening. Taken together, our findings support that stage-specific, precisely regulated mitochondrial dynamics are required for proper spermatogonial differentiation, thereby providing insights about how mitochondrial activities and functions are maintained so in order to support critical and unique events of germ cell development.

P-479 - ARRDC5 Regulation of Histone-to-Protamine Exchange During Spermiogenesis

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Spermiogenesis is the final phase of spermatogenesis when haploid round spermatids undergo a transformation to yield specialized elongated cells that are released from the seminiferous epithelium as spermatozoa. During this process, the paternal genome is condensed into toroid structures via an exchange of histones with protamines. Disruption in the histone-to-protamine exchange leads to sperm head malformations and elevated DNA damage, both of which are prominent abnormalities associated with the most common clinical description of male infertility, oligoasthenoteratospermia (OAT). In previous studies, we discovered that the molecule arrestin domain containing 5 (*Arrdc5*) is an essential regulator of spermiogenesis. Knockout of *Arrdc5* in mice leads to genesis of sperm with severe head malformations and a significant increase in DNA damage compared to sperm from wild-type mice. Here, we explored whether the histone-to-protamine exchange is abnormal in the absence of ARRDC5. Sperm from *Arrdc5* knockout mice were found to stain more intensely with the preferential histone binding dye aniline blue compared to sperm from wild-type mice. In addition, outcomes of immunoblot analyses revealed significantly greater amounts of histone variants in the DNA of sperm from *Arrdc5* knockout mice compared to sperm from wild-type counterparts. Furthermore, protamine content was found to be reduced and the ratio of protamine 1 and 2 variants altered in sperm of *Arrdc5* knockout mice compared to sperm from wild-type mice. Taken together, these data indicate that ARRDC5 influences the histone-to-protamine exchange process during mammalian spermatogenesis and aid in explaining the OAT phenotype that is caused by inactivation of the encoding gene.

P-480 - KDM2A Maintains H3K36me2/3 Deposition and Recruits HCFC1 and E2F1 to Orchestrate Male Meiotic Entry and Progression

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In mammals, the switch from mitosis to meiosis ensures the successive formation of gametes. However, the mechanisms regulating meiotic initiation remain unclear, especially in the context of

complicated histone modification. Herein, we show that KDM2A acts as a lysine demethylase targeting H3K36me3 in male germ cells and plays an essential role in modulating meiotic entry and progression. Conditional deletion of Kdm2a in mouse pre-meiotic germ cells results in complete male sterility, with spermatogenesis ultimately arrested at the zygotene stage of meiosis. Mechanistically, KDM2A maintains a close functional relationship with Stra8 and Meiosin in promoting the meiotic gene expression and binds a wide range of meiosis-related genes. Interestingly, KDM2A deficiency disrupts the deposition of H3K36me2/3 in c-KIT⁺ germ cells, characterized by a reduction of H3K36me2 but a drastic increase of H3K36me3. Furthermore, KDM2A could recruit the transcription factor E2F1 and its co-factor HCFC1 to the promoters of key genes required for meiosis entry and progression, such as Stra8, Meiosin, Spo11, and Sycp1. Thus, our study unveils an essential role for KDM2A-mediated H3K36me2/3 deposition in controlling the programmed gene expression necessary for mitosis to meiosis transition.

P-481 - A Mouse Testis-specific Long Noncoding RNA, LncRNA-HSVIII, Regulates the Testosterone Production and the Morphology of Testis, Cauda Epididymis, and Sperm

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Long noncoding RNAs (lncRNAs) are functional without being translated, and thousands of lncRNAs are expressed in various tissues. Among them, the testis expresses more lncRNAs than other tissues, but their functions are unclear. The mouse Prss/Tessp locus is composed of six testis-specific serine protease genes and three testis-specific lncRNAs and play important roles in the testis function. LncRNA-HSVIII, one of the lncRNAs at the Prss/Tessp locus, is specifically expressed in the testis, especially in Leydig cells and spermatocytes, but its function was unrevealed. An objective of this study is to elucidate the physiological function of LncRNA-HSVIII. To this end, we generated LncRNA-HSVIII knockout (KO) mice by the CRISPR/Cas9 system. Although LncRNA-HSVIII KO mice were not infertile, here we report the phenotype at 2 months and 6 months of age. We first performed the RNA-seq analysis using testes of wild-type and LncRNA-HSVIII KO mice and found more than 200 genes were differentially expressed in both 2-month-old and 6-month-old testes. Because five sex steroidogenic genes were downregulated in LncRNA-HSVIII KO testes, we analyzed the testosterone level by ELISA and found that LncRNA-HSVIII KO mice had lower testosterone levels. To see whether the low testosterone level and/or the differential gene expression affected the morphology, we performed the microscopic observation of testis and cauda epididymis as well as the sperm evaluation by the computer-assisted sperm analysis (CASA) system. In both 2-month-old and 6-month-old testes, the number of seminiferous tubule stages were altered, and the number of blood vessels was increased in the interstitial tissue by LncRNA-HSVIII KO. Interestingly, some phenotypes were observed specifically at 6 months of age. In 6-month-old LncRNA-HSVIII KO testes, we found clusters of germ cell degeneration, possibly leading to the vacuolation, and enlargement of round spermatids. The number of Sertoli cells was increased in the seminiferous tubule. In the mediastinum testis, the transition zone was elongated, and the interstitial tissue contained more fibroblasts. In 6-month-old cauda epididymis, the

extravascular leukocytes were increased in the lumen and interstitium. Furthermore, the sperm evaluation results showed sperm morphological defects mainly at the head and tail in 6-month-old mice, although the total sperm number and motility were not significantly different. Collectively, our results suggest that lncRNA-HSVIII regulates the testosterone production and the morphology of sperm, testis, and epididymis especially at an older age.

P-482 - Aging, Sperm Proteome, and Neurodegenerative Diseases: Incidental Observations or a Little Bit More than Meets the Eye?

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Aging associated wellbeing is of considerable importance due to the increasing aging population with related health issues including fertility problems and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Alzheimer's disease involves amyloid plaque accumulation and synaptic pathology, while Parkinson's disease is characterized by the presence of pathological intracellular misfolded α -synuclein aggregates in the aging brain. The clinical signs of these neurological diseases are manifested later in life, but the underlying indicators that might be present much earlier remain unknown. Understanding the underlying pathobiological mechanisms of age-related diseases is crucial for addressing health challenges in the aging human population. Our recent investigations on human semen proteome revealed deregulation of several proteins and pathways that are potentially associated with neurodegenerative disorders. Briefly, we examined semen samples from healthy fertile Arab/Saudi men across three age groups: a Young Adult group (21–30 years, n=6), a Late Adult group (31–40 years, n=7), and an Advanced Age group (40–51 years, n=5). The semen samples were centrifuged to separate seminal plasma and spermatozoa. Following separation, the spermatozoa were gradient purified, and both spermatozoa and seminal plasma were subjected to LC-MS/MS proteomic analysis. Total detected proteins were filtered for altered proteins among the groups and enriched annotations (ontologies and pathways) of differentially expressed proteins (≥ 2 -fold change) were analyzed. A total of 595 and 463 proteins, respectively, were identified in the spermatozoa and seminal plasma of the three age groups. Of these, 287 and 116 proteins were differentially expressed in spermatozoa and seminal plasma, respectively, in Advanced Age group compared to Young Adult group. In addition to sperm function-related processes, the functional and pathway analysis of these differentially expressed proteins indicated enrichment of pathways for neurodegenerative diseases such as Alzheimer's disease-presenilin pathway, Parkinson's disease pathway, Wnt signaling pathway, and Huntington's disease pathway. Specifically, in the Advanced Age group the differential changes were observed in the expression of Wnt2, ACTRT3, ACTA1, ACTB, ACTBL2, ACTG1, ACTG2, MMP7, PSMA3, PSMA5,

PSMA6, PSMA7, PSMB1, PSMB3, PSMB4, PSMB7, PKLR, HSPA1A, HSPA1L, HSPA2, HSPA5, HSPA6, HSPA8, SEPTIN4, YWHAQ, DYNLL, AR4, TUBB1, TUBB8, GAPDH, CAPNS1, and RAC1. Wnt signaling is a prominent pathway required for synaptic plasticity and maintenance in adult brain and is deregulated in aging brain and Alzheimer's disease. Similarly, the other differentially expressed proteins have been associated with the molecular portraits of the pathogenesis of Alzheimer's, Parkinson's, and Huntington's diseases. These interesting preliminary observations in the age-related proteomic profile of spermatozoa and seminal plasma made us wonder if there is any underlying consequential relationship with pathophysiology of neurodegenerative disorders. However, the fact that these might be incidental observations of overlapping proteomic pathways of sperm fertility and neurodevelopmental/degenerative pathways cannot be overlooked. Further studies in this area might help clarify this conundrum. Acknowledgement: The authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this research work through project number 1132.

P-483 - Specific DISRUPTION of Dcaf17 GENE in SPERMATIDS is DISPENSABLE for POST-MEIOTIC GERM CELL DEVELOPMENT and FERTILITY in MICE

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DDB1- and CUL4-associated factor 17 (Dcaf17) is a member of DCAF family genes that encode substrate receptor proteins for Cullin-RING E3 ubiquitin ligases and associates with cullin 4A / damaged DNA binding protein 1 ubiquitin ligase complex. Mutations in this gene are associated with Woodhouse-Sakati syndrome which is characterized by progressive malformations throughout the body. Our previous reports indicated that global Dcaf17 gene deletion in mice caused male sterility and impaired germ cell development with defective spermiogenesis, apoptosis, dysmorphic sperms and diminished sperm count. We hypothesized that Dcaf17 gene is critical for post-meiotic sperm development and attempted to induce spermatid specific knockout using Floxed Dcaf17 and protamine 1 (Prm)-cre mice breeding strategy. Fertility test, testis weight, sperm counts, motility, and testis histology were assessed in Spermatid specific Dcaf17 knockout mice after pairing with wild type (WT) C57BL/6J female mice. The spermatids specific Dcaf17 deletion was confirmed by tail and sperm genotyping. Spermatid specific Dcaf17 knockout mice are fertile and exhibiting normal sperm number and motility similar to control group. Moreover, histological sections stained with hematoxylin and eosin as well as periodic-acid Schiff stain in the spermatid-specific mutant and control testes were examined exhibited intact seminiferous tubules with normal spermatogenesis having all types of spermatogenic cells from spermatogonia to spermatozoa. Our results suggested that disruption of Dcaf17 in haploid spermatids stage of spermatogenesis does not influence spermiogenesis process and resulted in normal sperm production and male fertility. Despite anomalous Dcaf17 spermiogenesis in the global knockout mice, targeted deletion of this gene in spermatids does not affect mice fecundity and post-meiotic sperm development. Normal Dcaf17 gene expression is required before the development of round spermatids.

P-484 - The Germline Undergoes Genome-Wide DNA Methylation Changes During Human Spermatogenesis

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The Germline Undergoes Genome-Wide DNA Methylation Changes During Human Spermatogenesis Sandra Laurentino¹; Lara M. Siebert-Kuss¹; Verena Dietrich²; Sara Di Persio¹; Jahnavi Bhaskaran^{3,4,5}; Martin Stehling⁵; Jann-Frederik Cremers⁶; Sarah Sandmann²; Julian Varghese²; Sabine Kliesch⁶; Stefan Schlatt¹; Juan M. Vaquerizas^{3,4,5}; Nina Neuhaus¹

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Correct establishment of germline DNA methylation patterns is required for mammalian fertilisation and development. The mammalian germline undergoes two waves of genome-wide reprogramming during prenatal life. Studies in mouse and rat indicated that the male germline methylome undergoes remodelling during spermatogenesis, however it was unknown whether a comparable process takes in the human male germline. Here, we aimed at studying DNA methylation changes occurring during human spermatogenesis and evaluating whether DNA methylation patterns are disturbed in cases of male infertility. We isolated undifferentiated and differentiating spermatogonia, primary spermatocytes (4C), and spermatids (1C) from testicular biopsies from individuals with normal (n=3) and severely disturbed spermatogenesis (cryptozoospermia; n=2) by fluorescence-activated cell sorting. The methylome of each germ cell type was analysed by enzymatic methyl-sequencing (EM-seq). We found that the germline methylome underwent remodelling during spermatogenesis progression. This included a global decline in DNA methylation in primary spermatocytes followed by partial remethylation. The methylation remodelling did not affect imprinting control regions. Regions displaying differential methylation during spermatogenesis were enriched in short interspersed nuclear elements (SINEs), a type of transposable element. The remethylation of the germline genome following meiosis occurred at particular regions and resulted in a spermatid-specific methylome. These spermatid-specific hypomethylated regions were enriched at spermatid-expressed gene promoters and in binding sites for DMRT and SOX family members. The methylomes of cryptozoospermia-derived germ cells displayed significant DNA methylation changes compared to normal ones. Differentially methylated regions in germ cells from infertile

men were significantly enriched in genes involved in spermatogenesis and in transposable elements. Particularly, the evolutionarily young SVA (SINE-VNTR-Alus) D and F families of transposable elements displayed lower methylation in cryptozoospermia than in normal spermatogenesis. Our data hints at a possible association between abnormal germline methylation of transposable elements and failure of male germ cells to progress beyond meiosis, resulting in male infertility, and suggests a pivotal role for DNA methylation in the regulation of spermatogenesis and germ cell-specific gene expression.

P-485 - New Insights in Porcine Zonadhesin Protein Under Different Physiological Conditions

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Mammalian fertilization involves complex molecular and cellular interactions, and deviations from this orchestration can be detrimental to reproductive success. During spermatozoa-zona pellucida interaction, Zonadhesin (ZAN), a glycoprotein expressed in the acrosomal region of spermatozoa, plays a crucial role. A detailed study of zonadhesin may allow the improvement of artificial insemination protocols, the optimization of sperm selection methods, and an increase in the efficiency of swine breeding programs. Thus, the purpose of this study was to characterize pig zonadhesin protein under three different physiological conditions: noncapacitated (NCS), selected after a one-hour sperm capacitation via swim-up (CS1), and acrosome reacted (ARS1). The sperm-rich fraction was obtained from mature fertile tested Piétran boars (n=7). Sperm were capacitated by swim-up and the acrosome reaction was induced by calcium ionophore A23187. In each physiological condition, the immunofluorescence of the ZAN and acrosome status was determined using confocal microscopy. Negative controls were performed. The analysis of zonadhesin distribution revealed six different staining patterns. The first pattern (P1) was significantly observed in noncapacitated spermatozoa with fluorescence only in the periacrosomal region (NCS vs CS1 $p < 0.05$; NCS vs ARS1 $p < 0.01$). Pattern two (P2), also observed mainly in NCS, showed fluorescence in the periacrosomal region as well as in the postacrosomal region. However, the dominant pattern in NCS was pattern 3, with fluorescence in the periacrosomal, acrosomal and equatorial region (NCS vs CS1 $p < 0.01$; NCS vs ARS1 $p < 0.001$). Once the sperm are capacitated by selection (CS1), P1, P2 and P3 patterns decrease leaving a new dominant pattern named P4 (CS1 vs NCS $p < 0.01$). This fourth pattern displays fluorescence in the acrosomal and postacrosomal regions. Finally, after inducing the acrosomal reaction, fluorescence is mostly observed only in the postacrosomal region (P5; ARS1 vs NCS $p < 0.001$; ARS1 vs CS1 $p < 0.01$). A small percentage of spermatozoa in all conditions were unresponsive to anti-ZAN antibodies, resulting in pattern 6. In contrast to the literature, our results demonstrate the presence of ZAN both in the acrosome and in the postacrosomal region. This might be because our antibody targets a sequence located in the MAM domain (meprin/ A5 antigen/ mu receptor tyrosine phosphatase). Since the main function described to update of zonadhesin is the specific recognition of the zona pellucida, the presence of this protein in the equatorial and postacrosomal regions could indicate that this ZAN is presumably involved in various events of fertilization. Hence, further studies are needed to determine this new function. This work was supported by the project PID2021-123091NB-C22 funded by

MCIN/AEI/10.13039/501100011033 y por FEDER Una manera de hacer Europa; and University of Alicante (VIGROB-186). H.-F.M. predoctoral contract is part of the grant PRE2022-101681, funded by MCIN/AEI/10.13039/501100011033 and FSE+.

P-486 - Investigations into the Endocrine Regulation of Horse Testicular Polyunsaturated Fatty Acids Synthesis via Experimental Inhibition and Stimulation of Testicular Function.

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Polyunsaturated fatty acids (PUFA) metabolizing enzymes are expressed in the horse testis and epididymis suggesting an active PUFA metabolism during spermatogenesis and epididymal sperm maturation. The endocrine regulation of sperm PUFA synthesis is, however, unknown. The aim of the study was to fill this gap via an experimental inhibition (after GnRH vaccination) and re-stimulation (subsequent treatment with a GnRH agonist) of testicular function in stallions. Shetland stallions were assigned to a GnRH vaccination group (Improvac; 400 µg/animal at 4 weeks interval; n=6) or a saline control group (n=6). Each vaccinated stallion was hemicastrated together with an age-matched control when testosterone concentration was < 0.3 ng/ml (C1). Treatment of vaccinated stallions with the GnRH agonist buserelin (4 µg/day for 4 weeks and 8 µg/day for 6 weeks) started three weeks thereafter. The remaining testicle was removed when testosterone concentration increased to >0.5 ng/ml in vaccinated stallions (C2). Testicular tissues were stored at - 80°C for RT-qPCR and Gas chromatography–mass spectrometry (GC-MS) and fixed in formalin for histology. Statistical analyses were performed by non-parametrically by Mann-Whitney U test. Relative mRNA abundance for the Δ5 and Δ4-desaturases (FADS1 and DEGS1) was lower in testicular tissue of vaccinated than control stallions at C1 (p< 0.05 and p< 0.01 respectively). At C2, FADS1 and DEGS1 mRNA abundance no longer differed between groups. No difference in mRNA abundance for the elongases 5 and 2 (ELOVL5 and ELOVL2) was determined. Immunolabelling for ELOVL5, FADS1 and FADS2 in testicular tissue did not differ between the group. There were no significant differences between groups with regard to testicular tissue omega-3 and omega-6 PUFA content and the omega-3/omega-6 ratio at any time. In conclusion, GnRH-vaccination changed expression of PUFA metabolizing enzymes at the mRNA level in testicular tissue, but this did not influence omega-3 and omega-6 PUFA content of testicular tissue. These results suggest that the regulation of testicular PUFA synthesis in stallions may not depend solely on the functional hypothalamic–pituitary–gonadal axis.

P-487 - Characterization of Na⁺/H⁺Exchanger (NHE11) by Immunofluorescence and High-Resolution Microscopy in Porcine Sperm

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Na⁺/H⁺ exchangers (NHEs) encode a family of 13 proteins and are vital pH regulators in numerous cells types. Specifically, sperm function is particularly dependent on pH variations, and consequently, NHEs have been suggested to play significant roles in regulating the intracellular pH of these cells throughout fecundation process. In this context, the presence of the NHE11 exchanger has been studied in human and rat sperm, but there is no information about the presence and location of this exchanger in boar sperm. Therefore, the objective of this study was to characterize the immunolocalization of the NHE11 exchanger in boar sperm under different physiological conditions using fluorescence and high-resolution microscopy. The sperm-rich fraction was obtained from tested fertile Piétran boars (n=8). The sperm were capacitated by swim-up and the induction of the acrosome reaction in vitro was performed using the calcium ionophore A23187. In each sperm physiological condition, the NHE11 exchanger was characterized qualitatively by immunofluorescence using confocal microscopy and quantitatively by immunodetection using colloidal gold particle through the Field Emission Scanning Electronic Microscope (FESEM). Furthermore, in the fluorescence study, the integrity of the acrosome was evaluated by co-labeling using the lectin *Pisum sativum* agglutinin. As a result, a total of five fluorescence patterns were identified in the sperm head based on the location of the NHE11 exchanger: Pattern 1, periacrosomal region and acrosome region; Pattern 2, periacrosomal region with equatorial band; Pattern 3, equatorial region; Pattern 4, faint labelling; and Pattern 5, without labeling. Specifically, Pattern 1 was mostly significant in uncapacitated and capacitated sperm. While, the rest of the patterns were preferentially visualized in those sperm that had completed the acrosome reaction. In parallel, the data obtained by FESEM confirmed the presence and localization of the exchanger NHE11 in the different physiological conditions of porcine sperm. These data show for the first time the presence and localization of the exchanger NHE11 in porcine sperm. Overall, the detection of this exchanger could indicate a relevant role in the regulation of the capacitation process, as well as during the acrosome reaction or sperm/egg fusion events. This work was supported by the project PID2021-123091NB-C22 funded by MCIN/AEI/10.13039/501100011033 y por FEDER Una manera de hacer Europa; and University of Alicante (VIGROB-186).

P-488 - Phenotyping Differentially Abundant Proteins in Cryopreserved vs Fresh Bull Sperm

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Semen cryopreservation is increasingly utilized in the livestock industry to preserve and distribute genetics from high indexing genetic sires. This technology has provided the ability to share high value genetics around the world and decrease biosecurity concerns involved with transporting live animals. It is not well understood, though, how cryopreservation affects the quality of preserved cells, but it is known that quality markers such as motility and membrane viability are decreased upon thawing. Mass spectrometry (MS) analysis was employed to identify distinct proteomic differences between fresh and cryopreserved sperm cells. Extracts were submitted for MS analysis at the Iowa State University Proteomics Core. A total of 2411 proteins were identified between both treatments. Of those, 402 were unique to the fresh replicates and 57 were unique to cryopreserved replicates. Principle component and cluster analysis revealed 154 proteins with differential

abundances ($p < 0.05$). In regard to those, 106 proteins were less abundant in cryopreserved samples and only 48 more abundant. Based on these identified proteins, three were selected for further phenotyping. The three proteins selected were cytochrome c, which was more abundant in the cryopreserved samples, 40s ribosomal protein S3a (RPS3A) and dolichyl-diphosphooligosaccharide-protein glycotransferase (DDOST), which were less abundant in cryopreserved samples. Immunocytochemistry was performed to identify protein localization within formaldehyde fixed, fresh bull sperm cells. Samples were permeabilized with 0.1% Triton X-100 and incubated with anti-cytochrome-C, anti-RPS3A, or anti-DDOST overnight before incubating with a Tritc conjugated secondary antibody, nucleotide stain Hoechst H33342 to identify the sperm nucleus, and Alexa Fluor-488 conjugated lectin PNA to observe the acrosome. Cells were mounted on ploy-L-Lysine slides and images were acquired with a Leica DM6 THUNDER upright microscope. Localization was seen in the equatorial segment by cytochrome C, RPS3A, and DDOST. Additional localization was seen in the apical ridge by labeling with PRS3A and DDOST and unique labeling in the principal piece of some cells was seen by cytochrome-c. Future steps involve elucidating localization differences between these three proteins in fresh and cryopreserved bull sperm by additional immunocytochemistry and validation of differential abundances by western blot analysis. This research aims to identify protein targets for optimization of cryoprotectants to maintain optimum cell quality and thereby ensure the reproductive potential of cryopreserved semen used in artificial insemination. Supported by the National Institute of Food and Agriculture, U.S Department of Agriculture under grant number 2022-67015-36298 and Multistate Hatch Project 9835.

P-489 - Capacitation Signatures in Fresh and Cryopreserved Bovine Sperm

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Capacitation is an irreversible process sperm must go through prior to fertilization. Our hypothesis was that variation in capacitation status between bulls serves as an indicator of their fertility. Recently, it was discovered that zinc (Zn) signatures can be used to quantify capacitation status in bovine sperm as measured by flow cytometry. In vitro capacitation (IVC) and flow cytometry are long (5+ hours) processes, and sample collection can require extensive travel to a laboratory. Storing samples overnight and evaluating the following day would facilitate the workflow of sample analysis, but may provide different capacitation signatures than those of same day and cryopreserved samples. The objective of this study was to characterize Zn capacitation signatures on the day of collection (D0), stored overnight in OptiXcell extender (D1) or cryopreserved (FT) samples. Comparisons of capacitation signatures during IVC between D1 and FT samples were also made. Bulls (n=24) were collected by artificial vagina, and semen was either cryopreserved in a commercial egg-yolk based extender or diluted in OptiXcell extender and transported to the lab for analyses. Day 0 samples were analyzed for Zn signatures, viability, and acrosome integrity via flow cytometry approximately 7 hours after collection without IVC. Day 1 samples were stored at 40C

overnight and analyzed approximately 20 hours after collection. Day 1 and FT samples were in vitro capacitated for 3 hours and evaluated for Zn signatures, acrosome integrity, and viability via flow cytometry at times (T) 0, 1.5, and 3 hours. We observed that D0 samples had at least 21% more ($P < 0.001$) viable sperm cells than D1 and FT samples at T0, which did not differ ($P > 0.10$). An estimated 20% of sperm appeared to advance through capacitation signatures when stored in OptiXcell overnight and these cells also appeared to progress through the acrosome reaction. Freezing and thawing resulted in approximately 30% of sperm being cryocapacitated and not progressing through capacitation signatures. The capacitation signatures also differed during IVC in samples stored overnight compared to cryopreserved. Viable sperm cells that had not yet capacitated or were early in capacitation (signatures 1 and 2) were greater in FT compared to D1 ($P < 0.01$) and greater at T0 than T1.5 or T3 ($P < 0.01$). Cryopreserved samples had more sperm cells in a capacitated state (signature 3) during IVC when compared to D1 ($P < 0.0001$) and these cells did not progress further through capacitation. Dead cells with no Zn present (signature 4) were substantial in D1 samples and increased in FT and D1 samples from T0 to T1.5 ($P < 0.0001$). Both FT and D1 samples had fewer live sperm with an intact acrosome from T0 to T1.5 ($P = 0.002$). We conclude that storing cells overnight in OptiXcell extender does not prevent the progression of sperm from going through the process of capacitation. Cryocapacitation has been previously reported to occur in FT samples. These cells die without progressing through the final stages of capacitation. Capacitation signatures observed in bulls were highly variable during IVC for cryopreserved samples and samples stored overnight in extender. Further research is needed to understand how these signatures relate to bull field fertility. Funded by USDA NIFA Animal Reproduction award number 2022-67015-36300.

P-490 - Sperm RNA Characteristics in Stallion Fresh Semen are Influenced by Somatic Cell Lysis

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Increased chromatin condensation and the reduced content of RNA render sperm challenging for RNA extraction and species-specific protocols are required. We aimed to optimize RNA isolation techniques in fresh stallion sperm, with particular focus on the relevance of somatic cell lysis. Raw semen from 5 different stallions was diluted with EquiPlus extender. Sperm characteristics motility (CASA), morphology and DNA integrity (Aniline blue) were determined. With the remaining sample, density gradient centrifugation (Equipure) followed by two centrifugation steps in PBS was performed. One part was treated for somatic cell lysis (group SCL), the other was not (group NSCL). From each part, four portions were formed that underwent A) incubation with Trizol (room temperature); B) bead-based homogenization (3x) in Trizol; C) heated trizol (65°C 30 min); or D) bead-based homogenization (3x) + heated Trizol (65°C 30 min). In all samples, RNA was isolated using Direct-zol columns. RNA contamination by solvents (260/230 ratio) and by proteins (260/280 ratio) was considered, together with RNA integrity (DV200 Index) and RNA yield (TapeStation). Differences in the abundance of genes previously proven to be correlated with stallion fertility (PRM1, PRM2, PRM3, CRISP3, PLCz1, ACR, ZBPB, PRDX5, NOX5, SOD) with regard to treatment were determined by RT-qPCR (reference genes: ACTB and RPL32). Statistical analysis was

performed with two-way repeated measures ANOVA (IBM-SPSS statistics 29.0.1.0). Somatic cell lysis influenced ($p < 0.05$) RNA purity (260/280 ratio) and RNA integrity. Nevertheless, the relevance of 260/280 ratio remains questionable because numerical differences were low but still better for NSCL group considering 1.8-2.0 as the ideal range. With regard to RNA integrity, there was an effect of cell lysis ($p < 0.05$) In both groups, lowest DV200 index was determined with heated Trizol (SCL: 39.86 ± 3.12 ; NSCL: 46.22 ± 6.29). With regard to RNA abundance, some of the genes (PRM1, PRM3, PLCz1, ACR) were overexpressed ($p < 0.05$) in SCL, while SOD1 and CRISP3 were overexpressed ($p < 0.05$) in NSCL. The mRNA from genes ZPBP, NOX5 and CRISP3 was not detected in many samples in SCL. Results suggest that the RNA extraction protocol has considerably effects when analyzing mRNA abundance in sperm. The overexpression of CRISP3 in NSCL is therefore not considered reliable in this study. This study confirmed that in stallion sperm, the inclusion of somatic cell lysis in RNA extraction improves RNA characteristics. The use of heated Trizol cannot be recommended. These protocols should also be tested in frozen-thawed semen where an increase in RNA disruption caused by the cryopreservation procedure must be considered.

P-491 - Adenylate Kinase 9 Involvement in Sperm Flagellar Movement and Ependymal Cell Cilia of Mouse Brain Ventricles

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The extreme subfertility observed in a commercial dairy bull, with only a 10% pregnancy rate despite passing routine laboratory tests of semen quality, led to the discovery of an intronic variant in the bovine adenylate kinase 9 (AK9) gene (doi: 10.1073/pnas.2305712120). This variant activates cryptic splicing, resulting in a truncated protein. While AK9 is known to be involved in nucleoside homeostasis, its specific functions remain unclear. To further explore the role of the Ak9 gene in male fertility, we utilized CRISPR/Cas9 technology to generate mice deficient in Ak9. We established four lines of mice with deletions induced in exon 21 and between exons 21 and 23, causing a frameshift and premature termination of the encoded protein. This was confirmed through western blotting and immunohistochemistry. While both male and female edited mice exhibited normal development, males with homozygous mutations were found to be infertile. Despite the normal appearances of seminiferous tubules and acrosomal caps in knockout mice, the loss of Ak9 resulted in complete immotility of flagella. While sperm parameters such as testis size, gonadosomatic index, and sperm concentration showed no differences compared to wild-type mice, Ak9 knockout resulted in sperm with a high incidence of abnormalities, reduced ATP levels, and impaired fertilization capacity. RNA-seq analysis of testis samples from Ak9 gene knockout mice (Ak9^{-/-}) and wild-type mice revealed differential expression of genes associated with cilium- and flagellum-dependent motility, axoneme and flagellum assembly, microtubule movement, spermatid differentiation, sperm-egg recognition, energy state of the cell, and fertilization.

Ultrastructure analysis of sperm cells from Ak9^{-/-} mice via transmission electron microscopy showed severe disorganization of the (9 + 2) axonemal structure. These results indicated that the cause of infertility was the lack of sperm motility due to both a metabolic and structural deficit of the sperm flagella. Interestingly, AK9^{-/-} animals exhibited abnormal behavior, primarily hyperactivity and impaired balance on the hanging test. Analysis of AK9 expression in different brain regions using immunofluorescence revealed high expression in the ciliated ependymal cells of the ventricles, as well as in other regions of the hypothalamus and frontal cortex. Ultrastructure analysis of the cilia of the ependymal cells from Ak9^{-/-} mice performed by electron microscopy did not identify any disorganization of the axonemes. However, quantification of the lateral brain ventricles showed a significant increase in size in AK9^{-/-} mice, suggesting that the mutation in AK9 may impair ventricular multiciliated neuroepithelial cell function. Our results suggest a dual function of AK9 in fertility and brain functions, mediated by its role in ciliated cells. Our findings highlight the essential metabolic role of AK9 in sperm flagellar structure and motility. Additionally, alterations in mouse behavior and lateral ventricle sizes suggest that AK9 may be involved in the composition and movement of ependymal cilia, potentially affecting cerebrospinal fluid flow. Funding: pre-doctoral grant PRE2019-088813 from the Ministerio de Ciencia e Innovación to P.N.-L., grants FJC2021-047675-I to FM and PID2021-122507OB-100 by MCIN/AEI/10.13039/501100011033 and European Union NextGenerationEU/PRTR to FM.

P-492 - Validating Sperm Surface Lectin Targets for Use in the Nanopurification of Bull Semen

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The spermatozoon glycocalyx is the initial point of contact with the female reproductive tract epithelia and oocyte vestments. Composed of a heterogeneous multi-layered exocellular coating of glycans and glycosylated proteins, this sperm coating has been shown to influence sperm maturation, motility, and fertilization. By targeting the overall composition of the glycocalyx for superior bull sperm selection, our past work demonstrated that nano-purification of pre-processed semen by using lectin PNA-coated magnetic nanoparticles enriched sperm quality and decreased the number of spermatozoa required in an insemination dose by half without compromising pregnancy rates. In the current study, we have evaluated a new set of lectin-conjugated magnetic nanoparticles targeting different surface glycans, both alone and in combination. The lectins PNA, LBA, LCA, and UEA1 were included in our evaluations. We aimed to investigate how selective removal of spermatozoa based on their surface glycan motifs can influence pre- and post-cryopreservation bull sperm parameters. Twelve mature bulls (2.5-4 years old) from the Fort Keogh Livestock and Range Research Laboratory were used for bull semen purification. Semen was collected by electroejaculation, and two ejaculates were pooled per collection for each bull. Semen was assessed for sperm viability, membrane integrity, mitochondrial membrane potential,

oxidative potential, and capacitation status (zinc signatures) using conventional flow cytometry at three timepoints (neat semen, post purification and post thaw of cryopreserved samples). Motility and morphology were also assessed at all three timepoints by using an AndroScope computer-assisted semen analysis (CASA) and light microscopy, respectively. Purification using lectin PNA (targets galactose), or a mixture of lectins UEA1 (targets fucose) and LBA (targets N-acetylgalactosamine); Mix2 decreased ($P < 0.05$) the proportion of bovine spermatozoa with disrupted membranes post thaw when compared to non-sorted control (CON). Purification with Mix2 also increased ($P < 0.01$) the percentage of bovine spermatozoa that were able to effectively respond to an oxidative challenge post thaw when compared to CON, suggesting a greater potential to mitigate oxidative stress. Additionally, purification using a combination of PNA, UEA1 and LBA (Mix1) tended ($P = 0.08$) to improve post thaw motility. We plan to conduct a heterospermic field trial with bull semen from the best performing lectin-coated nanoparticles to assess the impact of the nanopurification on bovine pregnancy rates. Overall, this work will advance our knowledge of sperm surface glycans, their impact on bull sperm survival, fertilization, and potential use as bull sperm fertility biomarkers to improve bovine reproductive efficiency. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2022-67015-36300 from the USDA National Institute of Food and Agriculture.

P-493 - Single Cell RNA Sequencing Shed Light on the Cellular and Molecular Alterations Behind Distinct Types of Cryptozoospermia

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Incomplete understanding of the cellular and molecular alterations underlying male infertility leads to a high degree of diagnostic uncertainty, in fact over 70% of infertile men do not receive a causal diagnosis. The complexity of their reproductive disorder is often reduced to abnormal findings in their semen and hormone analyses. Consequently, patients with distinct etiologies are grouped together based on a similar descriptive diagnosis. An example for this, are men diagnosed with cryptozoospermia (Crypto), in which sperm is only found after centrifugation of the ejaculate (HP:0030974), who can though be highly heterogeneous in terms of testicular tissue composition. Here, we aimed at improving the classification of cryptozoospermic patients based on their cellular composition and evaluating potential distinct cellular and molecular alterations among different patient categories. To unravel the testicular tissue composition and transcriptional profile, we performed single cell RNA sequencing in testis samples from 13 infertile patients (obstructive azoospermia (OA), $n=4$, Crypto, $n=9$), who had undergone a testicular biopsy at the Centre of Reproductive Medicine and Andrology. We assigned the identity of over 65,000 cells, which - based on their transcriptional profile - included all the different germ cell subtypes (spermatogonia,

spermatocytes, and spermatids), as well as testicular somatic cells (Sertoli, Leydig, peritubular, endothelial, perivascular, and immune cells). We performed principal component analysis followed by hierarchical clustering on the principal components using the proportion of the different cell types as input. Interestingly, the clustering resulted in four groups. In group 1 (Control), which included 3 of the OA patients, the spermatids were the most abundant cell type. The Crypto patients were subdivided equally into three groups (n=3 each), which presented immune cells (immune-rich), spermatogonia (SPG-rich) and spermatocytes (SPC-rich) as the most abundant cell type, respectively. One of the patients diagnosed with obstructive azoospermia clustered together with the SPC-rich group. Using a combination of differential gene expression and gene ontology analysis, we evaluated common and distinct molecular changes among the different groups. Interestingly, we could not find any differentially expressed genes between the spermatids of the Control group and those of the three-cryptozoospermic groups, suggesting no transcriptional alterations in this cell type. The immune-rich group showed unique changes in the somatic compartment. Sertoli cells showed signs of reversion to a more immature state, as indicated by increased expression of the Inhibin Subunit Alpha (INHA) and genes involved in oxidative phosphorylation. Leydig cells showed higher expression of genes involved in the “steroid biosynthetic process”; however, the serum testosterone level in these patients did not differ from the controls. The SPG-rich group showed the highest number of differentially expressed genes in the germ cell stages up to diplotene spermatocytes. Gene ontology analyses revealed that the genes were involved in cell cycle as well as DNA repair. Finally, the SPC-rich groups showed the highest number of differentially expressed genes in the early spermatocytes, which were enriched in terms associated with meiotic cell cycle and chromosome segregation. In summary, our study revealed three subgroups of cryptozoospermic patients based on the testicular tissue composition and identified common and specific cellular and molecular alterations among them. Our approach can pave the way to more precise diagnoses for these infertile patients.

P-495 - Exploring The Fertility Rescue Effect of Transgenic Expression of Human PRSS55 in PRSS55 Null Mice

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The need for a non-hormonal male contraceptive pill for men still exists. Serine protease 55 (PRSS55) is a male reproductive tract-specific protein that when knocked out in mice leads to male infertility, making it a viable contraceptive target. The goals of our studies were to determine whether we could rescue fertility in Prss55 KO mice with human PRSS55, allowing for a more suitable animal model to test the efficacy of drug candidates. We used pronuclear injection to generate transgenic mice expressing tagged-human PRSS55 protein. We then crossed the transgenic mice with Prss55 KO mice to generate a rescue line. After generating our mouse lines, we then performed a fertility assessment, analyzed testes and epididymis grossly and histologically, and determined sperm parameters of each line. Morphological differences were observed between our rescue mouse lines and our WT mice when comparing body weight and testis weight, where humanized transgenic mice had lighter weights. Apart from hyperactivation, where rescue males

had decreased hyperactivation, all other sperm parameters were similar between the two groups. Our humanized transgenic mouse lines' poor ability to rescue fertility might be accounted for because of the low homology (57%) between human and mouse PRSS55. PRSS55 is required for male fertility and is a viable target as a small molecule for non-hormonal male contraceptive. This study has been supported by Eunice Kennedy Shriver National Institute of Child Health and Human Development T32 HD098069 (trainee subaward to C.M.S.), the Schepps Foundation, Bill and Melinda Gates Foundation INV-001902 (to M.M.M., and T.X.G.), National Institutes of Health (NIH) R01 HD088412 and P01 HD087157 (to M.M.M., and T.X.G.), NIH R01 HD106056 and R01 HD095341 (to T.X.G. and M.M.M.), Male Contraceptive Initiative David Sokal Innovation Award 2021-302 (to T.X.G.)

P-496 - Changes in Sialylation of Sperm Glycocalyx during in Vitro Capacitation

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Glycocalyx plays an important role in sperm cell physiology and its changes, especially during the capacitation process, can lead to sperm preparation for fertilization. The level of sialylation is crucial for mature spermatozoa giving the sperm a negative charge and protecting them from the immune system within the female reproductive tract. Sialylation may affect sperm motility, participate in the sperm oviductal reservoir formation and in the sperm binding to the zona pellucida. The aim of this work was to find differences in sialylation of sperm glycocalyx before and after in vitro capacitation using biotin-labelled lectins. Using indirect fluorescence, we detected the most significant changes in the flagellum after capacitation of boar spermatozoa showing a decrease in the MAL-II (recognizing α 2-3 sialylated Galb1-3GalNAc) and SNA (recognizing α 2-6 sialylated LacNAc) lectin binding signals. On the acrosome, an increase in percentage of spermatozoa with MAL-II and SNA lectin signals was observed only in the apical part of the acrosome. Unlike pig sperm, the SNA lectin does not recognize any glycans on bull spermatozoa before and after capacitation. MAL-II lectin labelled bull sperm flagella with the same intensity before and after in vitro capacitation. However, a weak non-uniform signal in the head region begins to appear on capacitated spermatozoa. Using MAL-II and SNA lectins in Western blot analysis, we detected a significant decrease in the number of various sialoglycoproteins after capacitation indicating the removal of sialic acids or the sialoproteins from the boar sperm. Similar to fluorescent labelling, an increase in sialylation detected by MAL-II of some bull sperm proteins is visible on Western blots. Our results confirmed desialylation of the boar sperm surface and redistribution of sialoglycoproteins on the sperm acrosome after in vitro capacitation for subsequent binding to the oocyte. The detection of sialylation, especially on the tail of boar sperm, could thus serve as a marker for the evaluation of capacitation status, and in addition, it appears that sialylation on the sperm surface is species-specifically different. Acknowledgments: This work

was supported by the Grant Agency of the Czech Republic (GA22-31156S), VEGA-2/0074/24, the support of the Institute of Biotechnology (RVO: 86652036), and project BIOCEV (CZ.1.05/1.1.00/02.0109) from the ERDF.

P-497 - Unmixing the [Fluorescent] Spectrum of Sperm Cellular Health Utilizing High-Parameter, Image-based, Spectral Flow Cytometry

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Sperm cell health evaluation is crucial for understanding potential male fertility status. Flow cytometry (FC) has been utilized to observe sperm characteristics since the 1970s; however, a limiting function of traditional flow cytometry is that fluorescence overlaps between fluorophores used can crowd one another and limit the number of biomarkers analyzed per assay. Recent advancements have enabled full fluorescent spectrum analysis to observe the unique fluorescent fingerprints of fluorophores. This advancement, called spectral FC, is achieved due to by having a high number of detectors (e.g. >25) compared to more traditional (e.g. < 15) FC along with computational unmixing of the fluorescent spectrum. Previously, the maximum number of fluorophores used in sperm quality analysis was reported to be five. We hypothesize that the use of spectral FC will enable the use of a high number (>10) fluorophores. The BD FACS Discover™ S8, equipped with 78 detectors and image and sorting capability, allows for high parameter subpopulation integration and analysis. Specifically, for sperm, this means the combination of high-throughput and high-parameter cell health and morphology quantification which was previously impossible. In this study, we aimed to multiplex the application of 19 fluorophores for sperm biomarker health determination. Sperm capacitation status; viability; plasma membrane integrity & potential; membrane fluidity; acrosomal membrane integrity; zinc signature; intracellular pH; intracellular calcium; cellular amines; mitochondrial mass, function, activity, and potential; reactive oxygen species (ROS) production (Nitric oxide and total ROS); lipid peroxidation; caspase activity; and cell death due to apoptosis were all targeted simultaneously in this assay. For this purpose, the following fluorophores were used, PNA-PE, LCA-Cy5, Sytox™ Red, DiSBAC2(3), Merocyanine 540, FluoZin™-3-AM, BCECF-AM, Rhod-5N-AM, Zombie Green, MitoTracker™ Green FM, MitoTracker™ Red CMXRos, MitoTracker™ Deep Red FM, JC-1, 4,5-diaminofluorescein diacetate (DAF-2 DA), CellROX™ Orange, BODIPY™ 581/591 C11, CellEvent™ Caspase-3/7 Green, and PE/Dazzle™ 594 Annexin V. Additionally, Hoechst 33342 was used as a nuclear dye for detection of single cellular events and exclude debris from downstream analysis. Following single stain sample acquisition, FlowJo™ (v10.10) software was used to calculate a spectral similarity matrix and spectral unmixing. All of the fluorophores used were able to be successfully spectrally unmixed except for Zombie™ Green, MitoTracker™ Green, and DAF-2 DA. These three fluorophores were too similar (similarity index ≥ 0.99), meaning two of the three fluorophores should be excluded. These results indicate that up to 17 of the 19 fluorophores in the present study can be used in combination to increase subpopulation segregation of sperm for detailed cellular health analysis. Results here support increased sperm quality parameters for the evaluation of fertility potential in livestock sires and men. This project was supported by Agriculture and Food Research Initiative Competitive Grant No. 2022-67015-36298 from the USDA National Institute of Food and Agriculture.

P-498 - Elucidating the Cellular and Molecular Mechanisms Driving Testicular Dysfunction Within in Utero Growth Restricted (IUGR) Boars

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To meet the increased global demand for pork, the industry has emphasized sow prolificacy. However, a direct consequence of greater litter size is an increased proportion of in utero growth restricted (IUGR) piglets due to uterine capacity limitations. Recent data demonstrates that IUGR negatively affects gonadal development and lifetime reproductive potential of breeding boars. For example, adult IUGR boars have smaller testes, reduced numbers of testicular somatic cells (e.g., Sertoli and Leydig), produce less sperm, and, therefore, generate fewer artificial insemination doses compared with normal birthweight (NBW) boars. However, the cellular/molecular mechanisms mediating this effect are poorly defined. Therefore, the objective of this study was to compare the testicular proteome and testis composition between neonatal NBW and IUGR boars. White crossbred littermates from nine litters were selected based on birth weight, resulting in two experimental groups: NBW (1.56 ± 0.03 kg; $n = 28$) and IUGR (1.01 ± 0.04 kg; $n = 14$). At 3 days of age, boars were castrated, and testis samples were processed for histological analysis and a subset ($n = 8/\text{treatment}$) were preserved for proteomics. A total of 1,494 proteins were identified. Of those, 491 proteins were affected by treatment; more specifically, 440 were significantly ($P \leq 0.05$) different between experimental groups, while the remaining 51 proteins tended to differ ($P \leq 0.10$). In total, 222 proteins were upregulated and 269 were downregulated in testes of IUGR boars compared with their NBW littermates. Upregulated proteins in IUGR testes included those involved in apoptosis (BAX, CASP3), fetal gonadal development (NRB0B1), and steroidogenesis (CYP19A3, HSD17B11). Other proteins with important reproductive functions, such as testis descent (INSL3), steroid metabolism (HSD11B2), and antioxidant functions (GSTK1), were downregulated in IUGR testes. Histological analysis revealed that IUGR testes had reduced Leydig cell numbers (per field) compared with NBW boars ($82.04 \pm 3.39 \mu\text{m}^2$ versus $102.30 \pm 2.06 \mu\text{m}^2$; $P < 0.01$), which is consistent with previous data from mature IUGR boars. Interestingly, Leydig cells from IUGR boars were hypertrophic compared with NBW boars ($143.17 \pm 2.52 \mu\text{m}^2$ versus $125.35 \pm 1.52 \mu\text{m}^2$; $P < 0.01$), suggesting a potential compensatory mechanism. The following did not differ between experimental groups: average seminiferous tubule area, interstitial area, number of seminiferous tubules (per field), or total seminiferous tubule area ($P > 0.10$). Notably, the concurrent downregulation of INSL3 and upregulation of BAX and CASP3 in IUGR boar testes may reveal a possible biological mechanism driving hypogonadism in adult IUGR boars. Others have reported that when INSL3, a protein secreted by Leydig cells, is neutralized in boars, an upregulation in apoptotic proteins such as BAX and CASP3 occurs within germ cells. Upregulation of these apoptotic proteins may be a causative agent of reduced germ cell numbers; thus, decreasing total sperm production in adult IUGR boars. Overall, these data suggest that neonatal IUGR boars have distinct cellular and molecular alterations within the testis that may drive poor reproductive

function in adulthood. Ultimately, this study elucidates porcine testis biology and exposes possible therapeutic targets to improve boar reproductive performance.

P-499 - Testis-Specific Actin-Like 7 Family Members A and B (ACTL7A/B) Are Key Components of Cytoskeletal Regulation in Spermatid Development and Are Required for Male Fertility.

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Spermiogenesis is a drastic cellular process required for male fertility where round haploid spermatids develop into functional sperm. This process requires the formation of many unique structures and organelles to both support this morphogenic development as well as to provide key sperm functions. These unique elements created during spermiogenesis include the acrosome (a specialized vesicular body essential for oocyte fertilization), the manchette (a tubulin cuff around the nucleus of spermatids required for intracellular transport and nuclear reshaping), and the flagellum (a predominantly cytoskeletal suprastructure responsible for the motility of sperm). Despite the well-established presence of cytoskeletal actin and actin related proteins (ARPs) in these structures, their specific function and involvement in spermatid development has remained enigmatic. Recent findings have tied several different human polymorphisms of testis-specific ARPs, like members of the ACTL7 and ACTRT subfamilies, to infertility in men. Through the use of mouse KO models, our data has concurred with these observations. We specifically show that ACTL7A plays a crucial role in coordinating the formation of perinuclear theca-associated F-actins for proper acrosomal attachment. Similarly, we have shown ACTL7B to be required for sperm flagellar stability and structural integrity. However, the mechanistic underpinnings of both ACTL7A and ACTL7B in spermatid cytoskeletal regulation and their importance of male fertility is still poorly understood. In this study, we seek to further hone our understanding of the mechanistic functions of these ARPs given their importance for fertility and the multifaceted nature of their respective KO phenotypes that remain unexplained. Herein, we further investigated the dynamic localization of these ARPs during the development of the germline, their molecular associations with other cytoskeletal components, and their direct impact on microtubule stability and PTM status, as well their effect on HDAC localization and nuclear transport. These findings have allowed us to better delineate the diverse impact these ARPs have across different cellular structures and refine already existing models for cytoskeletal dynamics in spermatids. Further study of the functions of proteins like ACTL7A and ACTL7B may lead us to better understand causes for male infertility as well as provide a new avenue towards non-hormonal contraceptive development.

P-500 - Nuclear Associations and In Silico Modeling Implicate Chromatin and Epigenetic Regulatory Mechanisms for Testis-Specific Actin Related Proteins in Spermiogenesis.

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Nuclear remodeling during spermatogenesis is critical for fertility with implications for germ-line epigenetic programming. However, the mechanistic regulation of nuclear dynamics and chromatin remodeling during germ-line morphogenesis to produce sperm is not well understood. Remodeling includes drastic chromatin rearrangements for unique transcriptional programming, meiotic recombination, and DNA repackaging in spermiogenesis. The latter includes transitions of unique histone variants and PTMs impacting transcription and chromatin organization. Here we explore the nuclear and molecular associations for testis-specific actin related proteins (ARPs). ARP variants are known to contribute to somatic chromatin master regulatory complexes conserved in eukaryotes. We and others have shown that the testis specific ARPs ACTL7A and ACTL7B are required for fertility impacting sperm structure through cytosolic and cytoskeletal contributions in animals and humans. Here we report *in vivo*, *in vitro*, and *in silico* evidence for localization to the nucleus. Step-specific spacio-temporal IHC localization, subcellular fractionation westerns, and immuno-EM all provide evidence for temporally regulated nuclear localization. A putative nuclear localization signal was identified through *in situ* overexpression of specific ACTL7B domains. We further identified ACTL7B associations with nuclear proteins including nuclear importins, histones, and the chromatin remodeling complex associated proteins. These included RUVBL1/2 which are known components of nuclear chromatin remodelers that also classically contain somatic ARP variants. Using AI facilitated *in silico* modeling approaches, we developed and validated a model of relative ARP binding affinity with two distinct ARP containing domains in these complexes, and identified site-specific associations for ACTL7A and ACTL7B in the DNA associating HSA domain of these large multimeric nucleosomal regulatory complexes. We propose that through subunit swapping in otherwise conserved chromatin regulatory complexes, novel testis-specific complexes are formed. Transcriptomic profiling of Actl7b KO testis revealed a general up-regulation of transcripts with a notable change in testis-specific transcription factors. Together these data suggest a role in spermatid-specific chromatin remodeling through epigenetic mechanisms. Nucleosomes are classically regulated through recognition of histone PTMs including acetylation. We had previously noted an increase in acetylation of tubulin and mislocalization of the tubulin deacetylase HDAC6 in both Actl7a and Actl7b KO spermatids. We show here that global acetyl-lysine nuclear localization observed in WT spermatids is also absent. Additionally, in the absence of ACTL7A or ACTL7B there is a loss of intranuclear associated HDAC1 and HDAC3, which are known regulators of epigenetic associated histone acetylation changes that in turn regulate gene expression. From a holistic perspective of the data presented, herein we propose mechanistic and structural models for testis-specific ARPs in germ-line nuclear chromatin dynamics. Further investigation into the ARP-driven mechanisms regulating specific chromatin states in the germline has exciting prospects in developing our understanding of heritability, specific disease states, and inter-generational epigenetics.

P-501 - Optimization of the Sperm Energy Restriction and Recovery (SER) Treatment in the Bovine Model

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In recent years, there has been a rise in the utilization of in vitro produced embryos within the global cattle industry. Enhancing embryo production to augment the quantity and quality of embryos capable of producing viable offspring holds significant promise for the agricultural sector. Our recent investigations have shown the efficacy of sperm energy restriction and recovery (SER) treatment in enhancing sperm function and increasing fertilization and early embryo development rates following in vitro fertilization (IVF) in mice. In addition, in the bovine model, SER treatment has shown to enhance the outcome of intracytoplasmic sperm injection (ICSI) without the need for exogenous chemical activation. When applied prior to ICSI, SER-treated sperm led to a 3-fold increase in the percentage of 2-cell embryos compared to non-treated sperm (control, 50% SER vs. 16% control). Notably, 17% of SER-derived embryos progressed to the blastocyst stage while none of the control-derived embryos did. Here, we focused on the optimization of the SER treatment for the bovine model and evaluated its impact on sperm function. Our findings reveal that a one-hour incubation under starvation conditions, followed by recovery in a complete media (containing lactate and/or pyruvate), significantly enhance total and progressive sperm motility (N&#3f4), as assessed by computer-assisted sperm analysis (CASA). This improvement in sperm motility is most pronounced under non-capacitating conditions (N&#3f4). Given the nutrient deprivation aspect of SER treatment, we examined the effects of the addition of either pyruvate, lactate, or a combination of both during the recovery phase. Our results indicate that the maximal improvement in sperm motility with SER occurs when sperm are incubated under starving conditions and subsequently recovered in a medium containing both lactate and pyruvate (N&#3f3). In summary, our findings underscore the efficacy of SER in enhancing sperm function and support its potential application for the in vitro production of bovine embryos. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2022-67016-36302 from the USDA National Institute of Food and Agriculture.

P-502 - Sperm Maturation Regulates Function and Binding of Sperm to Complex and Hybrid N-Linked Oligosaccharides

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Following insemination in mammals, sperm journey through the female reproductive tract to reach the site of fertilization. At the lower oviduct, a carbohydrate-mediated adhesion between sperm and the oviduct epithelium helps form the sperm reservoir. The sperm reservoir can extend the fertilization window by regulating sperm survival and the availability of fertilization-competent sperm at the time of ovulation. Carbohydrate-dependent interactions may also mediate the primary adhesion between spermatozoa and the zona pellucida in a similar carbohydrate-dependent fashion. However, the glycan molecular mediators involved in these interactions remain to be elucidated in detail. This study utilized a novel glycan array containing 140 hybrid and complex multi-antennary oligosaccharides to high-throughput-screen glycan structures that permit sperm tethering. We found that porcine sperm have a high affinity for multi-antennary glycans containing a bisecting GlcNAc and terminating Gal(β 1-4)GlcNAc or Neu5Ac(α 2-6)Gal motifs. Compared to bi-antennary glycans, porcine sperm tethering is enhanced by tetra-antennary and tri-antennary structures with a core GlcNAc(β 1-4)Man linkage. Sperm maturation did not impact glycan tethering except for capacitated sperm's increased preference for Neu5Ac-terminating glycans. Last, we found that sperm tethering by glycans in this array could impact sperm function. Sperm tethered by glycans in the array underwent lower rates of calcium ionophore-induced acrosome reaction when compared to free sperm. This suggests that a delay in sperm capacitation could be mediated by a glycan-mediated mechanism that can be replicated in-vitro. This work was supported by the National Institutes of Health [RO1HD095841 to D.M., T32EB019944 to L.M., and 1F31HD108959 to L.M.].

P-503 - New Multi-Parametric Flow Cytometry Analysis Unveils SCSA Limitations, Enhancing Sperm DNA Fragmentation Assessment.

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Introduction: Chances of natural conception or intra-uterine-insemination (IUI) success are significantly reduced when a sperm DNA fragmentation index (DFI) superior to 30% is observed. But this notion and the usefulness of the test remains controversial. The present set of experiments inspects the clinical performance of the test and provides explanations for the discrepancy in results. Method: 1) DFI scores from an ejaculate and serial dilutions of it were compared to identify any interference factors in the test. 2) In another set of experiments, sperm cells were either single labelled with Acridin (classic method) or double labelled with Acridin and Hoechst. DFI scores were then compared to verify if Hoechst addition creates any interference. 3) In a third set of experiments, the new multiparametric method (MPM) was set by a) double staining sperm with Hoechst and Acridin, and b) characterizing the constituents using a gating combination of forward/side scattering and Hoechst fluorescence. The MPM allowed to discriminate between debris, spermatozoa, and leucocytes without involving Acridin in the gating strategy. Then, the different constituents could be independently plotted into the classical analysis template for DFI calculation using their Acridin fluorescence. DFIs were measured from three spermatozoa-free samples (blanks) and twenty-two samples from different men using the classical method and the MPM. Results from the two methods were compared. Results: 1) Serial dilutions yielded different results compared to the undiluted sample, thus suggesting an interference in the test. 2) Hoechst-Acridin double stained samples had statistically identical DFIs (Paired T-test P value >0,76)

compared to the Acridin single stains, thereby ruling out Hoechst interference in the classical test. 3) MPM did not detect any spermatozoa in the blank sample, whereas $5.6\% \pm 1.4$ of the events from the blank were misidentified as spermatozoa with the classical method. The misidentified spermatozoa average DFI was $71.2\% \pm 16.6$. The number of events identified as spermatozoa and the average DFI were both significantly lower with the MPM when compared to the classical method ($P < 0,001$). Also, an average of $32.7\% \pm 8.7$ of events identified as spermatozoa by the classical method were not spermatozoa but mostly debris according to the MPM. Finally, a weak correlation between results obtained by the two methods ($r = 0,21$) was obtained. However, patients with abnormally high DNA fragmentation levels according to the classical method were not necessarily considered abnormal by the MPM and vice-versa. Conclusion: The classical method does not allow to discriminate spermatozoa from other sperm constituents. This flaw is likely to have a major impact on DFI results, their clinical value, and the choice of treatment. The present work suggests 1) that sperm DNA fragmentation tests as performed presently don't meet the clinical laboratory standards for diagnostic testing 2) a better way to perform the test and 3) the necessity to establish new clinical reference values. It raises the possibility that 1) debris prevalence in oligozoospermia samples compared to normal ones has skewed the results and led the scientific community to conclude to a correlation between oligozoospermia and poor DNA integrity, 2) testicular spermatozoa appear like having better DNA integrity than ejaculated ones only because less debris are present during the analyse, 3) that spermatozoa selection techniques improve DFI scores by removing debris rather than selecting optimal sperm. Further research is required to verify those hypotheses.

P-504 - Metabolic Characterization of Sperm after Early-life Exposure to Malnutrition

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Nutrition is one of the most significant environmental factors influencing both health and fertility. Experimental and epidemiological studies have highlighted the profound influence of dietary factors on male reproductive function. Furthermore, the maternal diet, spanning from pre-conception through pregnancy and lactation, plays a pivotal role in shaping offspring fitness, impacting body composition and the development of various organs, including the reproductive tract, ultimately affecting optimal performance. This study aimed to evaluate the impact of malnutrition during the early postnatal period on the metabolic status of sperm in sexually mature male mice. Newborn pups (F1) were assigned to either the control group (CON), where mothers were fed ad libitum, or the lactation undernutrition group (LUN), where mothers were provided with 50% of the daily portion of chow consumed by control dams. After weaning (postnatal day 21), F1 offspring was fed ad libitum. Sperm was collected from the cauda epididymis of sexually mature F1 males (>6 months) and suspended in EmbryoMax HTF medium. Flow cytometry was then utilized to assess mitochondrial potential (using JC-1 staining) and reactive oxygen species (ROS) production (using MitoSOX Red staining). High-resolution confocal microscopy was employed to visualize stained spermatozoa. Additionally, the Seahorse XF Cell Mito Stress test kit was used to evaluate the metabolic status of the collected sperm in the presence of either 5.6 mM glucose and

20 mM lactate or 5.6 mM glucose alone. The maternal diet during lactation had a mild effect on the sperm metabolic status of F1 male progeny. Although mitochondrial membrane potential was comparable in CON and LUN sperm, lower susceptibility to depolarization by 50 μ M carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a typical mitochondrial uncoupler, was observed in LUN males (vs. CON, $p \leq 0.04$; $n=4$). Additionally, the basal metabolic parameters of F1 offspring sperm (e.g., respiration, ECAR, OCR) were not affected by the maternal diet during lactation ($n=6$). However, in both CON and LUN spermatozoa, baseline respiration and OCR were higher in the presence of 20 mM lactate ($p \leq 0.0001$). Notably, basal ATP production in the presence of 20 mM lactate was increased in sperm collected from LUN males (vs. CON, $p \leq 0.0001$; $n=4$). MitoSOX staining ($n=7$), a superoxide indicator, did not reveal changes in ROS+ sperm counts under normal and heat stress conditions. In summary, it seems likely that early-life malnutrition in the F1 generation impairs the metabolic status of sperm. Further studies are necessary to elucidate the impact of maternal undernutrition on the reproductive performance of male offspring and their capacity to conceive healthy progeny. Research supported by Polish National Science Centre [2018/31/B/NZ4/03527] and basic Institute funds [19/FBW/2024].

P-505 - The Effects of Short-Term and Chronic Daily Propylparaben Exposure on Estrous Cyclicity and Fertility in Mice

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Parabens are a group of chemicals widely used as preservatives in cosmetics, prescription medications, and personal care products. Evidence suggests that parabens have weak estrogenic endocrine-disrupting properties. This is concerning because altered signaling of reproductive-related hormones can have marked effects on estrous cyclicity and fertility outcomes in females. The effects of propylparaben, one of the most commonly used paraben, on mouse estrous cyclicity and fertility are unknown. Here we hypothesized that propylparaben at low levels of exposure can negatively affect estrous cyclicity and fertility outcomes in mice. Thus, adult female CD-1 mice were treated with propylparaben at 0, 2, 20, and 200 μ g/kg/day over the course of one, three, and six months. Estrous cyclicity was evaluated over three weeks prior to the end of each dosing period to compare cycle lengths and numbers, and time spent in each estrous stage. Fertility studies were conducted after 6 months of chronic propylparaben exposure ($n=7-8$ per group). Analysis of variance of cyclicity outcomes (cycle length in days and numbers of cycles in 21 days) was performed with a linear mixed model for repeated measures. The model included treatment as a fixed effect and mouse as a random effect to account for repeated measures on the same mouse. The effect of treatment on the marginal means of the outcome variables was compared using Fisher's Least Significant Difference test. Overall, significant statistical differences were limited, likely due to relatively high biological variability between groups and small numbers of mice per group ($n=6-9$ /group). The mean ($\pm$ SEM) 'numbers of cycles' at 3 months differed between groups ($p=0.055$) and was higher in the control group (2.11 ± 0.27) compared to the 2 μ g/kg/day (1.27 ± 0.32 ; $p=0.055$) and the 200 μ g/kg/day (1.13 ± 0.33 ; $p=0.031$). Further, we observed reduced neonatal survival along with a higher incidence of pups' death per litter following propylparaben exposure. Specifically, percentages of total live pups/total pups were 91% (controls), 90% (2 μ g/kg), 77% (20 μ g/kg), and 83% (200 μ g/kg). Incidences of dead pups were 3/7 (controls), 6/8 (2 μ g/kg), 5/8 (20 μ g/kg), and 2/8 (200 μ g/kg). Yet, statistical analysis of fertility outcomes did not result in significant

differences between the groups, including averages of pup's weight (1.66gr-1.71gr), litter size (10-12), or number of dead pups per litter. Overall, our finding suggests that environmentally relevant propylparaben exposure had a limited impact on estrous cyclicity and fertility in female mice.

P-506 - Di(2-ethylhexyl) Phthalate Increases Expression of Several Oxidative Stress Markers in the Mouse Ovary

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Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer used in a wide range of consumer products including building materials, food and beverage containers, and children's toys. This is concerning because DEHP can leach out of products and enter the human body. Furthermore, DEHP has been shown to exhibit endocrine disrupting abilities after both short-term and long-term exposures. However, little is known about the effects of DEHP on the ovary. Thus, this study tested the hypothesis that DEHP alters gene expression in the mouse ovary. To test this hypothesis, female CD-1 mice aged 39-40 days were orally dosed with either vehicle control (corn oil) or DEHP (20µg/kg/day - 200mg/kg/day) for 10 days. The ovaries from these mice were then collected immediately post-dosing and subjected to gene expression analysis for regulators of oxidative stress. The results show that DEHP exposure at both 200 µg/kg/day and 200 mg/kg/day significantly increased ovarian expression of superoxide dismutase 1 (Sod1), catalase (Cat), glutathione peroxidase 1 (Gpx), glutathione S-transferase kappa 1 (Gstk1), and glutathione S-transferase alpha 1 (Gsta1). DEHP exposure at all doses significantly increased ovarian expression of superoxide dismutase 2 (Sod2), glutathione S-transferase theta-1 (Gstt1), and glutathione S-transferase theta-2 (Gstt2). Additionally, DEHP exposure at 20 µg/kg/day significantly increased ovarian expression of glutathione peroxidase 2 (Gpx2). DEHP exposure at 200 µg/kg/day significantly increased ovarian expression of glutathione S-transferase pi 1 (Gstp1) and DEHP exposure at 200 mg/kg/day significantly increased ovarian expression of glutathione S-transferase zeta 1 (Gstz1). Finally, DEHP exposure at all doses significantly decreased ovarian expression of glutathione synthetase (Gss). All of the selected genes are related to the regulation of oxidative stress in the body. Sod1 and Sod2 are genes that encode the enzyme superoxide dismutase, an enzyme that breaks down superoxide radicals in the body. Similarly, Cat is a gene that encodes the enzyme catalase, an enzyme responsible for breaking down hydrogen peroxide in the body. Gpx, Gpx2, Gss, Gsta1, Gstp1, Gstk1, Gstt1, Gstt2, and Gstz1 are all responsible for the production and transfer of glutathione, another prominent antioxidant in the body. Together, these data suggest that DEHP exposure for 10 days affects oxidative stress pathways in the mouse ovary. Supported by NIH RO1ES034112 and the Aarush M. Patel Foundation Fellowship.

P-507 - Prenatal Exposure to BPA and BPS Transgenerationally Alters the Transcriptome and Epigenome in Male Germ Cells

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Prenatal exposure to bisphenol (BP) A and BPS leads to transgenerationally maintained lower sperm counts and motility in male mice. Rigorous prior research suggests that certain epimutations sustained in the germline can potentially be transmitted to subsequent generations. Thus, we examined the transcriptomic and epigenetic impacts of prenatal exposure to BPA and BPS on the transgenerational murine male germline using single-cell multiomics. Pregnant CD-1 females (F0) were administered 50 µg/kg/day BPA or BPS orally from gestational day 7 to birth, and postnatal day 6 germ cells from F1, F2, and F3 males were collected for the multi-omics (scRNA-seq and scATAC-seq). In the F1 males, scRNA-seq analysis revealed that prenatal exposure to BPA and BPS induced upregulation of a large number of genes that are associated with the acceleration of spermatogonial stem cell differentiation and disrupt the balance of stemness in the neonatal germ cells. Pseudo-trajectory and Gene Ontology analysis further suggested that BP-induced accelerated stem cell differentiation was accompanied by metabolic changes. scATAC-seq analysis showed that differentially accessible chromatin sites were primarily in the promoter regions. Combined gene expression and transcription factor (TF) motif analysis, KLF/SP and DMRT family members were highly enriched and elevated motif activities by BP exposure. The candidate targets of top-active TFs were then predicted, and most genes showed a pattern of expression along with germ cell differentiation trajectory. In the F2 and F3 males, upregulated genes related to mitosis/meiosis and metabolic pathways were sustained in BP-exposed groups as in the F1 males. In contrast, fewer genes were explicitly identified in the F3 males. Interestingly, BPA exposure showed substantial effects on gene expression in the F1 offspring, whereas BPS exposure induced longer-lasting effects on the biological process associated with differentiation through F1 to F3 generations. Collectively, our findings provide the first systematic results for understanding the transgenerational transcriptomic and epigenetic alterations by prenatal exposure to BPA or BPS in male germ cells. Supported by NIH/NIEHS R21 ES031607.

P-508 - Altered abundance of proteins involved in chemical biotransformation in inguinal adipose tissue in lean and obese female mice during exposure to dimethylbenz[a]anthracene

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Lifestyle factors can lead to increased adiposity which can reduce female fertility. Obesity, which is associated with impaired oocyte quality, conception, and implantation, affects over 40% of US reproductively aged women. Many environmental toxicants are lipophilic and are sequestered into adipose tissue, including the polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene (DMBA), which is a known ovotoxicant. DMBA is generated during combustion of organic material present in tobacco products, smoked meats, and diesel exhaust fumes. Chemical biotransformation systemically is necessary to prevent toxicants from impacting the ovary and previous studies support that obese mice suffer increased DNA damage and a reduced ovarian protective response compared to lean counterparts during ovotoxicant exposure. The possibility that this differential ovotoxicity in obese compared to lean mice results from extrahepatic biotransformation in the adipose tissue remains unclear. This study investigated the hypothesis that DMBA exposure will alter adipose tissue proteins associated with chemical biotransformation in lean and obese female mice. Female KK.Cg-a/a (lean; n = 20) and KK.Cg-Ay/J (obese; n = 20) were provided a regular mouse chow diet ad libitum. When the obese group had ~30% increased body mass compared to lean mice (~10-weeks old), mice received an intraperitoneal injection with either corn oil (vehicle control; CT) or DMBA (1 mg/kg) for 7 d. Thus, there were four treatment groups:

lean-CT, lean-DMBA, obese-CT, and obese-DMBA (n = 10 each). Euthanasia occurred at day two of diestrus, upon which body, ovary, uterus, spleen, liver, and adipose tissue weights were recorded. Inguinal adipose tissue protein was isolated and western blotting determined that obesity increased (P < 0.05) AHR, GSTP1, and CYP1B1 but decreased (P < 0.05) CYP19 protein level. In lean mice, DMBA exposure increased (P < 0.05) AHR but, in contrast, in the obese mice, DMBA exposure decreased (P < 0.05) AHR protein level. Comparison between lean-DMBA and obese-DMBA mice identified increased (P < 0.05) EPHX1 and CYP1B1, and decreased (P < 0.05) AHR, CYP1A1 and CYP19 proteins. These findings support the hypothesis that the adipose tissue biotransforms chemicals with a differential response in the adipose tissue from an obese compared to a lean female, potentially affecting female reproductive and general health. Supported by 1R01ES030341-01 and 1R01ES030341-S2 from NIEHS.

P-509 - Molecular Mechanisms Underlying the Adverse Outcomes of DEHP Exposure on Human Preovulatory Follicles

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Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer known to possess endocrine disrupting activities. DEHP may enter the human body through leaching from everyday plastic objects. It is extensively metabolised upon uptake forming a range of hydrolysed and oxidized metabolites that are considered the active endocrine disrupting forms. While DEHP metabolites have been extensively investigated in relation to male reproductive disorders, rodent studies have also raised concern that DEHP may affect ovarian folliculogenesis and steroid hormone production, ultimately affecting fertility in females. In a previous study, we reported a significant association between DEHP metabolites in the human follicular fluid (FF) and reduced responsiveness to controlled ovarian stimulation during IVF treatments. Here, we investigated the mechanisms underlying these effects through molecular analysis of follicular fluid samples. To explore the molecular cues altered by DEHP exposure in humans, samples from 96 IVF patients from Swedish (N=48) and Estonian

(N=348) infertility clinics were analysed. The patients were stratified into high (mean $7.7 \pm \text{SD } 2.3$ nM, N=348) and low (0.8 ± 0.4 nM, N=348) DEHP exposure groups according to the molar sum of the measured levels of 4 DEHP metabolites MEHP, MEHHP, MEOHP and MECPP in their FF samples. Extracellular miRNA levels and 15 steroid hormone concentrations were measured from the same samples by small RNA sequencing and LC-MS/MS, respectively. In addition, FF somatic cell samples from the individual follicles were available in the Estonian cohort, and these were used to measure mRNA expression levels by RNA-seq. The results were compared between the high and low DEHP groups using the cohort as a covariate. In addition, data integration was used to understand the interactions between the disturbed miRNA, mRNA and steroid levels within a single follicle. Differential expression (DE) and miRNA:mRNA network analysis revealed that the expression levels of the majority of genes in the cholesterol biosynthesis and steroidogenesis pathways were significantly decreased ($\text{FDR} < 0.05$). The DE miRNAs ($\text{FDR} < 0.1$) were predicted to target a considerable number of key enzymes in these pathways ($\text{FDR} < 0.05$). The down-regulation of CYP17A1 ($\log_2\text{FC} = -2.34$, $\text{FDR} = 0.022$) in the somatic cells was linked to a reduced progesterone to 17-OH-progesterone ratio ($p = 0.046$) in the FF of women in the high DEHP group. The expression levels of genes involved in inflammatory processes were significantly elevated in the women from the high DEHP group ($\text{FDR} < 0.05$). Following a computational cell-type deconvolution approach, an indication for the infiltration of cells expressing leukocyte markers of M1 and M2 macrophages, T-cells ($p < 0.01$), as well as neutrophils ($p < 0.05$) was observed. In conclusion, exposure to DEHP metabolites significantly alters the follicular milieu within human ovaries: the inflammatory environment is stimulated, while lipid and steroid synthesis are obstructed. This study contributes to understanding the molecular mechanisms by which DEHP exposure may disrupt ovarian sensitivity to pituitary hormones in humans.

P-510 - Bisphenol A Impairs Brain Formation and Neurodevelopment by Enhancing HOX Genes

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Plastic pollution and exposure to synthetic chemicals are becoming a serious problem. There has been accumulating evidence that exposure to endocrine disrupting chemicals (EDCs) during development can lead to various adverse effects in humans. Brain and neuronal development are highly dependent on a combination of multiple signaling molecules and considered to be susceptible to EDCs. In fact, there are an increasing number of incidents of neurodevelopmental impairments such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD). However, the causal link between these neurodevelopmental disorders and EDCs is not clear. One of the reasons for this is due to the lack of a suitable testing method. Here, we used

human induced pluripotent stem cells (iPSC) and zebrafish embryos to investigate the effect of Bisphenol A (BPA) on brain and neuronal development. BPA is the most highly studied EDC and has been widely used in manufacturers of polycarbonate plastics and epoxy resins including food and drink packages, baby bottles, dental sealants, and thermal receipt paper. We found that BPA enhanced HOX gene expression in the presence of exogenous retinoic acid (RA), but not when BPA was used alone in iPSC. This effect was impeded with retinoic acid receptor (RAR) antagonists, but not with estrogen receptor (ER) antagonists, suggesting that ER is not involved in this signaling. To verify their physiological relevance, we also applied BPA to hindbrain formation in zebrafish. Spatial expression analysis of *hoxb1a*, *fgf8*, and *otx2* revealed that BPA enhanced RA-induced rostral shift of these brain markers, implying posteriorization of brain region, but not when BPA was used by itself. This rostral shift was successfully simulated with mathematical modeling. Co-exposure to BPA and RA also resulted in the duplication of Mauthner cells and the abnormal craniofacial cartilage formation. Transcriptome analysis revealed that 3'HOX genes and genes related to brain formation exhibited a similar expression profile between iPSC and zebrafish under these experimental conditions. We compared these transcriptomes using GO-based gene set enrichment analysis and found characteristic clusters, which consists of GO terms related to anterior/posterior pattern specification, brain formation, and skeletal system development. These GO terms were highly related to the adverse outcomes we observed. All the data we obtained are consistent with the notion that BPA potentiates RA signaling. Therefore, we are able to link the chemical treatment and detrimental outcomes with potentiation of RA signaling. As RA signaling plays a key role in many fundamental biological processes, pleiotropic effect of BPA showed good agreement with the interference of RA signaling. This result also implies that iPSC can be a useful model for predicting adverse effects in zebrafish and provide a new in vitro method in toxicology and environmental sciences.

P-511 - Diethylstilbestrol and Ketoconazole Alter Lateral Adherence of Cultured Bovine Oviduct Epithelial Cells

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Diethylstilbestrol and ketoconazole alter lateral adherence of cultured bovine oviduct epithelial cells. Barend M. Gadella 1,2, Konstantina Asimaki 1,2, Jonna van den Berg 1, Majorie B.M. van Duursen3,* 1. Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, the Netherlands2. Department of Biomedical Health Sciences, Faculty of Veterinary Medicine, Utrecht University, the Netherlands3. Amsterdam Institute for Life and environment, Section Environment and Health, Vrije Universiteit Amsterdam, the Netherlands The worldwide problem of increasing frequency of female reproductive disorders is considered to be partially related to the rise in endocrine disrupting chemical (EDC) production and exposure. EDCs can interfere with normal hormonal action and regulation, and the effect of the two well described EDCs diethylstilbestrol (DES; a synthetic estrogen agonist) and ketoconazole (KTZ; a steroidogenic enzyme inhibitor) are examined on bovine oviduct epithelial cells (BOECs). In this thesis work a 23-day BOEC monolayer culture in Transwell® inserts was established using the air-liquid interface (ALI) method to maintain the differentiated BOEC phenotype. BOEC-ALI cultures showed columnar epithelial cell monolayers that were confluent by cell-cell adherence at the apical side (>90%) of these cells. In this culture system four-day exposure to either DES or KTZ resulted in a substantial cell-cell adherence to a lower or even basal position in the still columnar epithelial that remained to

have full confluency properties (< 35 % of the cells remained apically adhered $p < 0.001$). In part the removal of DES led to a recovery of the cell-cell adherence to the apical area of the columnar epithelium (>50 %; recovery effect not studied for KTZ). Currently the transflux of both EDCs from the basolateral compartment of the Transwell® inserts over the BOEC into the apical compartment are determined by HPLC-MS/MS after cold ethyl-acetate extraction from both fluids. This provides insights in the possibility that exposure of DES or KTZ to the BOEC will attenuate potential toxic EDC effects in the lumen of the oviduct and thus have a more potential to influence the processes of fertilization and early embryonal development. *This project is funded by the European Union's Horizon 2020 research and innovation program "FREIA" grant number 825100.

P-512 - Iodoacetic Acid Alters the Expression of Oxidative Stress Genes in the Mouse Ovary

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The incorporation of water disinfectants into the main water supply has significantly decreased the presence of waterborne diseases such as cholera. However, the interaction between disinfectants and organic material generates water disinfection byproducts (DBPs). Iodoacetic Acid (IAA) is one unregulated DBP and has been reported as a reproductive toxicant. Our previous studies show that IAA inhibits follicle growth and alters the expression of steroidogenic factors in vitro, but its effects on the expression of oxidative stress markers are unknown. Thus, this study tested the hypothesis that IAA exposure alters gene expression of oxidative stress markers in the mouse ovary. To test this hypothesis, adult CD-1 mice were dosed with either water (control) or IAA (0.5 mg/L; 10 mg/L; 100 mg/L; 500 mg/L) in their drinking water for 35 days. Following this, ovaries were collected when the mice were in diestrus, RNA was extracted, reverse transcribed, and subjected to quantitative polymerase chain reaction (qPCR) to analyze the expression of oxidative stress markers with glutathione properties (Gstt1, Gstp1, Gsta1, Gsto1), antioxidant properties (Gpx1, Gsr, Cat), and superoxide properties (Sod1, Sod2). IAA exposure decreased expression of glutathione oxidative stress markers Gstt1 for the 0.5 mg/L, 10 mg/L and 500 mg/L treatment groups compared to the control and Gstp1 for the 10 mg/L treatment group compared to the control, but did not affect the expression of Gsto1 or Gsta1 compared to control. Further, IAA exposure decreased the expression of the antioxidant oxidative stress marker Cat for the 0.5 mg/L, 10 mg/L, 100 mg/L and 500 mg/L IAA treatment groups compared to the control, but did not affect the expression of Gpx1 or Gsr compared to control. In addition, IAA significantly decreased the expression of the superoxide oxidative stress marker Sod1 for the 0.5 mg/L, 10 mg/L and 100 mg/L IAA treatment groups compared to the control, but did not affect the expression of Sod2 compared to control. These data indicate that IAA exposure alters oxidative stress gene expression in the mouse ovary. This project is supported by NIH R21ES028963.

P-513 - Investigation into the effects of Sodium Valproate on Developing Fetal Gonads in Vitro

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One in every 200 pregnant women suffer from epilepsy, requiring treatment with anti-epileptic drugs (AEDs) which prevent seizures that can cause harm to the mother and fetus. Sodium valproate (SV) is an AED that was contraindicated in women of childbearing age in 2018 due to its significant teratogenic risks and in January 2024, The Medicines and Healthcare products Regulatory Agency (MHRA) issued a national patient safety alert advising that SV prescriptions for all patients under 55 will be required to be signed off by two independent specialists. Prior to 2018, SV was prescribed during pregnancy and around 20,000 children in the UK are estimated to have been prenatally exposed to SV. Approximately 40% of these children are at risk of developmental disorders, yet the assessment of its effects on the developing fetal gonads has been limited. We have investigated this using tissue culture of mouse fetal gonads. Mouse gonads were collected from embryonic day 13.5 (E13.5) CD1 embryos. Fetal ovaries were cultured for 12 days, with either DMSO or 10 µg/ml of SV added to the culture media for the first 6 days of culture only (n=6). Ovarian follicle number and health were analysed histologically, and levels of DNA double-strand breaks (DSBs) were examined by carrying out immunohistochemistry for γH2AX. Fetal testes were cultured for 96 hours with either DMSO or 10 µg/ml of SV for the whole duration of culture (n=5). ELISA was used to assess testosterone concentrations in culture media collected at 48 and 96 hours. Gross morphology of testicular tissue was examined histologically. SV treatment did not significantly impact the number or health of follicles in cultured ovaries, and did not impact the level of DSBs. Furthermore, testicular tissue health and seminiferous cord definition were not impacted by SV exposure. However, testosterone levels were significantly decreased between 48 and 96 hours of culture in SV treated testes ($p < 0.01$). Overall, our results suggest that exposure to clinically relevant doses of SV does not have a detrimental impact on the morphology of the developing fetal ovary or testis. However, SV does negatively impact testosterone secretion, suggesting a potential impact on the fetal Leydig cells. Further studies are needed to confirm these findings and to determine whether similar effects are observed in vivo and/or in human fetal gonads.

P-514 - Effect of Selective Serotonin Reuptake Inhibitor, Escitalopram, on Adrenal Cell Steroidogenesis

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Depression is a highly prevalent psychiatric disorder worldwide and lowers the quality of life. Selective serotonin reuptake inhibitors (SSRIs) are the first-line pharmacotherapeutic option for the treatment of depression and other psychiatric disorders. However, SSRIs are associated with a range of side effects, many of them linked to the endocrine system e.g. hormonal imbalances, breast enlargement, sexual dysfunction, and menstrual cycle disorders. Thus, they affect both male and female sexual development and fertility. Five drugs of the SSRI family, namely citalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline, were previously shown to alter the steroid secretion by the H295R cells. However, the effect of Escitalopram, another drug in the SSRI family, on adrenal cell viability and steroidogenesis has not been studied. Hence, we aimed to study the effect of Escitalopram on the viability and steroid production of adrenal cells. The human

adrenocortical carcinoma cell line, H295R, was grown in DMEM/F-12 medium supplemented with 7.5% FBS and 1% ITS+ Premix. Cells were seeded in 24-well plates or 96-well plates respectively for measurement of steroid production or cell viability. Cells were treated with different doses (0 – 400 μ M) of Escitalopram for 24 hours and spent culture media was harvested. Testosterone and progesterone were measured using Chemiluminescent microparticle immunoassay (CMIA) while androstenedione levels were measured by Enzyme-linked immunosorbent assay (ELISA). MTT assay was performed to assess the effect of Escitalopram on the viability of the H295R cells. It was observed that the production of progesterone was not affected by any of the drug doses. However, both the androgens measured showed reduced levels after treatment with Escitalopram. A significant decrease in the secretion of testosterone was seen only at higher doses of Escitalopram while androstenedione secretion was significantly decreased with all doses of Escitalopram. Interestingly, a 100 – 400 μ M dose of Escitalopram decreased cell viability also. Further experiments are required to decipher the molecular mechanisms underlying this negative impact of Escitalopram on adrenal cell steroidogenesis.

P-515 - Flavored E-Cigarettes Reduce Embryo to Placental Weight Ratios and Elicit Differential Gene Expression of Placental Growth, Invasion, and Antioxidant Response Pathways

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A rising 10% of women report using electronic cigarettes or e-cigarettes as a supposed safer substitute for traditional cigarettes during pregnancy. Cigarette use during pregnancy impairs embryo implantation, induces pre-term birth, and can result in fetal developmental abnormalities. Yet, few studies have examined how e-cigarettes impact pregnancy. Unlike cigarettes, e-cigarettes heat and aerosolize a base liquid consisting of propylene glycol and vegetable glycerine with additives such as nicotine and flavorings. During early pregnancy, the placenta develops after trophoblast cells invade and remodel the maternal artery network. Trophoblast cell invasion is promoted by the hormone peptide, adrenomedullin (ADM), and angiogenesis is induced by placental growth factor (PGF). As the placenta grows, reactive oxygen species can develop eliciting upregulation of antioxidants including the selenoprotein families: glutathione peroxidase (GPX) and thioredoxin reductase (TXNRD). Recent studies have shown e-cigarette liquids impaired in vitro trophoblast cell invasion; yet no studies have examined flavored e-cigarettes. Furthermore, the impact of e-cigarette use on placental development is unknown. We aimed to examine how e-cigarette liquid with flavoring can impact trophoblast cell gene expression and placental development. We hypothesized that e-cigarette vapors with flavoring impair placental growth pathways and induce an antioxidant response. To test this hypothesis, wildtype C57BL/6J animals were exposed to sham or e-cigarette vapors containing flavoring with and without nicotine (6 mg/mL) until day 12.5 of pregnancy. Concurrently, the same e-cigarette liquids were heated and condensed. These condensates were administered for 6 hours at a 1.5% concentration to the transformed trophoblast cell line, HTR8/SVneo. In the e-cigarette exposed animals, embryo to placental weight ratios were decreased significantly using one-way ANOVA with the student's t-test

(n=76 fetal-placental pairs). Furthermore, in the absence of nicotine, GPX3 and GPX4 mRNA was significantly reduced in female placentas using the student's t-test (n=3 placentas). Thus, e-cigarette exposure impairs fetal-placental growth and can dampen the GPX antioxidant response in a sex-specific manner. In the trophoblast cells, placental growth regulator PGF, was upregulated while ADM was downregulated using the student's t-test (n=3 replicates). In the absence of nicotine, TXNRD1 was upregulated. Hence, e-cigarette condensates may promote trophoblast cell growth and the antioxidant response via PGF and TXNRD1, but impair ADM-driven trophoblast invasion. Therefore, e-cigarettes with flavoring reduce embryo to placental weight ratios and modulate genes critical for placental development and the antioxidant response. Future directions aim to measure in situ levels of reactive oxygen species upon e-cigarette exposure and identify how nicotine contributes to the placental antioxidant response. These studies suggest a measure of caution be observed when using these devices during pregnancy. This work was supported by NICHD 1K99HD10490001, R00HD104900 to MWM.