ORAL PRESENTATONS

SSR Society for the Study of Reproduction **59TH**

ANNUAL MEETING

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Novel Technologies for Ovarian Biology

Monday, July 20 10:00 AM – 12:00 PM

Submission ID#2365678

Bridging Engineering and Biology: New Frontiers in Ovarian Function Restoration and Fertility Research

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Abstract Text

Premature ovarian insufficiency (POI) and infertility are irreversible and life-altering consequences of radiation and chemotherapy, significantly affecting young cancer survivors. Restoring reproductive function in women poses a unique challenge, as they are born with a finite and non-renewable reserve of ovarian follicles—the ovary's functional units, each containing a single oocyte and hormone-producing somatic cells. Current clinical options for restoring lost ovarian endocrine function are limited to hormone replacement therapy (HRT), which provides only a subset of gonadal hormones in a continuous fashion, unlike the cyclical and pulsatile hormone patterns found in healthy young women.

To address these limitations, my lab has developed a cell-based therapy that delivers the full spectrum of ovarian hormones at physiological levels and rates, and is capable of responding to feedback from the body. This innovative therapy uses an immune-isolating hydrogel capsule with a degradable core and a non-degradable shell to encapsulate and implant donor ovarian tissue. The design enables growth and expansion of the ovarian grafts while effectively preventing immune cell infiltration. In studies with immunocompromised and immunocompetent ovariectomized mice, implantation of encapsulated human ovarian tissue successfully restored estrous cycles and produced systemic estradiol levels, correlated with the presence of large antral follicles in the grafts—results comparable to non-encapsulated controls.

Our research also aims to enhance in vitro ovarian follicle development. We have engineered biomimetic matrices that support the deposition and retention of extracellular matrix (ECM) molecules secreted by cultured follicles and cells, replicating the fibrous structure of native ECM to better support folliculogenesis.

Lastly, the engineering efforts in my lab are closely integrated with biological discovery. Recently, we published a high-resolution spatial cell atlas of the human ovary and sequenced individual oocytes from human primordial follicles by combining spatial transcriptomics with single-cell RNA sequencing. By profiling over 18,000 genes in carefully selected ovarian regions, we revealed localized gene expression patterns and distinct cellular architectures. This spatially resolved atlas, highlighting new molecular markers and structure–function relationships critical to fertility, hormone production, and ovarian aging, serves as a valuable resource to optimize biomimetic platforms for follicle growth both in vitro and in vivo. Ultimately, our research aims to advance cures for infertility and loss of ovarian function through innovative therapeutic approaches.

Submission ID#2358228

Anti-Müllerian Hormone Protects Human Ovarian Follicles and Stromal Cells From Cyclophosphamide-Induced DNA Damage and Apoptosis *in Vitro*

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Abstract Text

A key adverse effect of chemotherapy on female fertility is damage to the primordial follicle pool. Loss of primordial follicles following chemotherapy is thought to occur through either direct cytotoxic effects, or through the premature growth activation of resting follicles. A promising candidate to protect the ovarian reserve from chemotherapy-induced damage is Anti-Müllerian hormone (AMH). We recently published data demonstrating that damage to human primordial & transitional (i.e. non-growing) follicles caused by 4-hydroperoxy-cyclophosphamide (4-HC – the active metabolite of cyclophosphamide) was mitigated by co-treatment with AMH after 24 hours *in vitro*. Here, we investigated both the immediate and longer-term effects of 4-HC on both human ovarian follicles and stromal tissue and examined the timing and duration of AMH-mediated chemoprotection in the human ovary.

Ovarian cortical biopsies from women undergoing elective caesarean section (25 – 35 years old) were exposed *in vitro* to 2μM 4-HC (or DMSO vehicle) ± 200ng/ml AMH. Tissue was treated for 4 hours (N = 5 - 7 biopsies), or 24 hours with ongoing culture for 7 days (N = 5 - 7 biopsies). Follicle stage and morphological health were assessed with H&E staining. TUNEL staining and immunostaining for γH2AX and cPARP were used to analyse follicle DNA damage and apoptosis, respectively. Ovarian stromal apoptosis was determined using TUNEL staining (mean grey value/mm^2). Granulosa cell proliferation was analysed using Ki67 immunostaining.

At 4 hours, there was an approximate two-fold increase in morphologically unhealthy non-growing follicles in 4-HC-treated tissue compared to vehicle control (vehicle: 24 ± 4% vs 4-HC: 46 ± 8%, $p< 0.0001$); this effect was prevented with AMH co-treatment (30 ± 6%, $p< 0.0001$). After 4 hours of 4-HC exposure, DNA damage (TUNEL+) in ovarian stromal tissue also increased (vehicle: 23 ± 6 mgv/mm^2 vs 4-HC: 53 ± 11, $p< 0.05$) and was also prevented by concurrent AMH treatment (22 ± 2, $p< 0.05$).

7 days following 24h 4-HC treatment, non-growing follicles remained morphologically unhealthy (vehicle: 32 ± 8% vs 4-HC: 47 ± 3%, $p< 0.001$), however 24h co-treatment with AMH conferred chemoprotection (27 ± 8%, $p< 0.05$). The proportion of non-growing follicles with DNA damage (TUNEL+ or γH2AX+) were approximately doubled in the 4-HC group compared to vehicle, (TUNEL; vehicle: 6.7 ± 3.8% vs 4-HC: 23.5 ± 12.9%, γH2AX; vehicle: 26 ± 7% vs 4-HC: 61 ± 14%, $p< 0.0001$), with DNA damage markedly reduced following co-treatment with AMH (TUNEL: 10.3 ± 4.4%, γH2AX: 30 ± 10%, $p< 0.001$). This corresponded with a four-fold increase in apoptotic follicles (cPARP+) in the 4-HC group compared to vehicle (vehicle: 8 ± 3% vs 4-HC: 35 ± 9%, $p< 0.0001$), which returned to vehicle levels with AMH co-treatment (4 ± 2%, $p< 0.0001$). There was no significant difference observed in either follicle stages or Ki67+ granulosa staining between any treatment groups 7 days post-4-HC exposure.

These data demonstrate that AMH protects human ovarian follicles and stroma from the direct cytotoxic effects of cyclophosphamide. We observed no evidence in this model to suggest that premature follicle activation occurred as a result of cyclophosphamide exposure. The protective effect of AMH is both immediate and persisting, suggesting it may have therapeutic potential as a fertility protecting agent during chemotherapy treatment.

Submission ID#2360929

Integration of ATAC-seq and RNA-seq Reveals Epigenetic Reprogramming Associated with Cholesterol–Lipid Metabolism in Granulosa Cells in Poor IVF Responders

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Abstract Text

Infertility impacts approximately 11-13% of couples globally and often requires assisted reproductive techniques like in vitro fertilization (IVF) for successful pregnancies. Due to lifestyle factors, women are delaying pregnancy into their mid-30s, when ovarian aging begins. Granulosa cells (GC), which surround the oocyte, are crucial for steroid production and oocyte maturation. Despite advances in technology, IVF remains physically, emotionally, and financially demanding, with generally low success rates and variable outcomes. This variability increases with age: some women respond well (retrieving ≥17 oocytes—good responders), while others respond poorly (≤8 oocytes—poor responders) to gonadal stimulation. As a result, patients are often classified based on ovarian response regardless of age. However, the molecular factors that differentiate these phenotypes, especially within the follicular microenvironment are not well understood. There is a knowledge gap about how age affects GC chromatin accessibility and response to ovarian stimulation. We hypothesize that GC of patients with good and poor IVF responses have different levels of chromatin accessibility and transcriptomic activity, which impact GC function.
To test this, we analyzed human granulosa cells (GC) obtained from older IVF patients (≥37 years), categorized as good responders (OG, ≥17 oocytes) and poor responders (OP, ≤8 oocytes). Freshly isolated GC were analyzed for changes in the cell cycle, cytokines and interleukins, apoptosis markers, and reactive oxygen species (ROS). GC of both OG and OP showed a stalled G0/G1 phase; however, OP had a significantly lower numbers od cells in S phase, and lower levels of mRNA for *CDK1* ($p< 0.05$), *CCND1* ($p< 0.01$), and *BCL2* ($p< 0.01$). Analysis of ROS levels, and antioxidant genes revealed higher levels of ROS in OP using the DCFDA probe, and significant lower levels of mRNA for antioxidant genes—*GPX1* ($p< 0.01$), *SOD1* ($p< 0.01$), and *CAT1* ($p< 0.0001$). Cytokines such as *IL-8, IL-6, IL-1B, and TNF* were significantly lower ($p< 0.01$) in poor responders. We performed bulk RNA-seq and ATAC-seq on GC samples to profile the transcriptome and genome-wide chromatin accessibility landscape. KEGG and Biological Process analyses on the differentially expressed genes (DEGs) (P-adj value < 0.01, Log2FoldChange| ≥ 0.05) demonstrate prominent immune response-related pathways in GC of OG patients and steroid metabolism pathways in GC of OP patients. Integration of ATAC-seq and RNA-seq data revealed a strong association between lipid-cholesterol-related genes and promoters in GC of OP patients, while immune-response-related genes and promoters predominated in OG, supporting the transcriptomics findings with chromatin alterations. IGV tracer analysis confirmed highly accessible chromatin in the promoter regions of genes responsible for cholesterol synthesis in GC of OP patients.
These results suggest that metabolic pathways promoting lipid synthesis and steroidogenesis are prominent GC of poor older responders, while periovulatory cytokine competence is preserved in GC of older good responders. Western blot analysis indicated significant upregulation of proteins in the lipid synthesis pathway in GC of OP compared to OG ($p< 0.05$). Overall, this study shows differential epigenetic modifications and gene expression patterns in GC based on patient response in IVF patients. This project provides novel insight into chromatin remodeling and gene expression in human GC and the potential development of biomarkers to better understand how older IVF patients respond to gonadal stimulation protocols.

Submission ID#2360870

Ovarian Follicles Direct Remodeling of the Stroma

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Abstract Text

The ovary is composed of follicles and a surrounding stromal microenvironment that supports their development. With advanced age, there is a decrease in follicle quantity and quality that occurs concurrently with a shift towards a fibro-inflammatory stroma. Follicles are signaling units exhibiting bidirectional communication and metabolic cooperativity between the oocyte and granulosa cells which are essential for folliculogenesis. Follicles also secrete factors which likely influence the stroma as several single-cell analyses predict direct transcriptional crosstalk between granulosa cell ligands and stromal cell receptors. However, it remains unclear whether follicles actively influence stromal homeostasis. We recently engineered an ovarian somatic cell (OSC) organoid model which recapitulates the cellular heterogeneity of the ovarian stroma. This model lacks follicles and is thus an inherent model of ovarian aging. Interestingly, culture of these organoids for 3-8 weeks resulted in fibroblast expansion and increased collagen accumulation, similar to what occurs in aging. These results suggest that the oocyte or follicles are critical for maintaining stromal homeostasis. To directly examine follicle-stroma interactions in a tightly controlled system, we established an ex vivo model in which we co-cultured either an OSC organoid with a multilayer secondary follicle, two OSC organoids, an OSC organoid with an inert glass bead, or two follicles. Strikingly, OSC organoids completely encapsulated the follicles within two days of culture forming a “micro-ovary”. This remodeling was not observed in the other experimental groups suggesting a unique interaction between follicle and stroma. To validate the cellular nature of this remodeling, we performed immunohistochemistry with markers for granulosa cells (FOXL2), fibroblasts (vimentin), and proliferative (Ki67) and apoptotic (CC3) cells. Staining confirmed that the follicular and stromal compartments remained distinct: Foxl2-positive granulosa cells were primarily localized within the follicular compartment surrounding the oocyte whereas vimentin-positive fibroblasts were detected within the organoid compartment and among the cells surrounding the follicle suggesting that fibroblasts originating from the organoid migrated around the follicle during stromal remodeling. Furthermore, the micro-ovary exhibited minimal cell death, and cellular proliferation was observed in both follicular and stromal compartments. These findings demonstrate that follicles are the instructive units driving stomal reorganization. Studies are ongoing to investigate the signals follicles secrete to regulate stromal homeostasis and how follicle quality influences stromal cell behavior and composition.

This work was supported by the Thomas J. Watkins Memorial Endowment and Department of Obstetrics and Gynecology Startup Funds (F.E.D).

Submission ID#2360522

Developing a Vascularized Ovary-on-a-Chip Platform to Study Follicle-Vasculature Signaling and Ovarian Angiogenesis

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Abstract Text

Expansion of ovarian follicles within the tissue microenvironment is essential for the production of sex hormones and meiotically competent oocytes, both of which are critical to systemic female health and fertility. Disruption of this process, as seen with gonadotoxic cancer therapy or age-related reproductive decline, can adversely affect endocrine function and elevate risks for cardiovascular, skeletal, neurological, and wound healing disorders. While traditional hormone replacement therapy delivers fixed estrogen and progesterone doses, it lacks physiological hormone cycling and dynamic ovarian feedback. To address these limitations, my project focuses on developing a vascularized ovary-on-a-chip platform (VASChip) that leverages live, growing follicles integrated within carefully engineered biomaterial matrices. The VASChip utilizes compartmentalized channels to co-culture ovarian follicles encapsulated in plasmin-degradable 5% w/v poly(ethylene glycol) hydrogels and 300 μm arteriole-scale endothelialized macrovessels embedded in 5 mg/mL fibrin gels, with each tissue compartment supported by its optimal media. Preliminary results show that the precise tissue-biomaterial interactions enabled by the VASChip's compartmentalized design support mouse secondary follicle growth from 120-135 μm to 300-400 μm and maturation, while also maintaining the structural integrity and functionality of co-cultured endothelialized macrovessels, which retain VE-cadherin-mediated cell-cell junctions post-culture. Building on prior transcriptomic and proteomic studies from our lab and others, which have demonstrated upregulation of pro-angiogenic genes in murine follicles and secretion of vascular endothelial growth factor (VEGFA) and angiopoietin-2 (ANGPT-2) to recruit endothelial cells, the VASChip provides an integrated platform to further investigate these signaling mechanisms and their impact on follicle-vascular crosstalk. We hypothesize that the VASChip will recapitulate ovarian angiogenesis, sustain long-term follicle culture, and restore physiologically relevant hormone cycles *in vitro*, providing a robust platform for studying ovarian biology and informing clinical fertility preservation strategies.

Submission ID#2434824

Ovarian Inhibins and the Endocrine Regulation of Reproductive–Metabolic Crosstalk

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Abstract Text

Gonadal hormones inhibin A and inhibin B (α/βA or α/βB heterodimers) are classically known for their abilities to constrain follicle stimulating hormone (FSH) production from pituitary gonadotroph cells. It is largely accepted that inhibins downregulate FSHB transcription by disabling receptor activation by related activins (βA/B/βA/B dimers). Beyond FSH regulation, roles for inhibins in female and male fertility have been poorly understood owing to a lack of appropriate physiological models. Here, armed with new mouse models of dysregulated inhibins we have uncovered novel roles for inhibins in gonadal function and fertility. Our, and supporting works by our collaborators at McGill University (Montreal), have shown that maintenance of inhibin physiological activity is important not only for FSH regulation, but also for embryo and foetal survival during pregnancy in female mice. Additionally, together we have uncovered evidence to support that partial inactivation of inhibins actually enhances fertility in female mice. More recently we have discovered that persistent inhibin inactivity in female mice is associated with the development of multi-cystic ovaries. Serendipitously, using these inhibin inactivated mouse models we have also identified roles for inhibins in the regulation of adiposity in females. Significantly, as these phenotypes pertain only to female mice, we are unveiling new sex-specific activities for the inhibins. Ultimately these findings signify inhibins as not only gatekeepers of female reproductive health, but also metabolic health.

Integrative Regulation of Sperm Physiology and Function

Monday, July 20 10:00 AM – 12:00 PM

Submission ID#2361303

The Centriolar Rod Protein POC1B Controls Rabbit Sperm Head-Kinking and Tail-Beating

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Abstract Text

Most somatic cells have a centrosome composed of two centrioles, which nucleate cilia and organize the mitotic spindle during cell division. In most mammals, sperms have one cylindrical (canonical) centriole and one splayed (atypical) centriole. The atypical centriole has two unique rod-like structures. During bovine sperm swimming, the rods slide, which correlates with sperm tail beating and head kinking. Here, we studied the essential function of rod-sliding in swimming by uniquely mutating the rod protein POC1B.

POC1B has multiple functions in sperm formation, and its loss results in abnormal sperm morphology and motility, which prevents it from being studied. To overcome this, we made a subtle mutation, deleting the POC1B C-terminal 78 amino acids. We used rabbits as a genetic model because, unlike mice, rabbit sperm centrioles resemble the human sperm function. The homozygote mutant sperm are fertile, swimming, and with normal morphology; however, their spermatozoa lacked POC1B at their centrioles, indicating the essential role of POC1B C-terminus for spermatozoa centriolar localization. Preliminary data suggest that mutant sperm have short, static, fused rods, indicating a critical role for POC1B C-terminus in rod formation and sliding.

Rabbit sperm, tails beat to the right, heads kink to the left, and the two movements correlate. This direction and correlation are the same in the mutant. However, the mutants have reduced ranges of tail beating and head kinking, indicating that rod fusion limits movement freedom. Additionally, the mutant sperm exhibit reduced tail-beating and head-kinking cycle times.

Analysis of control and mutant sperm trajectories using CASA reveals that the average path velocity (VAP) and curvilinear velocity (VCL) are unaffected in the mutant. Interestingly, the straight-line velocity (VSL) is higher in the mutant group, and the straightness (STR) and linearity (LIN) of the mutant sperm trajectories are also higher, suggesting that the mutant sperm trajectories are more linear.

Finally, the POC1B mutation's fertility in vivo was reduced in the sperm competition experiment, suggesting centriole rod sliding provides a reproductive advantage.

Overall, our preliminary data suggest that POC1B C-terminus and the rods control tail-beating and head-kinking amplitude, the ability to exhibit a circular trajectory, and are essential for sperm competition in double matings.

Submission ID#2350117

Transient Sperm Starvation Accelerates Porcine Sperm Capacitation In Vitro

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Abstract Text

Sperm capacitation is the terminal maturation event that endows spermatozoa with fertilizing ability. Occurring in the oviduct in vivo, this step involves a series of biochemical and biophysical changes that can be conveniently recapitulated in vitro. Sperm capacitation depends on the crosstalk between metabolic and signalling pathways. Hence, the choice of energy substrate as well as their transient withholding from the capacitation medium, referred to as sperm energy restriction and recovery (SER) was shown to have an impact on capacitation status and fertilizing ability of spermatozoa when compared to spermatozoa continuosly incubated in the standard, energy-rich capacitation media in mice and bovines. Starved and rescued mouse spermatozoa had improved fertilization and embryo development rates, in both the fertile WT mice as well as in the in vitro sterile FerT mouse model. The current study builds on our recent trials, showing that the inclusion of glucose in porcine capacitation medium (pCM) delayed the acquisition of the capacitated state, when compared to glucose-free control. The goal of this study is to optimize the SER protocol for porcine spermatozoa, particularly the duration of sperm incubation under starvation conditions. We used fresh ejaculates from two healthy, fertile Large White boars used for routine *in vitro* fertilization trials with high blastocyst yield. The sperm-rich fractions (n=6) were used. Spermatozoa were split into two groups and capacitated in pCM containing 2 mM bicarbonate under i) non-starvation conditions (11 mM D-Glucose, 5.2 mM Na-pyruvate, 10 mM Na-lactate, 12 mM sorbitol); and ii) starvation conditions (osmolarity was adjusted with NaCl). The capacitation-associated changes, including zinc ion efflux, acrosomal remodeling, membrane integrity, reactive oxygen species (ROS) production, motility, kinematic parameters, and proteasomal activity was measured every 30 minutes up to 4 hours. We observed a statistically significant decrease in sperm total and progressive motilities throughout the treatment course, significant reduction of velocities after one hour of incubation, and significant differences in movement patterns after 30 minutes of incubation in starved spermatozoa ($P< 0.05$). Further, we observed significantly increased sperm population with zinc signature 3 (capacitated state) after 30, 60, and 90 minutes of starvation, significantly decreased plasma membrane integrity after 30, and 60 minutes of starvation, significantly increased sperm population with remodeled acrosome at 30 minutes up to 2.5 hours of starvation, and a significant decrease in ROS production after 30 minutes of starvation. Sperm starvation did not have an impact on proteasomal activity. These results suggest that 30-60 minutes is sufficient to induce significant capacitation-related changes in starved spermatozoa when compared to the non-starved control. Since we used raw semen collected from two animals of the same breed, individual differences should be considered, as well as differences between breeds and raw vs extended semen. Including more animals in future studies will improve the robustness. Future studies include in vitro fertilization outcomes.

Keywords: porcine spermatozoa, in vitro sperm capacitation, sperm energy restriction and recovery, male fertility
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Submission ID#2412556

Ion Channels in Reproduction: Structural and Physiological Mechanisms of Kir7.1 and CatSper Regulation

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Abstract Text

Ion channels play a crucial role in regulating gamete fitness, fertilization, and embryo development. In sperm, the calcium channel CatSper is essential for hyperactivated motility and male fertility. Premature activation of CatSper can render sperm infertile, leading many species to evolve protective mechanisms that keep it inactive until sperm approach the egg. Given CatSper's critical role in sperm function and its ability to operate under diverse conditions within the female reproductive tract, we investigated key modalities of CatSper regulation. We provide a detailed characterization of its regulation in both the male and female reproductive tracts, with a particular focus on its modulation by seminal plasma components and the factors essential for successful fertilization.

The second part of this presentation will focus on the molecular mechanisms regulating the inwardly rectifying potassium channel Kir7.1, which is expressed in the mammalian myometrium and placenta during late gestation. Our findings reveal that Kir7.1 serves as a molecular target for select endogenous and synthetic steroids that modulate uterine excitability. Additionally, we will present structural insights into Kir7.1 regulation, as revealed by cryo-EM.

Submission ID#2346155

A clinical decision tool for sperm source selection in cryptozoospermia: integrating semen stability and peak motile count

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Abstract Text

Background: The management of cryptozoospermia presents a significant clinical challenge during assisted reproductive cycles, particularly in selecting the optimal sperm source on the day of oocyte retrieval. Current approaches lack reliable predictive tools that account for the dynamic nature of sperm production in these patients. This study aimed to develop and validate a clinically applicable prognostic tool that integrates semen stability and peak motile sperm count to guide this critical clinical decision.

Methods: We conducted a retrospective cohort study involving 93 cryptozoospermic men undergoing their first intracytoplasmic sperm injection (ICSI) cycle. A novel prognostic stratification system was developed based on preoperative semen analyses, defining three distinct prognostic groups: Group 1 (Favorable prognosis: semen stability >50% and peak motile sperm count ≥5), Group 2 (Intermediate prognosis: peak count ≥2 but not meeting Group 1 criteria), and Group 3 (Poor prognosis: peak count ≤1). The primary outcome was the successful retrieval of a sufficient number of motile sperm for ICSI from the fresh ejaculate.

Results: The prognostic model demonstrated a striking and statistically significant gradient in retrieval success rates across the three groups: 92.7% in Group 1, 37.5% in Group 2, and 0% in Group 3 ($p< 0.001$). Through a risk-adapted management approach utilizing either fresh or cryopreserved sperm from ejaculate or testis, we achieved ultimate success rates of 100%, 79.2%, and 64.3% for Groups 1, 2, and 3, respectively. Multivariable analysis confirmed the prognostic group as a powerful independent predictor of successful retrieval (adjusted OR per group decrease: 0.013, 95% CI: 0.001-0.109). Decision curve analysis further validated the model's robust clinical utility across a wide range of risk thresholds.

Conclusion(s) ： This validated stratification system effectively triages cryptozoospermic men into distinct management pathways, potentially preventing unnecessary surgical sperm retrieval in the majority (59.1%) of patients while ensuring a high ultimate probability of securing sperm for ICSI. Our findings provide a practical, evidence-based algorithm to resolve the longstanding clinical dilemma of sperm source selection, paving the way for more personalized and efficient patient management in assisted reproduction.

Submission ID#2359982

Sperm metabolic studies beyond glucose: Fructose-fuelled metabolic reprogramming

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Abstract Text

Successful fertilization depends on the ability of sperm to rapidly adapt their metabolism during ejaculation and their journey through the female genital tract. Unlike most mammalian cells that rely primarily on glucose, sperm are exposed to high concentrations of fructose during ejaculation - a unique energy environment that remains poorly understood. Despite being known for decades that fructose concentrations are high in semen, sperm metabolism studies have largely focused on glucose metabolism, overlooking the physiological relevance of fructose as a potential primary fuel source during sperm activation.

After quantifying the physiological concentration of glucose and fructose in mouse seminal fluid, we utilize ^{13}C stable isotope labeling to follow the individual steps of glucose and fructose metabolism through the sperm central carbon metabolic network before and after sperm activation. Moreover, we compare the effect of glucose and fructose on sperm functional parameters and fertilization capacity.

Comparing the two carbohydrates at equimolar concentrations (5.6 mM), we find that sperm more rapidly and efficiently metabolize fructose than glucose, resulting in higher ^{13}C labeling in glycolysis and TCA cycle metabolites. In line with increased metabolism, motility, capacitation, and fertility were elevated in fructose. At physiological concentrations (0.3 mM), however, glucose was not able to support the capacitation-induced increase in metabolic activity, whereas sperm metabolism and function were even further improved using fructose at its physiological concentration (7.5 mM).

Our study reveals that sperm energy production during ejaculation is adapted to fructose, suggesting that including fructose in sperm buffer might improve the outcome of assisted reproductive technologies.

Submission ID#2355926

Ram Seminal Plasma Extracellular Vesicles Show Distinct Compartmentalization of Capacitation Regulating Factors (RSVP14 & 20) and Differential Effects on Sperm Capacitation

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Abstract Text

In mammals, seminal plasma (SP) is a complex fluid that surrounds spermatozoa and plays a crucial role in regulating sperm function. However, it remains unclear whether the factors modulating sperm physiology circulate freely or are transported within extracellular vesicles (EVs) secreted by the male reproductive tract. Previous studies from our laboratory in ram demonstrated that SP extracellular vesicles (SP-EVs) carry capacitation regulating factors, including RSVP14 (BSP1) and RSVP20 (BSP5), and mediate their transfer to spermatozoa. As these proteins are known to be major components of SP and they bind to lipid membranes, we aimed to determine whether these capacitation regulating factors are located inside or outside SP-EVs, and further assess the potential influence of SP-EVs on sperm capacitation.

EVs were isolated and characterized according to MISEV 2023 guidelines using size exclusion chromatography, yielding two distinct fractions (P1 and P2). Western blot analysis after co-incubation of SP-EVs with proteinase K revealed that RSVP14 was protected from proteolysis in both SEC-P1 and SEC-P2 fractions, indicating an intravesicular localization. In contrast, RSVP20 was susceptible to proteolytic digestion, supporting its predominant external localization, possibly associated with the vesicle protein corona.

We next examined the impact of SP-EVs on molecular events associated with capacitation by incubating spermatozoa with EVs fractions under capacitating conditions. Western blot analyses revealed that SEC-P2 modulated tyrosine phosphorylation, resulting in a pY profile sperm similar to non-capacitating conditions. Importantly, none of the EVs treatments affected PKA activity. Given that tyrosine phosphorylation in the flagellum (specifically in the principal piece) is linked to capacitation-related changes, our subsequent analysis concentrated on the regional patterns of this phosphorylation. Using imaging flow cytometry, and limiting the analysis to events positive for both pY and PKA, we observed significant differences between conditions in the head, midpiece, and principal piece regions. The addition of SP-EVs selectively modulated these capacitation pathways in the principal piece: PKA substrate phosphorylation in SEC-P1, and SEC-P2, reaching levels comparable to non-capacitating conditions ($p < 0.05$). A similar reduction in pY was observed for SEC-P1 ($p < 0.05$), while SEC-P2 exhibited an intermediate response.

Taken together, these findings demonstrate that SP-EVs carry capacitation regulating proteins with distinct spatial localizations and modulate sperm capacitation signaling at specific subcellular levels.

Work supported by Society for the Study of Reproduction (SSR Emerging Investigator Award to L.Z)

Submission ID#2359888

Distinct Waveforms Determine Flagellar Beat Frequency and Amplitude in Sperm from the Mosquito, *Culex pipiens*

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Abstract Text

Sperm motility is essential for successful fertilization. Sperm that use a flagellum for motility can exhibit many changes in flagellar beating, from hyperactivation in mammalian sperm to backwards swimming in insects. Yet our understanding of factors that control flagellar beating patterns or regulate changes in flagellar beating are little understood.

In this study, we used a computer analysis system, SpermQ, to analyze various motility parameters of sperm from the mosquito *Culex pipiens*. Sperm motility was recorded at 100 frames per second using a high-speed video camera and subsequently analyzed using SpermQ. The SpermQ Preparator module, which generates a stacked image of multiple video frames, enabled us to identify several distinct waveforms exhibited by *Culex* sperm over time. The SpermQ analysis module measures many features of the beating flagellum, typically analyzed in 100 frame segments, and provides data to quantify numerous features including, but not limited to, flagellar beat frequency, curvilinear velocity, straight-line velocity, flagellar amplitude, asymmetry, and many other features.

Mature seminal vesicle sperm were isolated from male *Culex pipiens* and placed in Insect Ringers with trypsin. This buffer was previously shown to support vigorous forward progressive motility with >90% of sperm initiating motility. Ten second video recordings were captured every 2 min and sperm from recordings at 2, 4, and 6 minutes were used for this study. For SpermQ analysis, we used several (3 to 7) 100 frame segments from each time point. All in focus sperm with non-overlapping trajectories were analyzed (N=825).

Basic parameters (beat frequency, curvilinear velocity, and straight-line velocity) were obtained either directly from SpermQ output, or with minor calculations using SpermQ output. Amplitude of the flagellar beat was evaluated using two approaches: 1) the average of cAng_ampl (a value which relates to the angle of the sine wave formed by the flagellum) and 2) y-ampl (which directly measures the amplitude of the wave relative to a line bisecting the head lengthwise and parallel to the long axis of the sperm). Velocities and amplitudes were graphed relative to beat frequency and revealed the following trends: 1) both curvilinear and straight-line velocity increased with increasing beat frequency; 2) velocities plateaued with the highest beat frequencies; and 3) amplitudes were highly variable at low beat frequencies but converged to a narrow range at high beat frequencies.

Viewing the overlay image of motile sperm (generated by SpermQ Preparator) enabled us to identify three primary waveforms generated by *Culex* sperm, which we have named Wave, Ribbon, and Rocket, based on their appearance in the overlay images. We compared the average beat frequencies, velocities, and amplitudes of these waveforms to examine whether each waveform is delineated by a particular range of values.

By extending these studies to sperm treated with specific agents of known molecular targets, we may be able to determine the signaling pathways that are involved in specific aspects of flagellar waveform generation.

From Immune Modulation to Labor Onset: Mapping the Path to Parturition

Monday, July 20 10:00 AM – 12:00 PM

Submission ID#2358750

Melatonin Co-administration Partially Rescues Fetal Growth Restriction but Not Fetoplacental Stress Signatures in a Rat Model of Prenatal Oxycodone Exposure

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Abstract Text

Prenatal oxycodone (Oxy) exposure has been associated with adverse pregnancy outcomes and impaired fetal development, yet the mechanism remains unclear, and effective interventions are lacking. Therefore, we hypothesized that prenatal Oxy exposure would disrupt placental and fetal growth, while melatonin co-administration would mitigate these effects. To investigate this, female Sprague–Dawley rats received saline or Oxy by daily oral gavage for 15 days prior to mating, with dosing escalated to a final concentration of 15 mg/kg and maintained throughout gestation. From gestational day 12.5 (GD12.5) to GD19.5, half of the dams in each group additionally received oral melatonin (10 mg/kg), resulting in four experimental groups: saline, saline with melatonin (melatonin), Oxy, and Oxy with melatonin (OxMe). Placentas and fetuses were collected at GD19.5, and fetal anthropometric data were analyzed using a linear mixed model to nest the pups within their respective dams, while other outcomes were analyzed by a generalized linear model. Prenatal Oxy exposure significantly reduced placental weight ($p< 0.001$), fetal weight ($p< 0.001$), crown-rump length ($p< 0.001$), and fetal-to-placental weight ratio ($p=0.009$). Significant sex effects were also observed, with males exhibiting longer crown-rump length and greater abdominal diameter than females. The co-administration of melatonin with Oxy significantly increased placental weight ($p=0.018$), fetal weight ($p< 0.001$), crown-rump length ($p< 0.001$), head diameter ($p=0.002$), and head-to-abdominal ratio ($p=0.017$), indicating a significant fetal brain-sparing effect. These Oxy-induced physical changes were accompanied by molecular stress signatures identified via western blotting in both the placenta and fetal brain. In the placenta, Oxy induced a sex-dependent integrated stress response, with increased EIF2A phosphorylation in males ($p=0.001$) and increased CHOP expression in females ($p< 0.001$), along with an increased phospho-EIF2A/EIF2A ratio ($p=0.01$) in males, without changes in BiP, ATF4, or total EIF2A expression in both sexes. Importantly, OxMe placentas continued to show increased phospho-EIF2A levels relative to saline in both males ($p=0.012$) and females ($p=0.011$), as well as increased phospho-EIF2A/EIF2A ratios in males ($p=0.033$) and females ($p=0.025$), suggesting that melatonin treatment did not protect the placentas from the Oxy-induced damage. In the fetal brain, Oxy exposure increased EIF2A phosphorylation ($p=0.002$), phospho-EIF2A/EIF2A ratio ($p=0.001$), and CHOP expression ($p=0.005$) in both sexes. The brains of OxMe fetuses similarly demonstrated elevated EIF2A phosphorylation ($p=0.002$) and phospho-EIF2A/EIF2A ratios compared to those of saline-treated fetuses ($p=0.025$), with no significant differences between Oxy and OxMe groups and no sex-dependent effects observed. This indicated sustained activation of cerebral stress signaling despite melatonin administration. These changes were accompanied by elevated levels of cleaved caspase-3 ($p=0.002$) and cleaved caspase-9 ($p=0.004$), with no changes in cleaved caspase-8, BiP, ATF4, or total EIF2A expression in either sex. While melatonin co-administration reduced cleaved caspase-9 levels in the brains of males ($p=0.031$) and females ($p=0.024$) compared to the Oxy group, the OxMe group still exhibited increased levels of cleaved caspase-3 levels compared to controls ($p=0.012$). This pattern indicates that while melatonin partially modulates specific apoptotic signals, the persistent activation of the p-EIF2A/CHOP axis by Oxy still maintains a pro-apoptotic environment. Collectively, these findings demonstrate that prenatal Oxy exposure impairs placental and fetal development through the activation of integrated stress signaling and the intrinsic apoptotic pathway. While melatonin provided a partial rescue by mitigating some phenotypic outcomes, it failed to fully prevent neuroapoptosis and molecular stress, suggesting its limitations as a standalone therapeutic for opioid-related developmental impairment.

Submission ID#2361140

BK_{Ca} channel Sulfhydration in Human Uterine Artery: Effects of Estrogens and Pregnancy

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Abstract Text

Estrogen stimulates uterine artery (UA) vasodilation in pregnancy, in part, through selective upregulation of cystathionine β-synthase (CBS) that increases vascular production of hydrogen sulfide (H_2S) which in turn results in pregnancy-dependent UA relaxation via activating smooth muscle cell (SMC) large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels. BK_{Ca} are tetramers of the ubiquitous pore-forming α-subunits (BKα) whose activity is regulated by the tissue-specific, auxiliary β1-4 and γ1-4 subunits. UA SMC BK_{Ca} channels are known to be with the α/β1/γ1 stoichiometry, but it is unknown how H_2S activates BK_{Ca} channels to mediate estrogen-induced UA vasodilation.

H_2S acts a signaling molecule that converts free thiols ($-SH$) to persulfides ($-SSH$) at cysteine residues that regulates protein function. We hypothesize that H_2S-mediated protein sulfhydration serves as a major pathway to mediate estrogen-induced UA vasodilation. The goal of this study is to determine the effects of H_2S and estrogen on BK_{Ca} sulfhydration in UA.

Primary human UA SMCs (hUASMCs) and human UA tissues were used to evaluate BK_{Ca} subunit expression and sulfhydration. hUASMCs were treated with 17β-estradiol (E2β), the estrogen receptor antagonist ICI 182,780, the H_2S donor NaHS, or a nitric oxide sodium nitroprusside (SNP) for 30 min. Total sulfhydrated proteins were labeled and enriched by Tag Switch technique and used to detect total SSH-protein by immunoblotting with anti-biotin or to detect specific BK_{Ca} subunits by immunoblotting with specific antibodies.

E2β significantly increased total sulfhydrated proteins and BKβ1 and BKγ1, but not BKα in UASMC. These effects were attenuated by ICI 182,780, indicating estrogen receptor–dependent regulation. NaHS similarly enhanced BKβ1 and BKγ1 (but not BKα) sulfhydration, supporting H_2S as the proximal mediator. SNP did not produce comparable sulfhydration patterns, suggesting specificity of H_2S signaling. Total sulfhydrated proteins and sulfhydrated BKβ1 and BKγ1, but not BKα, were greater in pregnant compared to nonpregnant human UA.

Estrogens stimulation of UASMC BKβ1 and BK γ1 sulfhydration via specific receptor and H_2S-mediated but NO-independent mechanisms suggest sulfhydration of BKβ1 and BKγ1 as a key signaling mechanism for H_2S to exert its effects in estrogen-induced UA vasodilation. (Supported by NIH grants)

Submission ID#2360118

Maternal Ornithine Supplementation Alters Placental Morphology and Utero-Placental Angiogenic mRNA Abundance in a Sex-Specific Manner in Swine

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Abstract Text

Selective breeding of pigs for increased litter size has been accompanied by increases in the number of stillborn and low birth weight piglets, highlighting the need for strategies that enhance placental and fetal growth to improve piglet survival. Improved placental development and utero-placental angiogenesis may increase nutrient transport and support fetal growth. Ornithine is a precursor for the synthesis of polyamines (spermine, spermidine, and putrescine) that promote angiogenesis by stimulating endothelial cell proliferation and facilitating cytoskeletal remodeling and cell migration. The objective of this study was to determine if maternal ornithine supplementation during early–pregnancy alters placental development, fetal growth, and utero-placental expression of mRNA encoding regulators of angiogenesis.

Gilts (n=14) were bred by artificial insemination (day 0) and randomly assigned to control or ornithine treatment. All gilts received a basal diet formulated to meet gestational nutritional requirements. Ornithine-treated gilts (n=7) received 0.12% ornithine twice daily from gestational days 15–44. On day 45, a subset of gilts (n=4 control, n=4 ornithine) were euthanized for collection of fetal and placental measurements, fetal organ weights, and sampling of umbilical cords, placentae, endometria, allantoic and amniotic fluids. The abundance of candidate mRNAs with roles in angiogenesis (*CD31*, *VEGFA*, and *HIF1A)* were quantified in umbilical cords, placentae, and endometria using qPCR. Remaining gilts (n=6) were allowed to give birth, and litter outcomes were recorded at birth. Umbilical cord vessel diameters were evaluated using paraffin sections immunohistochemically stained for CD31. Data were analyzed using mixed-effects ANOVA with treatment and sex as fixed effects and gilt as a random effect.

Prenatal survival (number of live fetuses/number of corpora lutea) and placental efficiency (fetal weight/ placental weight) were not affected by ornithine treatment on day 45. Placentae from ornithine-treated gilts were longer than placentae from control gilts ($P \leq 0.05$), while placentae associated with male fetuses tended to be heavier than placentae associated with female fetuses ($P=0.068$). Males were heavier ($P \leq 0.001$) and longer ($P=0.06$) than female fetuses. Ornithine-treated gilts had greater volumes of allantoic fluid compared to control gilts ($P \leq 0.05$). Ornithine treatment had no effect on absolute or relative fetal organ weights or umbilical artery or vein diameters.

The abundance of candidate angiogenic mRNAs were not affected by treatment in any tissue investigated. Placentae associated with female fetuses had greater abundance of *CD31* mRNA compared to male fetuses ($P \leq 0.05$). Endometrial *VEGFA* mRNA abundance was greater in endometria associated with female fetuses compared to male fetuses ($P \leq 0.05$). The abundance of *HIF1A* mRNA was decreased in endometria from ornithine-females compared to control-females, whereas it was increased in ornithine-males compared to control-males (Treatment×Sex interaction $P \leq 0.05$). Similarly, there was decreased abundance of *HIF1A* mRNA in umbilical cords of ornithine-treated females compared to controls (Treatment×Sex Interaction $P \leq 0.01$). There was no effect of ornithine treatment on the percentage of piglets born alive. Males were heavier at birth than their female littermates ($P \leq 0.01$), and there was a Treatment×Sex interaction for birthweight ($P \leq 0.05$).

Maternal ornithine supplementation may alter aspects of fetal and placental development and the utero-placental abundance of mRNAs encoding angiogenic factors in a sex-specific manner. These findings support ornithine as a potential nutritional strategy to enhance fetal growth, particularly in male piglets, with implications for swine production efficiency and neonatal survival.

Funding: Pennsylvania Department of Agriculture and start-up funds from Pennsylvania State University.

Submission ID#2326340

FGF23 and KL: Regulators of Utero-Placental Phosphate Availability in Cattle?

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Abstract Text

Phosphate is essential for fetal and placental development, yet mechanisms regulating its availability within utero-placental tissues remain poorly understood. Fibroblast growth factor 23 (FGF23) and its co-receptor klotho (KL) are important regulators of phosphate homeostasis postnatally. However, their functional role in the regulation of utero-placental phosphate availability is unclear. This study aimed to characterize temporal changes in phosphate concentrations in bovine utero-placental tissues and fetal fluids across gestation and to investigate the expression and localization of FGF23 and KL in bovine utero-placental tissues.

Pregnant bovine uteri (n=95) were obtained from Nicolas Meat LLC and categorized into 7-day gestational age intervals based on fetal crown-rump length (day 46-136; n=3-10/group). Allantoic and amniotic fluids were aspirated and endometria and placentomes were either fixed in 4% paraformaldehyde or snap frozen in liquid nitrogen. Concentrations of phosphate in fluids and tissue homogenates were quantified spectrophotometrically. KL and FGF23 proteins were immunolocalized in endometrial and placentomal sections. Additionally, cotyledonary and caruncular tissues were collected from on gestational days 98 (n=3) and 111 (n=3). Explants (~180mg) were cultured for 22 h at 37°C with recombinant FGF23 (0, 1, 10, 50, or 100ng/mL) or KL (0, 0.5, 1, or 2µg/mL). Media and tissues were collected, and phosphate concentrations were quantified spectrophotometrically.

There was no effect of gestational day on phosphate abundance in utero-placental tissues. In contrast, phosphate concentrations increased in allantoic fluid and decreased in amniotic fluid as gestation progressed. KL protein immunolocalized to endothelial, luminal and glandular epithelial cells, with diffuse staining throughout the endometrial stroma. FGF23 protein immunolocalized to the luminal and glandular epithelia, with diffuse staining present throughout endometrial stroma, and was detected in blood within blood vessels. Both proteins immunolocalized to the chorionic epithelium, syncytium, and cotyledonary stroma within the placentome. Concentrations of phosphate were greater in caruncular ($P \leq 0.05$) and cotyledonary ($P = 0.07$) tissues on day 111 when compared to day 98. Similarly, phosphate concentrations in media from cotyledons were greater on day 111 compared with day 98 ($P \leq 0.05$). FGF23 treatment had no effect on phosphate concentrations in media or tissue from caruncular explants on either gestational day. In cotyledonary explants, phosphate concentrations in the media were affected by FGF23 treatment on day 111, with evidence of a treatment × day interaction ($P = 0.07$). On day 111, media from cotyledons treated with 1ng/mL ($P = 0.089$) or 100ng/mL ($P \leq 0.05$) had greater concentrations of phosphate compared to media from control (0ng/mL) FGF23. There was no effect of KL supplementation on the concentration of phosphate in media from caruncles or cotyledons on either gestational day investigated. However, there was an overall effect of KL concentrations on phosphate abundance in both caruncular ($P \leq 0.05$) and cotyledonary ($P = 0.09$) tissues. On day 111, caruncular tissue cultured with 1 µg/mL KL had greater concentrations of phosphate when compared with caruncular tissue cultured with 0 µg/mL ($P = 0.08$) and 0.5µg/mL ($P \leq 0.05$) KL. These findings provide evidence for local regulation of phosphate availability in bovine utero-placental tissues and fetal fluids during gestation and demonstrate that components of postnatal phosphate regulatory pathways are present at the maternal–fetal interface. Localization and functional data support a potential role for FGF23 and KL in regulating phosphate availability across tissues and gestation, warranting further investigation.

This project was supported by a new investigator grant from the Society for the Study of Reproduction and start-up funds from Pennsylvania State University.

Submission ID#2410134

Mechanisms of gestation length timing in mice

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Abstract Text

Current efforts investigating parturition timing mechanisms have focused on the proximal triggers of labor onset generated in late pregnancy. Our recent work suggests that parturition timing in mice is also regulated by events that take place in early pregnancy. These findings emerged from our dissection of the delayed parturition phenotype of mice with uterine fibroblast deficiencies in the histone H3K27me3 demethylase KDM6B. We found that uterine fibroblasts engage in a locus-specific epigenetic program immediately after copulation that abruptly adjusts H3K27me3 levels across their genome. In the absence of KDM6B, many of the adjusted loci over-accumulate H3K27me3. This over-accumulation leads to nearby genes being misexpressed in mid-to-late gestation, a delayed effect partly attributable to a second locus-specific but KDM6B-independent process initiated within uterine fibroblasts soon after implantation. This second process employs progressive H3K27me3 loss to temporally structure post-midgestational patterns of gene induction. Further dissection of the ways uterine programming controls parturition timing may have relevance to human pregnancy complications such as preterm labor.
Supported by grants from the Burroughs Wellcome Fund (Grant #1019789) and the National Institutes of Health (R01AI150191, R01AI143187, and F31HD106714).

Submission ID#2361329

Revisiting Placental Steroidogenesis: STARD3 does not Substitute for STAR in Mitochondrial Cholesterol Import

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Abstract Text

Mitochondrial cholesterol import is an essential step in steroid hormone biosynthesis and is mediated in gonadal and adrenal steroidogenic cells by the steroidogenic acute regulatory protein STAR (STARD1). In humans, placental progesterone production becomes essential for maintenance of pregnancy following the luteal–placental shift in early gestation, yet progesterone synthesis in the placenta has long been proposed to occur independently of STAR, based on reports that STAR expression is undetectable in trophoblasts, with STARD3 suggested to substitute for this function. Because prior functional studies of STARD3 were performed largely in non-steroidogenic systems, its ability to support mitochondrial cholesterol import in a physiologically relevant context remains unclear.

To directly test this question, we used CRISPR/Cas9-edited STARD1-null MA-10 Leydig cells, which exhibit a specific loss of steroidogenic capacity. Reconstitution with STAR restored steroidogenesis, confirming the functional specificity of the model. In contrast to current models, expression of full-length STARD3, as well as truncated forms lacking N-terminal regions (ΔN45-STARD3 and ΔN235-STARD3/MLN64), failed to rescue steroidogenesis, demonstrating that STARD3 cannot substitute for STAR in mitochondrial cholesterol import.

To determine whether the cholesterol-binding START domain of STARD3 is functionally competent to support mitochondrial cholesterol transfer, we engineered a forced-localization construct in which the STAR mitochondrial targeting sequence (MTS) was fused to ΔN235-STARD3/MLN64, directing the protein to the mitochondrial intermembrane space (IMS). This non-physiological targeting restored steroidogenesis, demonstrating that while the isolated START domain can support cholesterol transfer when artificially positioned, native STARD3 is inherently incapable of substituting for STAR in mitochondrial cholesterol import.

Because STAR expression has been reported to be absent in the human placenta, we examined its expression and regulation. Single-cell RNA-seq analysis of human placental tissue revealed STAR expression in trophoblast populations, indicating that STAR is present but may be tightly regulated. Consistent with this, in JEG3 trophoblast cells, inhibition of mRNA degradation increased both progesterone production and STAR mRNA levels, suggesting that STAR expression is regulated, at least in part, through mRNA stability and that rapid mRNA turnover may have contributed to its apparent absence in earlier studies.

Collectively, these findings demonstrate that STARD3 is not functionally redundant with STAR, and that STAR expression and mRNA stability contribute to progesterone production in trophoblasts. These results challenge the prevailing view of STAR-independent placental steroidogenesis and provide a revised framework for understanding mitochondrial cholesterol import and steroidogenic regulation in human pregnancy.

Submission ID#2360452

The Endotheliochorial Bridge: Mapping Feto-Maternal Dialogue in the Canine Placenta by Singe Nucleus Sequencing.

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Abstract Text

The placenta is a transient organ, composed of maternal and fetal tissues, and one of the most variable mammalian organs, differing in its macroscopic and microscopic properties among species. It serves as an important endocrine organ, mediates gas exchange, nutrient transfer, and the removal of metabolic waste products between mother and fetus. The interspecies structural diversity of the placenta reflects distinct evolutionary adaptations in the degree of maternal tissue invasion and remodeling. The zonary placenta of dogs represents the endotheliochorial type of placentation, which occupies an intermediate developmental position between the non-invasive epitheliochorial and the highly invasive hemochorial types. Despite the invasive nature of the trophoblast, maternal and fetal circulations remain separated, as the maternal endothelium and stroma-derived decidual cells withstand trophoblastic invasion. This makes the canine placenta an important and interesting comparative model for investigating how trophoblast and maternal decidual tissues interact in creating the morpho-functional barrier between the two counterparts. Recently, maternal decidual cells were shown to create a deciduo-chorial barrier between the fetal and maternal compartments of the canine placenta. Of functional importance, decidual cells are the only placental responders to progesterone via its nuclear receptor (PGR). Their role in the establishment and maintenance of pregnancy is vital, as interference with the P4/PGR signaling pathway leads to its termination. Although bulk transcriptomic analyses have delineated major gene expression changes in the canine placenta during gestation and preceding parturition, the cellular heterogeneity and spatial organization underlying these processes remain insufficiently characterized.

To address this complexity, we employed single-nucleus RNA sequencing (snRNA-seq) to resolve the cellular and molecular composition of the canine placenta and to define morpho-functional traits associated with transition from mid-gestation to termination of pregnancy. Fresh-frozen placental specimens were used, collected from clinically healthy bitches at mid-pregnancy and during luteolysis. Nuclei were isolated and processed using the 10x Genomics platform, yielding high-quality single-nucleus libraries. Maternal and fetal cell types were subsequently identified based on markers established in our laboratory. The resulting dataset revealed a remarkable diversity of cellular populations. Decidual cells displayed distinct subclusters, reflecting varying degrees of differentiation and functional specialization. Functional annotations suggested active participation in extracellular matrix remodeling, immune modulation, and signaling at the feto-maternal interface, emphasizing their role in maintaining the deciduo-chorial barier and regulating trophoblast behavior. During luteolysis, decidual nuclei exhibited a pronounced shift in transcriptional activity indicating a decline in PGR signaling, a withdrawal of epithelial transition, and re-acquisition of mesenchymal characteristics.

Trophoblast analysis identified several previously unrecognized lineages. Distinct cytotrophoblast clusters included an extravillous-like population characterized by an invasive molecular signature at mid-gestation. This group was transcriptionally distinct from villous cytotrophoblasts and showed enrichment in hypoxia-responsive transcripts, including HIF1A, consistent with adaptation to the low-oxygen environment at the labyrinth base, possibly contributing to trophoblastic invasive properties. Gene trajectory modeling provided initial insights into pathways leading toward syncytialization, indicating the origins of the multinucleated syncytiotrophoblast that embeds decidual cells and surrounds the intact maternal endothelium.

Our single-nucleus-based observations provide the first high-resolution atlas of the canine placenta. They highlight the dynamic molecular relationships between the maternal-decidual and fetal-trophoblastic compartments and reveal regulatory programs associated with progesterone withdrawal that shape placental function and drive parturition in the dog. By integrating molecular, cellular, and comparative perspectives, this work establishes a foundation for refined mechanistic studies and potential translational applications in placental biology.

Multiomics Led Systems Biology

Monday, July 20 10:00 AM – 12:00 PM

Submission ID#2432189

ECHOS Enables Spatial Epigenome Profiling at Subcellular Resolution

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Abstract Text

Biological structures and the epigenome are intertwined. For example, complex tissues are often the combined products of various groups of spatially patterned cell types with distinct epigenetic states. Furthermore, chromatin at various subnuclear locations within a cell often differ in their epigenetic properties. Thus, systematically understanding the relationship between the epigenome and its spatial distribution across biological scales would inform tissue and cellular functions as well as gene regulatory mechanisms. Yet, spatially resolved epigenome profiling—particularly at subcellular resolution—remains technically challenging. Here, we present Epigenetic CUT&Tag via High-resolution Optical Selection (ECHOS), a platform that combines high-resolution imaging and high-throughput sequencing to enable precise, spatially targeted epigenetic profiling across biological scales. At the cellular scale, ECHOS reveals distinct gene regulatory logic at different layers of human ectocervical epithelium. At the subcellular scale, ECHOS shows that human aging altered the epigenetic state of the inactive X chromosome located in the Barr body, a subcellular nuclear structure, which may contribute to genes escaping X chromosome inactivation during female aging. Together, ECHOS represents a scalable and generalizable framework for spatial epigenomic analyses, with broad potential applications in various domains of biology.

Submission ID#2361255

Preimplantation Development is Accompanied by Progressive Reconfiguration of Protein Domain Architecture Through Alternative Splicing

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Abstract Text

Successful preimplantation development requires rapid and coordinated cellular reprogramming, traditionally characterized through gene-level transcriptomic analyses. However, alternatively spliced transcript isoforms can reshape protein structure and function without altering gene expression, and the extent to which protein domain architecture is remodeled this way during early embryogenesis remains poorly defined. To investigate this, we developed FEATS (Function and Expression Analysis for Transitions in Splicing), a computational pipeline for RNA-seq data analysis that incorporates transcript isoform discovery, expression quantification, and functional domain annotations. We applied FEATS to RNA-seq data from mouse oocytes, zygote, 2-cell, 4-cell, 8-cell, and blastocyst embryos, performing progressive pairwise comparisons to identify stage-specific isoform transitions and associated changes in protein domain composition.

Across preimplantation stages, we identified 114 splice variants (representing 54 genes) that exhibited complete isoform switching, with 36 transcripts demonstrating shifts that altered predicted functional domain architecture. Clustering analysis revealed distinct stage-specific patterns of isoform expression and domain organization, with the highest frequency occurring at the zygote to 2-cell transition, aligned with embryonic genome activation. Oocyte-dominant isoforms exhibited minimal domain architecture and frequently lacked transcriptional, chromatin-binding, or interaction modules that appeared in later stages. Subsequent transitions were characterized by selective modulation of domains involved in chromatin engagement, transcriptional regulation, ubiquitin signaling, membrane interactions, and cytokinesis, indicating progressive expansion of regulatory capacity while core domain architecture remained largely preserved. By the blastocyst stage, dominant isoforms were generally longer and enriched for transcriptional and chromatin-associated domains, consistent with increasing regulatory and lineage-specific demands.

Representative examples illustrate how isoform switching reshapes regulatory potential across developmental transitions. During the zygote to 2-cell transition, *N4bp2* and *Wdfy3* acquired ubiquitin-binding and membrane-targeting domains, respectively, suggesting increased capacity for ubiquitin-mediated and membrane-associated interactions, whereas *Ncoa6* and *Zfp638* lost nucleic acid- or RNA-binding domains, indicating altered transcriptional regulatory potential. Chromatin regulators such as *Phf6* and Phf8 retained catalytic demethylase cores but exhibited reorganization of reader domains, consistent with altered chromatin engagement. Later transitions were dominated by refinement of regulatory and structural domains, as observed in *Itpk1*, *Tasor2*, *Ttc3*, *Shoc1*, and *Kif23*, while by the blastocyst stage dominant isoforms were generally longer and enriched for transcriptional and chromatin-associated domains. Notably, *Esr1* exhibited selective loss of the nuclear receptor DNA-binding domain while retaining the ligand-binding domain, suggesting stage-specific modulation of transcriptional regulatory capacity. These patterns indicate that isoform switching remodels regulatory and interaction domains across developmental transitions.

Collectively, these findings demonstrate that preimplantation development is accompanied by progressive reconfiguration of protein domain architecture through isoform switching, revealing a previously underappreciated mechanism of developmental regulation. A consistent principle emerged across developmental stages: regulatory, interaction, and targeting domains were selectively spliced-in or spliced-out with complete isoform switches occurring between stages. Early stages favor streamlined isoforms that maintained essential functions, whereas later stages exhibit increasing regulatory complexity associated with genome activation, proliferation, and lineage specification. These results indicate that gene-level analyses underestimate developmental remodeling and identify isoform switching as a critical layer of regulation in early embryogenesis.

Submission ID#2348116

EvolTESTIS-Zoo: A Comparative Single-Cell Atlas of Spermatogenesis across Model and Non-Model Mammals

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Abstract Text

Spermatogenesis is a highly coordinated developmental process involving more than 10,000 protein-coding genes that are dynamically regulated across distinct stages of sperm formation. Approximately one third of these genes are believed to be evolutionarily conserved, underscoring their fundamental roles in male fertility. However, despite the rapid expansion and widespread application of single-cell RNA sequencing (scRNA-seq) technologies, there is currently no accessible, integrated platform that enables systematic cross-species comparison of transcriptomic programs during spermatogenesis—particularly across model organisms, livestock, and wild species—thereby limiting the identification of conserved, stage-specific biomarkers. To address this gap, we developed EvolTESTIS-Zoo, an interactive R-based application (R v4.4.3) designed to reorganize, visualize, and compare testicular scRNA-seq datasets across multiple mammalian species. The application utilizes publicly available datasets from the NCBI Gene Expression Omnibus (GEO) and processes them using the Seurat framework (v5.1.0) for quality control, normalization, and downstream analyses. Depending on the species and dataset, approximately 2,000–20,000 individual cells were processed and manually annotated using selected conserved marker genes, enabling the identification of more than five major somatic cell types (including Sertoli cells, Leydig cells, peritubular cells, macrophages, and endothelial cells) and eight distinct germ cell stages (spanning spermatogonia, spermatocytes, and spermatids). Across species, approximately 24,000–51,000 gene features were defined, supporting robust intra- and inter-species comparisons to identify both conserved and novel gene markers. Dataset integration and interactive visualization are implemented using Shiny (v1.9.1), enabling intuitive exploration of integrated datasets through a web-based interface. EvolTESTIS-Zoo enables flexible exploration of gene expression patterns across cell types and spermatogenic stages among multiple species, including human (GSE109037), mouse (GSE104556), pig (GSE174782), and bovine (GSE206156), with planned expansion to additional model and non-model organisms—such as rat, horse, and chimpanzee—ultimately encompassing at least 13 mammalian species. Using this platform, researchers can readily identify conserved and species-specific transcriptional signatures, facilitating the discovery of stage-restricted candidate biomarkers relevant to sperm development. Cross-species analyses further enable comparative studies between experimental model organisms and economically important species, providing new insights into species-specific spermatogenic programs and evolutionarily conserved regulatory mechanisms. By leveraging publicly available scRNA-seq datasets from GEO, EvolTESTIS-Zoo is inherently extensible, supporting continuous expansion of its species coverage and long-term development as a universal reference resource for reproductive biology research. Overall, EvolTESTIS-Zoo represents a scalable and accessible platform for comparative single-cell analysis of spermatogenesis. It has broad potential applications in the study of spermatogenic regulation, the discovery of evolutionarily conserved biomarkers, the elucidation of the molecular basis of male infertility, and translational applications in animal breeding and species conservation.

Submission ID#2358288

A Multiomic Atlas of the Peri-Implantation Porcine Endometrium Reveals Cell-Specific Responses to Heat Stress

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Abstract Text

The severity and duration of heat waves are increasing globally. Heat stress during the peri-implantation period reduces conception rates and increases early embryo loss, with unknown life-course health implications for offspring. The endometrium plays a key role in mediating pregnancy success and exposure to stressors but cannot be studied in humans. Porcine embryos undergo similar embryonic patterning to humans, so we used a porcine heat-exposure model to 1) produce a single nuclei multiomic atlas of the peri-implantation endometrial heat stress response, 2) define endometrial cell-type-specific responses to heat stress, and 3) identify regulatory mechanisms driving these responses.

Estrus-synchronized gilts were exposed to heat stress (HS; THI 75-85) or thermoneutral conditions (TN; THI 60-70) from seven days prior to insemination to day 15 (D15) of pregnancy. Nuclei were isolated from n=2 endometria per group (pregnancy D8 and D15, TN/HS) and processed using 10X Multiome protocols to produce gene expression (GEX) and ATAC libraries. Sequencing was performed on a NovaSeq X plus and reads were aligned and aggregated using 10X Cellranger-arc and Ensembl Sus scrofa 11.1 genome. Quality filtering of nuclei, clustering, and downstream analyses were conducted using Seurat and Signac.

Across eight samples, we obtained 25,053 nuclei, of which 22,896 high-quality nuclei were used for analysis. UMAP and clustering identified multiple epithelial subclusters, and populations of ciliated, stromal, two endothelial, mural perivascular, and two immune leukocyte CD45+ clusters (lymphocytes [CD3+] and myeloid [CD3-]). Sub-clustering analysis found fourteen epithelial populations: six at D8 and eight at D15, including two glandular (GE) and four/six luminal (LE), respectively. Differential expression analysis (Wilcoxon rank-sum test, $p< 0.01$ and log2FC >1 or < -1) revealed expected transcriptional signatures from D8 (blastocyst stage) to D15 (elongated conceptuses), including increased expression of *SPP1* and decreased *PGR* on D15. In ciliated cells, heat stress increased response to type I interferon/virus (*IFNAR1, MX1, STAT1, FADD, RIGI*) and modified amino acid transport (*SLC7A11, SLC7A2*) genes on D8. Response to type I interferon transcripts (*IFIT1*, *MX1*) was also induced in endothelial cells on D8 by heat stress, alongside response to stress genes (*LRP11*, *THBS1)*, whereas on D15 cytoplasmic translation related transcripts were reduced, and altered response to oestradiol-related genes (including *PTGS2* decreased) under heat stress in endothelial cells. On D8, LE had increased expression of viral response (*CD55, MX1, OASL, RSAD2*), temperature homeostasis (*DIO2, IL1A,* TSHR), and response to LIF (*CACNB4, EFHC2*) transcripts, and decreased expression of response to oxidative stress (*GPX1, HBB, ND1,* PPARGC1A) and regulation of inflammatory response (*C1QTNF3, GPX1, IL1RL2*) genes. In D8 LE, altered expression of SLC transporters (6 increased, 2 decreased), increased *IFNGR2*, and decreased response to steroid hormone transcripts (including *PTGER2, PTGS2*) were identified. In D15 GE, increased response to virus (*MX1, USP18, DDX60*), and ephrin receptor signalling (*EPHA7, EPHB1*) which is associated with environmental stress, and decreased integrin adhesion (*ITGA8, TGFB2)*. On D15, increases in transcripts associated with response to type I interferon (*NLRC5, STAT1*) and perturbed SLC transport were observed.

We present the first single nuclei 10X Multiome atlas of porcine peri-implantation endometrium, integrating gene expression and chromatin accessibility. Our data reveal cell-type specific heat stress responses across the peri-implantation period which may contribute to reduced pregnancy success or impact embryo development and subsequent offspring health. Ongoing work will integrate ATAC-derived regulatory information to identify upstream mechanisms driving these transcriptional responses to heat stress.

Submission ID#2360693

Single-Cell Total-RNA-Seq Unveils Regulatory Network Underlying Mammalian Preimplantation Development.

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Abstract Text

Mammalian preimplantation development requires precise coordination of gene regulatory networks that drive maternal RNA clearance, zygotic genome activation (ZGA), and lineage specification. However, steady-state RNA expression measurements cannot directly distinguish whether observed changes arise from transcriptional regulation or from RNA processing and decay. RNA processing kinetics, captured by distinguishing unspliced and spliced transcripts, provide a quantitative framework to differentiate transcriptional activation from post-transcriptional regulation. Here, using our recently developed SnapTotal-seq, we simultaneously measured unspliced RNA (i.e., nascent RNA) and spliced RNA (i.e., mature RNA) in single oocytes and blastomeres (864 blastomeres in total) across bovine oocytes (GV and MII) and preimplantation embryos at the 2-, 4-, 8-, 16-, 32-cell, morula, and blastocyst stages.

RNA velocity analysis of the SnapTotal-seq dataset enabled us to establish RNA life-cycle kinetics during preimplantation development, revealing the dynamic regulation of maternal RNA decay, ZGA, and lineage specification at an unprecedented level. Comparative analysis of nascent and mature RNA identified a major kinetic transition in splicing dynamics between the 4- and 8-cell stages, coinciding with ZGA. Maternal RNA decay was largely completed during the 8- to 16-cell transition in bovine embryos.

Consistent with the importance of maternal mRNA clearance, we identified master regulators of RNA decay, including BTG4 and CNOT4. LASSO regression and motif enrichment analyses uncovered critical stage-specific ZGA transcription factors (TFs). Through integrative bioinformatic analyses and experimental functional validation, we categorized these TFs into master regulators (DUX4, POU5F1, MYC, ZSCAN4, FOS), core hubs (SP1, KLF17, KLF10, KLF4, NR5A2, ATF3), and terminal lineage regulators (TFAP2C, ESRRB, GATA6, NFAT5, GABPA, NKX6-1) across preimplantation development. Additionally, we identified master regulators associated with minor ZGA that contribute to the regulation of major ZGA and drive the earliest lineage specification at the morula stage in bovine embryos.

In summary, this study provides an invaluable resource establishing nascent RNA dynamics and regulatory hubs underlying mammalian preimplantation development and establishes a powerful framework for dissecting transcriptional and post-transcriptional control during the earliest stages of embryonic development.

Submission ID#

Title Coming Soon

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Abstract Text

Pituitary Gonadotropins

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Submission ID#2337842

Testosterone Gender-Affirming Hormone Therapy Rescues Changes in Reproductive, but Not Metabolic, Tissue in Gonadectomized Mice

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Abstract Text

An estimated 1.6 million Americans identify as transgender, non-binary, and gender diverse (TNG). Although gender-affirming hormone therapy (GAHT) is established as a safe and effective standard-of-care therapy for gender dysphoria experienced by many TNG patients, the long-term effects of GAHT on diverse body systems such as reproduction, metabolism, and bone health are critically understudied. Removal of ovaries and decreased estradiol without hormone replacement therapy in individuals assigned female at birth is associated with increased risk of cardiovascular disease, obesity, and decreased bone density with higher risk of fracture. However, there is very limited data on the health outcomes of removing versus retaining gonads if TNG patients using GAHT choose to undergo gender-affirming surgery. This study tested the hypothesis that ovary retention during testosterone (T)-GAHT is protective against adverse outcomes in metabolic, osteologic, and reproductive tissues using a preclinical mouse model of T-GAHT. 8-week-old adult female CD-1 mice were implanted with capsules containing either 10 mg T (Testosterone) or vehicle (Blank) and underwent either bilateral ovariectomy (OVX) or sham (Intact) surgery, resulting in four experimental groups: Blank+Intact (n=7, negative control), Blank+OVX (n=7, positive control), Testosterone+Intact (n=7), and Testosterone+OVX (n=8). After 6 weeks treatment, T-treatment induced higher levels of circulating triglycerides, increased heart weight, and increased femoral trabecular bone density and radius strength regardless of ovary presence (Two-way ANOVA with Tukey's post-hoc, $p< 0.05$). T-treatment was sufficient to rescue gonadectomy-induced decrease in glucose tolerance and hyperglycemia; however, all ovariectomized mice displayed elevated cholesterol levels and increased adipocyte size even with T-treatment, indicating that gonad retention is protective against these risk factors. As expected, uterine size and weight was greatly reduced in Blank+OVX mice and increased in Testosterone+Intact mice, with the greatest changes in the endometrium and lumen. Interestingly, Testosterone+OVX mice had uteri indistinguishable from negative controls in the parameters tested, suggesting a unique rescue interaction between these conditions in their effects on uterine biology. These findings indicate that, with the possible exception of changes in lipid metabolism, there are few increased metabolic or skeletal health risks associated with ovary removal in individuals receiving T-treatment. It also suggests that uterine morphology is not largely impacted by combined ovary removal and T-treatment, though more functional tests remain to be done. These findings support the safety of elective gender-affirming oophorectomy in transmasculine patients provided T-GAHT remains a part of the therapeutic regimen and indicate that transmasculine individuals may retain a functional uterus after oophorectomy and T-GAHT. Results from this study can help clinicians provide evidence-based, personalized medical advice to TNG patients considering oophorectomy, especially transmasculine individuals interested in pregnancy or surrogacy.

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Submission ID#2361380

Macrophages Regulate Pituitary Hormone Secretion

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Abstract Text

Chronic inflammation disrupts hormonal balance in the Hypothalamic-Pituitary-Gonadal (HPG) axis, contributing to reproductive disorders. While immune cells in the hypothalamus and ovaries have been extensively studied, their impact on the pituitary remains largely unexplored. Our research identifies macrophages as the primary pituitary immune cells, exhibiting unique transcriptional profiles for immune activation, phagocytosis, and hormone synthesis compared to other tissue-resident macrophages. Notably, these macrophages exhibit high levels of growth hormone (Gh) and prolactin (Prl) transcription. To investigate the role of pituitary macrophages in hormone regulation, we depleted macrophages from mouse pituitaries both *in vitro* and *in vivo*. *In vitro* macrophage depletion decreased secretion of gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) and cytokines, including IFN-γ and CXCL5. LβT2 gonadotrope cells responded to IFN-ɣ and CXCL5 in a dose- and time-dependent manner, which indicates a direct inflammatory impact on the pituitary. Furthermore, our *in vivo* macrophage-depletion mouse model validates FSH and LH reduction upon pituitary macrophage depletion using adeno-associated virus (AAV) with Cre- and Gh promoter-dependent caspase. Interestingly, estrous cycle length and average time spent per cycle stage remained unchanged upon depletion. These findings highlight the essential role of pituitary macrophages in hormonal regulation and inform potential immunotherapeutic strategies to restore hormonal balance in reproductive disorders.

Submission ID#2370543

Novel insights into gonadotropin regulation

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Abstract Text

The pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are key regulators of gonadal function. Though mechanisms driving FSH and LH synthesis and secretion by pituitary-derived activin B, hypothalamic gonadotropin-releasing hormone (GnRH), and gonadal inhibins and sex steroids have been the subjects of investigation for decades, many questions remain unanswered and some established 'facts' are being re-examined. For example, we recently reported that muscle-derived myostatin rather than intra-pituitary activin B is the primary transforming growth factor β family member driving FSH production by pituitary gonadotropes. GnRH signals through the GnRH receptor, a GPCR that canonically couples to the Gαq/11 subunits. Indeed, ablation of Gαq/11 in murine gonadotropes leads to hypogonadotropic hypogonadism and infertility. Unexpectedly, ablation of another subunit, Gαs, in gonadotropes decreases LH pulse and surge amplitude, and blocks post-gonadectomy increases in both FSH and LH. How GnRH signals via non-canonical Gαs to regulate the gonadotropins is an active area of investigation. In this lecture, I will review these and other unexpected results from the lab that have caused us to question what is fact vs fiction in our understanding of gonadotropin regulation.

Submission ID#2360626

Effects of Microenvironment Forces on Migratory Dynamics and Morphology of Immortalized GnRH Cells

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Abstract Text

Gonadotropin releasing hormone (GnRH) cells are essential central regulators of vertebrate reproduction. GnRH cells originate outside of the central nervous system and migrate into and colonize the preoptic area and hypothalamus. The GnRH cell migratory pathway passes through distinct microenvironments including the nasal placode, the nasal forebrain junction, and the developing forebrain, each of which differs in migratory substrates and physical constraints. We hypothesize that the physical interaction of GnRH cells with their migratory microenvironment results in alterations in cell morphology and physiology and is essential for proper cell maturation and function. To date, GnRH cell migration has been studied on 2-dimensional (2D) substrates which lack the ability to isolate and modulate the physical constraints encountered by GnRH cells in vivo. To address these gaps, we have utilized microfluidic culture technology. Polydimethylsiloxane (PDMS) microchambers are highly modifiable for cell migration through restrictive, 3-dimensional space and allow for live cell imaging for extended periods of time. In the current studies, PDMS microfluidic devices were designed with parallel microchannels aligned in a ladder-like configuration connecting 2-dimensional seeding and collecting chambers. Microchannel restrictions ranged from 10x10 μm^2, 6x10 μm^2, or 3x10 μm^2 (height x width) were used. Dimensions of 10x10 μm^2 were considered only mildly restrictive and 3x10 μm^2 were considered highly restrictive. GN11 cells were used as a model of immature and migratory GnRH cells. Cells were imaged every 15 minutes for 16 hours to determine migratory dynamics and morphological changes present during migration. Video images and statistical analysis were performed with ImageJ/Fiji, MatLab, and Prism. Increasing physical confinement decreased the migration speed of GnRH cells. Cells migrating in 10x10 μm^2 channels move significantly faster than cells migrating in 6x10 μm^2 channels (50.72±29.54 μm/hr v. 34.86±15.86 μm/hr, p=0.0178) and in 3x10 μm^2 channels (50.72±29.54 μm/hr v. 26.18±9.684 μm/hr, p< 0.0001). Restriction-induced changes in velocity were observed when the cells were exposed to a chemotactic gradient (10% FBS) or in devices with no gradient (static). Increasing restriction did not significantly alter cell persistence. As cells transitioned from 2D seeding channels into restrictive microchannels of all dimensions, cell aspect ratio was significantly reduced as cells converted to a bipolar migratory morphology. Surprisingly, a gradient of the defined GnRH cell chemoattractant factor hepatocyte growth factor did not alter cell persistence, cell velocity, or cell propensity to migrate in this system. GN11 cell migration was similarly unaffected by protein substrate. Coating microfluidic devices with different extra cellular matrix proteins did not alter migratory velocity (collagen I: 45.41±28.72μm/hr; collagen IV: 43.56±23.27μm/hr; fibronectin: 43.51±26.04μm/hr; laminin: 45.37±23.91μm/hr, p=0.5510). These findings suggest that when migrating in 3-dimensions, available space has the greatest effect on cell migration speed, more than even chemotactic or substrate signals. This ongoing project will develop and validate a new tool to enhance clinical relevance and translational impact of *in vitro* neural cell migration studies and allow for a more complete understanding of GnRH cell migration and the forces which affect their development.

Submission ID#2360195

The Microbiome Exerts Sex- and Estrous Stage-Specific Effects on Reproductive Parameters Relevant to Fertility

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Abstract Text

The gut microbiome plays a critical role in regulating reproductive function by modulating sex hormone homeostasis and enterohepatic steroid metabolism through microbial sterol conversion and deconjugation. Microbial communities at other reproductive sites, such as the vagina, also influence implantation success, suggesting that host-microbe interactions broadly shape reproductive physiology. Consistent with these observations, germ-free mice without any microbiome exhibit reduced circulating gonadotropins and lower copulation rates compared to conventionally raised mice with a natural microbiome. However, biological processes linking the microbiome to reproductive endocrine regulation remain unclear. This study aims to evaluate the impact of the microbiome on reproductive parameters and hormone regulation in both male and female mice. To investigate this, thirteen-week-old germ-free (GF) and conventionally raised (CONV) female and male C57BL/6 mice were euthanized for reproductive assessment. Females were euthanized during either estrus or diestrus to examine reproductive physiology at two distinct stages of the estrous cycle (n=6-9/sex and microbiome status; n=6-9/estrous stage and microbiome status). Estrous stages were monitored for 11 days prior to euthanasia to determine cyclicity. Circulating and gonadal sex steroids, reproductive organ weights, and sperm count were measured. Expression of genes involved in steroidogenesis was assessed in testes and ovaries, including follicle-stimulating hormone receptor (*Fshr*), luteinizing hormone receptor (*Lhr*), steroidogenic acute regulatory protein (*Star*), cytochrome P450 family 11 subfamily A member 1 (*Cyp11a1*), 3β-hydroxysteroid dehydrogenase (*Hsd3b1*), 17β-hydroxysteroid dehydrogenase (*Hsd17b1*), cytochrome P450 family 17 subfamily A member 1 *(Cyp17a1*), aromatase (*Cyp19a1*), and inhibin subunits α (*Inha*) and β (*Inhb*). In females, GF mice exhibited prolonged estrus and shortened diestrus compared to CONV mice, indicating altered estrous cyclicity. Uterine weight was reduced in GF mice, reaching statistical significance during diestrus (p=0.032) and showing a trend toward reduction during estrus (p=0.054). Circulating or ovarian estradiol levels did not differ by microbiome status at either estrous stage. During diestrus, ovarian expression of key steroidogenic genes, including *Lhr*, *Star*, *Hsd3b1*, and *Cyp19a1*, was significantly lower in GF mice compared to CONV controls. No differences in gene expression were detected during estrus, suggesting that microbiome effects on ovarian steroidogenic pathways are estrous stage dependent. In males, GF mice exhibited significantly lower testicular weight and sperm count compared to CONV mice. Interestingly, GF males had significantly higher circulating testosterone and a trend toward increased testicular dihydrotestosterone (p=0.069), while testicular testosterone did not differ between groups. In contrast to females, GF males showed significant upregulation of steroidogenesis-related genes, including *Fshr*, *Lhr*, *Star*, *Cyp11a1*, *Cyp17a1*, and *Hsd17b1*, along with increased expression of *Inha* and *Inhb*. These results suggest that lack of microbiome in males results in enhanced testicular steroidogenic signaling despite unchanged intratesticular testosterone levels. These findings support that the microbiome significantly impacts reproductive parameters and hormone regulation, with estrus stage-dependent effects observed in females and sex-specific differences shown in steroidogenic gene regulation. Further investigation of these relationships may inform novel microbiome-based strategies to support reproductive health.

Submission ID#2421325

Decoding Immune Dysregulation of Gonadotropins in Polycystic Ovary Syndrome

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Abstract Text

Polycystic Ovary Syndrome (PCOS) is one of the most prevalent endocrine disorders affecting women of reproductive age, yet its underlying pathogenic mechanisms remain incompletely understood. Emerging evidence suggests immune dysregulation plays a critical role in PCOS development. We demonstrate that both innate and adaptive immune pathways are mediators of PCOS pathogenesis. Using the letrozole induced model of PCOS-like symptoms in mice, we show that genetic knockout of toll-like receptor 4 (TLR4) or T cells ameliorates PCOS-like symptoms, specifically elevated luteinizing hormone, even in the presence of high androgen. By leveraging human datasets, we found that T cell derived TNF-β is a likely mediator of the disrupted sex steroid negative feedback in PCOS. Together, our data demonstrates that TLR4, T cells, and TNF-β constitute a cohesive program of dysregulation across both the innate and adaptive immune systems that are critical components driving PCOS pathogenesis, thereby providing potential therapeutic targets for this prevalent reproductive disorder.

Environmental Influences on Reproductive Epigenetics and Genetics

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Protein Citrullination is a Key Mediator of Developmental DES-Induced Gene Expression Changes in the Mouse Uterus

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Abstract Text

Neonatal exposure to estrogenic chemicals alters reproductive tract development and function and leads to uterine cancer in adulthood. Mice exposed neonatally to diethylstilbestrol (DES), a potent synthetic estrogen, have ~5,000 genes differentially expressed in the neonatal uterus. Among the highly upregulated genes are the PADI family of peptidyl arginine deiminases. PADI proteins citrullinate many cellular proteins including histones, which impacts chromatin accessibility. *Padi* genes, located in a single chromosome 4 locus, are expressed in an estrous cycle-dependent manner in the adult uterus, with *Padi1*, *Padi2* and *Padi4* increased >4-fold in estrus compared to diestrus; the other *Padi* genes are not expressed in the uterus. We hypothesized that in neonatal DES-exposed mice, PADI-mediated changes in histone citrullination are responsible for altered chromatin accessibility and the resulting gene expression changes that persist into adulthood and alter uterine function. To evaluate this idea, we generated mice with a uterine conditional deletion of *Padi1*, *Padi2* and *Padi4* (Padi ut-cKO). We then tested the impact of uterine *Padi* loss on adult mice that were either unexposed or exposed to DES on neonatal days 1-5. The outcomes assayed were uterine gene expression (bulk RNAseq), protein citrullination (mass spectrometry), and fertility (6-month breeding trial). Control (unexposed to DES) Padi ut-cKO uteri had 379 and 986 differentially expressed genes (DEGs) in diestrus and estrus respectively, relative to control wild type uteri. These wild type uteri had estrous cycle-dependent differential citrullination of 91 peptides (estrus/diestrus - 88 increased, 3 decreased); control Padi ut-cKO uteri lost citrullination of most of these peptides. A six-month breeding trial showed that Padi ut-cKO mice had a significantly reduced number of litters and cumulative pups compared to wild type mice. DES-exposed wild type mice, which are acyclic, had moderate expression of *Padi1* and *Padi2* mRNA and high expression of *Padi4*, with overall *Padi* expression midway between diestrus and estrus of control wild type uteri. There were 2,929 DEGs when comparing DES-exposed Padi ut-cKO to DES-exposed wild type uteri; 204 genes normalized toward control levels, while 478 genes showed enhanced DES-like changes. A comparison of wild type DES-exposed uterine proteins to those of controls at both diestrus and estrus revealed differentially citrullinated peptides (DES/diestrus - 26 up, 11 down; DES/estrus – 10 up, 72 down), consistent with the changes in *Padi* mRNA expression. Protein citrullination was lost in DES-exposed Padi ut-cKO relative to DES-exposed wild type uteri (44 peptides). These data demonstrate that in control mice, citrullination regulates dynamic estrous cycle gene expression and impacts fertility. Furthermore, neonatal DES-induced uterine dysfunction may be explained in part by an overall reduction in or loss of estrus cycle fluctuation in protein citrullination.

Submission ID#2353705

THC Alters the Epigenomic Landscape of Postejaculatory Sperm Revealing New Mechanisms of Action and Paternal Carryover Effects

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Abstract Text

Cannabis has surpassed alcohol as the most used recreational drug in the USA accounting for about 62 million users in 2024, of which 26% are of prime reproductive age (18-25 years). Delta-9 tetrahydrocannabinol (THC) is the principal psychoactive component of cannabis detected in human plasma and seminal fluids. Despite known effects at the testicular level, acute epigenomic consequences of THC on transient exposure to postejaculatory sperm during transport and subsequent carryover effects on embryo development have been severely overlooked. Our earlier study demonstrated that THC resulted in lower progressive motility, with significant structural damage to the sperm acrosome and chromatin. Utilizing a bovine embryo continuum model, the present study was designed to provide missing information on epigenomic and mechanistic effects of acute THC exposure to postejaculatory sperm function. Cryopreserved bovine sperm from three bulls were independently exposed to a physiologically relevant concentration of THC detected in human seminal plasma (32nM, n=2 rep/bull) for 24 h at 25°C. Changes to DNA methylation, relative abundance of methyltransferases (DNMT3A/1), select miRNAs and embryo development were quantified following IVF. Data were analyzed by logistic regression with a generalized linear mixed effect model. Alterations to the methylation landscape of sperm were determined after 24 h of THC exposure through Whole Genome Enzymatic Methyl-Sequencing. PCA analysis revealed distinct clusters unique to individual males regardless of THC exposure. Sperm exposed to THC revealed ~56 DMRs (39 hypomethylated and 17 hypermethylated) irrespective of male effect when compared to unexposed sperm. THC exposure elicited both individual and shared alterations in sperm DNA methylation across three males, indicating variability and susceptibility of epigenetic response to THC exposure. Gene ontology analysis indicated that common identifiable DMRs (56) were related to functional categories of binding (22%) and catalytic activity (15%), while 48% were not assigned due to lack of annotation. Canonical pathway analysis with common DMRs between THC and unexposed sperm mapped by IPA included mitotic G1 phase and synthesis of DNA and S phase senescence. Major biofunctions included developmental disorder, lipid metabolism, embryonic development, cancer and cell death. In agreement with IPA analyses, paternal carryover effects on embryos derived by IVF from THC-exposed sperm had reduced 2-cell cleavage, 8-16 cell morula development, and blastocyst formation compared to control (42% vs. 25%). Mechanistic studies investigating the basis of these phenotypes included a decreased in the relative abundance of methyltransferases and miRNAs that were characterized in sperm after 24 h of THC exposure. The data revealed a lower abundance of miR-449 (P=0.04) and miR-216 (P=0.02) in exposed versus unexposed sperm, both of which have distinct embryo development roles prior to zygotic genome activation. THC exposure to sperm also resulted in lower abundance of one DNMT3A variant (100kDa) and two variants of DNMT1 in all three males. The magnitude of this response differed among individuals, demonstrating that male-specific differences contribute to variability in the observed response to THC exposure. In conclusion, postejaculatory mammalian sperm exposure to THC results in changes to the sperm methylome while concomitantly lowering preimplantation embryo developmental potential. Collectively, these data implicate new considerations for transient cannabis exposure to postejaculatory sperm that impact cellular mechanisms important for sperm function with phenotypes that escape routine semen analyses but result in negative paternal carryover effects on embryo development. These data also provide new evidence and mechanisms for epigenetic modification to postejaculatory sperm.

Submission ID#2360600

Early Epigenetic and Transcriptional Reprogramming in Response to Phthalate Exposure Primes the Uterus for Fibrosis

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Abstract Text

Uterine fibrosis is a major contributor to impaired reproductive function, yet the early molecular events that program the uterus toward a fibrotic trajectory remain poorly defined. Our previous studies demonstrated that chronic exposure to an environmentally relevant phthalate mixture for six months induces uterine fibrosis, characterized by disrupted stromal architecture and excessive extracellular matrix ECM remodeling. To define the molecular events that precede overt fibrosis, we examined phthalate-induced alterations in uterine gene regulatory networks at an early time point of exposure. Adult female mice were exposed to vehicle control or two doses of a phthalate mixture for one month, followed by RNA sequencing of uterine tissues, with four independent biological replicates per experimental group. Transcriptomic analysis revealed a pronounced disruption of epigenetic and chromatin-modifying regulators prior to the induction of fibrotic markers. Phthalate exposure significantly altered the expression of genes governing histone modification and DNA methylation, including upregulation of the histone acetyltransferase *Kat2a*, the histone deacetylase *Hdac11*, and the DNA demethylase *Tet3*, along with downregulation of the de novo DNA methyltransferase *Dnmt3b* and the Polycomb repressive complex component *Eed*. These coordinated changes suggest early chromatin remodeling and destabilization of transcriptional repression, consistent with increased transcriptional plasticity at this stage. Concomitant with these epigenetic perturbations, phthalate exposure altered the expression of a network of transcription factors associated with developmental plasticity and lineage specification, including *Sox17, Sox5, Hopx, Msx1, Emx2, Tfcp2l1, Klf15, Gata2*, and *Ovol1*, indicating early stromal reprogramming toward a pro-fibrotic state. Notably, the epigenetic and transcriptional changes were detected prior to the robust induction of extracellular matrix and myofibroblast-associated genes, supporting a model in which epigenetic and transcriptional reprogramming precede structural fibrosis. Collectively, these findings identify early epigenetic and lineage-specifying mechanisms following phthalate exposure that reprogram uterine cell fate and establish a permissive transcriptional landscape for fibrosis. This work provides mechanistic insight into how environmental endocrine disruptors initiate uterine fibrosis and highlights epigenetic regulators as potential targets for early intervention prior to irreversible fibrotic remodeling. Funding: NIH R01ES034112

Submission ID#2361238

Female reproductive impacts of real-world environmental endocrine disruptor mixtures

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Abstract Text

Rivers are constantly subject to environmental exposures due to their close association with human necessity. Rivers support diverse ecosystems and serve as a source for drinking water and function for industrial, commercial, agricultural, and recreational activities. Increasing evidence has highlighted the presence of environmental organic pollutants in river systems, and many of them have been established as endocrine disrupting chemicals or EDCs. Female reproductive health is particularly vulnerable to disruption due to sensitive hormone and nuclear receptor-mediated signaling pathways and a finite ovarian reserve established early on in life. This study aims to determine the effect of exposure to environmentally relevant EDC mixtures obtained from real-world river water on female reproductive health.

Surface water was collected from Raritan River, a major river in central New Jersey (NJ). Total organic pollutant or EDC mixtures were isolated using solid phase extraction with double-distilled water as control. The chemical profile of the Raritan River organic extract was assessed using targeted LC-MS to determine concentrations of 72 commonly known EDCs. To examine female reproductive impacts of Raritan River EDC mixture, 8-week-old young adult CD-1 female mice were exposed to extracted EDC mixtures via daily drinking water at 0 (0.3% DMSO), 1x, 5x, and 10x the measured river concentrations, covering low to high contamination and exposure levels for 10 weeks. Mouse bodyweight, survival, water/food intake were monitored daily, and estrous cyclicity was assessed during the final three weeks of exposure through the day of sacrifice by daily vaginal smear. Complementary in vitro assessment was performed using a 3D encapsulated in vitro follicle growth (eIVFG) model to evaluate the ovarian toxic mechanisms of Raritan River EDCs mixtures. Follicles were exposed to the same concentrations used in the in vivo assessment.

Targeted LC-MS identified distinct concentrations of alkylphenols (50.0 ng/L), pharmaceutical and personal care products (PCPs) (35.2 ng/L), per- and polyfluoroalkyl substances (PFASs)(21.3 ng/L), and phthalates (10.0 ng/L) among other compounds in Raritan River water sample. Several of them exceed EPA recommended exposure limit, including PFAS mixtures and PFOA that surpassed the EPA's recommended hazard index and maximum contamination level (MCL) respectively. In vivo exposure results showed that there was a trend of dose-dependent decrease in animal body weight and no changes of other general health endpoints and estrus cyclicity. The results of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using antral follicles mice revealed a borderline increase of aryl hydrocarbon receptor (AhR) target gene Cyp1a1 and steroidogenic gene Star, and a decreased expression of several genes crucial for follicle development (Pappa, Lhcgr). Mechanistic data from the in vitro exposure experiment highlighted a dose-dependent increase of pre-ovulatory testosterone secretion and decreased post-ovulatory progesterone secretion. The results for in vitro RT-qPCR indicate that follicles treated with Raritan River EDCs had significantly increased expression of genes related to oxidative stress (Sod1), apoptosis (Bax), steroidogenesis (Star), and AhR target genes (Cyp1a1 & Cyp1b1). Single-follicle and Single-oocyte RNA sequencing analysis confirmed the RT-qPCR data and highlighted altered gene regulatory pathways related to cell-to-cell signaling and receptor binding.

In summary, our results demonstrate that exposure to real-world Raritan River EDC mixtures impairs female ovarian follicle development and expression of steroidogenic genes in a dose dependent manner. Future studies will include examination of serum hormone levels, morphological changes in ovarian structure, and counting of various stages of follicles from the in vivo experiment.

Submission ID#2358153

Germline Transmission of Endocrine Disruptor-induced DNA Epimutations to Offspring

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Abstract Text

Epigenetic inheritance of environmental endocrine disruptor- (EED) or lifestyle-induced phenotypes has been demonstrated in many model organisms in a laboratory setting, suggesting that individuals in current generations could be harboring ancestral environmental exposure-induced molecular memories or future generations may carry such memories due to exposure of the current generation. Ancestral EED exposure-related heritable epigenetic traits may be risk factors for future generations' health. It is essential to develop strategies to correct transgenerational epigenetic traits before they are transferred to offspring via germline transmission or to block the heritable pathways to diseases. Nevertheless, such strategies are yet to be developed. As a proof of concept, using medaka fish as an aquatic model organism of human and ecosystem health, we investigated whether the EED-induced transgenerational non-alcoholic fatty liver disease (NAFLD) can be reversed by epigenetic modifier treatments. Medaka embryos (8 hours post-fertilization) were treated with bisphenol A (BPA, an EED present in many consumables made of plastics) at the concentration 10 µg/L until 15 days post-fertilization and never thereafter. The window of BPA exposure was designed to avoid epigenetic reprogramming of post-fertilization embryo. Offspring phenotypes were verified histologically. DNA methylation was quantified by whole genome methylome sequencing and RNA by bulk RNA sequencing. Vitamin C was used as an epigenetic modifier. Ancestral BPA exposure at F0 generation caused transgenerational NAFLD leading to nonalcoholic steatohepatitis (NASH) in offspring at F2 generation, which persisted for five generations (up to F4 generation). The severity of the disease was sexually dimorphic- with females affected more than males. Past F2 generation, the transgenerational NAFLD started after puberty, developed in adulthood, and progressed toward NASH and fibrosis as the fish aged. We performed transcriptomic, metabolomic, and methylome analyses to understand the mechanisms involved. DNA methylation profile of the F1 paternal sperm predicted the DNA methylation and transcriptional landscape of the F2 offspring liver revealing distinct pathways associated with NAFLD. A larger percentage of transgenerational differentially methylated promoters present in the NAFLD liver at the F2 generation were removed in the F4 generation during germline transfer. Still, the phenotype persisted but with comparatively reduced severity. Treatment of the F4 generation females with 1 mg/L vitamin C, which acts as a two-edged sword- antioxidant and demethylating agent, resulted in: 1) A reversal of NAFLD phenotype to normal, 2) reprogramming of the majority of BPA-induced DNA epimutations, and 3) restoration of transcriptional network comparable to that in untreated controls. The present results suggest that transgenerational inheritance of EED-induced traits can be reprogrammed and offspring health can be restored. The results warrant studies focusing on the erasure of EED-induced heritable epigenetic traits in the parental germline prior to conception. *The study was supported by funds from National Institute of Environmental Health Sciences (R01ES032452, R21ES027123) and Eunice Kennedy Shriver National Institute of Child Health and Human Diseases (R21HD098621)*.

Submission ID#2361237

Decreased Ovarian Health from Environmental Contamination Leads to Female-Specific Cardiac Dysfunction.

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Abstract Text

Cardiovascular disease is the leading cause of death in men and women worldwide, and it is well established that estrogen is cardioprotective and, as a result, premenopausal women are less likely to experience cardiovascular disease when compared to men or aged matched post-menopausal controls. Although the ovary is the main producer of estrogen, ovarian function and fertility is not considered when assessing cardiovascular health in women. Humans are continuously exposed to environmental contamination which affects fertility; however, comparatively little is known about how an exposure which decreases female fertility may also impact cardiovascular health. We exposed juvenile zebrafish to an environmentally relevant concentration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a global and persistent environmental contaminant, known to decrease female fertility, or a vehicle control and assessed cardiovascular health between 3 to 12 months post-fertilization using electrocardiograms (ECG), echocardiograms, histology, and RNA sequencing. We discovered that juvenile exposure to TCDD leads to adult cardiac dysfunction in an age- and sex-dependent manner. Echocardiograms revealed that TCDD exposure causes diastolic dysfunction. To determine whether these effects are directly mediated by TCDD's canonical signaling pathway, we examined *ahr2* mutants. We found that the aryl hydrocarbon receptor mediates changes in the depolarization of the atrium in both males and females, while other elements of cardiac conduction show in a sex-specific differences. To disentangle the influence of ovarian dysfunction, we used the *Tg(piwil1:CFP-NTR)* zebrafish strain to ablate oocytes and phenocopy TCDD-specific infertility. We determined that ovarian health mediated changes in the heart rate. Sequencing revealed that estrogen responsive gene pathways, such as zona pellucida genes, were upregulated in the TCDD-exposed female hearts. Additionally, TCDD-exposed ovaries also exhibited elevated inflammation and stromal dysfunction relative to controls. Together, our results indicate that juvenile exposure to TCDD causes sex-specific changes in cardiac function by effecting estrogen signaling and ovarian health.

From Niche to Egg: Microenvironmental Drivers of Oocyte Health

Monday, July 20 1:30 PM – 3:00 PM

Submission ID#2338012

How Mechanical Forces Shape Follicle Development and Ovarian Aging

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Abstract Text

All tissues contain multiple cell types organized into compartments with unique mechanical properties that contribute to regulating their function. Yet we know remarkably little about how mechanical cues within the ovary regulate follicle development and oocyte quality. Mechanical forces might be important regulators of follicle development because follicles are assembled before birth and remain in the ovary for years, where they are continuously exposed to a highly dynamic mechanical environment. Additionally, multiple ovarian pathologies associated with infertility, including PCOS and POI, are characterized by increased ovarian stiffness. We and others have also found that ovarian stiffness increases with advanced maternal age in mice and humans, suggesting that altered tissue mechanics may be a previously underappreciated driver of ovarian aging. To begin exploring this, we utilized an alginate-based encapsulation system in which we synthesized gels that mimic the soft and stiff environments of reproductively young and old mouse ovaries, respectively. This system enables precise control of the mechanical environment *in vitro*, independent of other age-associated factors. Using this approach, we discovered that follicles rapidly sense increased stiffness and initiate a fibroinflammatory response. Prolonged exposure to elevated stiffness impairs follicle growth, reduces steroid hormone production, and compromises oocyte quality. These findings demonstrated that follicles are not passive recipients of their mechanical surroundings; rather, stiffness alone is sufficient to induce key hallmarks of ovarian aging. To build on this work, we are generating a high-resolution, multiscale mechanical map of the mouse ovary to define how spatial and temporal changes in tissue stiffness regulate ovarian function. In parallel, we are extending our studies beyond aging to investigate other conditions that may affect fertility by altering the ovary's mechanical properties. Together, our work positions ovarian mechanics as a fundamental regulator of ovarian function. Future research in the Amargant i Riera lab will focus on defining how follicles sense mechanical cues and identify mechanosensing and mechanotransduction pathways that can be targeted to preserve oocyte quality and extend ovarian function.

Submission ID#2359581

Establishing a Microinjection Platform for Mechanistic Studies of Meiotic Resumption in Feline Oocytes

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Abstract Text

The domestic cat is an important biomedical and veterinary model for assisted reproduction relevant to the conservation of endangered felines. Despite extensive use of this model *in vitro*, mechanistic studies of feline oocyte biology remain limited. Microinjection is a powerful and widely used approach for manipulating mammalian oocytes via protein depletion or RNA interference. An important microinjection technique used for rapid protein depletion is Trim-Away. It is particularly effective for oocytes since it acts at the protein level through antibody binding, ubiquitination, and degradation via the proteasome. Trim-Away may be more useful than other methods, such as RNAi, because oocytes are transcriptionally quiescent. However, Trim-Away has its own challenges when applied to feline oocytes specifically. Microinjection requires the removal of somatic cumulus cells attached to the oocyte to permit direct access to the cell membrane. In the cat, complete removal prevents meiotic resumption, making mechanistic studies of this stage impossible. The goal of this study was to determine if partial removal of cumulus cells to facilitate microinjection prevents meiotic maturation *in vitro*. High-quality cumulus-oocyte complexes (COCs) were obtained from domestic cat ovaries undergoing routine spay procedures. COCs were partially denuded by one gentle aspiration and release using a stripper pipette, resulting in COCs with two bare spots on the zona pellucida, one for a holding pipette and the other for the microinjection needle. After partial denudation, COCs were cultured in commercially-available human blastocyst culture media supplemented with 1IU/mL luteinizing hormone and 1IU/mL follicle-stimulating hormone for 26 hours, followed by fixation and DAPI DNA staining (n=22 cells). Maturation outcomes were assessed by chromatin configuration and polar body extrusion and compared to intact COC controls (n=23 cells) in 3 independent trials. There was no statistically significant difference in maturation rates between partially denuded and intact COCs ($p = 0.60$). Importantly, following microinjection of ~5pl of PBS, 77% of injected oocytes initiated meiotic resumption and 44% completed it (n=9). These findings demonstrate that partial, but not complete, cumulus cell removal is sufficient to support meiotic resumption and facilitate microinjection, establishing a platform for time-resolved protein depletion and functional interrogation of meiotic resumption pathways in the cat. This work advances the domestic cat as a tractable model for mechanistic studies of meiotic resumption and supports the application of targeted protein depletion in feline assisted reproduction and conservation biology.

Submission ID#2361365

A Biphasic Program of mcMTOC-nucleated Microtubule Dynamics Regulates Timely Spindle Migration in Mouse Oocytes

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Abstract Text

Aneuploidy arising during female meiosis I is the leading genetic cause of miscarriage and congenital disorders, yet the cellular mechanisms that safeguard accurate chromosome segregation in oocytes remain incompletely understood. Faithful division requires that the spindle assemble and remains centrally positioned until correct kinetochore–microtubule (MT) attachments are established, followed by timely spindle relocation toward the oocyte cortex to enable asymmetric division. While actin-based forces are known to drive spindle migration toward the cortex, how the timing of this migration is regulated—and how premature migration is prevented—remains a key gap in the field.

Here, we investigated the role of MTs nucleated by metaphase cytoplasmic microtubule organizing centers (mcMTOCs), a cytoplasmic MTOC pool that emerges after meiotic resumption to anchor the spindle to the oocyte cortex. We hypothesized that regulated changes in mcMTOC-nucleated MT dynamics constitute a timing "brake" that maintains the spindle at the oocyte center, thereby preventing premature spindle migration. To visualize mcMTOCs under physiological expression levels, we used Cep192-eGFP knock-in oocytes, in which the core MTOC component, Cep192, is endogenously tagged, together with EB3-based MT plus-end labeling to report polymerizing MTs. Quantitative live imaging analysis of MT nucleation rates and plus-end growth velocity (1-sec interval EB3 comet tracking) revealed coordinated changes in mcMTOC-nucleated MT dynamics across meiosis I: mcMTOC-nucleated MTs became progressively more prominent and dynamic as the spindle assembled and remained stably centered, followed by a marked reduction in mcMTOC-nucleated MT dynamics (1.5-fold decrease of MT nucleation rate and growth velocity, $p< 0.05$) prior to spindle migration. Fluorescence Recovery After Bleaching (FRAP) of mcMTOC-nucleated MTs further supported this dynamic switch (~1.3-1.5-fold increase of recovery at late prometaphase-I vs. early prometaphase-I or metaphase-I, $p< 0.001$).

To determine the functional importance of mcMTOC-nucleated MT dynamics, we implemented a localized optochemical MT stabilization strategy using a cell-permeable coumarin-caged epothilone (CouEPO). Targeted 405-nm uncaging at mcMTOCs generated short, densely EB3-decorated MTs with suppressed plus-end velocities (1-fold decrease, $p< 0.05$) and slowed FRAP recovery (1.3-fold decrease, $p< 0.05$), resulting in severing mcMTOC–spindle–cortex connectivity, compared to adjacent non-illuminated mcMTOCs. Importantly, there was no significant effect on spindle MT dynamics. In contrast to controls (non-illuminated mcMTOCs in CouEPO-treated oocytes and light-exposed mcMTOCs in non–CouEPO-treated oocytes), global uncaging at all mcMTOCs at prometaphase-I triggered premature (~50 min earlier than controls), faster spindle migration (0.20 mm/min vs. 0.10 and 0.11 in controls) and erratic spindle movements (1.4-fold increase in spindle orientation angle, $p< 0.01$).

Mechanistically, multiphoton laser ablation of MT connections (mcMTOC–cortex or mcMTOC–spindle linkages) demonstrated that mcMTOCs are under MT-dependent tension from both the cortex and the spindle. Moreover, we found that dynein is localized to the cortex and mcMTOC-nucleated MTs, and its inhibition by dynarrestin or expressing p150-CC1 dominant-negative mutant reduced mcMTOC-nucleated MT dynamics, disrupted spindle connections, and phenocopied premature/erratic migration. Finally, we found that Eg5 antagonized dynein to maintain mcMTOCs in the cytoplasm and reduce the aberrant increase of mcMTOC-nucleated MT dynamics.

Together, these results reveal a new model in which mcMTOC-nucleated MT dynamics act as a regulated timing mechanism that maintains central spindle positioning and gates the onset of spindle migration to the cortex, thereby promoting faithful chromosome segregation and robust asymmetric division in mouse oocytes.

Submission ID#2361117

IRX3 Impacts Oocyte size, Zona Pellucida Thickness, and Ability to Progress to Blastocyst Stage

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Abstract Text

The foundation of the ovarian reserve is established during fetal development in mammals and across animal phyla. Previously, we discovered that expression of two Iroquois family homeobox transcription factors, IRX3 and IRX5, are initially detected in the ovarian pre-granulosa cells before expanding into oocytes at the onset of germline cyst breakdown. IRX3 and IRX5 are traditionally localized to the nucleus and studied for their roles as transcription factors; however, we have established that oocyte expression of IRX3 is concentrated in the cytoplasm. Our previous studies using *Irx3*^{LacZ}/*Irx3*^{LacZ} *(Irx3* KO) and *Irx5*^{EGFP}/*Irx5*^{EGFP} (*Irx5* KO) genetically modified mouse models indicate both *Irx3* and *Irx5* contribute to fertility; however, their roles in oocyte quality and post-fertilization fidelity have yet to be determined. Therefore, we hypothesize that IRX3 is critical for oocyte quality and contributes to early embryonic development. To test this hypothesis, we subjected *Irx3* KO (n=9) and wild type littermates (WT) (n=6) to a superovulation hormone paradigm to collect released oocytes. Results show there were no differences in the number of oocytes ovulated between *Irx3* KO and WT mice, indicating that *Irx3* is not required for ovulatory competence. Next, *Irx3* KO (n=93) and WT (n=100) oocytes were imaged by brightfield microscopy and morphology was quantified using ImageJ with CellPose-SAM segmentation. Total oocyte-zona pellucida complex size did not differ between genotypes. Notably, while oocyte size was significantly increased ($p=0.0012$), the zona pellucida was thinner in *Irx3* KO compared to WT oocytes ($p< 0.0001$). These findings suggest that *Irx3* loss alters structural features of the ovulated oocyte despite normal ovulation. To determine whether these morphological changes affect developmental competence, oocytes were fertilized *in vitro* with WT sperm and cultured to 2-cell and blastocyst stages. The percentage of oocytes that progressed to 2-cell stage was not different between the groups, indicating that fertilization and initial cleavage occur normally in the absence of *Irx3* from the oocyte. A subset of *Irx3* KO (n=86) and WT (n=16) 2-cell embryos were cultured further to blastocyst stage. Results show a trend that fewer *Irx3* KO derived oocytes progress to the blastocyst stage compared to WT ($p=0.052$), suggesting compromised later developmental potential. Altogether, our results demonstrate that *Irx3* is dispensable for ovulation and fertilization but it contributes to oocyte structural integrity and optimal developmental competence. Future studies will be focused on additional genetic mouse models including *Irx5 KO* and an oocyte specific conditional knockout of *Irx3* (*Stra8*-cre;*Irx3*^{flox}/*Irx3*^{flox}) to further our understanding of *Irx5* versus *Irx3* contributions to fertility and oocyte quality.

Submission ID#2346387

A Human *CEP120* Gene Variant Impairs Oocyte Meiotic Spindle Building Causing Aneuploidy

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Abstract Text

Infertility is increasing, leading more women to seek assisted reproduction treatments than ever before. One of the main causes of infertility and pregnancy loss is aneuploidy, an incorrect number of chromosomes in the embryo or developing fetus, which hinders normal development. Aneuploidy overwhelmingly arises from the missegregation of chromosomes during the maternal meiotic divisions that produce haploid oocytes. Variants of genes involved in spindle building are of primary interest when searching for genetic causes of aneuploidy because the oocyte meiotic spindle is responsible for faithful chromosome segregation. We previously identified a genetic variant in a human centrosome gene, *CEP120*, as associated with high embryonic aneuploidy. To evaluate the functional significance of this genetic variant, we generated a knock-in mouse model and found that female mice had reduced fertility and increased egg aneuploidy. By assessing microtubule regrowth after cold temperature exposure and warming after vitrification, we found that oocytes from mice harboring the genetic variant had reduced microtubule nucleation. Because mouse and human oocytes present differences in spindle building, we used a humanized mouse oocyte spindle building assembly system to better mimic human oocyte meiosis and found that aneuploidy levels significantly increase in *CEP120* variant oocytes. Although PGT-A allows the deselection of aneuploid embryos, the development of biomarkers for predisposition to aneuploidy could be used to identify subfertile patients and model their aneuploid risk. Therefore, our data indicate that patients harboring common genetic variants in CEP120 may require additional counseling when considering egg cryopreservation procedures. This work was supported by a grant from the National Institutes of Health (NIH/NICHD R01-HD091331) to KS.

Mechanisms of Sperm-Egg Interaction and Fertilization

Monday, July 20 1:30 PM – 3:00 PM

Submission ID#2360914

Flow Cytometric Sorting Alters Subpopulations of Boar Sperm Motility and Increases Polyspermy During In Vitro Fertilization

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Abstract Text

Flow cytometric sorting enables selection of sperm subpopulations based on defined physiological or molecular traits; however, its impact on sperm function and embryo development in pigs remains unclear. The objective of this study was to evaluate the effects of fluorescence-activated cell sorting (FACS) on boar sperm motility, fertilization outcomes, and developmental competence following in vitro fertilization (IVF). In Experiment 1, pooled semen from three boars was divided into either sorted or unsorted (control) treatments. Sperm motility and kinematic parameters were evaluated using computer-aided sperm analysis (CASA) at both the population level and the single-cell level both before and after sorting. Oocytes were fertilized with either sorted or unsorted sperm, and cleavage (≥2-cell) and blastocyst development were assessed. Cleavage and blastocyst rates were reduced in the sorted group (48.8 ± 9.9% and 8.5 ± 4.2%) compared with controls (92.2 ± 1.5% and 28.4 ± 2.8%; $p < 0.001$). Population-level CASA detected statistically significant but biologically modest changes in motility following sorting. In contrast, single-cell CASA combined with k-means clustering identified four distinct motility subpopulations and demonstrated that FACS redistributed existing motility states, characterized by depletion of fast, progressive sperm and enrichment of less energetically demanding subpopulations. In Experiment 2, FACS was performed using four defined sorting positions (A-D) that vary in the voltage (V) placed on the cell upon sort (A = -47V, B = -18V, C = +16V, D = +45V) to evaluate potential differences in embryo development. Motility and kinematic analyses revealed modest population-level changes consistent with Experiment 1. Oocytes were allocated to unfertilized controls (TPEN-treated activation control & unfertilized control), fertilized unsorted controls (control & mock-sort), or sperm collected from FACS positions A-D. Cleavage and blastocyst development were evaluated at 48 and 144 h post-fertilization (hpf), respectively. Cleavage rates were higher in unsorted sperm treatments (control = 63.0 ± 5.6%, mock-sort = 61.2 ± 6.8%) than in sorted sperm treatments (A = 25.3 ± 2.4%, B = 31.0 ± 7.4%, C = 27.3 ± 1.0%, D = 27.5 ± 3.4%; $p < 0.001$). Similarly, blastocyst development was greater in unsorted controls (control = 23.5 ± 6.8%, mock-sort = 19.7 ± 3.3%) compared with sorted groups (A = 4.0 ± 1.9%, B = 13.8 ± 4.9%, C = 4.8 ± 2.4%, D = 6.6 ± 3.3%; $p < 0.01$), with position B exhibiting intermediate outcomes. Fertilization status and polyspermy incidence were assessed at 12 hpf via pronuclear staining (Hoechst 33342). Zona pellucida remodeling was quantified using *Lens culinaris* agglutinin (LCA) fluorescence intensity. Polyspermy incidence was higher in all sorted treatments (36.6-50.4%) compared with unsorted controls (≤5%; $p < 0.001$). LCA intensity in sorted sperm treatments more closely resembled unfertilized controls, indicating impaired zona pellucida hardening. Notably, FACS position B consistently aligned more closely with unsorted controls across multiple developmental and functional metrics. Phospholipase C zeta (PLCζ) immunocytochemistry localization was further evaluated using image-based flow cytometry. Two distinct PLCζ populations were identified, with unsorted controls having a population absent in sorted sperm ($p < 0.001$). These findings suggest that FACS alters sperm function in a subpopulation of cells, potentially impairing oocyte activation through disrupted PLCζ dynamics, contributing to increased polyspermy, reduced oocyte activation/cleavage, and reduced blastocyst development.

This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2022-67015-36298 from the US Department of Agriculture's National Institute of Food and Agriculture (K.K.).

Submission ID#2359074

Mitoquinol Alleviates Redox Imbalance and Modulates Early Transcriptional Programs after Assisted Oocyte Activation in Mice

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Abstract Text

Ionomycin, a calcium ionophore, is increasingly used as an assisted oocyte activation (AOA) agent to treat oocyte activation deficiency (OAD), however its efficacy and safety require further validation. Here, we evaluated ionomycin-based AOA with mitoquinol (MitoQ), a mitochondria-targeted antioxidant, in an ICSI-based OAD mouse model and characterized the associated transcriptomic changes. Individual normal (control) or 0.1M Na2CO3-inactivated sperm were injected into CF-1 oocytes by piezo-ICSI, followed by treatment with 10 μM ionomycin with or without 25 nM MitoQ (MitoQ+/MitoQ-) for 15 minutes. Without AOA treatment, Na2CO3-inactivated sperm resulted in no cleavage after ICSI, mimicking OAD in human IVF. Presumptive zygotes were cultured for 120 h at 37 °C under 6% CO_2 and 5% O_2. Embryo development was assessed by cleavage, day-4 blastocyst, and day-5 hatching, as well as blastocyst ICM and TE cell numbers. ROS levels were quantified and analyzed by H2DCFDA staining and JC-1 staining was used to compare the mitochondrial membrane potential (MMP) within AOA treatments. Early two-cell embryos (20-22 hpi) were collected for ultra-low-input RNA-seq, and a EU-based labeling assay was used to quantify nascent synthesized RNA at late two cell stage. All experiments were repeated at least three times. Developmental data were analyzed by Fisher's exact test, and fluorescence-based measurements (ROS, JC-1, EU) and cell-count data were analyzed by Student's t test or by one-way ANOVA followed by Tukey's test or Fisher's LSD. Compared with the control (n=119), MitoQ- group (n=79) showed markedly reduced day-4 blastocyst rate (40.0% vs 61.44%) and day-5 hatching rate (26.99% vs 57.76%), whereas a clear rescue effect was observed in MitoQ+ group (n=80) with improved day-4 blastocyst rate (62.94% vs 40.0%) and day-5 hatching rate (48.95% vs 26.99%). Consistent with these findings, cell allocation analysis showed that both AOA groups have reduced ICM（10.37 and 8.65） and TE cell numbers (83.15 and 88.4) compared with the control (13.35 for ICM, and 106.82 for TE), whereas a restored ICM-to-TE ratio was observed in MitoQ+ group (0.1235 in control, 0.0953 in MitoQ- and 0.1245 in MitoQ+). In addition, MitoQ+ group had significantly reduced ROS levels and increased MMP compared to the MitoQ- group, suggesting its beneficial effect in restoring redox balance of ionomycin treated oocytes. Transcriptomic analysis revealed that the control embryos preserve a transcriptional landscape more consistent with physiological zygotic genome activation (ZGA), characterized by strongly activated biosynthesis and developmental programs together with a coordinated protein-homeostasis, stress-response, and quality control axis. Meanwhile, MitoQ- embryos exhibit an over-stressed and misdirected ZGA program, with transcriptional signatures of exaggerated mitotic and cell-cycle activity, pronounced MAPK- and mitochondrial stress responses, and broad ectopic activation of axonogenesis/synaptic as well as Ca^{2+}/IP_3-driven morphogenetic pathways. In contrast, MitoQ+ embryos show attenuated stress signaling, largely avoid ectopic axonogenesis/synaptic and Ca^{2+}/IP_3-driven morphogenetic programs, and partially preserve the biosynthesis-oriented, ribosome/translation module characteristic of physiological ZGAEU labeling revealed higher nuclear EU incorporation in $MitoQ^-$ embryos than in controls, while $MitoQ^+$ embryos showed a lower signal than $MitoQ^-$ but slightly higher than ICSI; none of these differences were statistically significant.. Collectively, these findings reveal potential risks associated with ionomycin-based AOA, raising concerns about its clinical safety. Moreover, MitoQ supplementation partially restores developmental competence during ionomycin-based AOA, alleviates ionomycin-induced redox and mitochondrial stress, and rebalances the transcriptome toward a more physiological state, highlighting the potential for a more efficient and safer AOA strategy.

Submission ID#2412487

Calcium at the Intersection of Metabolism and Embryo Development

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Abstract Text

Calcium (Ca2+) signals initiate embryo development at fertilization and are frequently disrupted in human assisted reproduction, which is associated with metabolic abnormalities in children. In mice, excess or inadequate Ca2+ signals at fertilization impair embryo development and alter adult metabolism, but the mechanisms are unknown. We hypothesize that the impact of cellular Ca2+ signals on energy and metabolism may explain this connection. I will describe our use of orthogonal approaches to show that excess Ca2+ signaling in one-cell mouse embryos alters the production of epimetabolites required for nuclear reprogramming and zygotic genome activation. Imbalance in these metabolites, particularly a reduction in lactyl coenzyme A, alters how the embryo is epigenetically reprogrammed and impairs embryo development. Our results indicate that Ca2+ dynamics drive metabolic regulation of epigenetic reprogramming at fertilization, connecting Ca2+ signaling at the initiation of development to long-term metabolic function in adults.

Submission ID#2321497

Functional and Clinical Implications of Extrachromosomal Circular DNA in the Human Germline

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Abstract Text

Extrachromosomal circular DNA (eccDNA) originates from linear chromosomal DNA and has been widely detected across diverse species and cell types. Recent studies have also revealed the presence of eccDNAs in human sperms. However, it remains unknown how these eccDNAs are generated, maintained and transcriptionally regulated during spermatogenesis, whether they can be transmitted across generations. Moreover, as eccDNAs are often considered by-products of DNA damage, it is unclear whether physiological and pathological conditions during spermatogenesis lead to differences in their abundance.

We developed a sequencing strategy to simultaneously profile eccDNAs and their corresponding transcriptomes in human sperms to explore their transcriptional activity and disease relevance. In parallel, we employed mouse models to investigate the mechanisms of eccDNA generation in sperm, their paternal transmission to embryos, and their effects on offspring genomes.

We found the transcriptional activity of germline eccDNAs. Patients with chronic diseases such as hypertension and diabetes showed significantly higher sperm eccDNA levels than healthy controls. Using doxorubicin-induced apoptosis mouse models, we confirmed apoptosis contributes to eccDNA formation during spermatogenesis. Using eccDNA biosensor mouse model, we further demonstrated that eccDNAs containing complete expression elements can be transcriptionally active in germ cells, transmitted into offsprings, and integrated into sperm genomes, potentially altering the offspring genome.

These findings reveal that apoptosis-driven eccDNA formation is inherent to spermatogenesis, with transcriptionally active and heritable eccDNAs that may impact offspring genomes and serve as biomarkers of male reproductive health.

Submission ID#2361134

Role of WNT6 in the Pathogenesis of Oligoasthenoteratozoospermia (OAT)

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Abstract Text

Sertoli cells provide attachment and nourish the differentiating male germ cells. Negative elongation factor (NELF) regulates gene transcription by pausing RNA Polymerase II at the promoter. We generated Sertoli cell-specific knockout (cKO) of NELF-B (loss of subunit B leads to degradation of the NELF complex) mice to test the hypothesis that NELF in Sertoli cells regulates the expression of genes critical for Sertoli cell function and germ cell differentiation. We found that adult cKO mice (n=8) were infertile with phenotype closely resembling that observed in OAT in men. At postnatal day 90 (PND90, n=5), cKO sperm were decreased ($p < .001$, t-test) in concentration and motility, and showed morphological deformities, including misshaped head, bent tail, and loss of the annulus ring. Evaluation of PND21(n=3) and PND90(n=3) testicular cross sections indicated that, although quantitatively diminished with a decrease in pachytene spermatocytes ($p < .01$, t-test) and round spermatids ($p < .05$, t-test), germ cells completed meiosis. We observed that defective spermiogenesis and disorganization began at stage VIII by immunohistochemistry with SP-10 antibody (n=3). The apical ectoplasmic specializations (aES) formed by the Sertoli cells nestle the spermatids and aid in their differentiation. To test if cKO Sertoli cells are unable to form proper aES, we examined aES gene expression and found that *Jam3* ($p < .01$, t-test) and *Inga6* ($p=0.066$, t-test) were expressed at low levels in PND90 cKO (n=3). Immunofluorescence showed defects in the formation of the actin filaments and microtubule structures supporting the aES. 15P-1 Sertoli cell culture model showed that siRNA knockdown of NELF-B leads to disorganization of the cytoskeleton, which was quantified by mathematical laboratory (MATLAB) algorithm and Fourier transform analysis. Control cells exhibited directionally aligned actin filaments (CV:0.03) and tubulin microtubules (CV:0.16), whereas knockdown cells showed increased disorganization and reduced alignment of actin filaments (CV:0.19) and tubulin microtubules (CV:0.23). To understand the mechanistic basis for the above, we performed RNA-seq of primary Sertoli cells from PND12 mice (n=3) and found that *Wnt6* was upregulated (3.7 fold) in cKO. WNT/β-catenin pathway has been shown to be important for spermatogenesis. To test whether WNT6 acts via the canonical pathway, we performed immunohistochemistry using phospho-β-catenin antibody. Testis from PND90 WT mice showed nuclear β-catenin in germ cells but no staining beyond step 9 spermatids. In contrast, cKO testis showed comparatively more intense staining in germ cell nuclei. More importantly, steps 10-12 spermatids showed persistent β-catenin in their nuclei, suggesting this could lead to changes in gene expression (n=3). The manchette is a specialized structure that transiently appears during steps 8-12 of spermiogenesis. Its function is to shape the sperm head and transport proteins to the axoneme via intra manchette transport (IMT). We found that manchette formation and IMT genes, including *Camsap1*, *Hook1*, *Pacrg*, *Ift122*, and *Ift14,* were downregulated ($p < .05$, t-test) in PND90 cKO (n=3). Thus, the data show that upregulated WNT6 acts in an autocrine manner on Sertoli cells, leading to the disruption of cytoskeleton and malformation of aES. WNT6 acts in a paracrine manner on the germ cells and is critical for manchette formation. Loss of aES and manchette together contributed to the spermiogenesis defects leading to OAT-like phenotype. Our work found a potential role for the WTN6 signaling pathway in OAT pathogenesis. Future studies will explore whether suppression of WNT6/β-catenin signaling will rescue spermiogenesis and infertility phenotype of cKO mice.

Trophoblast Origins in Focus – Stem Cell Foundations to Placental Development

Monday, July 20 1:30 PM – 3:00 PM

Submission ID#2360894

Developmentally Regulated Expression and Localization of TEAD2 and TEAD3 in Human Trophoblast

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Abstract Text

The placenta is a transient yet essential organ that governs fetal development and pregnancy success. Proper placental development requires tightly regulated trophoblast lineage specification and differentiation. The Hippo signaling pathway regulates cell fate decisions through TEAD transcription factors. While TEAD4 has been defined as a stem-state regulator, the roles of TEAD2 and TEAD3 in trophoblast development remain poorly understood. We hypothesize that TEAD2 and TEAD3 function as lineage-associated regulators of trophoblast differentiation, exhibiting distinct expression patterns, subcellular localization, and regulatory dynamics across human trophoblast stem cells (hTSCs), syncytiotrophoblast (ST), and extravillous trophoblast (EVT) states. hTSCs were maintained and differentiated in vitro into ST and EVT lineages. TEAD expression was assessed using RT-PCR and Western blotting. TEAD2 and TEAD3 protein localization was examined via immunofluorescence (IF) across trophoblast states and further evaluated in first-trimester and term placental tissues. Functional interrogation of TEAD2 and TEAD3 is ongoing using shRNA-mediated knockdown with validation by RT-PCR. Differentiation of hTSCs into EVT and ST was confirmed by lineage-specific marker expression, with HLA-G enriched in EVT and CGB markedly increased in ST. TEAD4 expression was highest in hTSCs and reduced upon differentiation, consistent with its established stem-associated role. TEAD2 mRNA increased in EVT relative to hTSCs and was reduced in ST, whereas TEAD3 expression was elevated in differentiated states compared to hTSCs. At the protein level, TEAD3 was present in hTSCs and maintained across EVT and ST states, while TEAD2 protein was most abundant in hTSCs and appeared reduced following differentiation. IF analyses demonstrated predominant nuclear localization of both TEAD2 and TEAD3 across trophoblast states. TEAD2 exhibited strongest signal in hTSCs with reduced intensity in differentiated EVT and ST conditions. TEAD3 displayed nuclear localization across hTSCs, EVT, and ST, with maintained expression in differentiated populations, including invasive EVT cells and multinucleated ST clusters. In first-trimester placental tissue, TEAD2 and TEAD3 exhibited robust nuclear localization within KRT7-positive trophoblast compartments. TEAD2 signal appeared developmentally dynamic, with reduced intensity observed in term placenta, whereas TEAD3 remained detectable at term with comparatively reduced signal intensity. Analysis of first-trimester transcriptional datasets further supported differential TEAD expression patterns, with TEAD2 enriched in EVT-associated populations and TEAD3 broadly expressed across trophoblast states with relative enrichment in fusion-associated cells. Ongoing functional studies will define how TEAD2 and TEAD3 contribute to lineage-associated regulatory programs. Collectively, these findings establish distinct and developmentally modulated expression patterns for TEAD2 and TEAD3 within the Hippo signaling framework governing human placentation.

Submission ID#2354541

Unraveling the Developmental Trajectories of Syncytiotrophoblast Subtypes in a hESC-Derived Trophoblast Model

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Abstract Text

Single-nucleus RNA sequencing (snRNAseq) has proven to be an invaluable tool for studying multinucleated cell types. By using this technology on human embryonic stem cell (hESC)-derived trophoblasts (TB) in a BMP4-based differentiation (BAP) model, we previously identified two distinct syncytiotrophoblast (STB) nuclear populations, designated as cluster 5 and 6, from BAP Day 8 TB. We hypothesize that two developmental trajectories may contribute to STB formation, with cells potentially transitioning through different intermediate stages before developing into one of the two STB subtypes. Single nuclear RNAseq (snRNAseq) was performed across 11 time points, including the undifferentiated hESC state, hESC cultured in conditioned medium for 24 hours (BAPd0), and BAP days 0.5, 2, 3, 4, 5, 6, 7, 8, and 11. Notably, clusters organized into two distinct groups at every time point, including in undifferentiated hESCs. Trajectory analysis predicts that each group differentiates into a distinct STB subtype. Moreover, differentially expressed genes (DEGs) from Group-1 and Group-2 overlap with previously identified Cluster 5 and Cluster 6, respectively, suggesting that the two STB subtypes originate from these two groups of hESCs. To test this hypothesis, we sought to identify a marker that could be used to separate Group-1 and Group-2 cells in order to follow the developmental trajectory of each group. We identified 84 and 41 genes that are upregulated in Group-1 and Group-2, respectively, and that encode translation products localized on the cell surface. Among them, we choose CD24, whose transcript is upregulated in Group-1 and has been previously shown to discriminate between primed and naïve ESCs in both humans and mice. By using a fluorescent activated cell sorting (FACS) approach, hESC were sorted into CD24_high and CD24_low populations. Downstream experiments determined greater capacity for substrate attachment and more efficient STB differentiation following BAP treatment in CD24_high cells than in CD24_low cells. However, real-time PCR comparison of additional transcripts suggests that separating cells by CD24 protein level did not efficiently sort them into Group 2 and 1 nuclei. Nonetheless, our findings provide i. validation for our previously identified STB clusters< ![if !supportAnnotations] >[LS1]< ![endif] >, ii. further insights into the developmental trajectories of STB subtypes and iii. Demonstrate the differential capacity of CD24_high and CD24_low hES cells to differentiate into TB.

Submission ID# 2350045

Investigating the Role of Maternal Derived-Microbial Metabolites on Trophoblast Function

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Abstract Text

Alterations in the human maternal gut microbiome are increasingly recognized as contributors to pregnancy complications, which underlie millions of maternal and neonatal deaths each year. These complications often stem from impaired placental development, a process critically dependent on the proper differentiation of cytotrophoblast stem cells (CTBs) into syncytiotrophoblast (STB) and extravillous trophoblast lineages. However, the molecular mechanisms by which microbial metabolites influence trophoblast differentiation and placental function remain poorly understood, with few studies directly examining these effects. Therefore, we investigated the impact of short-chain fatty acids (SCFAs) and tryptophan-derived indoles, key gut-derived metabolites, on human primary trophoblast energy metabolism and CTB differentiation into STBs. We hypothesized that maternal gut-derived metabolites mediate microbial-host interactions during pregnancy by regulating trophoblast function and development.

We used physiologically relevant doses of SCFAs (acetate, propionate, and butyrate) and indole derivatives (indole-3-lactic acid [ILA] and indoxyl sulphate [IS]) on human primary trophoblast energy metabolism and differentiation. Microbial metabolite levels were quantified across gestation in maternal serum using LC-MS/MS (n=20). We isolated human primary trophoblast cells from term placentas (n=11) and treated them with physiological concentrations of SCFAs, ILA, and IS. Trophoblast differentiation was evaluated by measuring human chorionic gonadotropin (β-hCG) release and energy metabolism was assessed using a Seahorse XF Extracellular Flux Analyzer.

β-hCG release did not differ significantly between control and SCFA groups after 3 days in culture (acetate, $p=0.0812$, $n=8$; propionate, $p=0.1383$, $n=13$; butyrate, $p=0.508$, $n=13$). No significant differences in β-hCG release were observed between control and ILA or IS treated cells on days 3 and 4 ($n=4$; ILA day 3, $p=0.1025$; day 4, $p=0.0515$; IS day 3, $p=0.7720$; day 4, $p=0.8521$). Basal oxygen consumption rate (OCR) levels differed significantly across ILA treatment conditions ($n=11$, $p=0.0387$). However, post hoc analysis revealed no significant differences between the control and individual treatment groups. All other respiratory parameters measured for ILA, and for the IS group remained non-significant. However, ILA and IS were present in maternal and cord serum, with ILA significantly higher in cord than maternal serum across all gestations ($n=20$, $p \leq 0.0012$).

Preliminary data demonstrate that primary human trophoblast differentiation and function, assessed via β-hCG release and mitochondrial respiration, were not affected by treatment with physiologically relevant doses of SCFAs and indole derivatives. However, considerable variation was observed among placental donors, suggesting that microbial metabolites have heterogeneous effects on trophoblast function. This research contributes to understanding the complex interactions between microbial metabolites and placental physiology.

Submission ID#2357235

Trophoblast stem cells have an attenuated LPS response but mount a potent interferon-mediated antiviral defense

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Abstract Text

The peri-implantation period of pregnancy requires a finely tuned pro-inflammatory response. Dysregulated inflammation can cause placental dysfunction, leading to early pregnancy loss. In this study, we aimed to elucidate the mechanisms underlying immune imbalance during implantation using a trophoblast stem cell (TSC) model. TSCs represent the cytotrophoblast cells of the peri-implantation placenta and can differentiate into syncytiotrophoblasts (STB), the cells responsible for maternal-embryo communication. TSCs were cultured in either stem cell or STB differentiation medium. Human dermal fibroblasts (HDF) were used as a positive control for inflammation. Cells were treated with 250 ng/mL lipopolysaccharides (LPS) or 250 U/mL interferon beta (IFNB) for six hours prior to RNA isolation for RT-qPCR. Additional samples were collected for Western blotting at 0, 15, 30, 45 minutes and 24 hours post-treatment. We first used qPCR to assess mRNA levels of cells and found that LPS did not stimulate expression of the pro-inflammatory cytokines *ck* or *TNFA* in TSCs or STBs. In contrast, IFNB significantly increased levels of the interferon-stimulated genes, *ISG20*, *IFITM1*, *IFITM2*, AND *IFITM3* ($p < 0.0001$) in TSCs and STBs. We next assessed protein levels of phosphorylated ERK (pERK). Compared to HDFs, TSCs had a delayed, modest increase in pERK while STBs showed no response. Alternatively, IFNB treatment for 24 hours increased total levels of STAT1 in all cell types. Finally, we used immunofluorescence to visualize protein localization of NFkB and IRF3 at 0 and 90 minutes post-LPS treatment. At 90 minutes, HDFs exhibited nuclear translocation of NFkB and IRF3 while the STBs and TSCs did not. These findings suggest the peri-implantation stage placenta exhibits limited responsiveness to pathogen-associated inflammatory signals such as LPS but remain highly sensitive to interferons. This implies that the primitive placenta is not directly responsive to microbial stimuli, but rather to maternal immune activation, highlighting the importance of the maternal immune balance in early pregnancy success.

Submission ID#2370803

Development of the human villous placenta- in vitro insights into the peri-implantation stage

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Abstract Text

Developmental Processes and Epigenetic Control

Monday, July 20 1:30 PM – 3:00 PM

Submission ID#2355753

Toward Spatiotemporal Models of Mammalian Gastrulation

Author(s)

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Abstract Text

My research group aims to address the fundamental challenge of understanding how multicellular organisms achieve variation despite cells having identical genetic information. The development of a single fertilized egg into a complete mammalian embryo is an especially beautiful embodiment of this problem. During this process, cellular differentiation is defined by the capacity of the cell ensemble to acquire increasingly specialized internal states. It follows a coordinated and canonical trajectory in which, given sufficient sampling, embryos and cell states can be systematically measured and aligned across mammalian species. This perspective allows embryonic time to be treated as a quantitative metric, providing a powerful framework for modeling developmental dynamics. In recent years, we have established phenomenological quantitative models of the spatiotemporal process of mammalian gastrulation. Building on these models, we work to dissect epigenetic machineries regulating intracellular diversification, as well as signaling mechanisms that shape cell fate decisions and patterning in a non-cell-autonomous manner. In my talk, I will present recent progress by my group in these directions.

Submission ID#2360670

Ribosomal and Mitochondrial Dysfunction Underlies Lineage-Specific Loss of Trisomy 1/19 Cells in Embryonic Day 10 Human Embryos

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Abstract Text

Trisomy 1 and trisomy 19 are among the most lethal human autosomal aneuploidies and are strongly associated with implantation failure, yet the underlying cellular mechanisms remain poorly defined. A deeper understanding of how these aneuploidies perturb early human embryogenesis has important clinical implications for human implantation, early pregnancy loss, preimplantation genetic testing, and embryo selection. Here, we investigated lineage-specific tolerance to trisomy 1/19 in human embryos cultured in vitro to day 10 by integrating extended embryo culture (EEC), single-cell RNA sequencing (scRNA-seq), and immunofluorescence staining. Euploid and trisomy 1/19 blastocysts donated for research were cultured on ibid plates under EEC conditions until embryonic day 10, then profiled by scRNA-seq. Clustering and lineage marker expression were used to identify epiblast (EPI), primitive endoderm (PE), and trophoblast (TE) populations. In parallel, embryo outgrowth area, hCG secretion in spent medium, and immunostaining for POU5F1 (EPI), SOX17 (PE), and CGB (TE) were performed. Strikingly, while scRNA-seq revealed abundant EPI- and PE-like cells in trisomy 1/19 embryos, SOX17 immunostaining failed to detect any PE cells in these embryos, in contrast to clear PE staining in euploid controls. This discrepancy suggests that aneuploid cells can transiently adopt PE-like transcriptional identities but are not stably maintained as PE at the protein level. Differential expression and pathway analyses showed that trisomy 1/19 embryos exhibit prominent downregulation of ribosomal protein genes, including RPL41 and multiple small and large ribosomal subunits, together with reduced expression of mitochondrial rRNAs and oxidative phosphorylation genes. In parallel, RNA-binding, cell-cycle, and stress-associated genes were upregulated, consistent with a proliferative yet highly stressed cellular state. These transcriptional signatures are characteristic of aneuploidy-induced proteotoxic and energetic stress, implicating impaired translation and mitochondrial dysfunction as core vulnerabilities of trisomy 1/19 embryos. Given the high secretory and metabolic demands of PE, we propose that PE-fated trisomy 1/19 cells are particularly sensitive to this combined ribosomal and mitochondrial dysfunction. Consequently, they likely undergo apoptosis, fail to fully mature, or remain below the protein detection threshold of immunostaining, even though their transient PE-like transcriptional signatures are captured by scRNA-seq. Similar translational and mitochondrial defects were observed in the TE lineage of trisomy 1/19 embryos, correlating with markedly reduced outgrowth areas, decreased CGB protein levels by immunostaining, and diminished hCG production in spent medium. In contrast, euploid embryos maintain robust ribosomal and mitochondrial programs, show stable PE formation, and support proper TE differentiation by day 10. Together, these findings support a model of lineage-specific cell competition and metabolic vulnerability in which trisomy 1/19 cells are selectively eliminated from PE and functionally compromised in TE, despite transient passage through EPI/PE-like transcriptional states. This work provides a mechanistic framework for why trisomy 1/19 embryos rarely progress beyond early development and highlights ribosomal and mitochondrial dysfunction as key drivers of early pregnancy loss in these aneuploidies. More broadly, it underscores the value of integrating single-cell transcriptomics with protein-level and functional readouts to understand how specific chromosomal abnormalities reshape lineage differentiation and to refine clinical strategies for embryo assessment and selection.

Submission ID#2361104

Uncovering Requirements for Primordial Germ Cell Migration

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Abstract Text

In most sexually reproducing animals, germline development begins with the migration of Primordial Germ Cells (PGCs) to the gonad. This migration is essential for proper development of the germ cells, and in humans, failure to reach the gonads can lead to germ cell tumors. Despite the importance to health and fertility, many gaps persist in our understanding of the journey because PGC migration occurs early in embryogenesis, involves many tissues, and spans days to weeks in mammals. To overcome these barriers, our lab leverages genetic tools and live imaging capabilities of *Drosophila melanogaster*. Importantly, the migratory path PGCs take and the intracellular signaling cascades required are shared between flies and vertebrate model organisms. For example, we recently found that fly PGCs, like zebrafish and chicken PGCs, exhibit asynchronous, periodic calcium transients as they migrate to the gonad suggesting a conserved role for calcium signaling in PGC migration. To understand better how these calcium transients arise and whether they are required for PGC migration, we conducted a targeted genetic screen for calcium signaling factors. Among the factors we identified was the G-protein coupled receptor linked Phospholipase C at 21C (Plc21c). Using tissue-specific knockdown experiments, we found that Plc21c is autonomously required for PGC migration. To determine what activates calcium signaling in migratory PGCs, we performed a loss-of-function screen for G-protein coupled receptors that are expressed in migratory PGCs and have been linked to Plc21c in other biological contexts. This screen revealed a requirement for the opsin, Rhodopsin 4. Although classically associated with phototransduction, opsins are increasingly recognized for non-visual roles in reproduction. To understand the role of Rhodopsin 4 in PGC migration, we performed genetic epistasis experiments with known axes required for PGC migration and found Rhodopsin 4 genetically interacts with retinoid-like juvenile hormones. We are now testing whether juvenile hormones can modulate Rhodopsin 4 activity using the cell-based PRESTO-TANGO assay. Together, our investigations thus far have revealed the requirements of an opsin in PGC migration and calcium transients. We are excited to explore whether these new requirements are conserved in vertebrates. Indeed, like flies, one opsin is specifically expressed in mouse migratory PGCs and we previously found retinoids can induce mouse PGC migration *in vitro*. Together this comparative biology approach will close the gaps in our understanding of this crucial stage in germline development.

Submission ID#2347548

A Hexa-Species Single-Cell Transcriptomic Atlas of Symmetry Breaking

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Abstract Text

The establishment of the mammalian body plan is initiated during symmetry breaking, which specifies the anterior-posterior axis. Most current knowledge of this event derives from studies in the mouse, where symmetry breaking occurs in a three-dimensional egg cylinder. However, this developmental architecture differs substantially from the flat embryonic disc where symmetry breaking occurs in most mammals, including primates and ungulates.

Here, we aimed to construct a hexa-species transcriptomic atlas of symmetry breaking by integrating our own single-cell RNA sequencing dataset of *in vivo* sheep embryos at pre-primitive streak (PS) and early PS stages, with stage-matched, publicly available datasets from mouse, cow, pig, rabbit, and marmoset *in vivo* embryos.

Using the Evercode™ WT Mini kit (Parse Biosciences), we obtained scRNA-seq profiles from embryos collected *in vivo* from superovulated ewes at embryonic days (E) 11 (pre-PS; n = 15), and E11.5 (early-PS; n = 25). Following quality control, transcriptomes from 7,934 cells were retained. Unsupervised clustering (UMAP) and established lineage markers enabled the identification of epiblast (EPI), trophectoderm (TE), parietal hypoblast (PH), and visceral hypoblast (VH) populations in pre-PS embryos. At the early-PS stage, additional anterior and posterior VH populations (AVH and PVH), as well as the PS and nascent mesoderm (nMeso) emerged.

Equivalent developmental stages and lineages were extracted from mouse (E5.25-6.5), cow (E12-14), pig (E11-11.5), rabbit (E6-6.7), and marmoset (E14-16) datasets to match sheep pre-PS and early-PS cell populations. At the pre-PS stage, AVH and PVH clusters were already evident in cow and rabbit embryos, and the distal/anterior visceral endoderm (DVE/AVE) could be distinguished from the remaining embryonic visceral endoderm (emVE) in mouse. In contrast, VH cells in sheep, pig and marmoset pre-PS embryos had not yet segregated into anterior and posterior domains. By the early-PS stage, AVH/AVE and PVH/emVE populations were present in all species. Conserved AVH markers across species included *CER1*, *DKK1*, *LHX1*, *HESX1, HHEX, OTX2, FZD5*, and *GSC*. PVH markers conserved primarily between ungulates and rodents included *MSX1, FGB, FBLN2, SLC39A5,* and *RSPO3*, with *RSPO3* also conserved in primates.

Inter-lineage communication analysis using *CellChat* identified NODAL, BMP, WNT, and FGF as the major signalling pathways mediating symmetry breaking across species. However, species-specific features were observed, including divergent BMP and FGF ligand usage and distinct BMP sources. In mouse embryos, the TE-derived extraembryonic ectoderm was the principal BMP source, expressing *BMP4* from pre-PS stages to induce DVE/AVE formation. Conversely, in sheep, cow, pig, rabbit, and marmoset embryos, the TE did not contribute to inter-lineage signalling, and *BMP4* expression was largely absent at the pre-PS stage, with only low levels detected in cow and rabbit hypoblast and in marmoset extraembryonic mesoderm. Instead, the hypoblast acted as the primary BMP sender, with *BMP2* and *BMP6* representing the predominant ligands across non-rodent species.

Together, our work reveals both conserved principles and species-specific mechanisms governing mammalian symmetry breaking. These findings establish sheep as a powerful and accessible model for studying body plan establishment in mammalian embryos that develop a flat embryonic disc.

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Submission ID#2361427

CRISPR-Cas9 Electroporation Drives Efficient *KEAP1* Gene Editing and Reduces KEAP1-NRF2 Interaction in Bovine Embryos

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Abstract Text

Genome editing in livestock allows the improvement of desirable traits and the development of research models within shortened generational intervals. The CRISPR-Cas9 system is a versatile and highly specific genome-editing tool; however, its efficiency depends on the effective delivery into early embryos. The traditional delivery method relies on micromanipulation systems to individually microinject the CRISPR-Cas9 components into presumptive zygotes. This approach is time-consuming, requires expensive equipment, and demands highly skilled personnel. Here, we evaluated electroporation as an alternative CRISPR-Cas9 delivery method to generate embryos with biallelic mutations (fully edited) without compromising blastocyst development. This hypothesis was tested by targeting the *KEAP1* gene in bovine embryos. KEAP1 regulates NRF2 activity by promoting its degradation. The NRF2 pathway is a master regulator of cellular antioxidant defenses and is therefore of particular interest in vitro embryo development, where culture conditions have been shown to increase oxidative stress. Therefore, we expect that *KEAP1* disruption would reduce KEAP1-NRF2 interaction, enhance antioxidant responses, and potentially improve embryo resilience to oxidative stress during in vitro culture. Cumulus-oocyte complexes (n= 504) were in vitro matured for 22 hours and then fertilized. Three sgRNAs targeting the exon 2 of *Keap1* were individually complexed with Cas9 (200 ng/µL Cas9, 100 ng/µL sgRNA). At 10 hours post-insemination, presumptive zygotes (PZs) were denuded and electroporated in a 1 mm electrode gap slide using either 15V or 20V (5 pulses, 3 ms duration, 100 ms intervals). Following electroporation, PZs were cultured for 7 days at 38.5 °C, 5% CO_2, and 5% O_2. Control groups included non-electroporated PZs and PZs electroporated without sgRNA. Resulting blastocysts were Sanger-sequenced to determine editing efficiency and indel type. Results revealed no difference in blastocyst rates between the control (24.0 %) and the 15V (18.01%) groups, suggesting that 15V did not impair embryonic development. However, 20V significantly reduced blastocyst formation (7.07%) compared to the other treatments. Analysis of Sanger traces demonstrated that 96.23% of the embryos sequenced were edited at some degree, with sgRNA-3 producing the highest proportion of embryos with biallelic mutations (64.3% at 15V; 100% at 20V). Among the fully edited embryos, mosaicism was the predominant indel type (92.85%). Additionally, embryos electroporated with 15V and edited with sgRNA-3 were subjected to Proximity Ligation Assay (PLA) to determine if the induced mutations in fully edited embryos resulted in disrupted KEAP1-NRF2 interaction. PLA puncta per nucleus were quantified in control and edited embryos from confocal z-stacks using FIJI/ImageJ. We observed a significant reduction in puncta per nucleus in the fully edited embryos (1.38 ± 1.04) compared to the control group (11.95 ± 2.69). These findings suggest that electroporation is an efficient method for CRISPR-Cas9 delivery into bovine zygotes to generate embryos with biallelic mutations. Furthermore, the induced mutations in the *KEAP1* gene resulted in reduced KEAP1-NRF2 interaction, indicating the availability of free NRF2 protein.

Nutrition/ Energy Balance and Neuroendocrine Function

Monday, July 20 1:30 PM – 3:00 PM

Submission ID#2329527

Hypothalamic remodeling during pubertal development

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Abstract Text

Puberty is a critical developmental stage during which individuals acquire the capacity for sexual reproduction. This transition involves a series of complex biological events primarily orchestrated by the activation of the hypothalamo-pituitary-gonadal (HPG) axis. Central to this process are gonadotropin-releasing hormone (GnRH) neurons, which play a key role in regulating reproductive maturation and function throughout life. However, the precise mechanisms that trigger the pubertal increase in GnRH activity remain incompletely understood. Evidence from our laboratory indicates that a profound remodeling of the hypothalamus is crucial for sexual maturation. In this presentation, I will discuss findings from our studies utilizing a combination of RNA sequencing, conditional genetic manipulation in mouse models and viral vectors, and systems neuroscience approaches. Our results reveal that the pubertal transition involves changes in the chemical phenotype and site-specific innervation of key hypothalamic neurons. Among these neuronal populations, those expressing growth hormone-releasing hormone (GHRH), kisspeptin, or dopamine transporter (DAT) will be the focus of my discussion. Building upon data from other laboratories, our findings offer new insights into the neural and molecular mechanisms by which the hypothalamus orchestrates sexual maturation.

Submission ID#2360618

Gonadotropin-Releasing Hormone Stimulation of Luteinizing Hormone β Transcription via a Non-Canonical MAP Kinase Signaling Pathway in Gonadotrope-Like Cells

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Abstract Text

Reproduction is regulated by hormones in the hypothalamic-pituitary-gonadal axis. Hypothalamic gonadotropin-releasing hormone (GnRH) acts on pituitary gonadotropes to stimulate the synthesis and release of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The gonadotropins regulate diverse aspects of gonadal function. FSH and LH are dimeric glycoproteins that share a common α subunit that is noncovalently linked to hormone-specific β subunits (LHβ and FSHβ, encoded by the *Lhb* and *Fshb* genes, respectively). According to the current model, GnRH stimulates *Lhb* transcription by activating a canonical PKC-Raf-MEK1/2-ERK1/2 signaling cascade, which culminates in expression of the transcription factor early growth response 1 (EGR1). EGR1 binds to the *Lhb* promoter to drive its transcription. Consistent with this model, genetic ablation of ERK1/2 (*Mapk3/1*) or EGR1 (*Egr1*) in murine gonadotropes leads to reduced LH synthesis in both sexes, and anovulation in females (both KOs). Challenging this model, however, GnRH-mediated ERK1/2 phosphorylation (pERK1/2) was reportedly MEK1/2-dependent, but Raf-independent in the gonadotrope-like cell line, αT3-1.

In light of these results, we hypothesized that GnRH signals through a non-canonical MAP kinase pathway to stimulate *Lhb* transcription. Here, we investigated the roles of PKC isoforms, Raf kinases, MEK1/2, and ERK1/2 in GnRH-induced pERK1/2, EGR1 expression, and *Lhb* transcription in the mature murine gonadotrope-like cell line, LβT2.

LβT2 cells were transfected with a murine *Lhb* promoter-reporter, incubated for 20 min with 10 μM MEK1/2 (U0126), Raf (LY3009120), PKC (Gö6983), or ERK1/2 (FR 180204) inhibitors, and then treated with 100 nM GnRH for 6 hours. Protein lysates were used in luciferase assays. Next, LβT2 cells were incubated for 30 min with 10 μM U0126, LY3009120, Gö6983, or FR 180204, followed by 100 nM GnRH for 5 minutes (for pERK1/2) or 1 hour (for EGR1). Protein lysates were analyzed by western blotting for pERK1/2 and EGR1.

The MEK1/2 inhibitor greatly reduced GnRH-induced pERK1/2, EGR1 protein expression, and *Lhb* promoter-reporter activity. In contrast, PKC inhibition attenuated GnRH-induced *Lhb* promoter-reporter activity, but not pERK1/2 or EGR1 expression. The Raf inhibitor greatly reduced GnRH-induced EGR1 expression and *Lhb* transcription, but not pERK1/2. Consistent with the latter result, the ERK1/2 inhibitor failed to block GnRH-induced EGR1 expression or *Lhb* promoter-reporter activity.

These results suggest that GnRH stimulation of *Lhb* transcription does not depend on a canonical MAPK signaling cascade in LβT2 cells. Though the small molecule inhibitors implicate PKC, Raf, and MEK1/2 catalytic activities in GnRH-induced *Lhb* promoter activity, these kinases do not appear to function in the same linear signaling pathway. Unlike the MEK1/2 inhibitor, neither PKC nor Raf inhibition attenuated GnRH-stimulated pERK1/2. The PKC inhibitor also did not alter GnRH induction of EGR1 expression. Coupled with the absence of effects of the ERK1/2 inhibitor, the data indicate that ERK1/2 enzymatic activity is dispensable for GnRH-induced *Lhb* transcription. Moreover, whereas EGR1 expression may be necessary for *Lhb* promoter activity, it does not appear to be sufficient. Future studies are aimed at determining roles for the ERK1/2 proteins independent of their kinase activity, and mechanisms through which: 1) MEK1/2 is activated independently of Raf, 2) Raf induces EGR1 independently of MEK1/2 or ERK1/2 catalytic activities, and 3) PKC regulates *Lhb* transcription independently of EGR1 induction.

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Submission ID#2360928

Reproductive Hormone Dysregulation in Women After Concussion: A Preliminary WOMEN'S Health Concussion Study

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Abstract Text

A total of 128 participants comprising 70 women suffering concussions (CONCUSSED) and 58 with an orthopedic injury (OI CONTROLS) were evaluated in association with the Women's Multidomain Evaluation of Neurobiological Health Concussion Study (WOMEN'S Health Concussion Study). Participants were followed for 90 days using assessments of concussion symptom severity (i.e., Concussion Clinical Profiles Screening [CP Screen]), reproductive hormone levels and menstrual cycle patterns. ELISA assays of reproductive hormone levels in urine samples provided by participants every third day were performed for pregnanediol glucuronide (PDG), a metabolite of progesterone, which is required to prepare the uterus for embryo implantation; estrone, an estradiol metabolite; as well as cortisol, which regulates metabolism, blood pressure, blood sugar levels, and immune function. We also measured serum levels of luteinizing hormone (LH), which promotes progesterone production. Results of the ELISA assays indicated that 45% of CONCUSSED participants had average cyclical PDG peak levels 56% lower than OI CONTROLS ($p < 0.001$). We also found support for diminished cyclical peaks of estrone (31% decrease compared to OI CONTROLS, $p=0.137$) in the low PDG population. The population of CONCUSSED participants with low PDG also had reduced average cortisol levels over a 90-day post-concussion period (29% decrease, $p=0.0109$) and lower serum levels of LH (60% decrease, $p=0.0225$) assayed an average of 15 days after concussion. These results may be indicative of reduced function of the hypothalamus and/or anterior pituitary gland. The average length of menstrual cycles post-injury was similar for CONCUSSED and OI CONTROLS. However, CONCUSSED women with low PDG had increased variation in individual cycle lengths compared to controls. Initial results suggest that 80% of participants with severe hormone and menstrual cycle changes had concussions occurring on days 5-15 of the menstrual cycle (before and during ovulation). Patient clinical profiles (CP) survey measurements of concussion severity for all concussed participants returned to normal 90 days after injury, but hormone levels for 70% of the low PDG population did not recover. Thus, concussed women may not be aware of longer-term fertility and hormone changes associated with concussions. Together, these results highlight the need for clinicians to provide concussion patients with referrals to specialty care for hormone-related follow-up. Funding was provided by the Chuck Noll Foundation for Brain Injury Research and Magee Womens Research Institute.

Submission ID#2337690

Neural Regulation of Puberty in Female Sheep

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Abstract Text

Timing of puberty is determined by a combination of various external and internal signals such as gonadal steroids and nutritional cues. Ultimately, such inputs are transduced into a signal that accelerates the frequency of pulsatile GnRH and LH release, driving follicular development and estradiol secretion, culminating in a GnRH/LH surge and the first ovulation. The neural mechanisms underlying the escalation in GnRH secretion are not completely known, particularly in domestic livestock species. We focused on the role of a set of neurons located in the arcuate nucleus of the hypothalamus termed KNDy neurons as they coexpress kisspeptin, neurokinin B (NKB), and dynorphin. Kisspeptin and NKB stimulate GnRH/LH release while dynorphin inhibits it and, in humans, the lack of kisspeptin, NKB, or their receptors blocks puberty. Initially, we showed that kisspeptin and NKB protein expression was readily evident well before puberty and cell numbers did not change during development. Subsequently, we found that deleting the majority of KNDy neurons significantly delayed puberty and reduced the amplitude of the LH surge. We concluded that puberty was not dependent upon changes in kisspeptin or NKB expression, but that KNDy neurons were necessary for its onset. In a next set of studies, we showed that puberty-related increases in LH secretion were associated with increased activation of KNDy neurons. We further noted an increase in activation of arcuate proopiomelanocortin (POMC) neurons that produced alpha-melanocyte stimulating hormone (alpha-MSH) and a marked reduction in agouti-related peptide (AgRP) cell numbers and activation. These neurons, known collectively as the melanocortin system, are involved in nutritional regulation of reproduction, with alpha-MSH stimulating hormone evoking LH secretion while AgRP inhibits it. Thus, the melanocortins may serve to transduce signals reflective of energy balance and activate KNDy neurons, thereby connecting the timing of puberty with nutritional state.

I’m From The Future: Emerging Contraceptive Technologies

Monday, July 20 1:30 PM – 3:00 PM

Submission ID#2361059

Mapping the Ovarian MASTL/AGC-Kinase Landscape to Advance Cell-Cycle–Based Contraceptive Strategies

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Abstract Text

Unintended pregnancies remain a serious global health concern, stressing the necessity for safe and reversible contraception that does not interfere with natural hormonal balance. Thus, a better understanding of molecular regulators of female gametogenesis is needed. Greatwall kinase (MASTL-Microtubule-Associated Serine/Threonine Kinase-Like; human ortholog), a conserved AGC-kinase member, has recently been recognized as a pivotal regulator of cell-cycle function, ensuring proper meiotic progression and genomic stability in oocytes. The AGC-kinase family, comprising 23 subfamily members, orchestrates phosphorylation-mediated signaling essential for cellular growth, metabolism, and survival. Although their significance in cancer and metabolic syndromes is extensively acknowledged, their detailed function in ovarian biology, aging, and disorders remains poorly understood. Limited studies suggest that MASTL is essential for oocyte meiotic progression and metaphase II stability via regulating PP2A (Protein Phosphatase 2A) and its substrate, ARPP19/ENSA. MASTL deficiency disrupts spindle formation and meiotic control, leading to oocyte maturation failure and highlighting its importance for oocyte developmental competence. A coordinated input from upstream AGC-kinases (RSK/PKA), further fine-tunes this pathway by regulating PP2A inhibition and CDK phosphorylation during mitotic and meiotic progression.

However, our understanding of its regulation across ovarian compartments and its contraceptive potential remains unclear. To address these questions, we evaluated RNA-seq datasets from different species to map AGC-kinase expression across ovarian developmental stages and cell types. RNA-seq data from mouse ovaries at 21 days, 3–6 months, and >10 months were analyzed to define age-dependent AGC-kinase transcriptional changes. In these different-aged mouse ovaries, AGC-kinase expression was most dynamic in early follicles and more restricted in aged ovaries. To identify cell-type-specific AGC expression patterns, RNA-seq data were further examined across bovine ovarian cell types, including granulosa, theca, and luteal cells. Pathway enrichment (GO and KEGG) analysis indicated that, during the early phases of ovarian growth, AGC markers are linked to robust activity in cell-cycle progression and structural (cytoskeletal and junctional) dynamics. Conversely, later developmental stages (luteal stage) and the later reproductive period (>10 months) are characterized by marked downregulation of MASTL and its associated AGC-kinases, marking a transition in which suppression of mitotic drivers shifts the ovarian environment from active cellular proliferation to a more stable, quiescent state. Notably, MASTL is highly expressed in primordial and primary follicles relative to antral/luteal stages, as observed in bovine and murine ovaries. Furthermore, GKI-1: MASTL inhibitor, markedly reduces proliferation in human GC, therefore affirming its functional significance. In a pilot in vivo study, adult female mice (n≈3/group) received GKI-1 during a defined window and were subsequently housed with proven breeder males. Treated females failed to conceive during the treatment period but resumed normal fertility after drug withdrawal, suggesting that transient MASTL inhibition can induce a reversible contraceptive state by briefly halting oocyte maturation and granulosa cell support. Moreover, GKI-1 has been shown to avoid irreversible ovarian damage and has no adverse effects on subsequent litter size or pup viability, representing a significant translational advance.

Altogether, this study provides the first cross-species map of AGC-kinase signaling in the ovary, positioning the understudied MAST subfamily as a critical controller of reproductive function. The functional association found between MASTL activity and fertility regulation suggests a mechanistic rationale for cell-cycle–based, non-hormonal, reversible contraceptive interventions. Beyond its mechanistic insights, this work also identifies MASTL and its AGC-kinase associates as promising biomarkers of reproductive longevity and for targeted fertility interventions.

Submission ID#2360540

A phenotypic screening pipeline identifies novel inhibitors targeting Wee2 kinase activity to block egg activation for non-hormonal contraception

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Abstract Text

WEE2 is an oocyte-specific kinase crucial for meiotic resumption from metaphase II (MII) arrest and egg-to-embryo transition during fertilization. Indeed, loss of WEE2 activity due to homozygous and heterozygous mutations lead to infertility due to fertilization failure. Therefore, selective inhibition of WEE2 kinase activity without impacting other WEE family kinases, such as MYT1 and WEE1, offers a targeted strategy for non-hormonal contraception. We established a phenotypic screening pipeline using mouse and bovine eggs to screen for compounds that block egg activation via Wee2. This screening pipeline includes functional egg activation assays via parthenogenesis and *in vitro* fertilization (IVF), target engagement via Western blotting, and evaluation of reproductive safety via *ex vivo* follicle growth assay. Our initial screening assay used mouse treated with strontium chloride ($SrCl_2$) to trigger parthenogenetic egg activation in the presence or absence of chemical compounds. Staging of egg activation and early embryo development was determined using a closed time-lapse imaging system (EmbryoScope+™). In collaboration with Schrödinger, we treated eggs with highly potent and selective Wee2 inhibitors (B-1 and B-2, Ki < 30nM) along with an inactive enantiomer of analogue B-2 (B-3, Ki >1000nM). We found that treatment with these compounds at 10 μM still allowed for second polar body (PB) extrusion but reduced pronuclei (PN) formation (Control: 100%, B-1: 0%, B-2: 0%, B-3: 81%) and two-cell cleavage (Control: 100%, B-1: 0%, B-2: 0%, B-3: 59%). These phenotypes were dose-dependent and exhibited similar but less potent effects in egg activation via IVF. As WEE2 phosphorylates CDC2 to drive egg activation, we assessed target engagement by evaluating pCDC2 expression in activated oocytes treated with 10 μM B-1 and B-2 using Western blotting. While pCDC2 expression increased following egg activation, it was significantly inhibited by B-1 and B-2 exposure. Additionally, we evaluated the reproductive safety of these compounds by examining their impact on follicular growth, survival, and ovulation over an 8-day period. Follicles were treated with the minimum effective concentrations identified from the egg activation assay (B-1: 100 nM; B-2: 1 μM; B-3: 1 μM). Treated follicles exhibited no statistical differences in growth, survival, and ovulation rates compared to the control group. Lastly, preliminary studies in bovine eggs, which are more physiologically similar to humans, indicate that 10 μM B-1 treatment also blocks egg activation via parthenogenesis and IVF. These findings highlight our screening pipeline to comprehensively evaluate Wee2-specific inhibitors for non-hormonal contraceptive development. Studies are ongoing to evaluate more potent selective Wee2 inhibitors and establish similar functional assays within human eggs.

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Submission ID#2408912

Approaches to block sperm-egg fusion

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Abstract Text

Modern contraceptives are effective, yet systemic side effects contribute to discontinuation and avoidance of hormonal methods, particularly in low-resource settings where access and choice are limited. Expanding non-hormonal options is critical to meet global needs for safety, reversibility, and convenience. Here, we report a first-in-class non-hormonal strategy targeting the IZUMO1–JUNO axis, an essential and untapped step in sperm–egg fusion. We discovered several nanobodies that bound with nanomolar affinities, with lead candidates achieving up to 99% inhibition of human *in vitro* fertilization, even under sperm concentrations far exceeding physiological conditions. Structural models revealed epitope overlap with the IZUMO1 binding site, consistent with direct competitive blockade. These nanobodies demonstrated high specificity, exceptional stability, serum integrity, solubility, and efficient penetration of the cumulus-oocyte complex. Collectively, these findings establish proof-of-concept for IZUMO1-JUNO disruption and lay the foundation for next-generation non-hormonal immunocontraceptive innovation.

Submission ID#2352034

Advances in Nonhormonal Contraception: Inhibiting Prostate-Specific Antigen (PSA) Suppresses Semen Liquefaction In Humans and Nonhuman Primates

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Abstract Text

Human semen coagulates (gels) immediately after ejaculation. Liquefaction is a process that occurs after ejaculation, transforming the solidified semen into a liquid and releasing motile sperm. This process is unique to humans and nonhuman primates and does not happen in rodents or domestic species. Prostate-specific antigen (PSA) is a protease that liquefies semen, and men with mutations that impair PSA function tend to have hyperviscous semen and are infertile. The premise of this work is that selectively inhibiting PSA could offer a non-hormonal contraceptive method that acts within the vagina by preventing liquefaction. We tested whether small-molecule PSA inhibitors can block semen liquefaction and potentially function as female-controlled, non-hormonal, on-demand contraceptives. We evaluated the PSA inhibitors by examining 1) enzyme specificity in the lab, 2) how long and in what way they inhibit, 3) their binding via 3D docking, 4) their safety in mouse and rhesus vaginal epithelial cells, and 5) their effects on semen liquefaction in humans and rhesus macaques. Four proprietary inhibitors were initially screened against a panel of serine-proteases. Compounds B1 and 6B1MI were selected for further study because of their superior selectivity and ability to block liquefaction *in vitro*. Both fit within the PSA active site and showed no detectable toxicity in vaginal epithelial cells. B1 and 6B1MI significantly increased semen viscosity in human and rhesus macaque samples, indicating they effectively inhibit liquefaction. To move forward with in vivo studies in NHPs, we prepared a prototype vaginal film containing 15% polyvinylpyrrolidone (PVP) and 10% glycerol, with each film delivering 2 mg of 6B1MI. Macaques treated with this film showed a 30% pregnancy rate. However, we observed that the prototype film had a far slower than expected dissolution rate (>4 hours), resulting in incomplete dissolution within the required treatment window for an on-demand contraceptive trial. Trials using film formulations with 5% PVA, 10% glycerol, and 2 mg of 6B1MI—designed to dissolve within 30–60 minutes—are currently underway. We conclude that selective PSA inhibition effectively prevents semen liquefaction in both humans and macaques and can reduce pregnancy rates, making it a promising approach for female on-demand contraception. Development of a suitable delivery film is the next critical step in exploring this approach. By preventing sperm release from the seminal gel, PSA inhibitors provide a mechanistically distinct contraceptive method that avoids systemic hormonal exposure. This research lays the groundwork for developing on-demand vaginal films as a new form of female-controlled contraception.

Supported by R01 HD108198 and P51 OD011092

Submission ID#2360627

Targeting the ovastacin-fetuin-b interaction to induce premature zona hardening for non-hormonal contraception

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Abstract Text

During fertilization, sperm must penetrate the egg zona pellucida which triggers the hardening to prevent polyspermy. This process is mediated by the release of cortical granules containing the metalloendoprotease ovastacin which cleaves ZP2 of the zona pellucida. Notably, low levels of ovastacin are also released throughout oocyte maturation but are inhibited by the presence of the liver-derived fetuin B. Fetuin B-deficient knockout mice are infertile due to prematurely hardened zona pellucida but show no other defects. Consequently, blocking the interaction between fetuin B and ovastacin during oocyte maturation offers a promising mechanism for non-hormonal contraception by inducing premature zona hardening and thus blocking fertilization. Here, we established functional assays that can be used as readouts to determine the biological efficacy and potency of selective inhibitors. Our platform consists of (1) target engagement evaluating ovastacin enzymatic activity through detection of cleaved ZP2 and (2) evaluation of zona pellucida hardening via a sperm binding assay. Through our assays, we achieved reproducible and reliable detection of variations in sperm binding between eggs with unhardened zona (*in vivo* MII egg – negative control) and hardened zona (activated egg – positive control). Additionally, we confirmed loss of full-length ZP2 post-activation when comparing these two conditions via Western blotting. Notably, we observed that *in vitro* matured MII eggs had reduced sperm binding capacity comparable to activated eggs and that this phenotype is prevented by supplementation with recombinant fetuin B or fetuin derived from fetal bovine serum. Reduced sperm binding in *in vitro* matured MII eggs also corresponded with reduced but not complete loss of full-length ZP2. These findings demonstrate the ability to reliably detect phenotypic changes in zona pellucida hardening via ZP2 cleavage and sperm binding and that this process is inhibited by the presence of fetuin B. Further optimization of these functional assays is ongoing, and studies are underway to begin screening emerging inhibitors blocking ovastacin::fetuin B interactions.
This study was part of the Ovarian Contraceptive Discovery Initiative (INV-003385) funded by the Bill & Melinda Gates Foundation.

Folliculogenesis and Corpus Luteum Function

Tuesday, July 21 10:00 AM – 12:00 PM

Submission ID#2361391

Corpus luteum maintenance after classical maternal recognition of pregnancy: Mechanistic insights from a bovine pregnancy loss model

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Abstract Text

The corpus luteum (CL) is essential for the establishment and maintenance of pregnancy in cattle. Although mechanisms governing CL maintenance during classical maternal recognition of pregnancy (MRP) have been extensively investigated, those implicated in CL maintenance after MRP remain largely unknown. A growing body of evidence supports the presence of a local physiological mechanism that sustains CL function after the classical MRP period. Furthermore, there is evidence indicating that inappropriate luteolysis contributes significantly to the occurrence of pregnancy loss after the classical MRP period, highlighting the need for greater understanding of the processes governing the dichotomy between CL and pregnancy maintenance or CL regression and pregnancy loss. However, the inherent variability in the incidence and timing of spontaneous pregnancy loss poses a significant challenge for investigating these biological processes. Hypertonic-saline induced pregnancy loss (IPL) at ~35 days of gestation produces rapid conceptus demise and results in luteolysis within a predictable window (i.e. ~7-8 days later), providing for a robust model to investigate the CL maintenance or regression dichotomy. Moreover, the interval between IPL and luteolysis is stage-dependent and lengthens with advancing gestational age, suggesting ongoing conceptus-mediated support of the CL after the classical period of MRP. Importantly, the IPL procedure itself does not delay luteolysis in cyclic cows, confirming endometrial luteolytic integrity. Mechanistically, using an ablation and replacement approach, we demonstrated that uterine-derived prostaglandin $F_2\alpha$ ($PGF_2\alpha$) is the luteolysin responsible for CL regression following conceptus demise. Ablation of $PGF_2\alpha$ using flunixin meglumine, a COX inhibitor, prevented luteolysis following IPL, whereas replacement of intrauterine $PGF_2\alpha$ pulses induces luteal regression in most cows. Furthermore, preliminary results from a recent study indicate that luteolysis following IPL is temporally associated with a marked increase in circulating $PGF_2\alpha$-metabolite (PGFM) suggesting enhanced $PGF_2\alpha$ secretion. Because uterine blood flow increases and uterine $PGF_2\alpha$ pulsatility is restored after MRP, it has been hypothesized that the increase in utero-ovarian blood flow could be involved in maintenance of the CL by reducing the utero-ovarian transport of PGF. Consistent with this hypothesis, Doppler ultrasonography following IPL revealed a ~24% reduction in ipsilateral uterine artery perfusion beginning approximately six days before luteolysis, indicating that reduced uterine perfusion precedes CL regression and suggesting a functional role for elevated perfusion in luteal maintenance. To directly test whether enhanced perfusion is causally responsible for sustaining the CL during early pregnancy, uterine blood flow was pharmacologically increased using sildenafil citrate. In non-pregnant cows, sildenafil transiently increased uterine artery perfusion index and diameter by 31 and 22%, respectively, demonstrating its efficacy as a local vasodilator. In IPL cows receiving flunixin meglumine to suppress endogenous uterine $PGF_2\alpha$ secretion and undergoing controlled intrauterine $PGF_2\alpha$ pulses, sildenafil again increased uterine perfusion and even amplified the PGFM response to exogenous $PGF_2\alpha$. Despite these clear hemodynamic effects, sildenafil did not delay or prevent luteolysis. These results indicate that enhanced utero-ovarian blood flow alone is not sufficient to maintain the CL in the presence of a luteolytic $PGF_2\alpha$ stimulus. Collectively, these studies reveal that increased uterine perfusion is permissive but not sufficient for CL maintenance, underscoring uterine $PGF_2\alpha$ secretion as the dominant regulator of luteal fate and providing new insights into the yet unresolved mechanisms responsible for CL maintenance after classical MRP in cattle.

This project was supported by AFRI Grant no. 2021-67015-33838 from the USDA National Institute of Food and Agriculture.

Submission ID#2361305

Aire Deficiency Drives Ovarian T Cell Infiltration and Autoantibody Responses in Mice

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Abstract Text

The immune system's ability to distinguish between self and non-self is critical for preventing autoimmunity and for protecting fertility. During T cell development in the thymus, the transcriptional regulator Autoimmune Regulator (Aire) plays a pivotal role in establishing self-tolerance by promoting expression and presentation of tissue-restricted self-antigens, thereby facilitating the elimination of developing autoreactive T cells. In both humans and mice, genetic mutations in *Aire* lead to widespread multi-organ autoimmunity, including pathologies in reproductive tissues. In mouse models of Aire deficiency, the ovary is a target of this breakdown in tolerance, often leading to infertility. Using Aire-deficient mice created by CRSPR-Cas9 technology, we confirmed subfertility in the absence of Aire ($P < 0.0016$; n=14 *Aire*$^{-/-}$; n=7 WT). By 20 weeks of age, ovaries of most *Aire*$^{-/-}$ females were depleted of follicles and had evidence of lymphocyte infiltration; pregnancy, however, appeared to protect against follicular loss. Further, crossing immune-deficient *Rag*$^{-/-}$ mice to *Aire*$^{-/-}$ mice confirmed the immune-dependent mechanism of follicular loss in *Aire*$^{-/-}$ females. To understand the pathophysiology of oophoritis in Aire deficiency, we transferred splenocytes from 30-week-old *Aire*$^{-/-}$ or wild type control female mice into *Rag*$^{-/-}$ recipients (n=7). Mice receiving immune cells from *Aire*$^{-/-}$ donors exhibited robust infiltration of CD8+ cytotoxic lymphocytes into the ovaries. In parallel experiments, we profiled serum autoantibody reactivities in 30-week-old female *Aire*$^{-/-}$ mice (n=8) using Phage ImmunoPrecipitation Sequencing (PhiP-Seq). This approach identified a marked increase in autoantibodies targeting organ-specific antigens, including a significant enrichment of ovarian targets (n=8). Our findings suggest that Aire deficiency drives both ovary-specific T cell infiltration and a distinctive autoantibody signature. Ongoing studies will further clarify the role of immune cell types and autoantibodies in disease progression. Together, this integrated analysis highlights the multifaceted nature of autoimmune ovarian pathology in *Aire*$^{-/-}$ mice and provides a foundation for dissecting ovarian autoimmunity.

Submission ID#2361347

SMAD6 Upregulation is a Causal Determinant of Reduced Bovine Granulosa Cell Proliferation in vitro

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Abstract Text

The oocyte-derived TGFB family members, BMP15 and GDF9, are critical in regulating follicular development and particularly granulosa cell (GC) proliferation by activation of the SMAD intracellular signal transduction pathway. SMAD6 is an inhibitor of the BMP15-activated SMAD pathway, and we recently identified a high-fecundity allele in cattle, termed the Trio allele, that exhibits dramatic upregulation of SMAD6. In this study we investigated the role of SMAD6 in GC proliferation using bovine follicular fluid (bFF) as a native source for BMP15/GDF9. In all experiments we used GC collected from small follicles (~5mm) during a synchronized follicular wave using follicle ablation 60 h before collection in both cattle that were Trio allele carriers or noncarriers (n=3 or more replicates from different cows in each experiment). In Experiment 1, after cell attachment, GC from noncarriers were incubated for 60 h with different concentrations of bFF (0, 1%, 2%, 4%, 8%, 16%). The bFF dramatically increased (>10-fold) GC proliferation in a dose-dependent manner. In Experiment 2, treatment with FF activated a BMP-responsive element (BRE luciferase reporter plasmid [pGL3 BRE, Addgene #45126]) promoter (2543±137 RLU), to a level comparable to recombinant hBMP15 (2383±43 RLU), relative to the control group (346±43 RLU; $p < 0.001$) indicating the presence of BMP15 in bFF. In Experiment 3, BMP signaling activity was evaluated by treatment with LDN-193189 (0.1 μM, Selleck Chemicals), a selective small-molecule inhibitor targeting BMP type I receptors. LDN completely inhibited GC proliferation induced by hBMP15 or bFF indicating that effects of bFF on GC proliferation were mediated through BMP15 receptors. In Experiment 4, GC from Trio carriers and noncarriers were incubated with 0 or 4% FF from either carriers or noncarriers in a 2×3 experimental design. Noncarrier GC had greater proliferation than carrier GC but FF from carriers or noncarriers had a similar stimulatory effect, consistent with the hypothesis that differences in proliferation are not due to variation in BMP15 level between carriers and noncarriers. In Experiment 5, GC from carriers and noncarriers had *SMAD6* knockdown using transfection with SMAD6-targeted LNA GAPMERs (Qiagen). Treatment with 4% bFF induced greater *SMAD6* mRNA expression in GC from Trio carriers (~9-fold) than non-carriers (~4-fold) and this increase was completed prevented by SMAD6-targeted GAPMERs. Treatment with GAPMERs also further increased FF-induced GC proliferation but the increase was greater ($P< 0.01$) in Trio carriers (8,175 ± 233% to 17,610 ± 919%; 1.2-fold) than noncarriers (14,244 ± 867% to 19,848 ± 709%; 0.4-fold) indicating the greater role of *SMAD6* inhibition of GC proliferation in Trio carriers than noncarriers. Experiment 6 evaluated association of inhibitor of DNA-binding-1 protein (*ID1*; transcriptional regulator of proliferation/differentiation) with GC proliferation. Treatment with FF induced *ID1* with greater induction in noncarriers (4.09±0.2) than carriers (1.99±0.08) suggesting an association between *SMAD6* and *ID1* expression. Further, *SMAD6* knockdown increased *ID1* mRNA in GC of both carriers (1.99±0.08 to 4.19 ± 0.20) and noncarriers (4.09±0.2 to 5.74±0.29) confirming that SMAD6 functions as negative regulator of *ID1* expression. Thus, FF stimulates GC proliferation through the BMP15 pathway and *SMAD6* is important inhibitor of this pathway, particularly in Trio carriers. Intriguingly, the overexpression of *SMAD6* in GC of Trio carriers requires BMP15 stimulation. Furthermore, *SMAD6* inhibits *ID1* expression, and this may mediate the negative effects of *SMAD6* on GC proliferation.

Submission ID#2361420

Loss of *Adar* in Granulosa Cells Blocks Ovulation and is Associated with Upregulation of Innate Immune Regulatory Transcripts in Ovarian Stroma

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Abstract Text

Ovulation is a complex inflammatory-like process essential for reproductive success, and failure to ovulate is a common cause of infertility. Ovulation is also the most common physiologic contraceptive target in reproductive-aged women worldwide. Adenosine deaminases acting on RNA (ADAR) is an enzyme family responsible for Adenosine-to-Inosine editing of double-stranded RNA. Prior work showed that target deletion of ADAR1 *(Adar)* in mouse granulosa cells (GCs) with a human gonadal-specific aromatase promoter linked to Cre leads to delayed and inhibited ovulation resulting in infertility and upregulation of interferon pathway negative regulators such as IFIT1, IFIT3B and IFI44. ADAR1 is postulated to post-transcriptionally regulate gene expression by altering coding and non-coding sequences and regulating innate immune responses through its interaction with melanoma differentiation-associated gene 5 (MDA5). The primary objective is to gain insight into the specific mechanism of action by which loss of ADAR1 in follicles affects the ovulatory process. Using the Xenium spatial transcriptomics platform, central ovarian sections from PMSG + HCG treated control (n=3) and Adar flox/flox; AromCre/+ gcKO (n=3) mice were analyzed 4 hours post-hCG. Xenium Explorer software detected 97,507 and 84,359 cells in the control and knockout (KO) tissues, respectively. Graph-based clustering analysis revealed 34 and 30 distinct cell clusters in the control and KO samples. In line with the lack of ovulation, luteal cell populations were significantly reduced in the KO ovarian tissues, as were granulosa cell populations expressing *Cyp19a1*, *Fshr* and/or *Amh*. Analyzing gene overlap within distinct cell clusters revealed a significant upregulation of innate immune genes, including *Ifit3*, *Ifi44*, *Ifit1* and *Oasl2* in stromal cells in the KO ovaries, while ovarian stromal-residing macrophage and neutrophil populations were similarly distributed in both groups. Clustering spatial data using lymphatic endothelium markers, *Pdpn* and *Prox1,* revealed increased lymphangiogenesis in the ovarian stromal cells of the *Adar* KO mice. In summary, loss of ADAR1 within granulosa cells was followed by upregulation of innate immune genes in a currently undefined ovarian stromal cell population. These results point to an interesting crosstalk between the granulosa cells and ovarian stroma regarding the inflammatory-like ovulation process that may explain the block in ovulation in the absence of ADAR1.

Submission ID#2353262

Androgen Receptor Dysfunction Compromises Ovarian Angiogenic Responses in Polycystic Ovary Syndrome

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Abstract Text

Ovarian follicles require a robust vascular supply to grow and ovulate. Dysfunction of ovarian vasculature impairs follicle growth and ovulation. The ovarian vasculature is in close proximity to theca cells, which produce androgens. Women with Polycystic Ovary Syndrome (PCOS) have altered macrovascular and microvascular function as well as elevated androgens. We hypothesize that the response of ovarian microvascular endothelial cells to androgens may be impaired in PCOS. Ovarian microvascular endothelial cells from healthy oocyte donors (HOMECs) and women with PCOS (PCOMECs) were isolated from discarded follicular aspirates. HOMECs and PCOMECs expressed mRNA and protein for the classical androgen receptor AR and the plasma membrane androgen receptor SLC39A9. PCOMEC AR mRNA expression was 138% greater than that of HOMECs ($p < 0.05$), with no difference in SLC39A9 mRNA between HOMECs and PCOMECs. Androgen activation of SLC39A9 increases intracellular zinc flux. Testosterone (T) stimulated zinc influx in a dose dependent manner in HOMECs (93% increase, $p < 0.05$), but not in PCOMECs. Additionally, T, membrane impermeable bovine serum albumin-conjugated T (BSA-T), and the SLC39A9-selective agonist (-)-epicatechin each increased proliferation in HOMECs by 300-500% ($p < 0.05$), with no change in PCOMEC proliferation. These findings indicate that androgens increase zinc influx and proliferation via SLC39A9 in HOMECs but not in PCOMECs. HOMEC and PCOMEC responses via AR were determined by allowing cells to migrate through a semi-permeable membrane in the direction of an androgen stimulus gradient. HOMEC migration was increased by 62% in the presence of T ($p < 0.05$), but not BSA-T, whereas PCOMEC migration was not altered by any stimulus. siRNA knockdown of AR blocked T-stimulated migration in HOMECs ($p < 0.05$) while siRNA knockdown of SLC39A9 had no impact on basal or T-stimulated migration. PCOMEC migration in the presence of T was not affected by siRNA knockdown of SLC39A9 or AR. Live cell tracking showed that T increased speed and distance traveled by migrating HOMECs, while T had no impact on migrating PCOMECs. Androgens therefore stimulate migration via AR in HOMECs but not in PCOMECs. Overall, androgens act via both SLC39A9 and AR at HOMECs to stimulate proliferation and migration, which are key processes in angiogenesis. Importantly, although PCOMECs express SLC39A9 and AR, androgens do not stimulate angiogenic responses via SLC39A9 or AR in PCOMECs. Local androgen action may be central to ovarian vascular remodeling and ovulatory angiogenesis in healthy ovarian function. Absent or aberrant function of androgen signaling in PCOMECs may contribute to ovarian vascular dysfunction and may ultimately play an important role in the pathology of PCOS. This work was supported by the Ryan Family Foundation and the Jones Family Foundation.

Submission ID#2360994

TGFβ Drives Senescence in Luteinized Granulosa Cells and Reduces Progesterone Production

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Abstract Text

The ovarian follicle is exposed to an abundance of different signaling molecules produced by surrounding cells within the ovarian microenvironment. These molecules can influence the function of the follicle, specifically the granulosa and theca cells, which serve to produce sex steroids and nourish the oocyte during follicle maturation. We recently observed that luteal cells exhibit features resembling senescence, including increased expression of mRNAs of cytokines, chemokines, and extracellular matrix degrading proteins characteristic of the senescence-associated secretory phenotype (SASP). This study aimed to investigate the effects of molecules related to the SASP on bovine granulosa cells prior to and during luteinization, as well as a premature luteinized granulosa cell state. Granulosa cells were collected from healthy, non-atretic antral follicles (≥8 mm, n = 3 pools from 8-10 ovaries each). Cells were treated with interleukin 1 beta (IL1β, 10 ng/mL), tumor necrosis factor alpha (TNFα, 10 ng/mL), transforming growth factor beta (TGFβ, 1 ng/mL), a TGFβ receptor antagonist (SB431542, 10 μM), or no treatment as a control. Additionally, cells were subjected to a control or luteinization cocktail (10 μM forskolin, 20 nM Phorbol 12-myristate 13-acetate, 1x insulin-transferrin-selenium) for 96 h. Similarly, granulosa cells from early-stage follicles (2-5 mm, n = 3 pools of 8-10 ovaries) and were treated with or without TGFβ (1 ng/mL) and cultured under control or luteinizing conditions for 96 h to mimic premature luteinization of a developing follicle. Media was analyzed for progesterone and cells were stained for β-galactosidase, a marker of senescence, where blue staining is indicative of β-galactosidase activity. Exposure of antral granulosa cells to IL1β or TNFα did not significantly affect progesterone synthesis in control or luteinized cells. TGFβ decreased progesterone production in luteinized granulosa cells (2.4-fold, $P < 0.01$). When TGFβ signaling was blocked by treatment with a receptor antagonist, progesterone was rescued ($P < 0.01$) with no difference compared to luteinized controls ($P > 0.1$). Exposure of early-stage granulosa cells to TGFβ significantly decreased progesterone production in control (16.4-fold, $P = 0.011$) and luteinized granulosa cells (5.7-fold, $P < 0.05$). Both antral and early-stage luteinized granulosa cells appeared to have stronger β-galactosidase staining than the non-luteinized cells with or without cytokine treatment. Additionally, cells treated with the luteinization cocktail clustered together in a starburst pattern, with the positive staining concentrated towards the middle of the cluster. TGFβ further induced β-galactosidase in both luteinized and control antral and early-stage granulosa cells. Most positive β-galactosidase stain within these individual cells concentrated near the nucleus, however early-stage granulosa cells that had been luteinized and treated with TGFβ had the strongest positive β-galactosidase activity, turning completely blue. These studies suggest that paracrine signals from molecules (IL1β and TNFα) associated with the SASP may not affect progesterone production by granulosa cells during the transition to luteal cells. However, TGFβ together with other signaling molecules produced by follicular cells, may play a role in reprogramming the granulosa cells to become senescent as the follicle develops and eventually become large luteal cells.

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Submission ID#2354720

Luteinizing hormone-induced interfollicular communication in the mouse ovary

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Abstract Text

Luteinizing hormone (LH) acts on mammalian ovarian follicles to restart the oocyte meiotic cell cycle and to induce ovulation, resulting in the release of a fertilizable egg. However, it is unknown if responses to LH are initiated only by LH receptors within an individual follicle, or also by LH receptors elsewhere in the ovary. Here we investigated this question by co-culturing follicles from *Lhr*-knockout (*Lhr*-KO) mice with wildtype follicles. Isolated *Lhr*-KO follicles did not respond to LH, but when these follicles were placed in contact with wildtype follicles, LH caused the *Lhr*-KO follicle-enclosed oocytes to resume meiosis and cumulus cells to expand. The paracrine factors passing from wildtype to *Lhr*-KO follicles were present in media in which wildtype follicles had been incubated with LH, indicating that the interfollicular communication did not require follicle contact. LH-induced interfollicular communication was inhibited by the epidermal growth factor receptor (EGFR)- kinase inhibitor AG1478, and by antibodies neutralizing EGFR ligands. These findings indicate that LH elicits interfollicular communication, with individual follicles responding not only to LH directly, but also to paracrine signals, including EGF-like ligands, generated elsewhere in the ovary to ensure successful meiotic resumption.

Frontiers in Male Reproductive Biology

Tuesday, July 21 10:00 AM – 12:00 PM

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Single-molecule Mapping of the Protamine-Nucleosome Landscape in Human Sperm Reveals Protamine Lacunae as Potential Carriers of Epigenetic Information

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Abstract Text

A central event in mammalian spermatogenesis is the replacement of most nucleosomes with protamines, driving extreme condensation of the genome in the sperm head. Notably, however, a few nucleosomes are retained even in mature sperm. The locations of these retained nucleosomes across the genome, and their potential importance in fertility and epigenetic programming, remains incompletely defined. We used Fiber-seq, a method for mapping protein footprints on multi-kilobase chromatin fibers, to resolve patterns of paternal chromatin repackaging in sperm at single-molecule resolution and examine the relationship between protamines and nucleosomes across the genome in human sperm. We find that nucleosome retention occurs preferentially at promoters of spermatogenic genes, but is highly variable across sperm and across genomic loci. Centromere kinetochore binding regions also robustly retain CENP-A mononucleosomes, providing a mechanism for the focal transmission of paternal centromeres. However, we find that the predominant mode of paternal epigenetic information is protamine lacunae, accessible discontinuities in the protamine coat that preferentially mark regulatory elements. Importantly, we also find that the protamine-nucleosome balance is altered in low-motility sperm, with abnormally high protamine content associated with lower motility. Together, our study defines the protamine-nucleosome landscape in sperm at high precision, identifies protamine lacunae as a novel mode of epigenetic encoding in sperm, and reveals specific defects that correlate with infertility.

Submission ID#2361313

Investigating Sperm Chromatin Remodeling in the Absence of Autoimmune Regulator (Aire)

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Abstract Text

Inflammation of the male reproductive tract is an underappreciated driver of infertility, and the mechanisms linking inflammatory activation to sperm dysfunction remain poorly defined. To investigate this, we use an Aire-deficient ($Aire^{-/-}$) mouse model that develops spontaneous epididymal inflammation and fibrosis. The Autoimmune Regulator (AIRE) gene is required for central immune tolerance, and mutations in humans cause Autoimmune Polyglandular Syndrome Type 1 (APS-1), which is associated with a high incidence of male infertility. $Aire^{-/-}$ male mice mirror this phenotype, exhibiting infertility and pronounced epididymal pathology. Computer-assisted sperm analysis revealed a shift toward increased static and decreased motile sperm populations in $Aire^{-/-}$ males compared with controls ($p = 0.03$), while progressive and slow motility were unchanged. Hyperactivated sperm showed a reduction trend ($p = 0.08$), suggesting defective acquisition of capacitation-associated motility. Elevated DNA damage is characteristic of $Aire^{-/-}$ sperm, supporting known defects in chromatin remodeling. Together, these findings demonstrate that Aire deficiency disrupts functional and structural sperm maturation. During normal sperm maturation, histones are replaced by protamines to compact and protect the genome. We hypothesize that inflammatory disruption of histone-to-protamine exchange in $Aire^{-/-}$ males destabilizes sperm chromatin and thereby impairs fertilization capacity. To test this, chromatin condensation was assessed using Aniline Blue staining across all epididymal regions in wild-type and $Aire^{-/-}$ mice. Preliminary data show a higher proportion of Aniline Blue-positive sperm in $Aire^{-/-}$ mice, consistent with impaired histone-to-protamine transition. These findings identify a mechanistic link between inflammatory pathology and defective sperm maturation, revealing a pathway by which autoimmune disease can drive male infertility.

Submission ID#2360464

Regional Immune Niches in the Epididymis: CX3CR1+ Mononuclear Phagocytes as Gatekeepers of Immune Homeostasis

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Abstract Text

Male fertility relies on a finely balanced immune environment within the epididymis that simultaneously protects maturing spermatozoa from autoimmune reactions and defends against ascending urogenital pathogens. In contrast to the immune-privileged testis, the epididymis represents an immunologically active mucosal tissue. Consequently, when spermatozoa exit the protected testicular milieu, they enter an environment enriched with resident immune cells that closely interact with the epithelium and luminal content. Disruption of the tightly regulated immune equilibrium within the epididymis, particularly during infection, can therefore lead to region-specific inflammation, tissue damage, and long-term impairment of fertility.

We previously demonstrated that the epididymis is organized into distinct immunological niches along its proximal–distal axis. Proximal segments, where sperm first enter, display a restrained and rapidly resolving immune response to bacterial challenge, whereas distal regions mount pronounced proinflammatory reactions that predispose to tissue injury and persistent sub- or infertility. Single-cell RNA sequencing and high-dimensional flow cytometry revealed that this regional specialization is supported by strategically positioned immune cell populations, with CX3CR1^{+} macrophages representing the dominant epithelium-associated subset with striking regional characteristics.

To define their functional role, we employed a transgenic mouse model enabling selective depletion of intraepithelial CX3CR1^{+} macrophages. Under physiological conditions, loss of these cells resulted in focal epithelial alterations within the epididymis and impaired sperm maturation, leading to reduced sperm quality and quantity. Notably, partial depletion of the testicular macrophage pool was associated with transient defects in spermatogenesis and steroidogenesis, highlighting a coordinated immune contribution across both organs. Although the macrophage compartment repopulated over time, newly recruited cells exhibited altered phenotypes, coinciding with persistent abnormalities in sperm parameters.

Using a model of acute bacterial epididymitis induced by uropathogenic *Escherichia coli* (UPEC), macrophage-depleted mice developed exacerbated and earlier-onset inflammatory responses in both distal and typically protected proximal epididymal regions. This was accompanied by increased immune cell infiltration and more severe tissue damage compared to wild-type controls. Transcriptomic analyses indicated a failure to appropriately restrain proinflammatory and antibacterial gene programs, underscoring the importance of CX3CR1^{+} macrophages in limiting excessive immune activation.

Collectively, our findings identify CX3CR1^{+} macrophages as central regulators of epididymal immune homeostasis. They preserve epithelial integrity, support sperm maturation, and fine-tune immune responses to prevent excessive infammation and collateral tissue damage during infection. Ongoing work aims to clarify how additional immune cell populations and their interations contribute to sustaining this delicate immunological balance. In particular, we seek to define the factors that shape the region-specific imprinting of resident immune cells along the epididymal duct, which appears critical for coordinated immune regulation, optimal sperm maturation and male fertility.

Submission ID#2360706

Melatonin Administration Influences Semen and Preputial Microbiota that are Associated with Sperm Quality

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Abstract Text

Melatonin has been shown to modulate microbial community composition in the gut, however, the relationship between melatonin and microbes present in semen and the preputial environment has yet to be elucidated. Therefore, the objectives of this study were to investigate the effect of oral melatonin administration on the semen and prepuce microbiome and identify associations between microbes and factors relevant for fertility. Yearling Angus bulls (n = 21) were randomly assigned to melatonin (MEL; n = 11) or control (CON; n = 10) diets for 60 days. MEL bulls were supplemented with 200 mg/kg of body weight of melatonin dissolved in ethanol, while CON bulls received an equivalent ethanol vehicle control. Semen, obtained via electroejaculation, was aliquoted for further analysis along with preputial swabs on D0, 28, and 56. Semen samples were used for sperm quality analysis, testosterone and melatonin assays, and V3-V4 16S rRNA gene amplicon sequencing via the Illumina NextSeq2000. MicrobiomeAnalyst 3.0 was utilized to assess beta diversity while differences in alpha diversity, microbiota relative abundance at each taxonomic level, and Spearman correlations were performed using R Studio Version 4.3.1. Significant differences were noted at $P \leq 0.05$. There were no differences in semen or prepuce alpha diversity (Chao 1, Shannon) between CON and MEL ($P > 0.05$). Both semen ($P = 0.009$) and preputial ($P = 0.008$) beta diversity differed at D28 with MEL having more uniform community composition compared to CON. In semen, there was an overall effect of treatment at both the phylum and family level with MEL having lower relative abundance of the Gracilibacteria ($P = 0.04$), Acidaminococcaceae ($P = 0.01$), and Christensenellaceae ($P = 0.006$) compared to CON. In the prepuce, MEL had a lower relative abundance of the phylum Gracilibacteria ($P = 0.02$) compared to CON. Among semen and prepuce samples there were six unique genera that differed between MEL and CON groups. Interestingly, in D28 semen, melatonin administration decreased the relative abundance of *Comamonas* ($P = 0.03$) and *Lachnoclostridium* ($P = 0.0008$) which can act as opportunistic pathogens. At D56, *Helcococcus* ($P = 0.04$) and the *Ornithinimicrobium-Serinicoccus* group ($P = 0.02$) were decreased in MEL semen compared to CON. In contrast, the preputial genera *Flavobacterium* ($P = 0.02$) and *Leucobacter* ($P = 0.007$) were greater in the MEL group on D28 and D56 respectively. Several of these bacterial taxa were correlated with various physiological metrics. Melatonin concentrations in seminal plasma were negatively correlated with the relative abundance of Gracilibacteria, Acidaminococcaceae, Christensenellaceae, and *Lachnoclostridium* in semen ($P \leq 0.002$). There were various sperm quality parameters that were correlated with genera that did not have differential abundance between MEL and CON groups. Notably, *Actinomyces*, a common urogenital microbe, was positively correlated with VCL and ALH ($P \leq 0.001$) and negatively correlated with sperm linearity and wobble ($P \leq 0.001$). Additionally, Sphingobacteriaceae and *Sphingobacterium* had positive correlations with sperm motility ($P \leq 0.002$). Correlations with flow cytometric parameters revealed that both *Enterococcus* and *Staphylococcus* were positively correlated ($P \leq 0.01$) with the amount of acrosome reacted sperm. To the author's knowledge this study is the first to indicate that exogenous hormone administration modulates the semen and preputial microbiome. Furthermore, microbiota are not just passive components of the male reproductive environment but are associated both positively and negatively with sperm quality.

Submission ID#2360105

Multispecies Single-Cell Analysis and Functional Validation Reveal New Regulators of Spermatogonia Stem Cells Establishment

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Abstract Text

In sexually reproducing species, spermatogenesis is essential for male fertility and species continuity. Sustained sperm production depends on spermatogonial stem cells (SSCs), which arise during the perinatal period from prospermatogonial (ProSpg) precursors formed after fetal sex determination. Although the ProSpg-to-SSC transition has been described in mice, the molecular mechanisms regulating this process and their conservation across mammals remain poorly understood.

To help address this gap, our preliminary work used single-cell RNA sequencing to characterize gene expression in perinatal pig testes. Bioinformatic analysis identified several candidate regulators of the ProSpg-to-SSC transition that are highly expressed in human, pig, and mouse testes but whose functions have not yet been investigated. This project focuses on determining the role of one such candidate gene in the establishment of spermatogenesis. Prior research suggests that this gene may influence cell proliferation and survival in tissues with high metabolic demands, and it has also been implicated in regulating proliferation and apoptosis of cultured human SSCs. However, its *in vivo* function in male germline development remains unknown.

In this study, we used CRISPR-Cas9 technology to generate knockout mouse models and examine the gene's molecular role in male reproductive biology. The anticipated outcomes are expected to provide fundamental insight into mechanisms governing spermatogenesis, with broad implications for understanding male infertility and informing future diagnostic, therapeutic, and non-hormonal contraceptive strategies in humans and animals.

Submission ID#

Title Coming Soon

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Abstract Text

Back to Contraception: Integrating Biology, Technology, and Society

Tuesday, July 21 10:00 AM – 12:00 PM

Submission ID#2360146

Progress in the Discovery and Development of Nonhormonal Male and Female Contraceptives by Inhibiting Sperm-specific Ion Channels

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Abstract Text

We are targeting the genetically validated sperm-specific ion channels Na,K-ATPase-a4, CatSper, and Slo3 for male and female contraception. Development of Na,K-ATPase-a4 inhibitors is underway using two types of compounds, the natural product ouabain and the antihistamine cetirizine, which we identified through virtual high-throughput screening. We identified CatSper and Slo3 inhibitors through a phenotypic screen of 72,000 compounds. Ongoing efforts aim to optimize selective Na,K-ATPase-a4, CatSper, and Slo3 inhibitors and to develop dual-target inhibitors.

This research was supported by NIH/NICHD (R01 HD012623 and 1R61HD114221) and the Male Contraceptive Initiative.

Submission ID#2359085

CCL2 Directly Influences Human Oocyte Maturation and Cumulus–Oocyte Complex Gene Expression

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Abstract Text

Shortly before ovulation, the LH surge induces processes critical for fertility, including cumulus-oocyte expansion and resumption of meiosis. While some paracrine factors important for these events have been identified, the molecular mechanisms underlying their initiation remain poorly understood. Moreover, one of the biggest challenges for assisted reproductive technologies (ART) is to retrieve high-quality oocytes from patients. Thus, a better understanding of these two essential periovulatory processes would aid in identifying potential causes of infertility and/or novel markers of oocyte quality that could increase live birth rates in IVF clinics. Previous work from our laboratory identified the chemokine receptor CCR2 as a novel and critical intermediate in the ovulatory cascade, demonstrating that the CCR2 signaling pathway is downstream of the epidermal growth factor receptor (EGFR) pathway in a feline model. Based on these findings and the similarity of cat oocytes to their human counterparts, we hypothesized that signaling through the chemokine receptor CCR2, via its ligand CCL2, directly promotes human oocyte maturation. Therefore, to test this hypothesis, experiments were designed to: 1) study the involvement of CCR2 signaling in human oocyte maturation and gene expression in human cumulus–oocyte complexes (COCs) *in vitro,* and 2) assess possible crosstalk/interaction between EGFR and CCR2 signaling within human COCs. Individual human COCs aspirated from antral follicles (13-16 mm) from oocyte donors (mild stimulation without hCG, in compliance with IRB protocols and ethical standards) were cultured in SAGE IVM medium (Cooper Surgical). COCs (n=16 oocyte donors, 94 COCs) were cultured in the absence (Control) or presence of two concentrations of recombinant CCL2 (10 ng/ml and 100 ng/ml) at different time points. COCs were cultured for 3 h for transcriptomic analysis (RNAseq) and for 48 h to assess oocyte nuclear maturation. Also, a small cohort of COCs was randomly selected and fixed in 4% paraformaldehyde for indirect immunofluorescence to localize CCL2 and CCR2 proteins within human COCs. In a second experimental design, COCs (n=8 oocyte donors, 50 COCs) were cultured in the presence or absence of a specific EGFR inhibitor (1 μM), with or without CCL2 (10ng/ml) and/or gonadotropins (FSH+LH; 75 mIU/ml) to assess oocyte nuclear maturation. Recombinant CCL2 (10 ng/mL) in the IVM media significantly increased the percentage of MII-stage oocytes ($P < 0.05$). Although CCL2 trended towards reversing the inhibition induced by the EGFR inhibitor (FSH+LH: 80%; FSH+LH+EGFR INH: 40%; FSH+LH+EGFR INH+CCL2: 78%), this difference did not reach significance, likely due to a smaller sample size. Immunolocalization for CCR2 was observed in the cumulus cells, whereas the chemokine CCL2 was detected in both the cumulus cells and the oocyte. RNA sequencing revealed 1036 differentially expressed genes (DEGs) in response to CCL2 10 ng/ml vs untreated controls (676 upregulated, 360 downregulated). There were 865 DEGs in response to 100 ng/ml CCL2 compared with the control (399 upregulated, 466 downregulated). Between the two concentrations of CCL2, 972 DEGs were observed (277 upregulated and 695 downregulated). Pathway enrichment of p-adjusted genes revealed significant modulation of calcium ion transport, axon development, and the Golgi apparatus subcompartment. KEGG analysis indicated directional enrichment for calcium- and synapse-associated pathways and downregulation of MAPK signaling. Our results demonstrate that CCL2 directly affects human COCs by increasing the percentage of MII oocytes during spontaneous oocyte maturation and by modulating gene expression in vitro, suggesting that it could be beneficial in promoting IVM.

Submission ID#2349009

Lower Reproductive Tract Tissue Drug Levels of a Potential Non-Hormonal Contraceptive

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Abstract Text
In the lower female reproductive tract, cervical mucus viscosity regulates fertility throughout the menstrual cycle, promoting or preventing sperm advancement into the upper tract. Cystic fibrosis transmembrane conductance regulator (CFTR) is a principal ion channel that regulates the hydration and viscosity of mucus secretions. Previously, we demonstrated that INH-172, a CFTR inhibitor, thickens cervical mucus *in vitro*. Here, we utilized INH-172 in the non-human primate (NHP) model to evaluate the *in vivo* inhibition of CFTR in the lower reproductive tract. In this study, we investigated vaginal absorption of INH-172 by local epithelia (vaginal and cervix) as well as measured local and systemic concentrations of this molecule. To accomplish this, we placed INH-172 (2% w/w) prepared in a hydroxyethylcellulose gel (2.7% w/w HEC) into the vagina of the rhesus macaque (*Macaca mulatta*) both alone as well as soaked into sterile packing strips (Covidien Curity™). We obtained biopsies of both the cervix and vaginal fornix following INH-172 incubation for one to 24 hours and measured the INH-172 levels in the biopsies through high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). In animals receiving the gel formulation, median INH-172 levels were 75.74 ng/mg (N=16, IQR [50.46, 144.60]) and 88.35 ng/mg (N=17, IQR [69.13, 325.21]) in cervical and vaginal tissues, respectively. In the strip application study, levels were lower: 12.04 ng/mg (N=17, IQR [5.49, 28.92]) in cervical and 20.87 ng/mg (N=17, IQR [5.58, 73.90]) in vaginal tissues. The concentration of INH-172 did not significantly differ between the two delivery methods in the vaginal wall (p=0.22), although the gel formulation resulted in a higher concentration in the cervix compared to the strip application (p=0.032). Median INH-172 levels fell below the assay detection threshold in serum samples. In conclusion, we demonstrate that vaginal administration of an INH-172 gel results in elevated tissue drug levels both in the vagina and cervix of the rhesus macaque, confirming INH-172 absorption and distribution throughout the lower reproductive tract. In addition, the lack of serum drug level elevation indicates that vaginal dosing can result in local absorption with low risk of systemic effects.

Submission ID#

Title Coming Soon

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Abstract Text

Submission ID#2343179

Evaluating Channel Inhibition on Endocervical Mucus Hydration

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Abstract Text

In the epithelia of the endocervix, ion channels, water channels, and channel adjacent enzymes (channel supporting proteins) play critical roles in the mediation of mucus hydration and biophysical properties, directly impacting lower tract fertility. Selective perturbation of channels offers potential targets for disrupting natural fertility in the cervix. These channels in the cervix have not been fully characterized, and it is unknown if loss of function can affect endocervical mucus secretion. In this study, we utilize channel-specific small molecule inhibitors to evaluate a panel of channels in the endocervix and measure the resulting effect on mucus hydration. We cultured primary endocervical epithelial cells from the rhesus macaque (*Macaca mulatta*) and differentiated them at an air liquid interface (ALI). We evaluated nine potential channel targets (TMEM16A (ANO1), PDE4, SMPD3, NaK ATPase, AQP3, AQP4, NKCCQ, TRPV4, SLC34A2, and CFTR) using 21 unique small molecule inhibitors (CaCCinh-A01, MONNA, ANI9, T16Ainh-A01, Niclosamide, Benzbromarone, 1PBC, A9C, Roflumilast, GUDCA, GW4869, Ouabain, DFP00173, DFP00176, TGN-020, Bumetanide, HC067047, Compound 27, Inh-172, GlyH-101, and BPO-27). Measuring the change in Airway Surface Liquid (hydration layer above the cells) assessed the inhibitors effects on mucus hydration relative to the control using brightfield microscopy. We found 12 inhibitors altered ASL. Of the 12, two increased and eight decreased hydration; the last two had dose dependent effects. Of the 8 dehydrating inhibitors, two were specific for the cystic fibrosis membrane regulator (CFTR), three for the calcium chloride channel TMEM16A, two for aquaporin AQP3 and one to AQP4. Of the inhibitors that increased hydration, one was an inhibitor of phosphodiesterase 4 (PDE4) and the other sphingomyelin phosphodiesterase 3 (SMPD3). The final two inhibitors for TMEM16A demonstrated dose dependent hydration behavior. Of the inhibitors that rapidly dehydrated mucus, the largest effects were observed with CaCCinh-A01 and T16Ainh-A01, inhibitors of TMEM16A. Our study identified several ion and water channels that play a role in mucus hydration in the endocervix. Existing small molecule inhibitors offer the possibility for downstream drug development utilizing additional experimental models or platforms. Further studies are needed to characterize mucus altering properties between target/molecules as well as off-target effects.

Submission ID#2334373

Micronucleus-Mediated Ploidy Reduction in Triploid Mouse Embryonic Stem Cells

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Abstract Text

Diploidy is the default chromosomal composition of most mammalian cells including those in early embryos. However, errors such as polyspermy and failure of secondary polar body extrusion frequently occur which result in polyploid embryos and early developmental arrest. For example, triploid (3N) and tetraploid (4N) mouse embryos fail by embryonic day E10. Although electrofused 4N embryos can support extraembryonic tissues in 4N complementation, polyploid cells are largely excluded from the fetal lineage, indicating recognition of the intrinsic chromosomal defects. Despite these observations, the mechanisms underlying the elimination of polyploid cells from the fetal lineage and the arrest of polyploid embryos remain poorly understood. Here, we derived mouse embryonic stem cells (mESCs) from cytochalasin B induced 3N embryos as a model to study polyploid dynamics beyond the blastocyst stage. A total of 12 independent 3N mESC lines were established from 3N blastocysts. Flow cytometry cell cycle analysis revealed heterogeneous DNA content among the cell lines. Seven exhibited G0/G1 peaks between the expected 2N and 3N positions, suggesting a reduction in chromosome numbers from 3N toward 2N. Surprisingly the G0/G1 peaks of the remaining lines overlapped with the expected 2N peak, suggesting significant if not complete ploidy correction. To assess detailed chromosomal numbers, karyotyping was performed on five representative mESC lines from 3N blastocysts and three control 2N lines at 40 metaphase spreads per line. Regardless of their flow cytometry profiles, all five 3N lines displayed chromosome numbers below 60 with broad variations ranging from 5 to 60. Additionally, 91.3% of cells of the 3N lines lacked either exact 40 or 60 chromosome counts while 59.2% of 2N lines maintained the expected 40 chromosomes. These findings indicate that chromosome losses not only occurred in 3N but also in 2N mESCs. Immunofluorescence analysis of mitotic spindle (α-tubulin) on mitotic cells in both 3N mESCs and 3N blastocyst outgrowths revealed predominantly bipolar spindles (100% and 99.3%, respectively). This suggests that multipolar division is not the primary mechanism of chromosome loss in mESCs. We also examined mitotic spindles by immunofluorescence in 3N blastomeres, and observed chromatin bridges in anaphase spindles which are a hallmark precursor of lagging chromosomes and micronucleus formation. Consistently, numerous micronuclei were seen in the inner cell mass outgrowths of 3N embryos. To model this process dynamically, we stained 3N mESCs with a live DNA dye, SPY555, and performed overnight time-lapse imaging. Micronuclei repeatedly formed during mitosis, and some even underwent further division but most ultimately degraded. Together, these findings suggest that 3N cells of early development 1) recognized ploidy abnormality, and 2) attempted chromosomal reduction by continuous micronucleus formation and elimination rather than catastrophic mitotic failure, or tripolar mitosis. Our data also revealed that 2N mESCs can also undergo chromosomal losses by micronuclei.

From IVF to Embryogenesis

Tuesday, July 21 10:00 AM – 12:00 PM

Submission ID#2359913

The Power of Paralogues: *Sox2* and *Sox21* in Early Embryonic Development in Mice

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Abstract Text

Preimplantation development in mammals is marked by a series of precisely orchestrated molecular events from fertilization to implantation, and provides an excellent model for studying the initiation of pluripotency. In mice, SOX2 is the earliest marker of pluripotency. SOX2 labels pluripotent inside cells as early as the 16-cell stage, when other pluripotency factors such as OCT4 and NANOG are expressed in both pluripotent and non-pluripotent cell types. However, *Sox2* is dispensable for formation of pluripotent epiblast cells in the blastocyst, suggesting that closely related *Sox* paralogues may compensate for the loss of *Sox2* during blastocyst formation. We discovered that the closely related *Sox* paralogue *Sox21* is expressed at both the mRNA and protein levels alongside *Sox2* prior to blastocyst formation. Moreover, *Sox21* mRNA and protein persist in *Sox2* null embryos, indicating a potential compensatory role. Using knock-out mouse models, we showed that loss of any three alleles of *Sox2* and *Sox21* impairs blastocoel formation, suggesting a dose-dependent, combinatorial role of these paralogues prior to cavitation. Subsequently, these embryos frequently fail to hatch from the zona pellucida, suggesting an implantation defect. Consistent with this, loss of one allele of *Sox2* impairs survival of otherwise viable *Sox21* null mice after birth. Strikingly, *Sox2/Sox21* double knockout embryos show an even more severe phenotype: they completely lack a blastocoel and exhibit pronounced morphological defects, including reduced cell numbers and disrupted organization at the blastocyst stage. This preimplantation arrest underscores the essential, overlapping contributions of these paralogues to early embryo viability. Furthermore, we found that *Sox21* expression is independent of NANOG and OCT4, but, like *Sox2*, is regulated by TFAP2C, highlighting shared regulatory inputs. These data suggest that distinct yet overlapping regulatory pathways involving *Sox* paralogues converge to regulate early embryonic development. These studies will advance our understanding of how *Sox* paralogues cooperatively regulate gene networks that establish early cell identity during embryonic development and may further our knowledge of the pathways involved in early embryo viability. This work is supported by NIH R01 HD108722 awarded to A.R.

Submission ID#2360842

Replication Timing and Chromosomal Stability in Bovine Preimplantation Development

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Abstract Text

Replication stress is a critical challenge during early embryonic development, leading to chromosomal instability and segregation errors that result in aneuploidy and developmental arrest. Environmental stresses induced during in vitro culture are thought to increase this risk, yet systematic identification of dynamics of chromosomal stability between in vitro and in vivo produced embryos remain unexplored. Understanding how replication dynamics differ between these conditions is essential for improving culture systems and reducing the incidence of developmental failure. Here, we report the first analysis of replication dynamics during bovine preimplantation embryo development in vitro and in vivo by using EdU incorporation and single-cell whole-genome sequencing (scWGS). EdU labeling revealed S-phase progression at the 1-cell stage, with replication starting at about 9 hours post-fertilization, peaking between 12–17 hours, and then declining. The replication timing programs are already established at the 4-cell stage and remain broadly conserved through the 16-cell and blastocyst stages. Late-replicating domains are frequently observed in gene-poor and intergenic regions, whereas gene-rich regions tend to replicate early. Further analyses showed that open chromatin regions replicate earlier at the 4-cell and 16-cell stages. In addition, aneuploidy rates are about 32% at the 4-cell stage but decreased to 24% and 28% at the 16-cell and blastocyst stages, respectively, suggesting potential mechanisms of error correction or selective elimination of abnormal cells during development. We also collected different stages of in vivo produced embryos and performed scWGS analysis. When embryos from stages before (8-cell and before) and after (16-cells and beyond) ZGA stages are pooled, no significant difference in aneuploidy rate is observed between the in vivo and in vitro produced embryos. In summary, our work comprehensively measured DNA replication timing and chromosomal stability during preimplantation development in bovine. On-going comparative analyses with transcriptional activity, DNA methylation, histone modifications and genome-nuclear lamina interactions will further identify the mechanisms by which late and/or incomplete replication result in chromosome fragility, aneuploidy, and poor developmental potential in the early embryo.

Submission ID#

Title Coming Soon

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Abstract Text

Submission ID#2357285

A Single Amino-acid Mutation in MEIG1 Protein Impairs Normal Embryo Development

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Abstract Text

Normal embryonic development requires the fusion of a haploid spermatozoon and a haploid oocyte. Sperm defects account for at least 50% of abnormal embryogenesis. Intracytoplasmic sperm injection (ICSI) bypasses fertilization barriers such as low sperm count or motility and serves as both a clinical tool and a research platform to investigate genetic causes of male infertility. MEIG1 encodes a 10 kDa protein essential for normal spermatogenesis and embryo development. In early germ cells, MEIG1 is distributed throughout the cell bodies, but in elongating spermatids it is recruited to the manchette by PACRG. The manchette is a transient microtubule/F-actin structure implicated in sperm chromatin remodeling, a process involving replacement of histones first by transition proteins (TNPs) and then by protamines (PRMs). Proper chromatin packaging is critical for maintaining sperm DNA integrity and supporting embryo viability. Structural analysis identified 12 amino acids on MEIG1 predicted to mediate protein–protein interactions, particularly with PACRG. Mutation analysis revealed that tyrosine 68 (Y68) is essential for MEIG1–PACRG interaction. To assess its *in vivo* role, a single amino acid mutant mouse model (MEIG1^{Y68A}) was generated. Male mutants phenocopied global *Meig1* knockout mice, displaying abnormal spermatogenesis and infertility. Using ICSI, sperm heads from MEIG1^{Y68A} mutant mice were injected into wild-type oocytes. Embryos derived from mutant sperm exhibited poor fertilization rates and delayed development, with few reaching the blastocyst stage by day 4 and none progressing further. In contrast, control sperm supported normal embryonic development. Functional assays revealed severe sperm DNA damage in mutants via COMET analysis. Additionally, mutant sperm showed positive staining for Aniline blue and Chromomycin A3, indicating defective chromatin compaction and impaired protamination. These findings demonstrate that Y68 is critical for MEIG1's role in sperm chromatin remodeling, fertilization, and embryo development. In summary, this work reinforces the essential function of MEIG1 in maintaining sperm chromatin integrity. It also highlights Y68 as a potential molecular target for male contraception. Small molecules that inhibit MEIG1 or disrupt its interaction with PACRG may provide a novel strategy for male contraceptive development. Furthermore, MEIG1-associated pathways may serve as diagnostic markers for sperm quality in assisted reproductive technologies and infertility research

Submission ID#2360570

A Chemically Defined Bovine Trophoblast Stem Cell Platform Reveals Transcriptional Poising and Supports Blastocyst-Like Self-Organization

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Abstract Text

Trophoblast stem cells provide a powerful experimental system to understand early embryo–maternal communication, mechanisms of placental development, and pregnancy establishment in mammals. In cattle, the trophectoderm is indispensable for conceptus elongation and for secretion of pregnancy recognition signals, and it orchestrates the earliest fetal–maternal interactions that enable implantation and formation of a functional placenta. Consequently, disruption of trophoblast proliferation, differentiation, or signaling during this narrow peri-implantation window can directly compromise embryo survival and implantation success, coinciding with the high incidence of early pregnancy loss in dairy cattle. Although *in vitro* bovine TSC systems to study trophoblast development have been described, these platforms typically depend on feeder layers and/or conditioned media that introduce batch variability and constrain reproducibility, scalability, and standardization. To address these limitations, we established a serum-free, feeder-free, chemically defined platform for the derivation and long-term maintenance of bovine trophoblast stem cells (bTSCs) from IVF-derived blastocysts. bTSCs maintained stable morphology and molecular identity over extended passaging and remained developmentally competent, including the ability to differentiate in response to differentiation cues, recapitulating a hallmark trophoblast transition relevant to embryo–uterine attachment and placental function. Transcriptomic profiling of these cells demonstrated retention of an undifferentiated trophoblast stem state, with sustained expression of key trophoblast lineage regulators. Notably, the same custom-defined medium also supported derivation and maintenance of bovine placental cells from first-trimester placental tissue, indicating broader applicability across early placental cell types. To resolve the regulatory landscape underlying future lineage commitment, we performed precision run-on sequencing (PRO-seq) to map genome-wide RNA polymerase II activity at nucleotide resolution, revealing a subset of transcriptionally poised genes marked by elevated promoter-proximal pausing with limited productive elongation. In parallel, we established a three-dimensional trophoblast culture system (“trophocysts”) that resemble blastocysts lacking an inner cell mass, enabling interrogation of trophoblast–maternal communication and systematic testing of extracellular matrix–dependent effects on bTSC growth and differentiation. Finally, bTSCs assembled into blastocyst-like structures when co-cultured with primed embryonic stem cells in a custom-defined medium, demonstrating self-organization and interlineage interaction *in vitro*. Collectively, these platforms establish bTSCs as a robust, scalable model for mechanistic dissection of early placental development and embryo–maternal interactions, and they provide a foundation to define pathways that underpin reproductive success in livestock.

Submission ID#2353110

Anti-Müllerian Hormone is a Sexually Dimorphic Regulator of Gonadotropin and Steroidogenic Gene Expression in the Human Preimplantation Embryo

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Abstract Text

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive age women characterized by hyperandrogenism, irregular menstrual cycles, polycystic ovaries, and elevated Anti-Müllerian Hormone (AMH). While AMH primarily serves as a clinical marker of ovarian reserve, elevated levels in the follicular fluid of PCOS patients have been associated with altered oocyte maturation and embryo quality. Although AMH does not cross the placental barrier, the preimplantation embryo is exposed to maternal AMH during its transition from the oviducts to the uterus. Following our identification of transient expression of the AMH receptor, *AMHR2,* in the mouse and human pre-implantation embryo, we aimed to investigate the impact of exogenous AMH on the blastocyst transcriptome. Donated day-5 cryopreserved embryos were warmed and cultured for 24 hours in medium supplemented with recombinant human AMH (1.0 µg/mL) or PBS vehicle. Morphologically intact blastocysts were selected for single-embryo RNA-sequencing using the SMART-Seq v4 workflow and a Novaseq sequencer. Following digital karyotyping and sexing, a total of 45 embryos were analyzed for differential gene expression: 22 AMH-treated (9 XX, 13 XY) and 23 control embryos (14 XX, 9 XY). While no significant expression differences were found in XY embryos following treatment, AMH-treated XX embryos exhibited significant downregulation of human chorionic gonadotropin subunits, including *CGB8* (logFC = -7.90, FDR < 0.001), *CGB5* (logFC = -7.01, FDR < 0.0001), and *CGA* (logFC = -8.79, FDR < 0.001). Additionally, expression of aromatase (*CYP19A1*) was significantly reduced (logFC = -3.31, FDR = 0.034). Analysis of a published embryonic single-cell atlas confirmed that *CGA*, *CGB*, and *CYP19A1* are coexpressed with *AMHR2*, and that these cells are primarily localized to the trophectoderm. These results suggest that AMH exposure produces a sex-specific transcriptomic impact, selectively downregulating gonadotropins and aromatase in female blastocysts, functions that parallel its role in gonadotropes and granulosa cells, respectively. We hypothesize that supra-physiological AMH exposure may perturb trophectoderm differentiation and early maternal-fetal hormone signaling, developmentally priming the female reproductive axis for future dysregulation. Together, these findings provide a potential mechanistic link between elevated maternal AMH levels and altered placental steroid metabolism that could contribute to the transgenerational inheritance of PCOS.

Submission ID#2347317

Triiodothyronine (T3) Drives Lipid Remodeling via Epigenetic Regulation to Stimulate Metabolism in Preimplantation Embryos

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Abstract Text

Thyroid hormones are fundamental regulators of energy homeostasis, cellular differentiation, and organogenesis. Beyond their canonical metabolic roles, triiodothyronine (T3) has emerged as an epigenetic modulator that interacts with nuclear receptors to induce histone modifications. However, how T3 integrates metabolic signaling with epigenetic regulation during *in vitro* embryonic development remains unclear. In present study, transcriptomic analysis revealed distinct metabolic signatures between *in vivo* and *in vitro* embryos, particularly in fatty acids metabolism at the 4-cell and blastocyst stages. Supplementation with 50 nM T3 significantly enhanced blastocyst formation, with the most pronounced effect observed in late T3 treatment (from the 4-cell to blastocyst stages), accompanied by increased thyroid hormone receptor expression. T3 reduced lipid droplet size and intensity while enhancing mitochondrial activity and lipid–mitochondria colocalization, indicating improved lipid catabolism and mitochondrial coupling. Mechanically, inhibition of the histone acetyltransferase PCAF suppressed histone acetylation, reversed T3-induced developmental improvement, and altered lipid degradation pathways. T3 promoted cytosolic lipolysis, whereas PCAF inhibition redirected lipid degradation toward lipophagy. Furthermore, T3 enhanced fatty acid–mitochondria interactions and upregulated β-oxidation, thereby increasing mitochondrial membrane potential. T3 also elevated prostaglandin synthesis–related genes, suggesting enhanced implantation potential, which was diminished under epigenetic suppression. Collectively, these findings demonstrate that T3 promotes lipid remodeling and mitochondrial activation through PCAF-mediated histone acetylation, thereby enhancing developmental competence in porcine embryos and revealing a previously unrecognized metabolic–epigenetic axis in mammalian embryogenesis.

Environmental Causes of Pregnancy and Placental Disruptions

Tuesday, July 21 10:00 AM – 12:00 PM

Submission ID#2354203

Impact of Mild Heat Shock After Genome Activation for Development and Establishment of Pregnancy of the Bovine Preimplantation Embryo

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Abstract Text

Heat stress can have catastrophic effects on female fertility. The early preimplantation embryo is susceptible to detrimental effects of elevated temperature (i.e., heat shock) but acquires thermotolerance after embryonic genome activation (EGA). However, it remains unclear whether heat shock after EGA compromises blastocyst ability to establish pregnancy. Here, it was tested whether heat shock at day 4 post insemination impacts embryo development and competence to establish pregnancy. Experiment 1 involved embryos generated in vitro using abattoir-derived oocytes. Embryos were cultured continuously at 38.5°C (normal cow body temperature; n=600 putative zygotes in 10 replicates) or were heat shocked at 41.0°C for 6 h on day 4 (n=600 putative zygotes), with the remainder of culture at 38.5°C. Neither percent of putative zygotes becoming a blastocyst at day 7.5 (P=0.8077; 50.04 ± 9.00% for 41 vs 49.30 ± 9.0% for 38.5°C) nor percent of cleaved embryos becoming a blastocyst was affected by heat shock (P=0.5286; 58.21 ± 10.7% for 41 and 56.18 ± 10.6% for 38.5°C). For Experiment 2, Holstein embryos produced with abattoir-derived oocytes (Holstein) and semen from Holstein bulls were cultured in a time-lapse incubator and treated as in Experiment 1 (n=84 per treatment). There was no effect of temperature on percent development to the blastocyst stage but time to formation of the morula (P=0.056), compact morula (P=0.063), early blastocyst (P=0.058) and blastocyst (P=0.045) was later or tended to be later for embryos exposed to 41.0°C. For experiment 3, oocytes were collected by transvaginal ultrasound-guided follicular aspiration and fertilized in vitro. Cows were assigned to one of two treatments (38.5 continuously or 41.0°C for 6 h on day 4). Embryos were from two genetic groups: B. taurus (n=59 donors) or B. indicus and B. indicus crosses (n=30 donors). Blastocyst-stage embryos were transferred to cyclic recipient females (n=184) on day 7 after ovulation, and pregnancy diagnosis was performed on day 30 by ultrasound. Treatment at 41°C increased (P=0.058) the proportion of cleaved embryos becoming blastocysts for B. taurus (40.4 ± 4.7% vs 27.1 ± 4.7%) but not for B. indicus and B. indicus crossbred donors (40.2 ± 6.4% vs 42.3 ± 5.5%; P=0.731). There was no significant effect of temperature on pregnancy establishment although numerically, pregnancy rate for B. taurus embryos was higher for 41°C (44.7%, 22/46) than 38.5°C (36.7%, 11/30). Pregnancy rate for B. indicus and B. indicus crossbred embryos was similar for 41°C (44.7%, 21/47) and 38.5°C (44.6%, 25/56). Exposure to heat shock after EGA does not reduce blastocyst production and may improve competence of the embryo to establish pregnancy. This beneficial effect occurs for embryos from breeds whose cells are sensitive to heat shock (B. taurus) but not for embryos from breeds that are resistant to heat shock (B. indicus).

Submission ID#2360536

The Effects of Heat Shock on Bovine Endometrial Epithelial Cell Responses to Pyolysin, a Cholesterol Dependent Cytolysin

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Abstract Text

Following parturition, bacterial contamination of the uterus is ubiquitous in dairy cattle. This infection can develop into uterine diseases, including metritis and endometritis, which have negative consequences on production and reproduction. Heat stress in cattle is also associated with subfertility and increased morbidity, including uterine diseases. *Trueperella pyogenes* is a Gram-negative bacteria associated with prolonged and severe cases of uterine disease in cattle, and produces the cholesterol-dependent, pore-forming toxin, pyolysin. We hypothesized that heat shock would modulate the cellular response of bovine endometrial cells to pyolysin related to uterine disease. To test our hypothesis, a bovine endometrial epithelial cell line was exposed to recombinant pyolysin (rPLO) for 2 hours at either thermoneutral (38.5°C) or heat shock (40°C; emulating heat stress) temperatures in vitro to assess cell viability and cholesterol abundance. Bovine red blood cells were exposed to rPLO (18.8 – 250 ng/mL) for 30 min to evaluate cytolytic activity. Fifty percent lysis was achieved with 85.8 ng/mL of rPLO, which was defined as one hemolytic unit (HU). Next, an endometrial epithelial cell line was exposed to either thermoneutral or heat shock temperatures for 22 hours before a 2-hour exposure to rPLO (0.125 – 4 HU) to evaluate cell viability. Exposing cells to 1, 2, and 4 HU of rPLO caused complete cell lysis compared with controls ($P < 0.001$), while 0.5 HU resulted in 44.7% cell lysis. Exposing cells to 0.25 HU or below did not induce cell lysis. Heat shock did not affect cell viability in response to rPLO ($P = 0.461$). We also evaluated cellular cholesterol as the primary target of pyolysin. Cells were exposed to either thermoneutral or heat shock temperatures in normal serum (20% v/v) or reduced serum (2% v/v) medium for 24 hours. Heat shock did not alter cellular cholesterol ($P = 0.784$), while reduced serum decreased cellular cholesterol by 11.5% ($P = 0.013$). There was no interaction between reduced serum and heat shock ($P = 0.790$). Finally, we investigated the cellular response to sequential rPLO exposure with and without heat shock. Cells were exposed to two sequential periods of thermoneutral or heat shock temperatures for 10 hours followed by a 2-hour exposure to 0.5 HU of rPLO or fresh media in a cross over design. Cell viability was evaluated after each exposure. When cells were exposed to rPLO once, followed by control medium, heat shock increased cell viability ($P < 0.01$) by 91.75% compared to thermoneutral cells. Heat shock also tended ($P = 0.057$) to increase viability by 162% when cells were exposed to two sequential doses of rPLO. Together, these data show that acute heat shock does not modulate viability or cellular cholesterol of bovine endometrial cells in response to rPLO. However, heat shock does increase cell viability when cells are exposed to sequential, sublytic concentrations of rPLO. This suggests that heat shock modulates cytoprotective mechanisms of cells exposed to pyolysin that may be important in disease pathogenesis of heat stressed animals.

Submission ID#2361246

Exposure to Polystyrene Nanoplastics During Pregnancy Influences Placenta Function in a Sex Specific Manner in Mice

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Abstract Text

Plastic production has been increasing exponentially, surpassing 460 million tons last year. However, the large majority of this plastic is not recycled and instead degrades into smaller particles known as nanoplastics. It is estimated that the average human consumes approximately a credit card's worth of plastic particles each week. It was recently discovered that plastic particles can travel into the human placenta. The placenta is a temporary organ that develops at the maternal-fetal interface during pregnancy to support the developing fetus through the exchange and production of essential nutrients and . Our study aims to identify how these particles influence placenta function in the mouse model. We orally dosed pregnant female CD-1 mice with either vehicle control or 50 nm or 200 nm polystyrene nanoplastic particles at 5 mg/kg/day from embryonic day 8 to day 15. Placentas were then collected and sliced in half with one half frozen for gene expression analysis and the other half fixed for morphology analysis. For morphology, we looked at the area of the placenta layers including the decidua, the basal zone, and the labyrinth zone as each layer has a unique function that can point to potential disrupted pathways. We observed a statistically significant decrease in the area of the decidua in the placentas for the 200 nm treatment group and a borderline significant decrease in decidua area for the 50 nm treatment group compared to control. However, when we separated by sex, only the male deciduas were significantly decreased in the 200 nm group and borderline decreased for the 50 nm group. The labyrinth zone was borderline significantly increased for females in the 50 nm group. We also looked at the area of the maternal and fetal blood spaces and the length of the intrahemal barrier between them to ensure proper nutrient and waste exchange between the mother and fetus. These measurements revealed the maternal blood space was significantly decreased in size and the intrahemal barrier was borderline significantly decreased for the 200 nm males compared to controls. For gene expression, RNA was extracted, converted to cDNA and then evaluated using qPCR. Our analysis revealed that expression of *Smad3* and *Cfl1* borderline statistically significantly increased and *Prl3d1* and *Esr1* borderline decreased for the 50 nm group. In the 200 nm group, *Smad2* and *Smad3* borderline increased, and *Gdf15* borderline decreased when compared to the control. However, when we separated based on sex, placenta growth factor (*Pgf*) was borderline increased for the 200 nm particles and *Pparg* was borderline increased for the 50 nm treatment group females. For the males we saw a significant decrease in cell cycle gene *Cyp11a1* for the 50 nm treatment group and *Smad3* significantly increased for the 200 nm group. Additionally we observed a borderline decrease in *Tgfb1* and *Esr1* for the 50 nm group males. Ultimately, we learned that exposure to plastics can influence placenta function in a manner that is dependent on both the size of the plastic particle and the sex of the placenta. Future studies will evaluate changes in hormone production due to our findings related to the decidua and *Esr1*. We will also evaluate transforming growth factor beta (TGF-β) signaling due to alterations observed in *Tgfb1*, *Smad2*, and *Smad3*.

Submission ID#2357685

Investigation of the Effects of Phthalate Exposure on Human Trophoblast Development

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Abstract Text

During early pregnancy in humans, maternal uterine endometrial stromal cells and fetal extraembryonic trophoblast cells engage in bidirectional cell-to-cell communication to ensure successful placentation, making this period a particularly sensitive window for environmental toxicants to influence pregnancy outcomes. Phthalates are a class of commercially important endocrine-disrupting chemicals widely used in plastics, personal care products, and medical devices, leading to widespread human exposure. Phthalates have been detected in blood, urine, semen, amniotic fluid, and breast milk. Epidemiological studies have reported associations between urinary phthalate levels and adverse pregnancy outcomes. We previously reported that exposure to monoethylhexyl phthalate (MEHP), a major bioactive metabolite of the plasticizer di (2-ethylhexyl) phthalate, impairs the function of human endometrial stromal cells (HESCs) in vitro, disrupting uterine cell-to-cell communication. Given the bidirectional communication at the maternal-fetal interface, it is important to understand the impact of phthalates on human extraembryonic trophoblast development and function.

In this study, we used an in vitro human trophoblast stem cell differentiation system to investigate the effects of MEHP on trophoblast development, focusing on the differentiation of trophoblast stem cells into invasive extravillous trophoblast (EVT) cells, which are essential for placentation. MEHP was added at an environmentally relevant concentration during differentiation. The results indicated that MEHP exposure significantly disrupted gene expression patterns during differentiation in trophoblast cells compared with vehicle controls. Notably, MEHP exposure downregulated matrix metalloproteinase 2 (MMP2), a key marker of functional differentiation. Previous studies have shown that EVT cells rely in part on MMP2 to remodel endometrial tissue, enabling invasion and the establishment of the maternal blood supply to support fetal development. Consistent with this role, we demonstrated that MEHP-treated EVT cells exhibited impaired EVT invasion, as shown in a transwell invasion assay. Furthermore, HLA-G expression, a key mediator of immune tolerance at the maternal-fetal interface, was increased in the MEHP-treated group, suggesting disruption of the maternal-fetal immune balance. Additionally, we performed transcriptomic profiling of MEHP-treated EVT cells in vitro using RNA sequencing. Our results revealed that a large number of interferon-responsive genes (ISGs) were altered by MEHP exposure. Previous studies have shown that ISGs are integral to EVT differentiation and trophoblast invasion, and that dysregulated ISG expression is associated with placental disorders. Therefore, MEHP-induced impairment of trophoblast invasion may involve disruption of ISG signaling pathways. Overall, our findings indicate that environmentally relevant MEHP exposure impairs trophoblast invasion by suppressing MMP2 and interferon signaling while aberrantly increasing HLA-G expression, thereby disrupting the maternal–fetal interface during early pregnancy.

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Submission ID#2359693

Impact of Prenatal Cannabis Exposure on Fetal, Infant, and Placental Health

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Abstract Text

The prevalence of prenatal cannabis use continues to rise, which is concerning because studies have shown that it is associated with preterm birth and small for gestational age infants. However, the impact of prenatal cannabis use on fetal developmental biology and programming is not well understood. The objective of our study was to further characterize the deleterious effects of cannabis exposure during pregnancy on fetal, infant, and placental health, and the epigenome.
Here we leveraged our established non-human primate model of edible delta-9-tetrahydrocannabinol (THC) consumption that recapitulates human medical cannabis use. Pregnant rhesus macaques consumed a daily edible containing either THC (2.5 mg/7 kg/day, equivalent to a heavy human medical cannabis dose) or placebo (CON) pre-conception and throughout pregnancy. Animals underwent serial ultrasound and MRI at gestational days 85 (G85), G110, G135 and G155 (full term is ~ G168). Half of the cohort underwent cesarean delivery and placental collection at G155 for histologic, RNA-Seq, and DNA-methylation analysis. The remaining half of the cohort delivered naturally at term with serial postnatal infant brain MRI on postnatal day (P)30 and P180, and behavioral testing (i.e., motor and emotional) performed on P30 and P120. Tissue collection was performed at P180 for histologic and molecular analysis.
THC-exposed pregnancies had significantly decreased amniotic fluid volume ($p < 0.001$), placental perfusion ($p < 0.05$), and fetal oxygen availability ($p < 0.05$), all indicators of placental insufficiency. Placental and fetal brain histological analysis demonstrated evidence of ischemic injury with microinfarctions present in THC-exposed animals only. Bulk RNA-seq demonstrated that THC alters the placental transcriptome and pathway analysis suggests dysregulated vasculature development and angiogenesis pathways. In half the cohort, DNA methylation was measured in 5 tissues collected at cesarean delivery (placenta, lung, cerebellum, prefrontal cortex, and right ventricle of the heart) using the Illumina MethylationEPIC platform. In utero exposure to THC was associated with differential methylation at 581 CpGs, with 573 (98%) identified in placenta. Loci differentially methylated with THC were enriched for candidate autism spectrum disorder (ASD) genes from the Simons Foundation Autism Research Initiative (SFARI) database in all tissues.
In the other half of the cohort, at P180 infants exposed to THC *in utero* displayed signs of increased stress and anxiety, including higher cortisol levels, significantly greater vocalizations in response to novel stimuli ($p=0.0017$), and difficulties with parental separation. Brain MRI at P180 revealed higher fractional anisotropy in the corpus callosum and centrum semiovale in THC-exposed infants ($p< .05$). Whole-genome bisulfite sequencing showed regions differentially methylated with THC in the cerebellum were involved in synaptic organization and brain development and ASD genes from the SFARI database. RNA-seq analysis of cerebellum identified 10 differentially expressed genes (DEGs) at FDR significance, pathway analysis of nominally significant DEGs (935, $p< 0.05$) identified enrichment of genes related to axon development and glutamatergic synapse.
Overall, our findings reveal that prenatal THC exposure alters placental and fetal/infant DNA methylation at genes involved in neurobehavioral development that was associated with an adverse effect on infant emotional behavior as well as brain microstructure changes that may have long-term implications for offspring outcomes. The data from this study add to the limited existing literature to help guide patient counseling focused on prenatal cannabis use.

Submission ID#2361016

Maternal Effects of Low Intensity Exercise from Early to Mid-Gestation in a Sheep Model

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Abstract Text

Despite the knowledge that exercise is beneficial for overall health, how maternal exercise indirectly benefits the fetus and/or placenta is not well understood, and cannot be easily studied in humans. Sheep are widely used as a model for human pregnancy and can be utilized to study molecular mechanisms during gestation. The objective of this study is to determine the impact of low-intensity exercise during gestation, and we hypothesize that exercise improves maternal health which will subsequently benefit fetal-placental growth. Nulliparous polypay ewes (n=20) were bred and randomly assigned to either an exercised (n=11) or sedentary group (n=9) and housed in individual pens. All ewes are fed the same diet of grain and orchard grass pellets to meet or slightly exceed 100% NRC requirements, and the refusals are weighed and recorded daily to determine feed intake. The exercised ewes are subject to 15 minutes of low-intensity walking twice daily starting prior to gestation and continuing thereafter. Blood glucose concentration and body weight are determined biweekly. At gestational day 120, ewes will be humanely euthanized, and maternal organs, fetal organs, and placental tissues will be collected, weighed, and stored for future molecular analyses. Existing daily feed intake, blood glucose concentration, and maternal body weight have thus far been analyzed out to day 45 of gestation using a mixed linear model with repeated measures. Feed intake increased with gestational age ($P < 0.001$), but no significant treatment or treatment by time interaction was observed. Blood glucose levels also changed across gestation ($P < 0.001$) without a significant effect of exercise. Body weight increased over gestation ($P < 0.001$), and there was a tendency for greater weight gain in the exercised group ($P = 0.056$). These findings indicate that low-intensity maternal exercise during early to mid-gestation does not disrupt evaluated metabolic parameters. Future analyses will investigate the effect of low-impact maternal exercise on salivary and circulating maternal cortisol concentrations, as well as organ-specific gene and protein expression to characterize the tissue-level adaptations and physiological responses in maternal and fetal samples.

Submission ID#2361125

The Effects of Chronic Exposure to DEHP and DiNP on Uterine Endometrial Glycogen in Mice

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Abstract Text

Phthalates are endocrine-disrupting chemicals (EDCs) mainly used as solvents and plasticizers and, hence, are present in many consumer products. Di-2-Ethylhexyl phthalate (DEHP) and Di-isononyl phthalate (DiNP) are widely used in food and beverage packaging and have been linked to several reproductive disorders. Successful establishment of pregnancy involves dramatic metabolic changes in the embryo and uterus during the first weeks of pregnancy. Glucose is one of the most important nutrients for developing embryos. Glucose uptake by the embryo increases approximately 50-fold as the embryo enters the uterus. Within the endometrium, glucose is metabolized via glycolysis and the pentose phosphate pathway (PPP). It can be used to glycosylate proteins, which is important for embryo attachment and stromal decidualization. Uterine glucose must come from maternal circulation, as the uterus does not express gluconeogenic enzymes, and uterine glycogen is believed to act as a buffer for maintaining optimal glucose levels in the uterine epithelium and decidua. Phthalates have been shown to alter glycogen metabolism in rat liver, but their effect on glycogen in the endometrium is unknown. Thus, the objective of this study was to explore the effects of DEHP and DiNP on uterine glycogen metabolism. Phthalates were administered to mice (12 to 14 per group) ad libitum in the rodent chow. Three levels of exposure for DEHP and DiNP were used at 0.15 ppm, 1.5 ppm, and 1,500 ppm for 9 months. Uteri were collected at diestrus. In DEHP-treated mice, periodic acid Schiff (PAS) staining showed that glycogen level in the glandular epithelium (GE) was significantly lower in all three DEHP groups compared to the control. In the luminal epithelium (LE), only the 1.5 ppm DEHP-treated group had significantly lower levels of glycogen compared to control. Stromal glycogen content was lower in both 1.5 and 1500 ppm DEHP-treated groups compared to the control ($P< 0.05$). qPCR indicated no significant differences among the groups in gene expression for the following enzymes: hexokinase1 (HK1, the enzyme that phosphorylates glucose), glycogen synthase1 (GYS1), glycogen phosphorylase (PYGM), and glucose-6-phosphatase (G6PC, the enzyme that dephosphorylates glucose-6-phosphate). To look at tissue-specific levels of these enzymes, immunohistochemistry was performed. Stromal immunostaining of HK1 in all three treated groups appeared more intense compared to the control, while it was similar among groups in GE and LE. Immunostaining for GYS1 was similar in all 4 groups. In contrast, immunostaining levels for PYGM appeared more intense in GE, LE, and stroma of mice treated with 1500 ppm of DEHP compared to the control. Immunostaining for G6PC3 appeared not to be different from control. In the DiNP-treated mice, all three concentrations of DiNP significantly decreased glycogen levels in GE, LE, and stroma. The qPCR results did not show significant differences in gene expression of the glycogen-metabolizing enzymes among groups. Stromal immunostaining of HK1 in all three groups appeared more intense than the control, while GE and LE immunostaining appeared similar to control. Immunostaining of GYS1 did not appear different than control. Interestingly, similar to DEHP-treated groups, immunostaining level for PYGM appeared more intense in GE, LE, and stroma of mice treated with 1500 ppm of DiNP compared to the other concentrations of DiNP and control. Immunostaining for G6PC3 was also not different compared to the control. These results collectively suggest that both DEHP and DiNP impact glycogen metabolism and may alter early pregnancy success

Shaping Implantation: From Glandular Origins to Uterine Models

Tuesday, July 21 1-:00 AM – 12:00 PM

Submission ID#2360961

An Epigenetic SIN3A Program Governs Postnatal Uterine Morphogenesis and Female Fertility

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Abstract Text

Postnatal uterine morphogenesis and gland development are critical processes in most mammals and involve coordinated organization of endometrial stromal cells, myometrial differentiation, and endometrial gland formation. Here, we identify Switch-Independent 3A (SIN3A) as a previously unrecognized epigenetic regulator of uterine morphogenesis and adenogenesis. SIN3A is a highly conserved scaffold protein and transcriptional coregulator that modulates chromatin architecture and gene expression through context-dependent recruitment of chromatin-modifying complexes. Depending on associated cofactors, SIN3A can mediate transcriptional repression or activation by altering chromatin accessibility. Analysis of human and mouse uterine tissues revealed that SIN3A is expressed in both epithelial and stromal compartments, with elevated expression during the window of implantation and dynamic regulation during stromal decidualization, including age-dependent changes. Given that SIN3A mutations are driver events in uterine corpus endometrial carcinoma, we generated a progesterone receptor-driven conditional knockout mouse model, *Pgr*$^{Cre/+}$*Sin3a*$^{f/f}$ (*Sin3a*$^{d/d}$). Unexpectedly, rather than developing endometrial hyperplasia, adult *Sin3a*$^{d/d}$ females exhibited markedly reduced uterine size and complete infertility. Histological and molecular analyses demonstrated that *Sin3a*$^{d/d}$ uteri display reduced uterine diameter, diminished stromal compartment, and a profound loss of endometrial glands compared with controls, despite preserved FOXA2 expression in glandular epithelium. *Sin3a*$^{d/d}$ uteri also exhibited aberrant epithelial-stromal organization, including ectopic localization of vimentin-positive stromal cells within the luminal epithelium and pseudostratified epithelial morphology. Notably, the *Sin3a*$^{d/d}$ uterine phenotype partially recapitulates features observed in Wnt7a-null (e.g., a small stromal layer) and Pgr-Cre:Wnt4 conditional knockout (e.g., a pseudostratified LE with decreased uterine gland number) models, suggesting convergence on shared developmental pathways. Because Pgr-Cre mediated recombination occurs in both epithelial and stromal compartments beginning in early postnatal life, we examined neonatal uterine development. *Sin3a* mRNA was robustly expressed in luminal epithelium and stroma as early as postnatal day (PND) 5. Compared with controls, *Sin3a*$^{d/d}$ neonates exhibited significantly smaller uteri by PND 15 and 21, accompanied by shortened and poorly branched glands, while ovarian development remained unaffected. These findings indicate that loss of SIN3A disrupts epithelial-stromal crosstalk required for proper uterine morphogenesis and gland development during the hormone-independent phase of neonatal uterine maturation. Collectively, our results establish SIN3A as a critical epigenetic regulator of postnatal uterine development and reveal a previously unappreciated role for chromatin remodeling in coordinating epithelial-stromal interactions during uterine morphogenesis and adenogenesis. This work provides a conceptual framework for dissecting epigenetic mechanisms underlying uterine development and fertility.

Submission ID#2359022

An ESR2-Lineage Subpopulation of Uterine Epithelial Cells Is Essential for Estradiol-Dependent Uterine Homeostasis

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Abstract Text

Estradiol (E2) signaling is indispensable for uterine development and adult tissue homeostasis and is mediated primarily by estrogen receptor alpha (ESR1), with increasing evidence supporting an important regulatory role for estrogen receptor beta (ESR2). Although ESR1 is broadly expressed in the uterine epithelium, the identity and functional significance of ESR2-expressing uterine epithelial cells remain poorly defined. Here, we tested the hypothesis that a discrete subpopulation of uterine epithelial cells belongs to the ESR2 lineage and that loss of ESR1 specifically within this lineage disrupts epithelial homeostasis, predisposing the uterus to estradiol-dependent pathology. To address this, we utilized a novel mouse model in which ESR1 is selectively ablated in ESR2-expressing cell lineages (Esr1-flox/flox; Esr2-iCre, herein referred to as Esr2-Esr1KO). Uterine phenotypes were compared with wild-type (WT) mice using longitudinal 9.4T magnetic resonance imaging (MRI), histological analysis, and immunohistochemistry across developmental stages. These phenotypes were compared with the known uterine phenotype of global ESR1 knockout mice, which exhibit severely hypoplastic uteri with a thin endometrium, impaired gland development and absent epithelial proliferation. At postnatal day (PND) 40, immunohistochemical staining revealed a loss of ESR1 expression in a small subset of luminal and glandular epithelial cells in Esr2-Esr1KO mice, whereas ESR1 was uniformly expressed in WT epithelium, providing evidence for an ESR2-lineage epithelial subpopulation in the uterus. MRI analysis demonstrated significant uterine enlargement and luminal fluid accumulation in all (100%) Esr2-Esr1KO mice examined beginning as early as PND25 (n=3-4). Histological evaluation revealed progressive, age-dependent uterine pathology emerging after puberty (as early as PND45), characterized by epithelial disorganization, reduced glandular integrity, immune cell infiltration, and accumulation of luminal exudate (n=10). These findings implicate estradiol signaling in the onset of pathology and indicate a failure of epithelial homeostatic regulation following disruption of ESR1 in the ESR2 lineage. Further molecular characterization showed increased epithelial proliferation, as indicated by elevated Ki67 expression in the luminal epithelia in Esr2-Esr1KO compared to WT uteri ($p=0.03$, n=3). Between PND 25-40, SOX9 expression was observed in the glandular but not luminal epithelia, and was not different between WT and Esr2-Esr1KO uteri (n=3). Older adult (2-5 months old) Esr2-Esr1KO mice exhibited increased SOX9 in the luminal epithelia ($p=0.001$, n=3), suggesting altered epithelial differentiation or expansion of progenitor-like populations. Although FOXA2 expression in glandular epithelia was preserved at PND40 (n=3), gland architecture progressively deteriorated with age. Immunostaining for F4/80 and neutrophil elastase identified macrophages and neutrophils as the predominant immune populations infiltrating the endometrium and uterine lumen (n=4-5), consistent with chronic inflammatory activation. Notably, these pathological features were distinct from the phenotypes of global ESR1 or ESR2 knockout models, highlighting a lineage-specific requirement for ESR1 in maintaining uterine epithelial integrity. Collectively, these findings provide functional evidence for an ESR2-lineage subpopulation within the uterine epithelium and demonstrate that loss of ESR1 in this lineage disrupts estradiol-responsive epithelial homeostasis, leading to hyperproliferation, architectural remodeling, and chronic inflammation. This study identifies a previously unrecognized epithelial lineage critical for uterine physiological balance and reveals a novel mechanism by which estrogen receptor lineage interactions regulate uterine health.

Submission ID#2361060

Defining Lineage Dynamics and Plasticity During Endometrial Gland Regeneration in the Adult Uterus

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Abstract Text

The endometrium is a highly dynamic tissue composed of stroma, vasculature, immune cells, and two main epithelial populations, luminal epithelium (LE) and glandular epithelium (GE), that coordinate implantation, stromal decidualization, and placental development. In most mammals, uterine glands arise postnatally through differentiation of LE-derived precursors, and defects in gland formation or function contribute to infertility and endometrial diseases including endometriosis and endometrial cancer; however, the cellular dynamics governing GE homeostasis and regeneration in the adult uterus remain poorly understood. To address this gap, we generated a triple transgenic mouse model (*Cxcl15-iCre; iDTR; nTnG*) enabling lineage tracing and conditional ablation of GE cells in the adult uterus. *Cxcl15-iCre*, a glandular-specific Cre recombinase, induces diphtheria toxin receptor (iDTR) expression for selective GE ablation following diphtheria toxin (DT) administration, while the *nTnG* reporter permits lineage labeling through a nuclear tdTomato-to-GFP fluorescent switch. Lineage tracing across multiple pregnancies revealed minimal contribution of lineage-labeled GE cells to luminal epithelial regeneration. Next, GE ablation was performed to directly test the impact of GE on endometrial remodeling. Administration of DT at postpartum day (PPD) 0 resulted in complete loss of uterine glands by PPD2. The loss of GE did not impact tissue remodeling and by PPD4 both LE and regenerating FOXA2 positive GE cells were present. To determine whether GE regeneration occurs independent of the environment of postpartum uterine regeneration, GE ablation was performed in cycling females. Histological and immunofluorescent analysis confirmed apoptosis restricted to the GE at 24 hours post DT administration and the complete loss of the GE, confirmed by absence of FOXA2 and GFP-positive (*Cxcl15*-expressing) cells. Of particular interest, 3–4 days after DT administration, a population of RFP-positive, GFP-negative luminal epithelial cells acquired FOXA2 expression. During this early regenerative phase, widespread LE proliferation was observed, marked by Ki67 expression, which subsequently became restricted to FOXA2-positive epithelial cells associated with gland regeneration. By days 5–6, there was a progressive expansion of GFP-positive gland cells from the RFP-positive LE, and by day 10, the endometrium appeared histologically indistinguishable from controls, with regenerated GE expressing FOXA2 and *Cxcl15*. Finally, functional assessment of DT-treated females demonstrated that regenerated glands supported the establishment and maintenance of pregnancy. Ongoing single-cell transcriptomic analyses aims to resolve the cellular trajectories and molecular programs governing GE regeneration. Together, this study establishes a robust *in vivo*platform for understanding the role of the GE in tissue homeostasis, revealing epithelial plasticity and lineage dynamics in the adult endometrium and providing a framework for future mechanistic studies to understand uterine development and disease progression. This study is supported by NIH R01HD112315.

Submission ID#2344652

Contributions of the Glandular Epithelium to Luminal Epithelial Regeneration Following Parturition and Aging

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Abstract Text

Several models have been proposed to account for the tremendous regenerative capacity of the endometrial luminal epithelium (LE) including activation of an as-yet poorly defined and assumed epithelial stem/progenitor cell population, mesenchymal-to-epithelial transition, dedifferentiation of committed epithelial cells to reinstate plasticity and their subsequent redifferentiation, and glandular epithelial (GE) cell transdifferentiation to LE. To evaluate the latter model, we used a novel *Cag-Flox-STOP-Flox-Pgrmc2-3XFlag* (*Pgrmc2-3XFlag*) reporter mouse coupled with a recently developed GE-specific cre recombinase mouse (*Prss29-Cre*) to determine if and to what extent the GE contributes to regeneration of the LE following parturition and during aging in nulliparous mice. *In situ* hybridization revealed that *Prss29* was exclusively expressed in GE on gestation day 7 and following parturition, as well as in aging nulliparous mice. *Prss29* was undetected in LE or stroma regardless of parity status. To initiate regeneration in the endometrium, control (*Pgrmc2-3XFlag*) and *Prss29-Cre;Pgrmc2-3XFlag* mice were placed with males for one pregnancy. Postpartum (pp) uterine tissues were then collected 5 days (1x5dpp), 3-weeks (1x3wpp) and 7-weeks (1x7wpp) later. A fourth set of uterine tissues was collected 3-weeks after three pregnancies (3x3wpp). The number of glands did not change at any time point. Indelibly marked $FLAG^+$ GE cells were detected by immunohistochemistry and immunofluorescence in GE as expected. About 5 GE-LE junctional points were observed in each 4 mm longitudinal 1x5dpp uterine section, and this did not change at 3 and 7 weeks pp. Approximately 80% of these points showed GE breaching the LE as if releasing glandular content into the luminal space. The remaining 20% of the GE-LE junctional points showed transition of $FLAG^+$ GE cells into $FLAG^+$ LE cells as confirmed by morphological conversion of cuboidal GE cells into simple pseudostratified LE cells, an absence of the GE marker FOXA, and induced expression of the LE marker calbindin-1. 1x5dpp tissues also showed developmentally-derived $FLAG^-$ PGR^- LE cells, $FLAG^+$ PGR^+ GE cells throughout the antimesometrial pole and at GE-LE junctional points, as well as transitioned $FLAG^+$ PGR^- LE cells. About 17 $FLAG^+$ LE cell islands were also observed in 1x5dpp 4 mm longitudinal sections at sites distant from GE-LE junctional points. Interestingly, the number of islands decreased (p=0.03) linearly from 1x5dpp to 1x7wpp. Likewise, the total number of GE-derived $FLAG^+$ LE cells observed in each island decreased from 1x5dpp to 1x3wpp, but then tended (p=0.07) to increase by 1x7wpp. There was no difference in the number of GE-LE junctional points or GE-derived $FLAG^+$ LE islands between 1x3wpp and 3x3wpp tissues. However, a significant increase in the total (p=0.02) and average (p=0.02) number of $FLAG^+$ LE cells was observed in 3x3wpp LE islands highlighting the proliferative expansion of these cells over the intervening ~80 days. GE-LE junctional points were also observed in 5-month old nulliparous *Prss29-Cre;Pgrmc2-3XFlag* mice at diestrus, but not in younger 6-week old mice. These findings support the concept that GE initially helps restore LE mucosal barrier function through a GE-to-LE transdifferentiation during natural cycles of regeneration after parturition and aging. However, while most (~80%) GE-derived $FLAG^+$ LE cells are not retained long-term, those that are retained do clonally expand into $FLAG^+$ LE cell islands. It remains to be determined if these GE-derived FLAG+ LE cells are functionally equivalent to developmentally-derived LE cells.

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Submission ID#2360850

Role of ADAR1 mediated RNA editing in the regulation of endometrial thickness and fertility

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Abstract Text

Women with a thin endometrium/endometrial insufficiency experience increased implantation failure and pregnancy loss and, an overall 22% decrease in live birth rate. However, the underlying causes remain unknown, and suitable models to study this phenomenon are lacking. Interestingly, immunofluorescence detection of Adenosine Deaminase Acting on RNA 1 (ADAR1), which catalyzes the post-transcriptional adenosine-to-inosine (A-to-I) editing of cellular RNAs, was lower ($p< 0.05$) in thin human endometrium samples than thick samples. Targeted uterine deletion of ADAR1 using the progesterone receptor (PR)-Cre rendered female mice infertile with a thin endometrial lining. Moreover, uterine abnormality appears to be the sole cause of infertility, as there were no differences in estrous cycle patterns or ability to ovulate fully competent oocytes with normal developmental potential following *in vivo* or *in vitro* fertilization. Morphological analyses of H&E-stained uterine tissues showed that the stromal compartment was significantly diminished in PR-Cre Adar1 knockout (KO) mice compared to wild-type mice, with minimal glandular epithelium, but the luminal epithelium was normal. Global transcriptomic analyses of uterine tissue from PMSG-stimulated PR-Cre Adar1 KO (n=4) and control littermate mice (n = 4) reveal alterations in SEMA3, TWEAK, EGF, and a number of inflammation, interferon, antioxidant, and ERK/MAPK signaling molecules. Altered expression of these genes in PR-Cre Adar1 KO mice correlate with transcriptomic profiles of endometrial biopsies from women with thin endometrium (< 7mm?) and associated infertility. Our results indicate that ADAR1-mediated A-to-I RNA editing is essential for maintaining uterine endometrial homeostasis and fertility in mice and humans.

Submission ID#2357944

Endometriosis Impairs the Eutopic Endometrial Epithelial Hormone Response in Endometrial Epithelial Organoids

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Abstract Text

The inner lining of the uterus, the endometrium, completely remodels in response to ovarian steroid hormones during the menstrual cycle. Endometriosis is a chronic, debilitating gynaecological disorder that affects more than 10% of women, with 30-50% suffering from infertility likely due to disruptions in hormone-driven processes critical for endometrial receptivity to embryo implantation. The underlying mechanisms of endometriosis-associated infertility remain largely unknown.

Our objective was to utilise human endometrial epithelial organoids (EEO) to investigate the impact of endometriosis on the hormone-responsiveness of endometrial glands and their secreted products.

EEO derived from the eutopic endometrium of patients diagnosed with minimal-mild (stages I-II, n=7), moderate-severe (stages III-IV, n=5) and without endometriosis (control, n=7) were treated with either vehicle control, 17β-estradiol (E2) (proliferative phase mimic), or E2 and medroxyprogesterone acetate (MPA) (secretory phase mimic). Hormone receptors PGR, ESR1 and ESR2 in EEO were examined by RT-qPCR and in full-thickness hysterectomy tissue by immunofluorescence. The basolateral secreted proteome of hormone-treated EEO was analysed by mass spectrometry.

E2+MPA treated EEO demonstrated progesterone-mediated downregulation of *PGR* gene expression, resembling *in vivo* cycle phase-dependent patterns, in control patients (n=4, $p< 0.05$) and stage I-II endometriosis (n=7, $p< 0.01$) compared to vehicle treatment, but not in patients with stage III-IV endometriosis. Glandular PGR localisation was downregulated in secretory phase endometrium of control patients, but sustained in all patients with endometriosis, showing varying degrees of intensity between epithelial cells and between patients. 157 significantly ($P< 0.05$, Fold change ≥ 2.8 or ≤ -2.8) differentially abundant secreted proteins were identified, including IGFBP4, SEPTIN2, AKR1C3, PAMR1, ANXA1, ASRGL1 and RARRES2. 33 (2%) proteins were unique to controls, 76 (4%) proteins were unique to stage I-II endometriosis and 274 (16%) unique to stage III-IV endometriosis. RARRES2 and ANXA1 were secreted at significantly ($P< 0.05$) lower levels in E2+MPA treated EEO from stage I-II endometriosis patients compared to control patients, while PAMR1 was secreted at significantly ($P< 0.05$) higher levels in E2-treated EEO from stage I-II endometriosis patients compared to controls. Genes previously associated with endometrial receptivity or recurrent implantation failure including HABP2 (FC = -7.45, $p< 0.05$) and GPX3 (FC = -2.79, $p< 0.05$) were secreted at altered levels in EEO from patients with endometriosis, regardless of stage, compared to controls, while IGHG1 and C4BPA had altered secretion in stage I-II endometriosis EEOs.

EEO from endometriosis patients demonstrate an impaired response to hormones, leading to an altered steroid hormone receptor expression pattern in patients with moderate-severe endometriosis, and an aberrant secreted proteome in endometriosis patients, with stage-specific differences. These findings are critical in determining the impact of endometriosis on the hormone-driven processes required for endometrial receptivity and embryo implantation. This work provides new insights into endometriosis stage-specific differences within the eutopic endometrium, facilitating the future development of targeted treatments for endometriosis-associated infertility.

Submission ID#2340529

Mesenchymal and Contractile Forces regulate Uterine Epithelial Folding for Implantation Success

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Abstract Text

Epithelial sheets fold cell-autonomously using mechanisms like buckling and apical constriction. Recently non-cell autonomous mechanisms of epithelial folding such as dewetting fluid behavior of subepithelial mesenchyme have been uncovered in the intestine. Shape of uterine epithelial folds is critical for embryo implantation, chamber formation, embryo-uterine axes alignment and successful murine pregnancy progression. However, the mechanisms by which uterine luminal epithelium folds changes during the preimplantation phase of pregnancy remains unclear. Using superovulated mice as a model with aberrant epithelial folds we tested if uterine folding is an epithelial cell-autonomous process or if it relies on mesenchymal forces and muscle contractions. Imaging-based contractility assays and myographs displayed differential contractility in superovulated uteri in the pre-implantation phases of pregnancy. Application of a muscle relaxant resulted in a rescue of folding patterns in ~50% of the uteri. When we performed single cell RNA-sequencing in the pre-implantation stage uteri we observed < 100 differentially expressed genes in the epithelium, but ~1500 differentially expressed genes in the mesenchyme of the superovulated uteri. Models of aberrant epithelial folding also displayed upregulated mesenchymal receptor tyrosine kinase signaling. Inhibition of the excess receptor tyrosine kinase signaling combined with modulation of uterine contractility rescues aberrant epithelial folding in 100% of the superovulated uteri. Thus, similar to the intestine, epithelial folding in the uterus is regulated by non-cell autonomous pathways. Short term modulation of both mesenchymal signaling and uterine contractility results in altered folding patterns suggesting that these pathways are good targets for therapeutic interventions to develop contraceptives or for treating infertility.

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Trainee Platform Competition

Tuesday, July 21 1:30 PM – 3:30 PM

Submission ID#2360634

Machine Learning Classification and Single-Cell Transcriptomic Analysis Identify Genes Determining Developmental Competence in 8-Cell Cattle Embryos

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Abstract Text

Early embryonic development is characterized by tightly orchestrated molecular events that determine whether an embryo will successfully reach the blastocyst stage or undergo developmental arrest. In cattle, maternal-to-zygotic transition occurs when the embryo reaches approximately eight cells, a critical stage marked by a major genome activation in which the embryo produces transcripts required for further development. In the first study, we hypothesized that 8-cell embryos can be classified based on their developmental potential for blastocyst development. In a follow-up study, we hypothesized that there are significant differences in the transcript profiles of 8-cell embryos that develop into blastocysts versus those that undergo developmental arrest. Our main goal was to identify genes that determine developmental competence at the 8-cell stage in cattle. First, we used time-lapse images from 8-cell embryos to classify embryos as developing to blastocyst (N=99) or arrested before cavitation (N=907). Images were taken 20 minutes apart, yielding 962,531 still images of 8-cell embryos for deep learning evaluation. The images were split in a 75:15:15 ratio for training, evaluation, and testing. An ensemble model (YOLOv8, RNN+CNN, and XGBoost) achieved 100% accuracy in classifying 8-cell embryos that arrested development, whereas 8-cell embryos that developed to blastocyst were identified with 71% accuracy by YOLOv8. After adjusting for blastocyst development percentage, the classification accuracy of 8-cell embryos based on their potential to develop into blastocysts was 90%. In the second study, time-lapse images were obtained from embryos up to the 8-cell stage, and blastomeres were isolated and preserved individually from each embryo. Following machine learning classification of the embryos, we performed single-blastomere RNA sequencing of 8-cell embryos classified as having the potential to develop into a blastocyst (N=5, 34 blastomeres) or to arrest development (N=5, 30 blastomeres). Rigorous filtering of genes yielded a robust quantification of 2965 protein-coding genes and 64 long noncoding genes. A robust analysis identified 100 genes with differential transcript abundance (FDR< 0.05). The biological processes “mRNA transport”, “proton motive force-driven ATP synthesis”, and “tRNA aminoacylation for protein translation” were enriched (FDR< 0.1) among the genes showing dysregulated transcript abundance based on the developmental potential of 8-cell embryos. Fifty-nine genes had greater transcript abundance in 8-cell embryos classified with low developmental competence. Notably, genes were enriched for the following biological processes: “negative regulation of apoptotic process” (*MCL1*, *MSX1*, *PLAUR*, *TEX11*), “protein import into nucleus” (*AKIRIN2*, *EIF4ENIF1*, *KPNA7*), and regulation of DNA-templated transcription (*E2F4*, *MSX1*, *NR1H2*, *NR3C2*, *RNF141*, *SAP130*, *UNCX*). Among the 41 genes with lower transcript abundance in 8-cell embryos classified as low developmental competence, there was an enrichment for the biological process “translation” (*MRPS18C*, *RPS17*, *RPS20*). A query of the mouse knockout database with the 41 genes with lower transcript abundance in low-competence 8-cell embryos identified that the absence of *AASDHPPT*, *MRPS18C*, *SMARCA5*, *TAF1B*, and *UQCR10* results in lethal phenotypes during pre-implantation development. In conclusion, the analysis of images of 8-cell embryos using a machine learning architecture can classify embryos based on their developmental competence to produce a blastocyst. A transcriptome analysis, at single-blastomere resolution of 8-cell embryos classified using this innovative approach, identified 100 genes whose dysregulation can compromise embryo development. This is an innovative approach for determining developmental competence at the time of embryo genome activation.

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Submission ID#2360017

The Vagina contains Functional Epithelial Stem Cells that Self-Renew and Differentiate In Vitro and are altered after Chemotherapy or Ovariectomy

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Abstract Text

The vagina undergoes cyclical epithelial remodeling during each menstrual cycle and self-repairs after vaginal birth; however, it loses this regenerative capacity after menopause resulting in vaginal atrophy. The regenerative cells of the human vagina have not been well-characterized. Given the intrinsic regenerative capacity of the vaginal epithelium, we hypothesize vaginal epithelial stem cells are present, responsible for tissue maintenance and sensitive to estrogen-loss. We generated a novel transcriptomic atlas from single-cell RNA sequencing of healthy, premenopausal human vagina (n=6, 25-43 years). We utilized this atlas to capture and validate compartment-specific markers which span from basement membrane (ITGA6) to luminal epithelium (KRT4, TGM1). We observed a subpopulation (5.2% of epithelial cells) of ITGA6+ cells marked by nerve growth factor receptor (NGFR). NGFR+ITGA6+ progenitor cells isolated via FACS from the human vagina were more efficient at forming colonies (3.32±2.42%) and creating organoids (2.58±1.76%) compared to NGFR-ITGA6+ epithelial cells (0.02±0.02% and 0.06+0.09%, respectively, $p< 0.05$), demonstrating NGFR+ITGA6+ cells self-renew and differentiate, in vitro. We used immunohistochemistry to confirm that organoids contain both undifferentiated basal cells and terminally differentiated cells, when seeded with ITGA6+NGFR+ cells but not NGFR-ITGA6+. Taken together we discovered NGFR marks a population of human vaginal epithelial progenitor cells with stem cell activity. Because the vaginal epithelium loses the ability to differentiate after estrogen-loss resulting in atrophy, we developed mouse models with reproductively young (6-8 weeks) cycling (control), ovaries-removed (OVX), ovaries-ablated (CHEMO) and a combination of OVX+CHEMO mice and harvested reproductive organs 7, 30 and 90 days. While estrous cycling was lost immediately in OVX and OVX+CHEMO animals, cyclicity (0.00 cycles/14 days) and follicle depletion (0.00 follicles/mm^2) occurred 90-days after injection in CHEMO mice compared to control (2.83±0.41 and 3.52±1.64, respectively, $p< 0.05$). Further serum-E2 was reduced in CHEMO (1.30+0.53 pg/ml E2), OVX (0.69+0.00), and OVX+CHEMO (1.01+0.7) compared to control (3.27±2.38, $p< 0.05$), confirming loss of ovarian function. However, while epithelium thickness was permanently reduced in OVX and OVX+CHEMO (24.2±9.62 µm), CHEMO animals displayed a transient thinning at 30 days (47.8±9.23 µm) and thickening at 90 days (96.7±15.3 µm) compared to control (93.6+19.2 µm). Regardless of mode of estrogen-loss, OVX, CHEMO and OVX+CHEMO epithelium displayed abundant NGFR+ cells (0.11±0.05 cells/mm,) compared to control (0.06±0.05, $p< 0.05$). We used spatial RNAseq to identify the cell populations in the vaginal epithelium after OVX and CHEMO treatment. Although the vaginal epithelium thickness recovered in CHEMO treated animals, it contained only cells expressing progenitor maintenance and expansion genes, lacking the sequentially differentiating cells that are characteristic of the control vaginal epithelium. We identified NGFR as a stem/progenitor marker in the vaginal epithelium. Our disparate results in OVX and CHEMO treated animals suggest that vaginal stem/progenitor cells persist and proliferate (CHEMO only) in the absence of estrogen, but estrogen is required for terminal differentiation. This work advances our understanding of the basic biology of vaginal degeneration/regeneration and establishes avenues for further investigation that may lead to new treatments for vaginal dysfunction.

This work was supported by the Richard King Mellon Foundation (MP002) and MWRI (K.E.O.).

Submission ID#2357042

A multi-species map of the ovarian ECM identified EMILIN1 as a modulator of follicle growth

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Abstract Text

A deeper understanding of the human ovarian microenvironment is necessary to inform and biofabricate technologies that restore fertility and ovarian hormones. Throughout folliculogenesis, the ovarian microenvironment, particularly the extracellular matrix (ECM), is remodeled. The ECM contributes to rigidity, sequesters paracrine or endocrine factors, and participates in biological signaling. We aim to elucidate ECM-dependent mechanisms that contribute to follicle growth to inform the development of defined ECM-based hydrogels

The ovary is compartmentalized into two regions: the cortex, containing primordial follicles, and the medulla, containing growing follicles. Fertility and endocrine restoration are dependent on the ability to modulate follicle growth. It is well known that pressure and rigidity are important for maintaining the quiescence of murine follicles and modulating growth *in vitro*. Therefore, it is important to consider the physical properties of the human ovary when developing biomaterials. We first performed micro-indentations in a 500µm x 500µm matrix across 9 human ovaries (age range = 20-43 years) to measure rigidity around quiescent and growing follicles. The average ovarian rigidity was 2.88 kPa (5,991 indentations), although the rigidity differed donor to donor and regionally across the ovary. The rigidity around primordial and transitional follicles is similar, with an average Young's Modulus of 7.63 ± 5.00 and 7.61 ± 4.77 kPa, respectively. A non-significant trend in decreased rigidity was identified around follicles of increasing stage (primary = 5.64 ± 4.58, secondary = 2.34 ± 0.91, and antral = 2.75 ± 1.34 kPa).

Next, we established a comprehensive multi-species map of the ovarian ECM to identify compartmental differences by performing proteomics on decellularized human, bovine, and porcine ovaries in multiple anatomical planes. We determined a list of conserved ECM proteins and performed gene-ontology analyses, which identified "elasticity", "compression resistance", and "sequestration of TGFB" as top processes. Proteins involved in these processes are less abundant in the medulla than in the cortex of bovine and human ovaries. We then identified elastin microfibril-interface located protein 1 (EMILIN1) as a protein of interest. EMILIN1 inhibits TGFB signaling, plays a role in elastogenesis, and reduces cell proliferation via integrin occupation in dermal and cardiac fibroblasts and other cell types. Additionally, EMILIN1 is more abundant around primordial follicles in humans, which may suggest it contributes to follicle quiescence or growth.

We first assessed the impact of EMILIN1 on murine folliculogenesis by staging and counting follicles in 8-week-old EMILIN1 knockout (KO) and wild-type (WT) mice. EMILIN1 KO mice have significantly fewer primordial follicles and antral follicles but significantly more atretic follicles compared to WT mice. Next, we cultured early secondary murine follicles in the presence or absence of recombinant human (rh)EMILIN1. Follicles grown in alginate + COL1 and in the presence of rhEMILIN1 had significantly larger diameters at days 6-12 (239.30 ± 9.09 vs 220.66 ± 5.73µm, at day 12) compared to those without rhEMILIN1. These data suggest that EMILIN1 may contribute to follicle growth *in vivo* and *vitro*. Future work will focus on elucidating the mechanism by which EMILIN1 increases follicle growth by interrogating the role of EMILIN1-mediated TGFB and integrin signaling.
This work will direct efforts to engineer biomaterials that mimic the physical properties of the human ovarian microenvironment, increase our understanding of EMILIN1's role in ovarian folliculogenesis, and provide a foundation for the generation of informed technologies to expand fertility and hormone restoration for all patients.

Submission ID#2361007

A Cross-Species 3D Atlas of Uterine Gland Architecture in Menstruating and Non-Menstruating Mammals

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Abstract Text

Menstruation, characterized by cyclical shedding of the endometrial functionalis layer, is a distinctive feature of human reproduction and is shared with less than 2% mammals, whereas commonly used model organisms, such as mice, do not menstruate. Recent reports that perform 3D imaging of human endometrial biopsies describe a plexus of interconnected branched glands in the basalis and tubular, coiled glands in the functionalis that link the basalis glands to the lumen. By contrast, mouse uterine glands arise postnatally from the lumen and mature into tubular ducts with branched end pieces. In humans, functionalis glands regenerate each cycle, and this is thought to depend largely on the basalis glands; however, lineage-tracing studies in mice suggest that the epithelial stem cell niche resides at lumen-gland intersection. This suggests that functional glandular epithelium stem cell niches are distinct depending on the species and endometrial cyclicity. To begin to address these fundamental developmental differences in uterine gland origins during the cycle, we performed a cross-species comparative study to examine 3D gland architecture in menstruating and non-menstruating mammals. Specifically, we asked whether menstruating mammals have interconnected basalis glands and tubular, coiled functionalis glands, whereas non-menstruating mammals retain mouse-like glands with a gland duct and a branched end piece. Using a combination of tissue-clearing, confocal imaging, fluorescence staining, and 3D reconstruction of endometrial glands, we analyzed endometrial glands from menstruating mammals (human, n=1; baboon, n=3) and non-menstruating mammals (marmoset, n=4; cat, n=1; kitten, n=3; naked mole rat, n=8; mouse, n=3). We successfully cleared whole-uterine thick transverse slices from human, baboon, marmoset, cat, and kitten (~400-1600 µm), and whole uterine horns from naked mole rat and mouse (~1100-1300 µm). In humans, we observed predominantly tubular glands with some branched glands, but few interconnected branches. In baboons, we identified glands were tubular with likely secretory phase-dependent coiling and side-branching, but these branches did not interconnect with adjacent glands. This data suggests that interconnected basalis networks may be unique to humans. In marmosets, short tubular glands were present near the fundus, whereas elongated tubular glands were observed in the uterine body, with only a small number of bifurcating glands. In kittens, we detected gland buds, while in adult cats we observed limited number of branched glands. Naked mole rats exhibited a mixture of gland buds and occasional branched glands. Consistent with prior descriptions, non-pregnant mouse uteri showed gland ducts with a branched end piece. Diestrus glands appeared longer and more coiled compared to estrus glands. Among the non-menstruating mammals examined, mice displayed the highest number of branches per gland. Collectively, our 3D reconstructions reveal systematic architectural differences between menstruating and non-menstruating mammals and, importantly, show that the human-like interconnected basalis network is not present in the baboon despite menstruation. Given that menstruating mammals undergo spontaneous decidualization, we plan to examine stem cell marker expression during artificial decidualization in mice and compare these patterns to menstruating species. This comparative 3D analysis across mammals can help identify which regenerative features are shared across species and which are unique to humans, laying essential groundwork for understanding endometrial regeneration.

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Submission ID#2349677

AMH Delivery in Non-Human Primates Using AAV9 Gene Therapy and Recombinant Protein

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Abstract Text

The ovary is a central regulator of female reproductive and overall health, coordinating reproduction and systemic physiology through follicular hormones. Anti-Müllerian hormone (AMH), produced by growing follicles, is the only known paracrine factor that restrains primordial follicle activation and growth. Studies in poly-ovulatory species have shown AMH's potential as a tool for ovarian reserve protection and contraception. However, these species do not fully recapitulate human ovarian physiology, leaving it unclear how this therapeutic potential translates to women. Non-human primate (NHP) models, which closely mirror human ovarian function, are therefore critical. Here, we aim to compare AAV9-based gene delivery and recombinant AMH (rAMH) administration in NHPs to gain preliminary physiological insights.

To establish an NHP-compatible gene therapy tool, two rhesus macaque AMH (rmAMH) constructs were generated: version 1 (rmAMHv1), incorporating signal peptide and cleavage site modifications to enhance secretion and processing, and version 2 (rmAMHv2), preserving the wild-type sequence while incorporating three prevalent single nucleotide variants from the Oregon National Primate Research Center (ONPRC) population. Both constructs produced biologically active AMH, as demonstrated by luciferase reporter assays and in vitro bioassays. In vivo experiment was performed in immunodeficient nude female mice (n = 5/group) injected IM with AAV9 vectors either empty (control) or encoding rmAMHv1 or rmAMHv2 at a dose of 1×10^{13} vg/kg. Circulating AMH levels were measured every 15 days, with an endpoint at 2 months post-injection. The rmAMHv1 vector produced higher circulating AMH levels, while rmAMHv2 resulted in ~23% lower concentrations; however, both constructs achieved sustained supraphysiological, contraceptive-range AMH levels (>500 ng/mL) and were associated with a significant reduction in ovarian size compared with controls, consistent with impaired folliculogenesis. Based on these results, rmAMHv1 was advanced to a pilot study in ovariectomized female rhesus macaques. Three middle-aged animals received a single IM administration of AAV9-rmAMHv1 at two dose levels (2.5×10^{11} vg/kg, n = 2; 1×10^{12} vg/kg, n = 1). Circulating AMH levels and immunogenicity were assessed by longitudinal blood sampling. AAV9 administration successfully induced detectable circulating AMH in all animals; however, anti-drug antibodies (ADA) developed in two of three animals (one at each dose level), resulting in an abrupt loss of detectable AMH by 27 days post-injection. Consequently, a second pilot study using rmAMHv2 was initiated.

To obtain physiological insights on recombinant protein delivery, two adults female cynomolgus monkeys were treated with human rAMH (OVI586, 90 µg/kg/day) for 90 days concurrently with immunosuppression using a CD20 depleting antibody (NHP21). Menstrual cyclicity, menses, and reproductive hormones were monitored before, during, and after treatment. OVI586 administration did not disrupt menstrual cycles, menses, or circulating steroid and gonadotropin levels. However, endogenous AMH and inhibin B were markedly reduced by treatment and converged back to pre-treatment levels within two cycles following wash-out, consistent with a reversible suppression of follicle growth.

Together, these studies establish foundational tools to study sustained AMH administration in NHPs and provide initial evidence that elevated AMH can modulate folliculogenesis without abolishing menstrual cyclicity. This platform will enable future long-term studies of AMH action relevant to fertility preservation, contraception, and ovarian aging.

Submission ID#2361447

Optimizing Calcium Signaling in Vitrified Mouse Eggs by Modifying Culture Conditions During Insemination

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Abstract Text

The use of egg cryopreservation has increased dramatically worldwide reflecting societal needs for flexible family planning alongside advances in techniques like vitrification. Despite substantial improvements, vitrified–warmed (V/W) eggs exhibit reduced developmental potential compared with fresh eggs. One contributing factor may be impaired egg activation, a process initiated by sperm-induced calcium signals at fertilization. These calcium signals transform the egg into a zygote, are essential for early embryo development and long-term offspring health. We hypothesize that altered calcium dynamics contribute to the reduced developmental competence of V/W eggs and represent a modifiable target to improve outcomes. Calcium signals at fertilization can be modulated by changing the extracellular calcium and magnesium concentrations. Notably, current human IVF media show up to fourfold variation in calcium/magnesium (C/M) ratio. This study aims to define the impact of vitrification on calcium dynamics after conventional in vitro fertilization and to develop strategies to improve post-fertilization development of V/W eggs by optimizing the C/M ratio in fertilization media.

V/W eggs and fresh eggs were sticked side by side in a dish and inseminated while recording calcium dynamics. Following sperm entry, V/W eggs ($n = 40$) exhibited a much more prolonged initial calcium transient than controls ($n = 86$, $p < 0.0001$). Moreover, the subsequent oscillations were faster in frequency (~2 times, $p < 0.0001$) but shorter in amplitude, resulting in a similar global calcium exposure. To evaluate for internal calcium stores, V/W and fresh eggs were treated with ionomycin. V/W oocytes ($n = 31$) exhibited a significantly higher peak amplitude ($n = 44$, $p < 0.0001$) and an impairment in calcium clearance than fresh eggs, strongly demonstrating that vitrification impacts calcium homeostasis in the egg.

To restore the sperm-induced oscillatory frequency to physiologic levels, the fertilization media was supplemented with magnesium. While magnesium supplementation did not alter the length of the first transient, the oscillatory frequency in V/W eggs was reduced to the same level as controls and the total calcium exposure was comparable between groups. To evaluate whether magnesium supplementation during fertilization improved egg activation and embryo development, fresh and V/W eggs were fertilized with/without magnesium supplementation, and 4h after insemination all fertilized eggs were cultured in KSOM media until the blastocyst stage. Fertilization rate was comparable between all 4 groups. The number of blastocysts in regular fertilization media was significantly lower with V/W eggs than with fresh eggs, as previously reported (59% vs 87%, $p< 0.05$). Interestingly, after magnesium supplementation, the V/W group had comparable developmental rates to controls and tended to be higher than V/W with no supplementation (72% vs 59%, $p=0.1$). These results strongly suggest that abnormal calcium dynamics at fertilization is a main contributor to poor embryo development of V/W eggs and improvement in fertilization media can restore calcium oscillation frequency and mitigate negative outcomes. Future directions will assess the mechanism by which magnesium supplementation during the first 4h after insemination improves blastocyst rates when using V/W eggs.

Submission ID#2360238

Oral Administration of Polyamide-12 (Nylon 12) Micro and Nanoplastics Affect Ovarian Follicle Development

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Abstract Text

The environmental degradation of bulk plastic through weathering and abrasion produces micro- and nano-plastics (MNPs, < 5 mm - < 0.1µm), which have become ubiquitous in the environment. Through ingestion and inhalation, MNPs have been established to enter systemic circulation and accumulate in the human liver, brain, and reproductive tissues or fluids. Unlike classical environmental toxicants with defined ligand-receptor interactions, the molecular targets of MNPs remain poorly understood and appear dependent on the particle physicochemical properties (i.e., particle size, surface properties, polymer composition, and adsorbed chemicals). Across diverse polymer types, exposure routes, and experimental models, oxidative stress and disruption of redox homeostasis emerge as a conserved biological response to MNP exposure. In the ovary, redox balance is physiologically important for follicle development, ovulation, and oocyte maturation, rendering the ovary uniquely vulnerable to MNPs. Polyamide-12 (PA-12), also known as nylon, has a long-chain aliphatic backbone and polar amide groups, which enhance its affinity for lipid bilayers. Several prior studies show that exposure to MNPs disrupts ovarian functions, but the ovarian impacts of PA-12 and related reproductive outcomes remain poorly understood. We hypothesize that exposure to PA-12 MNPs at environmentally relevant levels results in oxidative stress and activates the oxidative stress response pathway, which perturbs physiological redox balance in the ovary, impairing ovarian functions and female reproductive outcomes. Eight-week-old young adult female C57BL/6 mice were orally exposed to 2.5 mg/kg/day PA-12 MNPs mixed with peanut butter for 2 months. PA-12 MNPs did not affect general health parameters in mice, including survival, body weight gain, food and water intake, or overt behavior. Daily vaginal smears during the last three weeks of the exposure period showed comparable estrous cycle length and patterns between vehicle and PA-12 MNP-exposed mice. ELISA data showed that circulating anti-Müllerian hormone (AMH) levels, an established marker of ovarian reserve, were significantly decreased in PA-12 MNP-treated mice, suggesting compromised ovarian reserve. Consistent with this observation, ovarian histology and follicle counting data showed a borderline reduction in the number of total preantral follicles ($p = 0.0973$) with a significant reduction of secondary follicles. The results of qRT-PCR using isolated antral follicles demonstrated significantly reduced expression of genes involved in gonadotropin-dependent folliculogenesis, including *Fshr* (follicle-stimulating hormone receptor) and *Ccnd2* (cyclin D2, a cell proliferation marker). Several genes involved in peptide hormone synthesis, including inhibin A subunit α (*Inha*), subunit βA (*Inhba*), and subunit βB (*Inhbb*), showed a tendency of decreased expression. Moreover, *Gpx*, a gene encoding glutathione peroxidase (GPX) and an oxidative stress marker, was significantly elevated ($p=0.0480$) in antral follicles from PA-12 MNP-treated animals. Together, our results indicate that oral exposure to high yet environmentally relevant human consumption levels of PA-12 MNPs exhibits ovarian-disrupting effects by diminishing ovarian reserve, altering key genes involved in gonadotropin-dependent folliculogenesis, and inducing oxidative stress response. (This work is supported by R01ES032144 and EOHSI Pilot Fund)

Submission ID#2360816

Hnf1b Deficiency Drives Spontaneous Endometriosis Through Integrated Immune–Fibrotic–Neurogenic Pathways

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Abstract Text

Endometriosis affects 10% of women globally and is a leading cause of infertility and chronic pelvic pain yet remains the only major gynecologic condition without curative therapy. No new therapeutic class has been introduced in last thirty years. Disease pathogenesis is incompletely understood, in part because endometriosis occurs spontaneously only in humans and certain primates. Current rodent models rely on surgical transplantation, which confounds results by triggering artificial repair responses and fails to recapitulate key human features including spontaneous fibrosis and neurogenesis. Fibrosis is a prominent but understudied hallmark of endometriosis and represents a promising yet inadequately modeled therapeutic target.

In humans, HNF1B variants are significantly associated with endometriosis and Müllerian anomalies. We hypothesized that specific Hnf1b ablation in Müllerian-derived tissues would phenocopy spontaneous human pathology, improving translational opportunities. This study aimed to overcome current modeling limitations, identify mechanistic drivers of spontaneous endometriosis, and evaluate the presence of key disease hallmarks, including inflammation, fibrosis, and neo-neurogenesis. This approach leverages human HNF1B genetic associations and uses Wnt7a-Cre to target Hnf1b deletion to Müllerian-derived tissues, recapitulating human pathology without surgical intervention.

Leveraging human genetics data, we developed the $Wnt7a^{Cre+}$;$Hnf1b^{fl/fl}$ mouse model. We analyzed Hnf1b mutant and littermate controls using single-cell spatial transcriptomics, as well as biochemical and immunohistological analyses of eutopic uteri, ectopic lesions, and ovaries.

Hnf1b mutant mice developed Müllerian anomalies including obstructive cervicovaginal defects. Progressive accumulation of uterine tissue led to the spontaneous formation of endometriotic lesions expressing uterine epithelial and stromal markers as well as robust fibrotic and inflammatory features. Lesional epithelium expressed endometrial markers (FoxA-2, Pax-2), and elevated α-SMA+ myofibroblasts mediated fibrosis. Spatial transcriptomics revealed fundamentally altered cellular landscapes. While control uteri showed stromal, luminal and glandular epithelial, smooth muscle, and endothelial populations, mutant tissues were significantly enriched for fibrosis-associated clusters, immune cells, and neuronal populations. Epithelial compartments in mutant uteri showed enhanced signatures of vascularization, antigen presentation, and leukocyte, lymphocyte, and T-cell recruitment, accompanied by reduced retinoic acid receptor signaling and integrin-mediated adhesion pathways, consistent with a pro-inflammatory, immune-recruiting milieu with impaired differentiation and adhesion. Stromal and smooth muscle clusters exhibited upregulation of neutrophil and leukocyte recruitment pathways, phagocyte infiltration, and IGF1 transport. Conversely, cell viability, proliferation, and wound healing pathways were downregulated, suggesting a dysfunctional reparative microenvironment. Distinct myofibroblast subclusters expressed factors involved in fibroblast activation, extracellular matrix deposition, and remodeling, with fibrosis markers including Tgfb, Col4a1, Col6a1, Postn, and Fn1. Chronic inflammation was a consistent feature, supporting a functional interplay between immune activation and fibrogenesis. Neuronal cells were spatially organized adjacent to myofibroblasts, suggesting potential paracrine interactions functionally connecting pain to fibrosis.

This work establishes a new study model of spontaneous endometriosis without the need for confounding surgical or pharmacological interventions. Using spatial biology and histological analyses, we found evidence of inflammation, fibrosis, and neo-neurogenesis, together driving endometriosis complex pathology. Our Hnf1b model of spontaneous endometriosis replicates the complex immune–fibrotic–neurogenic interplay characteristic of human endometriosis and provides a new potential platform for mechanistic investigation and development of cell type–specific therapeutic strategies

Mechanisms and Modulators of Ovarian Aging

Wednesday, July 21 10:00 AM – 12:00 PM

Submission ID#2354710

Unraveling the influence of mitochondrial genotype on ovarian aging

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Abstract Text

Ovarian aging is characterized by the progressive loss of primordial follicles, reduced endocrine function, and emergence of age-related hallmark, including aberrant inflammation immune cell accumulation/activation, fibrosis, multinucleated giant cell formation, and mitochondrial dysfunction. These phenotypic changes coincide with increased risk for oocyte aneuploidy, miscarriage, and trisomic pregnancies. It remains unclear if natural variation in mitochondrial DNA (mtDNA) sequences acts as determinants of the pace and trajectory of ovarian aging. Mitochondrial abnormalities are established regulators of the aging process, yet most experimental models lack mitochondrial genetic diversity, thereby limiting insights into how mtDNA variation shapes age-related tissue declines. To address this gap, we generated a genetically heterogeneous rat model using a 4-way cross of inbred strains (Brown Norway [BN], Fischer 344 [F344], Lewis [LEW], Wistar Kyoto [KY]). Reciprocal matings generated two cohorts with distinct mitochondrial haplotypes: OKC-HETB (BN mtDNA) and OKC-HETW (KY mtDNA), differing at 95 sites, which is similar to human mitochondrial variation. We then sought to determine how these distinct mitochondrial haplotypes affected ovarian aging across the reproductive window. We found that OKC-HET^{B} and OKC-HET^{W} females begin with comparable follicle numbers at 4 months of age, suggesting that follicle development is similar across genotypes. In contrast, OKC-HET^{W} ovaries exhibited an accelerated decline in the primordial follicle pool by 9 months, indicating that the KY mtDNA genotype is associated with premature depletion of the ovarian reserve. Interestingly, ovarian collagen deposition was found to be higher in OKC-HET^{W} vs. OKC-HETB by 4 months of age, which was further exacerbated by 9 months of age. The OKC-HET^{W} rats were also found to have greater multinucleated giant cell accumulation and macrophage abundance at 9 months of age, indicating that the KY mtDNA genotype promotes accelerated ovarian aging when compared to the BN mtDNA genotype. Ovarian transcriptomic and proteomic analyses supported the histological data by revealing that pro-inflammatory and pro-fibrotic pathways are activated earlier in OKC-HET^{W} rats. Subsequent analyses revealed that the aforementioned phenotypes were associated with differences in ovarian mitochondrial energetics between the genotypes. Mitochondria from OKC-HETW ovaries were found to have severely diminished mitochondrial membrane potential by 9 months of age, which was mirrored by declines in ATP production and TFAM binding to mtDNA. Subsequent testing revealed that OKC-HETW rats also have impairments in ovulatory capacity and oocyte spindle organization following hormonal induction of ovulation. Collectively, our findings demonstrate that mitochondrial genotype not only influences ovarian aging at the molecular and bioenergetic levels, but also profoundly impacts oocyte quality and ovulatory capacity.

Submission ID#2361374

Dysregulated Corpora Lutea Regression Drives Fibro-Inflammation in the Aged Ovary

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Abstract Text

The ovary is one of the first organs to age, characterized by a decline in oocyte quantity and quality, leading to infertility and loss of endocrine function that negatively impacts overall health. This early loss of function coincides with a highly inflammatory environment and may be due in part to the ovary's incredibly dynamic physiology and high requirement for debris clearance. Impaired debris clearance is a driver of age-related pathologies. Therefore, we hypothesized that ovarian debris clearance mechanisms are disrupted with age and serve as a primary driver of ovarian inflammaging. To investigate this, we focused on corpora lutea (CL), which are large transient endocrine structures that undergo cyclic clearance. Using a novel CL-tracking system, we found that CL regression was significantly delayed in reproductively old mice (11-13 m) compared with young (6-12 wk). While approximately 80% of CLs from an induced ovulatory event were cleared within 3 wks in young ovaries, old ovaries show no decline, indicating impaired CL clearance. Aged ovaries exhibited an accumulation of non-regressed CLs, hereafter termed persistent CLs (pCLs), representing a newly defined ovarian aging phenotype. Of interest, pCLs accumulation coincided with the emergence of a unique macrophage population, termed multinucleated giant cells (MNGCs). Given the canonical role of macrophages in debris clearance, we next sought to define the relationship between pCLs and MNGCs. Laser capture microdissection and RNA sequencing were used to molecularly define pCLs and compare them to early-stage CLs and MNGCs. While pCLs retain some molecular features of early-stage CLs, their transcriptomic profiles more closely resemble those of MNGCs, suggesting a direct cellular or transitional relationship between these structures. While ongoing work is defining the mechanisms underlying this relationship, we have determined that MNGCs can modulate the ovarian environment in ways that recapitulate ovarian aging. Specifically, MNGC-enriched stromal fractions secrete factors that alter both follicular and stromal compartments, *in vitro*. Conditioned media from ovarian MNGC-enriched stroma induced inflammatory gene expression in cultured secondary follicles, ultimately resulting in reduced oocyte quality upon ovulation. Additionally, MNGC-conditioned media promoted the upregulation of genes associated with tissue and extracellular matrix remodeling in ovarian stromal organoids. As decreased follicle quality and remodeling of the ovarian microenvironment are central hallmarks of ovarian aging, these findings identify dysregulated CL clearance and resulting MNGCs as potential core mediators of ovarian inflammaging. This work was supported by the Global Consortium for Reproductive Longevity and Equality (AC & MTP), R01HD105752 (FED), and Northwestern University Startup funds to FED.

Submission ID#2361272

Single-Oocyte Transcriptomic Analysis Reveals Follicle Stage-Dependent Pathway Dysregulation in Human Ovarian Aging

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Abstract Text

Ovarian aging is characterized by a progressive decline in both oocyte quantity and quality, contributing to subfertility, pregnancy loss, and increased aneuploidy, with a marked acceleration after approximately 36-37 years of age. Although DNA repair pathways have been linked to ovarian aging, the regulatory pathways in early-stage oocytes that contribute to ovarian reserve decline are not fully understood. It remains unclear whether age-related reproductive decline differs across oocytes from early follicle stages. To date, oocytes from primordial (PD) and primary (PY) follicles have not been systematically examined at single-oocyte level to define stage-specific aging features. This study addresses this gap by directly comparing age-related (young and old) human PD and PY oocytes at single-oocyte level.

We analyzed human ovarian cortical tissue from young (18-25 years) and older (35-40 years) donors (n=12) using an integrated approach including histology, proteomic analysis, and single-oocyte RNA sequencing following laser-capture microdissection. Differential expression and pathway enrichment analysis were performed to identify age-related and follicular stage-specific molecular changes.

Histological analysis demonstrated significant depletion of the PD pool in older ovaries, with effects observed across both PD and PY follicles. Single-oocyte transcriptomic profiling identified stage- and age-associated differentially expressed genes ($P < 0.05$, $FDR < 0.25$), revealing distinct molecular signatures across early follicular stages. Pathway and upstream analyses indicated that ovarian aging involves stage-specific molecular pathway regulation. In PD oocytes, aging was preferentially associated with mitochondrial dysfunction, oxidative phosphorylation impairment, and mTOR pathway perturbation, whereas PY oocytes showed predominant enrichment of age-related alterations in eukaryotic translation and protein synthesis pathways.

This study integrates quantitative histology with single-oocyte transcriptomics to uncover stage-dependent molecular and pathway signatures of ovarian aging and demonstrates that ovarian reserve decline involves distinct molecular programs across early follicle stages, providing single-oocyte–level insight into ovarian aging.

Submission ID#2361185

Role of Long Non-Coding RNA in Ovarian Aging

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Abstract Text

Ovaries in mammals age earlier than other organs, inducing fibrosis and inflammation which disrupt hormone production and folliculogenesis. This can cause fertility issues in women trying to achieve pregnancy. Ovarian aging can be partially attributed to age-related changes in regulation and expression of the ovarian transcriptome. Long non-coding RNA (lncRNA) are non-protein coding RNA that can regulate transcription of other RNA, sponge microRNA, and bind to proteins. We hypothesize ovarian lncRNA expression changes with age, regulating the expression of other genes, thus contributing to the aging ovary phenotype. Ovaries from four age groups (n=4 per group) of CD1 female mice (21 days, 3-6 months, 10-14 months, and 18-20 months) were collected for RNA isolation. The isolated samples were sequenced, trimmed, and mapped to the GRCm38 mouse reference genome. The transcripts per million for each gene were analyzed to identify differentially expressed genes (DEGs) and lncRNAs (P-adjusted value< 0.05, |log2FoldChange| ≥ 1) between each age group. Ingenuity Pathway Analysis (IPA) was used to evaluate biological pathways impacted by DEGs (P< 0.05, |z-score| ≥ 2). A self-organizing map (SOM) clustering analysis was performed on the DEGs to identify gene expression patterns with age, resulting in 8 SOMs. Differentially expressed lncRNA were filtered for transcript length of 200 or more nucleotides, two or more exons, and not overlapping with coding exons on the same strand. Cis-targets were classified as genes located 100 kb upstream or downstream of the lncRNA and had a Pearson correlation coefficient |r| ≥ 0.7. Trans-targets were predicted by LncRTPred, then filtered for predicted targets with a cumulative model score of 100% and |r| ≥ 0.95. The combined cis and trans-targets were used for input for IPA to predict biological pathways for each age comparison and SOM. There were 208 DE lncRNAs with 1277 unique targets in total across age comparisons. Nuclear enriched abundant transcript 1 (*Neat1*), a lncRNA necessary for corpus luteum formation, increased 11-fold (P< 0.001) with age (SOM 5, upregulated genes). Known pathways regulated by *Neat1* such as metabolism and EMT were upregulated. Expression of *H19*, a lncRNA upstream regulator of *Star*, decreased 90% (P< 0.01) in in aged ovaries, while *Star* expression peaked at 3-6 months, indicating other age-related regulators of *Star*. EGFR long non-coding downstream RNA (*Eldr)* is a lncRNA known to stabilize transcription factor CTCF which also upregulates transcription factor *Foxm1* to induce cell cycle genes. Expression of both *Eldr* and *Foxm1* were highest at 21d and decreased 73 and 79%, respectively, in older ovaries. Inhibition of *Eldr* is a predicted upstream regulator of SOM 1 (56% of genes that decrease with age) with canonical pathways associated with inhibition of translation, cell cycle and mitosis as ovaries age. Other lncRNAs targeted regulators of inflammatory genes and apoptotic genes. When comparing lncRNA targets at 21 days to any older age, pathways involved in cell cycle, transcription and translation were inhibited in older ovaries while senescence, inflammatory and immune system pathways were activated, agreeing with the full RNA-seq analysis. These results implicate lncRNA in the ovarian aging process by regulating the cell cycle and inflammation, leading to senescence and decreased cell proliferation as the ovaries age. Further understanding of how lncRNA regulate their targets may lead to intervention strategies to slow ovarian aging and improve fertility.

Submission ID#2361378

In Vivo Ovulation Kinetics and Temporal Transcriptomic Regulation of Cumulus-oocyte-complexes are Disrupted with Advanced Reproductive Age

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Abstract Text

Ovulation is the process by which a mature metaphase II (MII)-arrested egg within a cumulus-oocyte-complex (COC) is released from a large antral follicle and is essential for fertilization. We previously demonstrated that fewer eggs are ovulated and corpora lutea formed in mice of advanced reproductive age compared to young counterparts following superovulation due to an increase in failed ovulatory events, including unruptured luteinized follicles and large antral follicles that did not rupture. To further understand the underpinnings of the age-dependent decrease in ovulatory capacity, we performed an *in vivo* time course of ovulation in reproductively young (6-12 week old) and old (12-14 month old) mice and compared the kinetics of ovulation. To do so, we hyperstimulated young and old mice and collected one ovary and oviduct from each animal for histologic analysis and the contralateral ovary and oviduct for COC collection at 0, 4, 8, 11, 12, or 13h post-hCG. Fewer COCs were collected from reproductively old mice at all time points examined. The kinetics of ovulation was also altered, with the majority (51.6%) of COCs having ovulated at 12h in young mice compared to only 7.3% in old mice. Moreover, in response to the ovulatory cues, there were age-dependent changes in the follicular response. Relative to young controls, follicles from reproductively old mice did not accumulate as much follicular fluid or expand to the same terminal size, did not undergo thinning of the mural granulosa cell layer in preparation for follicular rupture, and did not have COCs that expanded to the same degree or contain mature MII-arrested eggs. To distinguish whether there was an age-dependent delay or impairment in ovulation, we performed an additional extended time course (0, 13, 16, and 20h post-hCG) in reproductively young and old mice. While few COCs (7.9%) from reproductively old mice ovulated by 13h, the majority (85%) of COCs in young ovaries had ovulated by this point. However by 16h, most (68.8%) COCs from reproductively old mice had ovulated. To elucidate the molecular underpinnings of this age-related impairment of antral follicle development and ovulation, we performed bulk RNA-sequencing of individual COCs collected from reproductively young and old mice at 0, 4 and 8h post-hCG. COCs from young mice expressed a greater number of DEGs at 4-8h post-hCG compared to 0-4h post-hCG, and the majority of these genes were unique to their age group, whereas most genes upregulated from 0-4h post-hCG were conserved across age groups. Pathway analysis of uniquely upregulated genes in young COCs from 4-8h post-hCG revealed that young COCs upregulate metabolic pathways, while this upregulation is not observed in old COCs. Through Weighted Gene Co-expression Network Analysis (WCGNA), we further determined that the co-regulation of gene networks involved in oxidative phosphorylation, extracellular matrix organization, cell adhesion, and angiogenesis at 8h post-hCG lose connectivity with advanced reproductive age. Overall, our results thus far indicate the temporal regulation of the COC transcriptomic profile from 4-8h post-hCG is dysregulated with age, and impaired upregulation of metabolic pathways may contribute to age-related reduction in ovulatory capacity.

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Submission ID#2360636

Effect of Maternal Age on the Cortical Actin Layer and Associated Endoplasmic Reticulum Clusters in Oocytes: A Species Comparison

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Abstract Text

Ovarian aging results in reduced oocyte quality and has been linked to numerous changes in this limiting and long-lived cell, including alterations to the cytoskeleton, calcium release, and mitochondrial function. Specialized smooth endoplasmic reticulum clusters (ER) 'clusters' that reside immediately beneath the cortical actin layer of oocytes are thought to provide Ca2+ essential for fertilization, and dysmorphisms in smooth ER clusters in human MII oocytes have been associated with diminished developmental competence, particularly in embryo quality and clinical pregnancies. Here, we compare the effects of maternal age on ER cluster structure and its interaction with fenestrae of the cortical actin layer in MII oocytes from mice of different ages (6-7w, n=42; 31-35w, n=22; 45-49w, n=22; 53-57w, n=18; 62-66w, n=13) to MII oocytes isolated from 6 young (5-14y; n=27), and 4 aged (18-20y; n=15) rhesus macaques. Primates underwent standard IVF stimulation, while mice were superovulated; MII oocytes were collected by ovarian aspiration (primates) or oviductal dissection (mice). Samples were immediately fixed in 2% PFA, 1% Picric Acid solution, followed by staining for chromatin (DAPI), ER clusters (Calreticulin), and F-actin (phalloidin). Normal MII oocyte morphology for young mice consists of small, round ER clusters consistently aligned at the cortex beneath fenestrae of the F-actin layer. In contrast, oocytes from aged mice contained slightly enlarged fenestrae and ER clusters that tended to be enlarged, displayed abnormal morphology (nonspherical), and were dispersed in location compared to oocytes from young mice. To assess if the abnormal ER cluster morphology observed is associated with ER stress, MII mouse oocytes were immunolabeled with GRP78. GRP78 is an ER chaperone protein cooperatively regulated by all three Unfolded Protein Response (UPR) pathways and is commonly used to assess ER stress; its expression overlapped almost perfectly with ER clusters. Like the oocyte morphology observed in the mouse, rhesus macaque oocytes from young females contained cortical ER clusters that aligned beneath fenestrae of the cortical F-actin layer. MII oocytes from the aged rhesus macaque showed altered ER cluster morphology parameters, including decreased presence, inconsistent dispersal and localization at the cortex, an increase in nonspherical shapes, and misalignment with fenestrae. This study shows striking changes in ER cluster morphology with age and evidence of ER stress in murine oocytes, which could impact proper Ca2+ release at fertilization. This indicates that a systematic study of the mouse and primate systems could be useful for identifying age-dependent functional deficits associated with ER clusters and Ca2+ release and alludes to potential effects on fertilization in aged oocytes.

Submission ID#2349558

Anti-Müllerian Hormone Prevents Gonadectomy-Induced Adrenal Lesions in Mice

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Abstract Text

Adrenal glands and gonads share a common steroidogenic function and embryonic origin from the adrenogonadal primordium (AGP), first detectable around embryonic day 9.5, which bifurcates into adrenal and gonadal primordia by E10.5. This developmental relationship may underlie the well-documented effect of gonadectomy (GDX) on adrenal pathogenesis. Indeed, since the 1940s, GDX has been known to induce adrenocortical tumors in mice. These lesions are sex steroid-producing tumors thought to arise from capsular progenitor cells that normally maintain adrenal cortical homeostasis.
Here, we show that the anti-Müllerian hormone receptor (Amhr2), mostly known to be expressed by granulosa and Sertoli cells within the gonads, is also expressed in the adrenal capsule and is strongly enriched in subcapsular lesions following GDX. We hypothesized that AMH was an inhibitor of the pathological transformation of the subcapsular adrenal progenitor cells, and that its loss following GDX promotes tumorigenesis. To test this hypothesis, we administered AMH via AAV9 vector immediately after GDX in DBA/2J mice. This approach completely prevented adrenal lesion formation, whereas all control GDX mice receiving the empty vector developed tumors. To investigate the mechanisms underlying this robust phenotype, we performed single-cell RNA sequencing of adrenal glands from control, GDX, AAV9-AMH, and GDX + AAV9-AMH mature female mice, harvested two months post-treatment. Across all groups, we identified canonical cell clusters including fasciculata–glomerulosa cells (*Nr5a1, Cyp11a1, Star)*, capsular cells (*Wt1*), endothelial cells (*Pecam1, Kdr*), lymphoid cells (*Cd7*), macrophages (*Cd68*), and medullary cells (*Chga, Chgb*).
Detailed analysis of the capsule revealed three distinct clusters: *Tcf21*$^{+}$ mesenchymal progenitors, *Rspo3*$^{+}$WNT-niche stromal cells, and *Amhr2*$^{+}$ cells. AAV9-AMH treatment immediately following GDX prevented differentiation and expansion of *Amhr2*$^{+}$ capsular cells, which histologically corresponded to adrenal lesions and co-expressed granulosa cell markers such as *Inha* and *Kctd14*, as validated by RNA in situ hybridization.
GDX also triggered ectopic LHCGR expression in adrenal subcapsular cells. We speculate that this, in turn, induces *Cyp17a1* and enables androgen production, a capacity normally absent in rodents. Conversely, qPCR analyses confirmed that early AAV9-AMH treatment prevented *Lhcgr* and *Cyp17a1* induction and maintained capsular progenitors in an undifferentiated state.
In summary, our results revealed that AMH secreted by the ovary plays a critical role in maintaining adrenal cortical identity by restraining capsular progenitor differentiation into androgen-producing lesions. We observe similar lesions in aged mice with depleted ovarian reserves, suggesting loss of AMH during aging may promote adrenal pathologies. Collectively, these findings suggest a previously unappreciated gonadal-adrenal axis mediated by AMH, linking ovarian aging to loss of adrenal homeostasis and progenitor cell fate regulation.

Molecular and Cellular Control of Testis Biology

Wednesday, July 21 10:00 AM – 12:00 PM

Submission ID#2358678

Open Channel Culture Systems Generate Murine Testicular Assembloids With Resilient Germ Cell Populations and Enclosed Seminiferous Tubule-like Structures

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Abstract Text

Advancements in cancer therapies have resulted in an 85% 5-year survival rate for pediatric cancer patients. Unfortunately, many of these therapies are gonadotoxic, often leaving these patients infertile as adults. Post-pubertal male patients can preserve their fertility through sperm cryopreservation. Pre-pubertal patients, who do not yet produce mature sperm, are unable to utilize this option. Testicular tissue cryopreservation is one fertility preservation option for these patients, with the hope that the tissue can be thawed in the future and used to restore fertility. However, there are no technologies that have successfully restored fertility using this tissue in humans. Technologies that support in vitro spermatogenesis from cryopreserved testis tissue would enable future childhood cancer survivors the opportunity to have biological offspring, which is often cited as an important quality of life metric. Testicular organoids are one such technology, and the preliminary successes of previously published models suggest they are a viable platform for achieving in vitro spermatogenesis. Our goal was to create the appropriate microenvironment to support in vitro spermatogenesis in organoids generated from prepubertal primary murine testis cells. Specifically, we aimed to improve upon previously published testicular organoid models by establishing murine testicular organoids that maintain native-similar testicular architecture, contain enclosed seminiferous tubule-like structures (TLSs), and a mature spermatogonial stem cell (SSC) niche. To do so, we engineered 3D-printed open channel culture systems (OCCSs) of varying widths and oxygen tensions that facilitated the merging of prepubertal murine testicular organoids into larger assembloids. Histological analysis indicated that the OCCS design regulated assembloid ellipticity, TLS density, TLS diameter, and the formation of luminal compartments within TLSs. Assembloids cultured within submerged OCCSs of 400- and 600-micron widths more consistently contained enclosed TLSs, germ cells localized adjacent to Sertoli cells, and Leydig cells localized outside TLSs than assembloids cultured at the air-liquid interface of OCCSs of identical widths. More specifically, the 400 and 600-micron submerged OCCSs produced assembloids containing the largest percentage of DDX4-expressing germ cells (1.0% and 1.5% of total cells, respectively) and of these, 50-60% were localized adjacent to Sertoli cells within TLSs. Furthermore, our OCCSs were able to support the survival of proliferating spermatogonia, including SSCs, over 13 days of culture. Whole mount imaging of assembloids cultured in submerged OCCSs indicates that TLSs are largely enclosed structures. Quantitative measurements of 29 tubules within a 400-micron submerged assembloid were taken, with the minor diameters ranging from approximately 50-150 microns and the major diameters ranging from approximately 75-225 microns. The significant difference in major and minor diameters for each TLS indicates that the TLSs within assembloids are elongated and elliptical, despite not forming long cord structures as in the native testes. In summary, we have generated a robust protocol for forming murine testicular assembloids that contain native-similar cellular organization, enclosed TLSs, and resilient germ cell populations. Ongoing work aims to characterize the hormonal output of assembloids throughout culture, test the effect of vascularization on TLS formation, improve TLS elongation, and promote the formation of a mature stem cell niche. These advancements bring us one step closer to achieving in vitro spermatogenesis.

Submission ID#2359986

Dumb Spermatozoon, Smart Spermatozoa: Toward a Unifying Theory of Sperm Physiological Function

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Abstract Text

Sperm competition theory established a powerful link between reproductive physiology and evolutionary dynamics, explaining how mating systems shape traits such as average sperm velocity. Yet assisted reproductive technologies (ART) still lack quantitative gamete selection strategies because the relationship between measurable sperm phenotypes and fertility potential remains undefined at the cell-population level. Current semen analysis relies on qualitative associations among motility, acrosomal exocytosis, and fertility, and is predictive primarily in extreme cases (e.g., azoospermia), but not in most idiopathic infertility. Following on recent advances in biophysics, we propose that fertilization is a multiscale stochastic search process operating in physical space (motility-mediated exploration) and molecular recognition space (state transitions during capacitation and acrosomal exocytosis). Here, “stochastic” refers to experimentally measurable heterogeneity in trajectories and molecular states across millions of cells. To test this framework, we developed computer vision tools that track large numbers of individual sperm over observation windows >300-fold longer than standard Computer-Assisted Sperm Analysis (CASA), enabling quantitative characterization of long-timescale exploratory dynamics. We integrate these measurements with a theoretical movement model to determine how motility heterogeneity alters the spatial search efficiency for an egg. In parallel, we used high-resolution flow cytometry to resolve millions of sperm undergoing irreversible transitions among mutually exclusive physiological states during in vitro capacitation. We attempt to unify these spatial and molecular data within a mathematical framework that predicts fertility competence as a statistical function of sperm subpopulation compositional dynamics, microenvironmental chemistry, and time. Predictions are evaluated using controlled in vitro fertilization assays. Together, this work reframes fertilization from a ‘competitive race to the egg’ to a more nuanced stochastic process in which egganticipation in space and time emerges from the statistical behaviors of millions of sperm. These findings can be applied to emerging imaging technologies for automated semen analysis, which has long underperformed as an accurate fertility diagnostic.

Submission ID#2412793

MAGE Cancer-Testis Antigens Link Cancer and Spermatogenesis through Metabolic Stress Responses under Nutritional Stress

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Abstract Text

Background: We are investigating melanoma antigen genes (MAGEs), a family of cancer-testis antigens (CTAs) that are normally expressed in the testis but are aberrantly activated in cancer. This unique expression pattern raises a fundamental question: what biological features are shared between cancer and spermatogenesis? In our previous work, we demonstrated that distinct *MAGE* genes are expressed at specific stages of spermatogenesis, from spermatogonia to haploid spermatids. However, their molecular functions in these germ cells remain largely unknown. Genetic knockout models have begun to provide initial insights. MAGE-A family members are expressed in spermatogonia and are aberrantly activated in many cancers, where their tumor-promoting roles are well established. We previously showed that *Magea* knockout (KO) mice show increased germ cell sensitivity to chronic food restriction, but the underlying molecular mechanisms remain unclear. Here, we apply single-cell transcriptomic and multiomic analyses to investigate how MAGE proteins regulate adaptation to metabolic stress in spermatogonia and highly metabolically active pancreatic cancer cells.

Approach: WT and Magea KO mice were subjected to 100 days of chronic food restriction and subsequently analyzed for fertility, sperm quality, and single-cell RNA sequencing profiles of the testes. To test whether MAGEA confers similar effects when overexpressed in cancer cells, we treated the pancreatic cancer line MIA PaCa-2, stably expressing MAGEA3/6, with metabolic stressors (glycolysis inhibition in combination with glutamine deprivation). RNA sequencing of MAGEA3/6-overexpressing and GFP control cells was followed by proteomic and metabolomic analyses to identify stress- and metabolism-related genes altered in both models and to uncover shared or inverse regulatory pathways.

Results and Conclusions: Cross-analysis between the testis and cancer datasets revealed both convergent and divergent responses. Metabolic regulators such as HKDC1, HYPM, and SPATA18 were consistently upregulated in Magea-KO testes under food restriction but downregulated in MAGEA3/6-overexpressing cancer cells under nutrient stress, suggesting a role for MAGEA in energy-stress–induced metabolic remodeling. In contrast, autophagy-associated genes including DNAJA1, SPATA4, TRIM36, and GABARAPL1 were upregulated in both systems, representing a distinct adaptive response to metabolic challenge. Together with sperm and fertility analyses, these findings suggest that MAGEA proteins play a dual role in buffering metabolic stress and sustaining essential stress-resilience pathways to ensure the production of robust, high-quality sperm, some of which are hijacked by cancer.

Submission ID#2361123

Gene Expression during Spermatogenesis is Regulated by Proximal Promoters: Analysis of publicly available ATAC-seq and H3K27ac ChIP-seq datasets.

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Abstract Text

We have long been interested in understanding how developmental stage and germ cell-specific gene expression in the seminiferous epithelium is regulated at the transcriptional level. An initial candidate gene approach using promoter reporter assays in transgenic mice identified the key role of the proximal promoter in this process. Our recent work using a combination of bulk RNA-seq and PRO-seq (Precision-Run On-Sequencing) showed that genes expressed in spermatocytes (SC) are paused in spermatogonia (SG), whereas, for the majority of round spermatid (RS) specific genes, Pol II recruitment and transcriptional elongation occurred simultaneously. Typically, cell type-specific gene expression is regulated by the coordinated action of enhancers and core promoters. The role of enhancers in male germ cell-specific gene expression has not been fully addressed. Our *Acrv1* gene model showed that a 50-bp region located within the proximal promoter acts as an enhancer driving round spermatid-specific expression of a reporter gene in vivo. In fact, the same trend was seen for 15 other male germ cell-specific promoters characterized in transgenic mice. To assess the location of enhancers relative to core promoters in SG, SC, and RS, we used publicly available ATAC-seq (open chromatin) and H3K27ac (active enhancer marker) ChIP-seq datasets. For genes expressed in all three cell types, we found the enrichment of ATAC-seq peaks within the TSS±1kb proximal promoter region, indicating that chromatin is open for transcription. Surprisingly, even the H3K27ac peaks overlapped in the same region (50-65%), and 5-9% of peaks were found within the 3kb upstream region. This is consistent with the in vivo promoter reporter data, with 60-70% peaks enriched at the proximal promoter region. The transcription factor A-MYB has been shown to be critical for pause release and gene expression in male germ cells. We found A-MYB ChIP-seq peaks within the TSS±1kb regions for eight of the in vivo defined promoters. Taken together, the above findings consolidate the idea that male germ cell-specific genes contain enhancers within their proximal promoters and that the recruitment of specific transcription factors, such as A-MYB regulates the spatiotemporal expression of these genes during spermatogenesis.

Submission ID#2356616

Germ Cell-Specific Fatty Acid Beta-Oxidation is Required for Quantitatively Normal Spermatogenesis in the Mouse

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Abstract Text

Over the past several decades, there has been a growing concern that both sperm quality and quantity in men living in developed countries have declined. Although the extent and causes of this decline remain unclear, there are indisputable links between reduced male fertility and consumption of the 'Western diet'. Although high-fat diets (HFDs) are linked to male subfertility and infertility, the mechanistic roles of dietary fatty acids (FAs) on spermatogenesis and male fertility remain largely undefined. FAs are oxidized in mitochondria as both a rich fuel source for ATP production and to generate critical cellular intermediates, including the pool of acetyl groups for crucial chromatin remodeling, which in male germ cells can have a considerable impact on both gene expression and transgenerational epigenetic inheritance. Surprisingly, we discovered levels of long chain FA oxidation in testes were similar to other organs with known preference for FAs as a fuel source, such as the liver. To examine the requirement for FA oxidation in spermatogenesis and male fertility, we deleted in male germ cells one of the three enzymes required for transporting long chain FAs (>12 carbon) into mitochondria for oxidation, 'carnitine palmitoyltransferase 2' (CPT2). *Cpt2* germ cell-specific knockout (*Cpt2* gcKO) male mice had ~25% smaller testes, disrupted timing of male germ cell development and loss of condensing spermatids, and a dramatic ~50% reduction in cauda epididymal sperm. Despite these considerable defects, *Cpt2* gcKO mice were fertile, revealing FA oxidation is an important, but not essential, contributor to normal male germline development and sperm production. Ongoing work to define mechanistic roles of male germ cell FA oxidation will be informative for refining FA dietary recommendations for men who wish to maximize sperm quantity and quality, for improving efficiency of *in vitro* spermatogenesis, and for analyzing effects of FAO on acetylation of retained histones in sperm that transmit diet-induced changes to future generations.

Submission ID#2357099

Positive Selection Analysis of DC-STAMP Domain Proteins Essential for Sperm-Egg Fusion in Rodents and Caenorhabditis Nematodes

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Abstract Text

Reproductive proteins evolve rapidly and often show signs of positive selection, including well-characterized examples necessary for sperm-egg fusion such as IZUMO1 and JUNO. This study analyzed DC-STAMP-containing proteins essential for fertilization: DCST1 and DCST2 from mouse and SPE-42 and SPE-49 from *C. elegans*. *Dcst1* and *Dcst2* knockout mouse sperm exhibit sperm-egg fusion defects, with *Dcst2* knockout males being sterile. Similarly, *C. elegans spe-42* and *spe-49* mutants are sterile despite normal sperm morphology and egg contact.

We analyzed 15 orthologs each of DCST1 and DCST2 across Rodentia, and 18 orthologs each of SPE-42 and SPE-49 within *Caenorhabditis* using CODEML from the Phylogenetic Analysis by Maximum Likelihood (PAML) package. Site-specific models (M1a, M2a, M7, and M8) were employed to detect positive selection. All four proteins showed statistically significant evidence of positive selection. SPE-42 showed the most sites under selection, with 60 sites at posterior probabilities >0.95 (including 29 sites >0.99). DCST1 also showed strong evidence with 23 sites under selection (13 sites >0.99, 10 sites >0.95 posterior probability). SPE-49 showed 24 sites under selection (1 site >0.99, 23 sites >0.95), while DCST2 showed the fewest sites under selection with only 2 sites >0.95.

These results suggest differential selection pressures on DC-STAMP domain proteins across species, with *C. elegans* SPE-42 and mammalian DCST1 experiencing the strongest positive selection based on the number and confidence of sites identified. We are using these predictions to model how mutations at these sites may influence gamete recognition, sperm-egg fusion, and drive speciation.

Submission ID#2357985

Dissection of molecular mechanisms of COPS5 in the regulation of male germ cell development by OMICS studies

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Abstract Text

COPS5 is the fifth subunit of the COP9 signalosome (CSN) complex and plays essential roles in cellular and developmental processes, particularly in protein degradation through modulating protein ubiquitination. It also functions as a transcriptional coactivator that enhances the specificity of JUN/AP1 transcription factors. Conditional disruption of the Cops5 gene in male germ cells results in male infertility, characterized by increased germ cell apoptosis, defective acrosome biogenesis, and impaired progression from round to elongated spermatids.

Histological examination of testes during the first wave of spermatogenesis revealed that abnormalities emerged after postnatal day 16. To investigate the underlying mechanisms by which COPS5 regulates germ cell development, we performed comparative proteomics, RNA-seq, and small RNA-seq analyses using testes collected at postnatal day 16, a stage when cellular composition remains comparable between control and conditional Cops5 mutant mice.

Proteomic analysis identified 2,978 proteins, of which 399 were significantly downregulated and 65 were significantly upregulated in Cops5 mutant testes. The downregulated proteins are involved in autophagy, apoptosis, vesicular trafficking, cilia and motile cilia biogenesis, methylation, ubiquitination, proteasomal degradation, RNA trafficking and localization, and pre-mRNA splicing, among other processes. Many of these proteins are key regulators of spermatogenesis. RNA-seq analysis identified 9,912 transcripts, with 441 significantly decreased and 11 significantly increased in mutant testes. Notably, 240 genes were downregulated at both mRNA and protein levels, while 32 genes showed reduced protein expression without corresponding mRNA changes, suggesting post-transcriptional regulation. Selected genes were validated by quantitative real-time PCR and Western blotting.

Furthermore, major regulatory components of the piRNA pathway were downregulated in Cops5 mutants. Small RNA-seq analysis revealed global upregulation of miRNAs and downregulation of piRNAs, accompanied by increased expression of LINE1 and IAP retrotransposons. Collectively, our findings support a dual-axis model in which COPS5 directly sustains spermatogenic gene programs required for meiotic progression, while indirectly safeguarding genome integrity through maintenance of piRNA biogenesis and transposon silencing. Disruption of either axis results in meiotic failure and defective spermatogenesis. This study identifies COPS5 as a critical upstream regulator linking proteostasis, small RNA pathways, and transposon control in the male germline, and highlights COPS5 as a potential molecular target for understanding the etiology of male infertility.

Imaging and Engineered Biological Systems

Wednesday, July 21 10:00 AM – 12:00 PM

Submission ID#2358875

mFISH3D: Spatiotemporal Profiling of Uterine Epithelial Structure and Function via Rapid 3D mRNA Imaging

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Abstract Text

Understanding the complex topology of the uterine epithelium is currently hindered by a "dual bottleneck": 2D Fluorescence *In Situ* Hybridization (FISH) lacks volumetric context, while 3D antibody-based staining is restricted by the scarcity of validated antibodies for thick tissues. To bridge these gaps, we developed mFISH3D, a rapid volumetric imaging pipeline powered by Hybridization Chain Reaction (HCR). By leveraging nanoscale DNA hairpins for deep tissue penetration, mFISH3D advances uterine imaging from 2D sections to comprehensive 3D reality, enabling multiplexed visualization of mRNA targets in whole-mount uteri within a single week. We applied mFISH3D to map epithelial functional heterogeneity from postnatal development through early pregnancy. This approach revealed distinct 3D expression patterns, particularly for secreted factors like *Wnt7a*, that remain inaccessible to 2D sections or traditional proteomic methods. In conclusion, mFISH3D overcomes spatial and proteomic limitations, providing a high-resolution platform to dissect uterine biology with unprecedented speed and clarity.

Submission ID#2360919

Engineering an ECM Sequestering Fibrous Hydrogel to Promote Ovarian Folliculogenesis and Investigate Follicle-Nascent Matrix Interactions

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Abstract Text

Work towards the *in vitro* culture of ovarian follicles aims to expand fertility preservation options for women with hormone-responsive cancers and pediatric patients. Follicle culture requires a 3D biomimetic environment that degrades with follicle growth and retains extracellular matrix (ECM). Previously, modifying hydrogels with ECM-sequestering peptides improved follicle growth but failed to replicate dense structure of native ovarian tissue, which we hypothesize is critical for primordial follicle culture. Here we developed a novel fibrous biomimetic material combining degradable hyaluronic acid-vinyl sulfone (HA-VS) hydrogels and electrospun dextran-vinyl sulfone (DEX-VS) fibers, offering enhanced ECM templating and promoting ovarian follicle maturation. Immature murine ovarian follicles were encapsulated in HA-VS hydrogel with electrospun Dex-VS fibers modified with 2 mM ECM sequestering peptide BMB. To visualize nascent protein production across culture, we performed metabolic labeling and immunofluorescence staining. *In vitro* maturation was performed on day 8 of follicle culture to assess oocyte competence. Follicles cultured in hydrogels containing BMB-modified fibers showed substantial templating of nascent matrix along the fibers and retained significantly more ECM than hydrogels with unmodified fibers, or BMB-bulk hydrogels without fibers ($p < 0.001$) ($n=9$), more closely mimicking the ECM distribution in the native ovarian environment. Immunofluorescence staining for specific ECM proteins showed a cascading sequestration of collagen and laminin at the follicle fiber interface in ECM sequestering fibrous hydrogels. *In vitro* maturation revealed that 100% of follicles grown in ECM-sequestering fibrous hydrogels that reached over 275 µm resumed meiosis and reached metaphase II compared to only 62.5% of follicles in non-sequestering fibrous hydrogels. Here we have developed a platform with ECM sequestering fibers to retain nascent follicle-produced matrix and help recapitulate the fibrous nature of native ovarian ECM providing a richer, more sophisticated landscape of deposited sequestered ECM. Initial results indicate that such ECM retention may promote ovarian follicle maturation *in vitro*. Ongoing work includes utilizing this platform to probe follicle-matrix interactions throughout folliculogenesis. Ultimately, engineering a biomimetic culture system to sequester nascent extracellular matrix and study *in vitro* follicle-ECM interactions, may pave the way for the development of improved culture systems for early-stage ovarian follicles expanding fertility preservation options.

Submission ID#2342930

In Vivo Biodistribution of Extracellular Vesicles after Intrauterine Delivery

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Abstract Text

Extracellular vesicles (EVs) are nanoparticles secreted by cells for intercellular communication via transfer of bioactive molecules, such as lipids, proteins, and nucleic acids. EVs are being used increasingly as delivery vehicles for pharmaceuticals, CRISPR-cas9 constructs, and other therapeutic treatments. Notably, the location of where these delivery vehicles traffic among organ systems is affected by the route of administration. Our objective was to evaluate the biodistribution of EVs when administered via an intrauterine (IU) route. Our hypothesis was that EVs administered to the female reproductive tract will remain within the reproductive tract rather than circulating to other organ systems. EVs were isolated from sheep serum via differential ultracentrifugation, labeled with IVISense 680 near-infrared lipophilic fluorescent dye, and concentrated using ultrafiltration (Amicon 100 kDa MWCO). Labeled EVs were administered to mice, and the mouse organ systems were evaluated ex vivo 24 h after treatment for EV localization using an IVIS Spectrum (PerkinElmer) in vivo imaging system. Six treatment groups (n=3-4 mice per group) were compared: 1) mice that did not receive any injections or infusions (untreated control), 2) EV-free PBS IU, 3) $500x10^9$ EVs IU, 4) $1x10^{12}$ EVs IU, 5) $500x10^9$ EVs intraperitoneal (IP), and 6) $1x10^{12}$ EVs IP. The PBS control treatment also was labeled with IVISense 680 and subjected to identical purification steps as the EVs. Total radiant efficiency was quantified for brain, heart, lung, liver, spleen, kidney, and reproductive tissues. Data were analyzed using linear models for each tissue type followed by Tukey-adjusted post-hoc comparisons. Post-hoc analysis revealed higher fluorescence in the kidneys after administration of $1x10^{12}$ EVs IP compared to all IU and control groups. No significant statistical differences in total radiant efficiency were observed in other tissues; however, fluorescence showed a trend toward increased signal in reproductive tissues in the IU EV treatment groups compared with untreated and PBS control groups, without concomitant increases in other organ systems. Further studies with a larger sample size are planned to confirm these findings, as lack of significance may be due to insufficient power. Understanding IU EV biodistribution may guide development of localized EV-based therapies for reproductive applications. 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.

Submission ID#2361323

Reconstructing Feline Ovarian Physiology on a Chip to Decode Hormone-Driven Extracellular Vesicle Signaling

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Abstract Text

Foundational ovarian physiology demonstrates that endocrine signals shape somatic cell support of the oocyte; however, mechanisms by which hormonal cues influence extracellular vesicle (EV) signaling within the follicle remain unclear. Follicular EV cargo has been implicated in oocyte maturation, modulation of recipient cell transcripts, and enhanced blastocyst development, suggesting a role in developmental competence. Using a microfluidic chip platform, we tested the hypotheses that 1) feline granulosa- and cumulus-associated transcript markers can be defined to characterize the starting somatic cell culture population, and 2) bio-mimetically produced granulosa cell-derived EVs (gcEVs) exhibit molecular and functional features comparable to native follicular fluid EVs (ffEVs). This system enables modeling of EV-mediated signaling under defined hormone conditions *in vitro* to inform artificial reproductive technology efforts. To enable future assessment of phenotypic stability in microfluidic chip culture, we tested feline granulosa- and cumulus-associated transcript markers. Granulosa cells were mechanically scraped from domestic cat (*Felis catus*) antral follicles (>1.5mm), and cumulus cells collected following cumulus oocyte complex (COC) stripping. Candidate transcripts (*FOXL2, LUM, DCN, GREM1, COL1A1, PTGS2, PTX3, FSHR,* and *GDF9*) were quantified by RT-qPCR (five biological replicates; N = 24 cats). Both *LUM* and *GREM1* were significantly enriched in granulosa cells relative to matched cumulus cells (paired t-test, $p < 0.05$), supporting their utility as potential markers to distinguish the two cell types. Next, we evaluated the functional capacity of gcEVs generated under differing gonadotropin stimulation protocols. Granulosa cells were cultured in microfluidic chips (P2) for five days (2µl/min flow) under a natural estrus mimetic (NE-gcEV; pulsatile release of 3-5IU/L FSH, 2.5-20IU/L LH), baseline stimulation (B-gcEV; 3IU/L FSH, 2.5IU/L LH), or no hormone control (C-gcEV). EVs were isolated from spent medium and follicular fluid via ultracentrifugation. Immature COCs (N=14 cats; *n*=76 COCs, 5-6 COCs/treatment/replicate) underwent IVM±EV (NE-gcEV, B-gcEV, C-gcEV, ffEV, or media-only control [Quinns]), IVF, and embryo culture. The highest maturation rate (MII/oocyte) occurred in NE-gcEV (81%), whereas cleavage per MII was greatest in ffEV-treated COCs (86%). Morulas were observed in all groups, most frequently in Quinns (60% morula/MII). Blastocysts were detected in C-gcEV (17%) and NE-gcEV (8%) groups. These findings indicate stage-specific effects of EV origin and hormonal conditioning on oocyte maturation and early embryogenesis. To further compare gcEVs and ffEVs, small RNA sequencing was performed on ffEVs, a subset of gcEVs, and post-culture granulosa cell pellets. GcEVs contained fewer identified miRNAs than ffEVs and cells (7, 59, and 68 total miRNAs, respectively), consistent with the broader cellular and circulatory contributions to ffEV populations. Two miRNAs were uniquely detected in gcEVs relative to granulosa cells (miR-423-5p and miR-199c), previously associated with ovulation, oocyte quality, and granulosa cell function. Gene Ontology enrichment analysis of predicted miRNA target genes revealed overrepresentation of transcripts associated with enzyme, nucleotide, kinase, and RNA binding functions in both EV groups. Differential enrichment indicated ATP binding-associated genes were more prominent among ffEV targets, whereas ubiquin-protein ligase-associated genes were preferentially represented among gcEV targets. Six of seven miRNAs identified in gcEVs were also detected in ffEVs, indicating similarity in messages produced by gcEVs to native ffEVs. Together, these findings demonstrate that hormonally defined microfluidic culture supports production of bioactive gcEVs with miRNA profiles and developmental effects similar to those of native ffEVs. This platform provides a controlled system to investigate endocrine regulation of EV signaling and its impact on oocyte competence

Submission ID#

Title Coming Soon

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Abstract Text

Submission ID#2359812

Establishment of a Camel Endometrial Organoid Platform to Elucidate Cytokine-Mediated Embryo–Maternal Crosstalk During Extended In Vitro Culture

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Abstract Text

Endometrial organoids (EOs) derived from the Arabian camel (*Camelus dromedarius*), represent a powerful in vitro platform for deciphering the molecular and cellular mechanisms underlying maternal–embryonic crosstalk during early pregnancy. The present study, for the first time, aimed to establish camel EOs, evaluate their suitability for co-culture with camel embryos as a model of implantation, and characterize cytokine-mediated communication between embryonic and maternal compartments. Uteri (n = 3) were collected from the left uterine horns of non-pregnant she-camels immediately after slaughter, and endometrial tissues were aseptically isolated. Tissues were enzymatically digested with collagenase for 2 h, filtered through a 70-µm cell strainer, and resuspended in chilled, reduced growth factor Matrigel. Matrigel domes containing camel endometrial cells were plated in Petri dishes, overlaid with organoid formation medium, and incubated at 38.5°C in a humidified atmosphere of 5% CO_2. The culture medium consisted of advanced Dulbecco's Modified Eagle Medium supplemented with a defined cocktail of growth factors, hormones, and additives tailored for camel endometrial growth, modified after Saadeldin et al. (2024, *Reproduction*; PMID: 38112579) and Saadeldin et al. (2025, *Theriogenology*; PMID: 40408800).

Day 7 in vitro–produced camel embryos (n = 30; 6 replicates) were co-cultured with camel EOs. Embryonic cell numbers, embryo diameter, and attachment rates to polystyrene-coated culture dishes or Matrigel domes were compared with control embryos. Attachment ratios were analyzed using the chi-squared test, whereas embryo cell numbers and gene expression data were evaluated using Student's t-test, with significance set at $P < 0.05$.

Camel EOs with a characteristic spherical morphology and diameters ranging from 200–400 µm were observed by Day 7 of culture. Organoids were maintained within Matrigel domes for up to 20 days, exhibiting progressive growth to approximately 1 mm in diameter. Individual organoids were successfully harvested and expanded on tissue culture plates, forming colonies and epithelial outgrowths. Immunofluorescence analysis demonstrated cytoplasmic expression of canonical endometrial markers (mucin-1, pan-cytokeratin, and vimentin) and nuclear localization of Ki67, with over 80% of nuclei exhibiting proliferative activity.

Co-culture with camel EOs resulted in a fivefold increase in embryonic cell numbers and a sevenfold rise in trophoblast outgrowth formation compared with controls ($P < 0.05$). Gene expression analysis revealed upregulation of OCT4, c-MYC, CDX2, NTF3, and GDNF in co-cultured embryos. Cytokines CCL2, CCL17, CCL24, IGF-1, IFNG, IL1α, IL12, and IL-8 were significantly increased at both transcript and protein levels in embryos co-cultured with EOs. Analysis of embryo-derived conditioned medium through metabolomics revealed 36 differentially upregulated metabolites, which were related to the immunosuppression of the maternal tissues. Furthermore, embryos cultured in EO-derived conditioned medium exhibited extended development up to Day 21 post-cleavage, reaching a mean diameter of 2.4 mm. On the other hand, EOs cultured with the embryos showed increased expression of CCL2, CCL17, IL1, and IL2 at both transcript and protein levels.

Collectively, this study establishes, for the first time, camel endometrial organoids and demonstrates their functional relevance as a physiologically representative platform for investigating embryo–maternal interactions, cytokine-mediated signaling, and implantation biology in the dromedary camel.

Submission ID#2361178

Apical-Out Bovine Oviductal Organoids for Embryo Co-Culture

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Abstract Text

The oviductal epithelium is a crucial regulator of early embryonic development, providing stage-specific luminal and epithelial signals that shape cleavage dynamics, developmental competence, and blastocyst quality. The oviductal lumen contains a dynamic milieu of secreted proteins, extracellular vesicles, metabolites, and growth factors that directly interact with the developing embryo. Organoids are three-dimensional, self-organizing epithelial cell clusters that recapitulate key structural and functional features of their tissue of origin; however, conventional oviductal organoids adopt an apical-in configuration, sequestering the apical cell surface and associated secretory interface within the organoid lumen.

Here, we introduce a novel embryo–organoid co-culture platform using apical-outward-facing bovine oviductal organoids (BOOs) that were hormonally conditioned to mimic a diestrus endocrine state. This polarity configuration enables direct contact between embryos and the oviductal luminal epithelial cell surface and secretory milieu during the early post-fertilization cleavage window, restoring epithelial-embryo interactions absent from standard in vitro embryo culture systems. Following bovine in vitro fertilization, presumptive zygotes were co-cultured with diestrus-stimulated apical-out BOOs for 48 h to model the in vivo oviductal environment during early cleavage (n = 272 zygotes across 4 independent replicates; embryo: organoid ratio of 2:1). Embryos were subsequently transferred to conventional culture conditions and allowed to develop to the blastocyst stage. Developmental progression was assessed using cleavage rate and stage distribution, blastocyst formation, and blastocyst stage and grade. An integrated embryo competency index (ECI), derived from weighted gene expression-based developmental and quality metrics, was calculated to capture developmental performance across multiple endpoints.

Preliminary analyses indicate that embryos exposed to apical-out BOO co-culture during the first 48 hours show directional trends toward improved early cleavage kinetics, blastocyst formation, and overall developmental performance, as measured by the ECI, relative to embryos maintained under standard in vitro culture conditions. Together, this study establishes an apical-out BOO and early-embryo co-culture system as a functional and physiologically relevant model of luminal oviductal epithelial support during the earliest stages of embryonic development. This tractable platform enables direct interrogation of how the oviductal microenvironment shapes embryonic developmental competence and offers a translational framework to enhance embryo quality and resilience in assisted reproductive technologies.

How the Environment Impacts Female Reproductive Health and Beyond

Wednesday, July 21 10:00 AM – 12:00 PM

Submission ID#2327906

Di(2-ethylhexyl) Phthalate and Diisononyl Phthalate Exposures Alter Hox Gene Expression in the Ovary

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Abstract Text

Phthalates are a class of environmental contaminants found in plastic food containers, medical plastics, and personal care products. Di(2-ethylhexyl) phthalate (DEHP) and diisononyl phthalate (DiNP) are two of the most widely used phthalates globally. Because phthalates leach from plastics, humans are continuously exposed to these toxicants. According to the National Health and Nutrition Examination Survey, the majority of the US population is exposed to phthalates. Studies have shown that phthalate exposure negatively affects female reproductive health; however, the effects of DEHP and DiNP exposure on the ovary are less established. Thus, this study investigated the effects of phthalate exposure on the ovary in female mice. To investigate phthalate exposure in vivo, adult female CD-1 mice were dosed with either vehicle control (corn oil), DEHP (20 µg/kg/day or 200 µg/kg/day), or DiNP (20 µg/kg/day or 100 µg/kg/day) for 10 days. To study the direct effect of phthalate exposure on the antral follicle in vitro, adult female CD-1 mouse antral follicles were isolated and cultured in a vehicle control (DMSO) or mono(2-ethylhexyl) phthalate, (MEHP: 0.4, 4, 40, or 400 µM), the primary DEHP metabolite, for 96 hours. Following DEHP exposure in vivo, three DEHP metabolites were found by liquid chromatography-tandem mass spectrometry to be significantly higher in the ovary compared to control: MEHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate, and monobutyl phthalate. Phthalate metabolites were not detected in the ovaries of mice treated with vehicle control. To assess ovarian gene expression with an unbiased approach, RNA sequencing was used to determine differentially expressed genes (DEGs) in the ovaries of control and phthalate treated mice. Of the 15 common DEGs between DEHP and DiNP treatment groups, three genes belonged to the Hox family of transcription factors: *Hoxb3, Hoxc10, and Hoxd10*. Hox genes are a class of homeobox transcription factors that regulate animal development. Although Hox genes have been extensively studied in the developing embryo, much less is known regarding Hox genes in the adult ovary. Investigation of Hox genes by quantitative polymerase chain reaction (qPCR) revealed the following Hox genes to be differentially regulated between vehicle control and at least one phthalate treatment group: *Hoxb2, Hoxb7, Hoxb3os,* and *Hoxd11*. To characterize Hox genes in the ovary, RNAscope in situ hybridization was used to determine the spatial expression of *Hoxb2* and *Hoxd11*. Analyses revealed primordial follicles to have the lowest expression of *Hoxb2* and *Hoxd11* compared to primary, secondary, and antral follicles in the ovaries of vehicle control treated mice. Primordial follicle *Hoxd11* expression was significantly lower than expression in primary, secondary, and antral follicles, and *Hoxb2* expression was significantly lower in primordial follicles compared to primary and secondary follicles. Phthalate exposure did not alter expression of *Hoxb2* and *Hoxd11* in primordial, primary, secondary, or antral follicles compared to control. In vitro, qPCR revealed three Hox genes differentially expressed in the antral follicle between control and MEHP treatment: *Hoxa1, Hoxb8,* and *Hoxb2*. These data indicate DEHP and DiNP exposures aberrantly regulate ovarian gene expression. Further, DEHP and DiNP exposure may negatively impact ovarian health by affecting the Hox family of transcription factors.

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Submission ID#2360446

Follicular fluid fatty acid composition programs granulosa cell signaling and metabolic flexibility to shape ovarian steroidogenesis

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Abstract Text

Increased levels of circulating and follicular-fluid non-esterified fatty acids (NEFAs) are a common feature of metabolic stress associated with reduced fertility in humans and animals. Granulosa cells (GCs) are sensitive to nutrient cues as they integrate gonadotropin signaling with metabolic pathways that support steroid hormone synthesis. However, it has remained unclear how the composition of saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs) present together in follicular-fluid determines the net effects on GC metabolism and steroidogenesis. Across three complementary studies in primary bovine GCs, we tested the hypothesis that physiological NEFA mixtures coordinate GC signaling and energy metabolism to shape steroidogenesis, and we identified key molecular nodes linking lipid exposure to cellular metabolism and steroidogenesis.

First, we established the *in vivo* context by profiling follicular-fluid free fatty acids around the peri-ovulatory transition. Gas chromatography analysis revealed no differential accumulation of free fatty acids between pre- and post-LH surge follicles, indicating that LH does not appear to create major shifts in the overall follicular NEFA pool that could independently explain changes in estradiol production during the cycle. We then modeled the energy-imbalanced follicular environment *in vitro* using the predominant NEFA species that collectively represent the majority of their follicular-fluid fraction. UFAs (oleate and α-linolenate) reduced estradiol production by downregulating gonadotropin receptors and aromatase, whereas co-supplementation with SFAs (palmitate and stearate) rescued estradiol production by restoring these markers and antagonizing UFA inhibition. Mechanistically, UFAs activated ERK1/2 and AKT signaling pathways, with ERK1/2 acting as a negative regulator of estradiol synthesis. This UFA-induced signaling was suppressed by SFAs. Together, these data support a model in which SFAs exert counter-regulatory effects that prevent UFA-driven disruption of gonadotropin signaling and estradiol biosynthesis under physiological conditions.

Second, we asked whether the same compositional antagonism governs GC glucose metabolism. UFAs markedly increased glucose consumption and shifted carbon flux toward aerobic glycolysis with increased lactate production and extracellular acidification. This response partly required the insulin-independent glucose transporter encoding *SLC2A10* and activation of AKT and ERK signaling to sustain *SLC2A10* expression. When SFAs and UFAs were combined to mimic physiological mixtures, SFA cotreatment abolished UFA-stimulated glucose uptake, glycolytic reprogramming and the *SLC2A10* expression via AKT and ERK pathways, demonstrating that SFAs regulate the net metabolic outcome of NEFA exposure.

Third, to identify transcriptome-level regulators connecting lipid exposure to steroidogenesis, we performed global gene-expression analysis after NEFA treatment and focused on pyruvate dehydrogenase kinase 4 (*PDK4*) as a metabolic regulator. Silencing *PDK4* increased glucose utilization via upregulation of glucose transporters (*SLC2A1* and *SLC2A10*) and glycolytic enzymes, while downregulating fatty-acid oxidation genes, indicating a shift toward glucose oxidation even in the presence of NEFAs. Importantly, PDK4 knockdown reduced expression of key progesterone biosynthetic genes (*STAR*, *HSD3B1*, *CYP11A1*) and progesterone secretion. PDK4 was also enriched in large luteal cells versus GCs, which indicates luteal cell specialization for fatty acid oxidation may support steroidogenesis.

Collectively, these integrated findings define a composition-dependent antagonism in which SFAs counteract UFAs at the ERK and AKT signaling hubs to stabilize gonadotropin responsiveness, glucose uptake, and steroidogenesis, while PDK4 acts as a key metabolic node linking fatty acid metabolism and progesterone production. These pathways provide mechanistic targets to understand and potentially mitigate fertility impairments associated with metabolic stress.

This study is funded by DFG, Germany via grant No. BA6909/1-1.

Submission ID#2360707

Environmentally Relevant Phthalate Mixture Exposure Disrupts Ovulatory Outcomes in Multiple Human Ovarian Cell Models

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Abstract Text

Phthalates are solvents and plasticizers incorporated in common consumer goods, including food/beverage containers, cosmetic products, and building/housing materials. Therefore, daily exposure to multiple phthalates is unavoidable. This represents a public health concern because phthalates are known endocrine-disrupting chemicals, measurable levels of phthalates are detected in human ovaries, and exposure to phthalates is increasingly being recognized as contributing to the prevalence of infertility in women. Previous studies established that phthalate exposure disrupts the critical ovarian processes of steroidogenesis and folliculogenesis. However, major limitations to these previous studies are that they focused on individual phthalate exposures in animal models without investigating direct impacts on the ovulatory process. Thus, little is known regarding the mechanistic effects of phthalate mixture exposures on ovulation in women, and this is alarming since ovulatory defects are the leading cause of female infertility. To address this critical gap in knowledge, the Hannon laboratory utilizes environmentally relevant phthalate mixture exposures in primary human ovarian cell models to investigate how phthalate exposure disrupts ovulatory outcomes in women. Specifically, the phthalate mixture (MPTmix) contains six phthalate metabolites at percentages derived from urinary measurements in women, and the MPTmix is applied to primary human mural granulosa cells, cumulus cells, and ovarian microvascular endothelial cells that are obtained from women undergoing *in vitro* fertilization. This approach was used to test the hypothesis that environmentally relevant phthalate mixture exposure inhibits ovulatory prostaglandin (PG) production and function. Ovulation is characterized by immense production of PGs, and the three cell types mentioned above cross talk via the PG pathway to mediate follicle rupture, cumulus-oocyte complex (COC) expansion, and angiogenesis. Specifically, PGs are synthesized by mural granulosa cells and cumulus cells within the ovulatory follicle. PGs bind to their receptors on cumulus cells to establish a stabilized matrix for egg transport to the site of fertilization (COC expansion) and to receptors on endothelial cells to form new vascular networks in the ovulatory follicle/newly forming corpus luteum (angiogenesis). Main findings indicate that the ovulatory increase in PG levels was inhibited by MPTmix exposure compared to controls in mural granulosa cells and cumulus cells due to decreased synthesis and increased metabolism (n=4-19 patients/group; $p \leq 0.05$). This coincided with decreased mRNA levels of PG receptors by the MPTmix on both cumulus cells and endothelial cells. This disrupted PG function likely led to functional defects in COC expansion- and angiogenesis-related events. MPTmix exposure decreased levels of hyaluronan, which is the primary component of the expanded COC extracellular matrix, in cumulus cells, and MPTmix exposure decreased viability in endothelial cells. These findings suggest that environmentally relevant phthalate mixture exposure disrupts the essential PG pathway in three human ovarian cell types that collectively coordinate the ovulatory process, and these defects led to impairments in COC expansion and angiogenesis. These mechanisms appear to be conserved in mouse models, where MPTmix exposure also decreased ovulation rates directly in cultured mouse antral follicles and in mice treated *in vivo* (n=4-12; $p \leq 0.05$). Thus, exposure to phthalates may impair ovulation and fertility in women.
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Chronic Propylparaben Exposure Promotes Apoptosis and Fibrotic Remodeling in the Murine Oviduct

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Abstract Text

The oviduct is the site of fertilization and pre-implantation embryo development, and its morphology and function are tightly regulated by the female sex steroid hormones. However, this hormone-dependent regulation also makes the oviduct susceptible to endocrine-disrupting chemicals, such as parabens. Parabens are a class of chemicals widely used as preservatives in cosmetics, pharmaceuticals, and personal care products and are known to exhibit weak estrogenic endocrine-disrupting activity. Despite extensive human exposure, the effects of propylparaben, one of the most used parabens, on the oviduct remain poorly understood. We previously found that chronic propylparaben exposure reduces the abundance of oviductal luminal ciliated epithelial cells, however, the mechanisms by which propylparaben disrupts the oviduct remain unknown. Here we hypothesized that chronic exposure to environmentally relevant doses of propylparaben promotes oxidative stress and apoptosis, leading to dysregulated extracellular matrix remodeling in the mouse oviduct. Adult female CD-1 mice were orally dosed daily with vehicle control (corn oil) or propylparaben (2, 20, or 200 μg/kg/day) for six months. Oviducts were collected at diestrus for analysis of expression of genes related to oxidative stress, apoptosis, and fibrosis by RT-qPCR (n=6/group), collagen deposition was assessed by Masson's trichrome staining (n = 5 6/group), and microstructural organization by second harmonic generation (SHG; n = 2/group). Data were analyzed by one-way analysis of variance followed by Dunnett's 2 sided post-hoc comparisons. Our results show that propylparaben at 20 μg/kg/day significantly upregulated the pro-apoptotic genes Bax (mean ± SEM; 1.41 ± 0.12 vs. 1.02 ± 0.09 in controls; p=0.01) and Casp3 (2.62 ± 0.33 vs. 1.03 ± 0.12; p=0.0005). Exposure to 200 μg/kg/day also significantly increased Casp3 expression (2.60 ± 0.29; p=0.0005). The antioxidant gene Cat was significantly downregulated at 200 μg/kg/day (0.83 ± 0.07 vs.1.07 ± 0.04; p=0.02). Propylparaben at 200 μg/kg/day significantly downregulated the extracellular matrix remodeling genes Mmp2 (0.67 ± 0.09 vs. 1.01 ± 0.07; p=0.02), and Mmp9 (0.64 ± 0.11 vs. 1.03 ± 0.12; p=0.04). Profibrotic factors were also reduced by propylparaben at 200 μg/kg/day, including Tgfb3 (0.74 ± 0.11 vs. 1.02 ± 0.08; p=0.09), Tgfbr1 (0.79 ± 0.05 vs. 1.02 ± 0.08; p=0.04), Fgf2 (0.72 ± 0.07 vs. 1.02 ± 0.10; p=0.03), Bgn (0.69 ± 0.07 vs. 1.06 ± 0.17; p=0.07). Histological analysis showed significantly increased collagen deposition at 20 μg/kg/day (3.72 ± 0.83; p=0.04) and 200 μg/kg/day (4.02 ± 0.81; p=0.02) compared to controls (1.13 ± 0.35). Preliminary SHG analysis revealed altered collagen architecture following propylparaben exposure, with heterogeneous forward-to-backward SHG signal ratios suggestive of thickened or densely packed fibrils commonly associated with fibrotic collagen remodeling. Collectively, these findings suggest that chronic propylparaben exposure disrupts antioxidant defense responses and promotes apoptosis and fibrotic remodeling in the murine oviduct, suggesting a potential risk to female fertility.
Supported by NIH/NIEHS R03ES032887-01A1

Submission ID#2361012

Impact of Perfluorooctanoic Acid exposure on maturation and abundance of superoxide dismutase 1, Bcl-2-associated-X protein, and apoptosis inducing factor in the porcine oocyte.

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Abstract Text

Understanding factors contributing to pre-ovulatory oocyte health is important to assisted reproduction technologies for humans and animals. The antral follicle is highly vascular, permitting toxicants to partition and accumulate within the follicular fluid. Perfluorooctanoic acid (PFOA) is detectable in follicular fluid of humans and animals and implicated in endocrine disruption, infertility, ovarian senescence, and impaired embryonic development. The objective of this study was to evaluate the effects of PFOA on oocyte maturation and abundance of superoxide dismutase 1 (SOD1), Bcl-2-associated-X protein (BAX), and apoptosis-inducing factor (AIF). Oocytes (n=100 per replicate; 3 replicates) were exposed to either 0 ppt, 0.04 ppt (low), 4 ppt (medium), or 40 ppt (high) PFOA for 41h maturation period before being fixed and evaluated using fluorescent confocal microscopy. The maturation rate of oocytes exposed to low, medium, or high PFOA level did not differ from the control (P = 0.92, P= 0.71, P = 0.60). In MII oocytes, SOD-1 was reduced in low and high PFOA groups (P < 0.0001, P < 0.0001), while there was a tendency for lower SOD-1 in the medium PFOA group (P = 0.10). Decreased total BAX was noted in metaphase II (MII) oocytes exposed to low PFOA compared to the control (P = 0.02) and there was a tendency for reduced BAX in medium PFOA exposed oocytes (P = 0.06), but no impact on BAX in high PFOA exposed oocytes (P=0.68). In polar bodies of MII oocytes, BAX was noticeably present at all PFOA levels and in the control with no difference detected (low: P = 0.40, medium: P = 0.99, high: P > 0.99). Decreased total AIF was observed in MII oocytes exposed to low and medium levels of PFOA compared to the control (P < 0.0001, P = 0.0002), while there was no impact on AIF in high PFOA exposed oocytes (P = 0.14). These polar bodies were also positive for AIF at all PFOA levels and in the control with no difference detected (low: P = 0.78, medium: P = 0.97, high: P = 0.99). These findings suggest that PFOA does not directly impact oocyte maturation rate but reduces SOD-1 and apoptotic proteins in the oocyte. In addition, BAX and AIF are both localized to the polar bodies of mature control and treatment oocytes. Thus, PFOA may be detrimental for oocyte viability.

This work was funded in part by the Iowa State University W.S. Martin research fund.

Submission ID#2360703

Embryotoxicity of 0.05µm and 1µm Micro- and Nanoplastics in *Bos taurus*

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Abstract Text

Produced from the breakdown of plastic goods, the toxicity of micro- and nanoplastics (MNPs) has been of growing concern. In both male and female mammalian models, MNP exposure is correlated with the disruptions of the reproductive system, including accumulation in sex organs, gamete damage, and hormonal dysregulation. Cattle (Bos taurus) are an emerging species of interest to investigate the threats MNPs pose to reproductive function. In this study, we investigated if exposure to polystyrene MNPs affects bovine preimplantation embryo development and survival. Bovine embryos produced through in vitro fertilization were incubated for eight days in culture media containing 10µg/mL to 100µg/mL of 0.05µm or 1µm polystyrene MNPs. Embryos were cultured in groups of up to 30 (GC) or individually in a timelapse incubator (TC). Cleavage and blastocyst rates of embryos from GC IVF rounds (control: n = 27) exposed to 0.05µm (10µg/mL: n = 8, 25µg/mL: n = 3, 50µg/mL: n = 7, 100µg/mL: n = 5) and 1µm (25µg/mL: n = 4) MNPs were compared using an ANOVA test. The interaction between cell division timing in TC embryos and 0.05µm MNP treatment groups (control: n = 219, 10µg/mL: n = 94, 25µg/mL = n = 54, 50µg/mL: n = 61) was assessed using a two-way ANOVA. TC embryo arrest frequencies at each developmental stage were analyzed using a chi-square test. In both GC groups, embryos exposed to 0.05µm ($p = 0.019$) and 1µm ($p = < 0.001$) MNPs showed a reduction in cleavage rate among treatment groups. In contrast, only embryos exposed to 1µm MNPs showed a significant reduction in blastocyst rate ($p = 0.020$). The two-way ANOVA showed a significant interaction between TC treatment group and cell division timing ($p = < 0.001$). Embryos exposed to 10µg/mL of 0.05µm MNPs took significantly longer to reach the t7 and t8 stages compared to the control (t7: $p = < 0.001$, t8: $p = < 0.001$). Embryos took significantly longer to reach the morula stage when exposed to 10µg/mL ($p = 0.006$), 25µg/mL ($p = < 0.001$), and 50µg/mL ($p = 0.023$) of 0.05µm MNPs. Significant differences in the proportion of TC embryos arrested at the zygote ($p = 0.028$) and t7 ($p = < 0.001$) stages were found among treatment groups. Our study is the first of its kind to investigate the effects of MNP exposure on bovine preimplantation embryos. The reproductive success of bovines is essential to the continuation of cattle industries. This work will deepen understanding of how anthropogenic activities affect bovine species, allowing for informed decision making to support the longevity of the industry.

Submission ID#2352996

Chronic Short-Term Exposure to an Environmentally Relevant Phthalate Mixture Induces a Specific Ovarian Immune Response and May Alter Ovarian Autophagy and Extrinsic Apoptosis Pathways in Mice

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Abstract Text

Phthalates are endocrine disrupting chemicals that are found in numerous products, including food packaging, medical tubing, and personal care products. Humans are ubiquitously exposed to a mixture of phthalates daily, primarily through ingestion. Previous research indicates that chronic short-term exposure to an environmentally relevant phthalate mixture (Mix) increases the number of atretic follicles in the ovaries of mice compared to controls. Follicular atresia is essential for proper ovarian function; however, excessive atresia can lead to reduced fertility and accelerated reproductive aging. The primary mechanism underlying atresia is apoptosis through the intrinsic pathway; however, emerging evidence indicates that atresia can be driven by other cell death mechanisms such as extrinsic apoptosis and autophagy pathways. Further, autophagy and extrinsic apoptosis pathways are commonly initiated by signals from local immune cells. However, little is known regarding the impact of chronic adulthood Mix exposure on the ovarian immune state or the cell death mechanisms that may contribute to Mix-induced follicular atresia. Thus, this study tested the hypothesis that chronic short-term Mix exposure significantly dysregulates the ovarian immune state and alters expression of genes involved in autophagy and extrinsic apoptosis pathways in mice. To test this hypothesis, young adult female CD-1 mice were exposed to Mix (0.15, 1.5, or 1500 ppm) through the chow for 1 month. Following exposure, ovaries were collected for use in cytokine arrays to examine the immune state as well as qPCR analyses to examine the expression of genes involved in autophagy (*Becn1, Gabarap, Lamp1,* and *Map1lc3b*) and extrinsic apoptotic pathways (*Cflar, Fadd, Fasl, Tnfrsf1a, Tnfrsf10b,* and *Xiap*). Mix exposure increased between 14 and 25 cytokines and decreased between one and three cytokines depending on the dose compared to controls. Further, nine cytokines were commonly upregulated between doses, including ANGPTL3, CD105, CD40, CX3CL1, CXCL16, OPN, PCSK9, PDGF-BB, and RBP4. Mix exposure significantly increased *Gabarap* expression (1.5 and 1500 ppm) and borderline increased *Map1lc3b* expression (1500 ppm), but it did not alter *Becn1* or *Lamp1* expression compared to controls. Exposure to Mix (1500 ppm) significantly increased *FasL* and *Cflar* expression and borderline increased *Tnfrsf10b* expression, but it did not alter *Fadd, Tnfrsf1a,* or *Xiap* expression compared to controls. Together, these data indicate that Mix exposure may cause immune dysregulation that ultimately may lead to increased follicular atresia through upregulation of autophagy and extrinsic apoptosis pathways.

Embryo-Maternal Signaling; Molecular Beginnings to Implantation Success

Wednesday, July 21 10:00 AM – 12:00 PM

Submission ID#2361156

Retinoic acid receptor signaling orchestrates uterine receptivity, decidual competence, and immune homeostasis during early pregnancy

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Abstract Text

Successful implantation requires precise coordination of uterine epithelial receptivity, stromal decidualization competence, and immune homeostasis during a narrow peri-implantation window. Although retinoic acid (RA) signaling has been implicated in female reproduction, the endogenous and isoform-specific roles of retinoic acid receptors (RARs) during early pregnancy remain poorly defined. Here, we integrate isoform-specific conditional knockout mouse models, a dominant-negative RAR model, compartment-specific transcriptomic profiling, and functional assays in mouse and human stromal cells to define how uterine RAR signaling governs fertility. These analyses identify *Rarg* as the dominant RAR isoform required for early pregnancy in mice. Whereas loss of *Rara* or *Rarb* causes only modest or no fertility defects, loss of *Rarg* severely impairs fertility, and combined deletion of all three RAR isoforms results in absolute reproductive failure. Mechanistically, RAR deficiency disrupts implantation through multifactorial defects in uterine receptivity due to unsuppressed estrogen receptor activity, intrinsic loss of stromal decidualization competence, and the emergence of a pronounced innate inflammatory state, revealing a multilayered requirement for or RA signaling during early pregnancy. Extending these findings to humans, we show that repression of *RARA* and possibly *RARG* but not *RARB*, consistently impairs decidualization in primary human endometrial stromal cells, establishing RARA as the dominant isoform in human decidual responses. Together, these findings position RAR signaling as a central integrator of epithelial, stromal, and immune programs essential for early reproductive success, and reveal conserved yet species-specific isoform dominance underlying implantation and decidualization.
Successful implantation requires precise coordination of uterine epithelial receptivity, stromal decidualization competence, and immune homeostasis during a narrow peri-implantation window. Although retinoic acid (RA) signaling has been implicated in female reproduction, the endogenous and isoform-specific roles of retinoic acid receptors (RARs) during early pregnancy remain poorly defined. Here, we integrate isoform-specific conditional knockout mouse models, a dominant-negative RAR model, compartment-specific transcriptomic profiling, and functional assays in mouse and human stromal cells to define how uterine RAR signaling governs fertility. These analyses identify *Rarg* as the dominant RAR isoform required for early pregnancy in mice. Whereas loss of *Rara* or *Rarb* causes only modest or no fertility defects, loss of *Rarg* severely impairs fertility, and combined deletion of all three RAR isoforms results in absolute reproductive failure. Mechanistically, RAR deficiency disrupts implantation through multifactorial defects in uterine receptivity due to unsuppressed estrogen receptor activity, intrinsic loss of stromal decidualization competence, and the emergence of a pronounced innate inflammatory state, revealing a multilayered requirement for or RA signaling during early pregnancy. Extending these findings to humans, we show that repression of *RARA* and possibly *RARG* but not *RARB*, consistently impairs decidualization in primary human endometrial stromal cells, establishing RARA as the dominant isoform in human decidual responses. Together, these findings position RAR signaling as a central integrator of epithelial, stromal, and immune programs essential for early reproductive success, and reveal conserved yet species-specific isoform dominance underlying implantation and decidualization.

Submission ID#2360411

Role of Iroquois-Class Homeodomain Protein IRX-3 in the Uterus During Early Pregnancy

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Abstract Text

Successful placentation depends on tightly coordinated interactions among endometrial stromal, endothelial, and trophoblast cells to establish a structurally organized, vascularized, and metabolically supportive uterine microenvironment. However, the stromal factors that integrate these processes remain poorly defined. Here, we identify the homeobox transcription factor Iroquois 3 (IRX3) as a critical stromal regulator of placentation. Using the *Irx3* conditional mutant mouse model, we demonstrate that loss of IRX3 results in defective decidual angiogenic network formation within the uterine bed and impaired trophoblast differentiation, leading to placentation failure and pregnancy loss. Transcriptomic profiling by RNA sequencing revealed that IRX3 deficiency disrupts gene networks governing cellular architecture, extracellular matrix (ECM) organization, protein trafficking, and mitochondrial function. Notably, Septin 7 (Sept7), a core septin scaffold essential for cytoskeletal organization and membrane compartmentalization, was markedly downregulated, accompanied by coordinated dysregulation of actin-associated genes including Actn1, Ezr, Anln, Myh9, Cfl1, Pfn1, and Tubb3. These changes indicate a collapse of septin-actin-based cellular architecture in IRX3-deficient stromal cells. This structural disruption was associated with an impairment of vesicle-mediated protein trafficking pathways involving Cltc, Arpc1b, Arpc2, and multiple Rab family members, consistent with defective paracrine signaling from stromal cells to endothelial and trophoblast compartments. In parallel, IRX3 loss was accompanied by excessive ECM deposition and increased tissue stiffness, which are key determinants of endothelial sprouting and trophoblast invasion. Further, IRX3-deficient stroma exhibited a robust mitochondrial dysfunction signature, characterized by reduced expression of oxidative phosphorylation and metabolic genes (Ndufa, Cox, Atp5 family members, Idh3, Sdh, Mdh2), mitochondrial ribosomal proteins (Mrpl/Mrps), and redox regulators (Sod2, Prdx3). Collectively, these findings identify IRX3 as a critical stromal factor that integrates septin-mediated cellular architecture, ECM mechanics, vesicular trafficking, and mitochondrial metabolic capacity to support decidual angiogenesis and trophoblast differentiation during placentation.
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Submission ID#2357423

Conceptus-Maternal Interactions: Characterization of the Cervical Cell Transcriptome During the Peri-Implantation Period in Cattle

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Abstract Text

Previous data from our laboratory have demonstrated that at least some classical ISG exhibit a temporal expression profile in the cervix similar to that expected in the endometrium suggesting the potential for using cervical cells to indirectly assess endometrial cell function and embryonic-maternal interactions during early pregnancy. Importantly, collection of cervical cells can be performed multiple times without causing pregnancy interruption, making it a powerful *in vivo* tool for understanding the dynamic conceptus-maternal crosstalk over time. While the endometrial transcriptome has been extensively characterized in both cyclic and pregnant cattle, gene expression in the cervix has not been widely explored beyond a small panel of pregnancy-related markers. Therefore, the objective of this study was to characterize the complete transcriptome of cervical cells throughout the peri-implantation period. Angus beef cows (*Bos taurus*) underwent estrus synchronization and were artificially inseminated (d0). Using a cytological brush, cervical cells were collected on d18 (n=17) and from pregnant cows on d24 (P24; n=7) and d32 (P32; n=11). For cows on d18, pregnancy status was determined retrospectively based on endometrial fluid IFNT and ISG abundance. Cows were subclassified into experiencing early embryonic mortality (EEM18; low IFNT and low ISG; n=4) or pregnant (P18; high IFNT and high ISG; n=13). The RNA from cervical cells was extracted and subjected to bulk RNA sequencing using the Illumina Novaseq X Plus platform. Downstream analyses were performed using Qiagen Ingenuity Pathway Analysis. The threshold for differentially expressed genes (DEG) was an absolute log2 fold change ≥1 and false discovery rate ≤0.05. The threshold for significant canonical pathways was a Benjamini-Hochberg p-value ≤0.05; z-score was used to determine predicted activation/inactivation of canonical pathways. There was a total of 574 DEG (336 upregulated, 238 downregulated) for P18 versus EEM18 with activated pathways related to interferon signaling. Accordingly, the top upregulated genes in P18 included classical ISG recorded in cattle (*ISG15*, *OAS2*, *MX2*, *MX1*) as well as other interferon-inducible genes less documented in relation to ruminant pregnancy such as *IFI6*, *IFI44, RTP4*, *SLFN11*, and *SLFN14*. There was a total of 1995 DEG (1083 upregulated, 912 downregulated) for P24 versus P18 with activated pathways related to cytokine signaling, such as IL10 and IL17, while the interferon signaling pathways were inactivated. There was a total of 889 DEG (468 upregulated, 421 downregulated) for P32 versus P24 with cell cycle-related pathways inactivated; there were no activated pathways. The top upregulated genes in P24 and P32 included those encoding for secreted proteins implicated in placentation (*PLGF*, *PLET1*, *SPP1/OPN*, *PAG*). Moreover, we observed upregulation of genes implicated in mucin biosynthesis (*TFF1*, *LGALS3*, *CLCA1*, *MUC5AC*, *AGR2*) across the days examined, consistent with the crucial biochemical function of the cervix. Taken together, these findings indicate that the cervix is responsive to conceptus signaling and reflect, at least partially, endometrial transcriptomic changes related to conceptus development and pregnancy establishment. Future research is aimed at exploring genes similarly modulated in both cervical and endometrial cells to better understand the extent to which cervical cells reflect endometrial cell function and expand the panel of pregnancy marker genes detected in the cervix that may be used in subsequent studies.

Submission ID#2361107

Metabolic Reprogramming During In Vitro Decidualization of Human Endometrial Stromal Cells

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Abstract Text

Decidualization of human endometrial stromal cells (hESCs) is an essential differentiation process that prepares the endometrium for successful embryo implantation. Since endometrial receptivity is closely linked to reproductive success, understanding the cellular processes underlying this transition is of significant interest. This process entails extensive cellular remodeling and alterations in functional output, necessitating corresponding metabolic adaptations. The present study investigates changes in cellular metabolism during this differentiation under controlled in vitro conditions. To minimize confounding effects from serum or insulin and to more accurately model physiological conditions, a defined culture system was used. Immortalized hESCs were grown in medium containing 5% serum until they reached approximately 80% confluent. They were then transitioned to a serum-free, phenol red–free DMEM/F12 medium with physiological glucose levels (5 mM). Non-decidualized controls were maintained without hormonal treatment, while decidualization was induced using medroxyprogesterone acetate (MPA), estradiol (E2), and dibutyryl-cAMP. Media were replaced every 48 hours to ensure a stable environment for the differentiation process. Expression of decidualization markers, prolactin (PRL) and insulin-like growth factor-binding protein-1 (IGFBP-1), was analyzed by qPCR every two days to confirm the progression of differentiation. Cells were harvested at Days 0, 2, and 6 post-induction for untargeted GC-MS–based metabolomic profiling. Metabolomic data analysis was performed using MetaboAnalyst 6.0, and pathway enrichment was performed using KEGG pathway annotations. Metabolites with a false discovery rate (FDR) below 0.1 were considered significantly altered. Successful differentiation was confirmed by a progressive, time-dependent increase in PRL and IGFBP-1 expression, reaching statistically significant levels by Day 6 relative to uninduced cells (PRL: ~150-fold; IGFBP-1: ~40,000-fold). Metabolomic profiling identified 105 metabolites across all samples. On Day 2, no metabolites were significantly altered compared to controls. However, by Day 6, decidualized hESCs demonstrated pronounced and distinct metabolic remodeling compared to controls, indicating that metabolic remodeling develops progressively during decidualization. Seven metabolites were significantly altered at this stage (FDR < 0.1). Pathway enrichment analysis revealed significant changes in core metabolic pathways, including pyrimidine and purine metabolism, glycolysis and pyruvate metabolism, and nitrogen metabolism. Elevated levels of phosphoenolpyruvate, uridine, uracil, and hypoxanthine suggested increased nucleotide synthesis and glycolytic activity, whereas decreased levels of lactic acid, glutamine, and ethanolamine indicated a shift in energy production and amino acid utilization. The reduction in glutamine, a key nitrogen donor, together with changes in nitrogen metabolism intermediates, implies coordinated regulation of nitrogen handling to meet biosynthetic demands. Collectively, these findings indicated a coordinated metabolic reprogramming that integrates central carbon metabolism, nucleotide biosynthesis, and amino acid metabolism to support the energy, biosynthetic, and signaling requirements of stromal cell differentiation in vitro. Although untargeted metabolomics identifies changes in metabolite abundance and pathway enrichment, it does not directly measure metabolic flux. To address this limitation, ongoing studies employing ^{13}C-labeled glucose are underway to elucidate the distribution of glucose-derived carbon within central metabolic and biosynthetic pathways. These future studies will further refine our understanding of the metabolic requirements for endometrial receptivity and successful implantation.

Keywords: Decidualization, Differentiation, Endometrium, Enrichment, Metabolic reprogramming
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Submission ID#2408484

The evolving role of the endometrium in pregnancy and health in mammals

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Abstract Text

The uterine endometrium plays a key role in pregnancy success and life course health of the offspring. There is still a lack of understanding of the regulatory networks that are conserved across placental mammals and what species are specific and drive differences in pregnancy morphologies observed. Moreover, understanding the molecular mechanisms that are conserved allow us to use specific species to test hypothesis that can't be tested in either human health, are important for food production species, or indeed in conservation of at-risk species. Three big questions we are aiming to answer currently are: 1) What are the regulatory networks involved in pregnancy success in mammals and how have they evolved, 2) How have the male and female systems evolved their interactions -specifically the role of microRNAs, and 3) How does heat stress associated with climate change impact on maternal and offspring health in humans. To help answer these questions we use comparative approaches, develop novel 3D in vitro tools, and use multi-omics including single cell/nuclei to understand fundamentally how both protein coding and non-coding parts of the genome regulate endometrial function for health, fertility, food production, and conservation.

Submission ID#2360777

The Early Diestrus Uterine Luminal Metabolome Reflects the Capacity to Retain Pregnancies in Cattle

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Abstract Text

Fertility in cows is the ability to retain pregnancies. Overarching hypothesis is that fertility requires an optimal uterine luminal composition during preimplantation embryo development. Experimentally altering composition of the uterine luminal fluid (ULF) causes pregnancy failure. Biochemical characteristics of the ULF associated with fertility remain poorly known.

The objective was to test the hypothesis that abundance of ULF metabolites differs between fertile and subfertile beef cows. Fifty *Bos indicus*-influenced crossbred, primiparous, non-lactating, cyclic cows were enrolled. Cows were estrous synchronized to receive embryo transfer (ET) on eight consecutive rounds. Only cows displaying estrus followed by ovulation were included in each round. Estrus was designated as D0. On D4, ULF was collected from the uterine body using a cytobrush. On D7, cows received ET, and pregnancy (Preg) was diagnosed on D46. Cows were classified as fertile or subfertile by >65% or < 40% D46 Preg/ET across rounds, respectively. Non-cellular, PBS-soluble contents of cytobrush were analyzed by targeted metabolomics via mass spectrometry. Concentrations of 624 metabolites across 23 biochemical classes were measured.

Of the 624 metabolites detected, 213 were analyzed for the effect of fertility class by ANOVA. Fertile cows showed increased concentration of 9 and decreased of 78 metabolites ($P \leq 0.1$). Concentrations of 9/19 of the amino acids, 7/19 of the amino acid-related, 42/48 of the phosphatidylcholines and 10/13 of the sphingomyelins decreased, while 3/8 of the triacylglycerols, and 4/23 of the diacylglycerols increased in fertile cows.

Fertile cows exhibited 34.5% lower amino acid concentrations on average, ranging from 20.1% for asparagine to 58.3% for tryptophan. Asparagine synthesis was 28.8% less, and concentrations of aromatic (Phe + Trp + Tyr) and branched-chain amino acids (Ile + Leu + Val) were 58.7% and 48.9% less, respectively, in fertile cows. In contrast, diacylglycerol and triacylglycerol concentrations were on average 50% and 52.4% greater in fertile cows, respectively, with increases ranging from 20.2% (DG 18:1_20:3) to 112.5% (DG 18:1_18:2) for diacylglycerols and 43% (TG 20:1_32:1) to 60% (TG 22:4_32:2) for triacylglycerols. Concentrations of unsaturated diacylglycerols were 10.41% greater in fertile cows. The subfertile cows showed greater phosphatidylcholine concentrations (average increase 58.5%, range 12.5% to 99.2%) and greater sphingomyelin concentrations (average increase 51.4%, range 36.8% to 63.9%).

Pathway enrichment analysis identified spermidine/spermine biosynthesis as the top enriched pathway ($P < 0.05$), consistent with the greater spermidine concentrations in fertile cows. Subfertile cows displayed greater arachidonic acid concentration and enrichment of arachidonic acid metabolism, indicating increased production of pro-inflammatory eicosanoids that may impair embryo survival and implantation. Decreased amino acid uptake and transport, and increased synthesis, and enrichment of amino acid metabolism and degradation pathways in subfertile cows suggested dysregulated nutrient availability in the uterine lumen.

In summary, the uterine luminal biochemical milieu varies accordingly with cow fertility. The lumen of subfertile cows displayed a pro-inflammatory microenvironment potentially less favorable for conceptus survival, contrasting with enhanced polyamine biosynthesis and neutral-lipid support for cell growth, proliferation and differentiation in the uterus of fertile animals.

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Metabolic Control of Endometrial Stromal-Endothelial Communication is Essential for the Maintenance of Pregnancy

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Abstract Text

Successful establishment of pregnancy requires coordinated metabolic programming within the naturally hypoxic maternal uterus to support embryo implantation, stromal cell differentiation into a secretory decidual phenotype, and communication with neighboring endothelial cells to drive vascular remodeling. Our previous work identified hypoxia-inducible factor 2α (Hif2α) as a critical regulator of embryo implantation and pregnancy establishment in mice. We previously showed that embryos in conditionally Hif2α-mutant mouse uteri fail to maintain stable attachment to the uterine epithelium, resulting in infertility. Here, we show that Glucose Transporter 1 (GLUT1), induced downstream of Hif2α in decidual stromal cells at the implantation site, is not required for initial embryo attachment but is essential for maintaining pregnancy. Using a conditional knockout mouse model in which the Glut1 gene is deleted in uterine stromal cells, we demonstrate that loss of GLUT1 results in severe subfertility, driven by impaired decidual stromal cell function rather than by an intrinsic endothelial defect. In control uteri, metabolically competent stromal cells orchestrate robust expansion and remodeling of the decidual vascular network. In contrast, Glut1 mutant uteri exhibit abnormal endothelial cell clustering and defective sprouting, consistent with impaired stromal-derived instructive signaling within the decidual microenvironment. RNA sequencing of Glut1 mutant and control uteri revealed significant downregulation of Max-Like Protein X (Mlx), a glucose-sensing transcription factor that regulates Hif2α expression and, in turn, the vesicle trafficking regulator Rab27b. We also observed marked downregulation of synaptotagmin-like protein 1 (SYTL1), a Rab27-binding protein that mediates energy-sensitive docking of secretory granules, enabling regulated exocytosis in response to metabolic and physiological cues rather than constitutive trafficking demands. These alterations suggest that GLUT1-deficient decidual stromal cells exhibit impaired regulated protein secretion, resulting in defective delivery of paracrine cues required for endothelial sprouting and extracellular matrix remodeling. In support of this concept, we observed that although transcripts for Matrix Metalloproteinase 2 (Mmp2), a critical regulator of the extracellular matrix, were unchanged, MMP2 protein secretion was markedly reduced in Glut1-deficient stromal cells cultured in vitro, indicating a post-transcriptional secretory defect and reinforcing a cell-autonomous requirement for glucose-dependent secretion. Collectively, these findings identify a stromal-intrinsic HIF2α-GLUT1-MLX feed-forward metabolic-secretory axis that enables decidual stromal cells to function as a central signaling hub within the implantation niche, coordinating crosstalk among stromal, endothelial, and other cell types in the uterus that is necessary for vascular remodeling during early pregnancy.

The Wild West: Reproductive Technology in Model Organisms and Conservation

Wednesday, July 21 10:00 AM – 12:00 PM

Submission ID#2361405

Understanding the Reproductive Biology of the Critically Endangered Red Wolf (Canis rufus): From the Proteomics of Sperm Cryopreservation to the Non-Invasive Hormone Monitoring of Ovulation

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Abstract Text

The Red Wolf (*Canis rufus*) is a critically endangered North American canid with fewer than 290 animals under human care and approximately 25 in a reintroduced population in the wild. Due to the critical importance of the managed population for species survival, there is interest in developing an artificial insemination protocol utilizing the genetically valuable semen previously cryo-banked for the Red Wolf. Toward these goals, we aim to 1) improve on current sperm cryo-banking techniques via understanding the changes to Red Wolf sperm proteins following cryopreservation and warming, and 2) identify the optimal timing for future AI efforts via non-invasive fecal monitoring.

For the first aim, Red Wolf semen was collected via electroejaculation. A portion of sperm cells were washed, pelleted and frozen at -80C as fresh controls, with the remainder of the ejaculate processed for cryopreservation using a pellet method in Tris-egg yolk buffer with 4% glycerol. Paired fresh sperm and post-thaw sperm samples from the same Red Wolves (n = 15) were subjected to LC-MS/MS, aligned to the domestic dog proteome in MetaMorpheus, and differential expression analysis performed on filtered and imputed data using LIMMA. A single differentially expressed protein, outer dense fiber 1 (ODF1; $p = 0.04$), a key structural component of sperm and a small heat shock protein family member, was identified between fresh versus post-thaw sperm. This change in ODF1 expression, potentially in response to cryo-stress, represents an important target for future study to improve functionality of post-thaw Red Wolf sperm.
Toward the second aim, fecal samples were collected from nine paired female Red Wolves from pre-breeding season through gestation (January–April), steroid hormones extracted, then evaluated via enzyme immunoassay. Timing of first observed breeding was 13.0 ± 2.6 days following the elevation of fecal pregnane to 2x baseline values for pregnant females (n = 5), whereas for females who were not identified as having successfully carried a litter to term (n = 4), breeding was first observed 16.0 ± 4.5 days following this elevation ($p > 0.05$). More consistently the 2x pregnant elevation was 13.0 ± 1.2 days before fertilization (based on a 63 day gestation), indicating potential utility for AI timing.
Cumulatively, these studies contribute to our understanding of the reproductive biology of the critically endangered Red Wolf and advance our toolkit to support species sustainability.

Submission ID#2360893

CCL14 and FLI Supplementation Improve In Vitro Bovine Embryo Development by Regulating Oocyte Competence–Associated Gene Expression

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Abstract Text

In vitro oocyte maturation involves coordinated nuclear and cytoplasmic events required to produce mature oocytes and generate high-quality embryos. Because current systems remain suboptimal, growth factor and cytokine supplementation have been used to enhance quality of in vitro–derived oocytes. In pigs and cattle, supplementation of FGF2, LIF, and IGF1 (FLI) into IVM enhances oocyte competence and embryo quality. Chemokines, also known as chemotactic cytokines, play critical roles in reproductive events. Among all chemokines, CCL14 is involved in trophoblast migration and trophectoderm development and may influence oocyte maturation by affecting MAPK signaling pathway - its receptor CCR1 is present in bovine oocytes and early embryos. This study evaluated CCL14 (10, 50, and 100ng/ml) supplementation with or without FLI during bovine IVM, generating eight experimental groups. After 22h of IVM (5% CO_2, 38.5°C), oocytes were denuded and assessed for nuclear maturation or fertilized (1×10^6 sperm/ml, 18h). Embryos were cultured to Days7 and 8, when blastocyst rates were recorded. Gene expression of oocyte competence–related genes was evaluated in metaphase II (MII) oocytes and cumulus cells from groups exhibiting superior or inferior developmental outcomes. RNA was extracted and reverse-transcribed, and relative transcript abundance was quantified by real-time qPCR using the $2^{-\Delta\Delta Ct}$ method (three biological replicates; n=90/group). Data were analyzed using a generalized linear model (binomial distribution) in SAS. FLI (77% ± 3.70) and FLI100 (77% ± 4.72) significantly improved maturation rates compared with NOFLI (65% ± 0.40) and NOFLI50 (67% ± 3.03) ($p< 0.01$). The FLI100 group consistently demonstrated the highest developmental potential, achieving 79% ± 3.47 cleavage versus NOFLI100 (65% ± 2.62; $p< 0.01$). On Days7 and 8, FLI100 reached 31% and 46% blastocysts, respectively, which were significantly higher than NOFLI (Day7: 18% ± 2.90, $p< 0.01$; Day8: 33% ± 7.53, $p< 0.01$). In contrast, 50ng/ml CCL14 had a negative impact on embryo development regardless of FLI, with only 10% (NOFLI50, $p< 0.05$) and 21% (FLI50, $p< 0.05$) on day7 blastocysts. Key oocyte competence genes (BMP15, GDF9, NLRP5 and OOEP), were upregulated in FLI100. In MII oocytes, the transcript abundance of BMP15 and GDF9 was higher in FLI100 compared with FLI50 and chemokine-only groups ($p< 0.05$). In the cumulus cells, FLI100 exhibited markedly higher levels of GDF9, in comparison with NOFLI100. CCL14 also modulated PTGS2 and AREG expression, primarily in the presence of FLI. Supplementation of CCL14 at 50ng/ml or 100ng/ml in the absence of FLI showed no detectable transcription of PTGS2 and AREG in cumulus cells, suggesting the presence of FLI is crucial for the activities of CCL14. Changes in the level of CCNB1 and CCR1 further supported these findings. The expression of CCNB1, a gene coding for a subunit of maturation promoting factor, in the cumulus cells was reduced in NOFLI50 and NOFLI100 groups, with higher expression in FLI100 ($p< 0.05$). The level of CCR1, the main receptor of CCL14, was elevated in FLI100 compared with NOFLI, while absent in NOFLI50 and NOFLI100 ($p< 0.0001$). In conclusion, FLI combined with 100ng/ml CCL14 enhanced oocyte maturation and embryo development by regulating oocyte competence- and signaling-related genes, representing a promising strategy for improving bovine *in vitro* embryo production. Further studies are needed to clarify how CCL14 modulates signaling pathways, metabolism, and bovine embryo implantation.

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Submission ID#2360123

Species-Specific Sperm Binding: The Role of Oviduct Glycans in Reproductive Compatibility

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Abstract Text

In mammals, sperm must pass through the lower oviduct after mating before reaching the fertilization site, the ampulla. Females retain and store sperm in the lower oviduct, forming a sperm reservoir that extends sperm lifespan and reduces the risk of polyspermy. Carbohydrates (glycans) on oviduct epithelial cells help retain sperm in the reservoir, but oviduct glycans differ between species. To identify glycan motifs that bind sperm, we used an array containing 377 mammalian glycans printed on a slide and found that porcine sperm exhibit specific affinity for two glycan motifs: branched 6-sialylated N-linked glycans and Lewis X trisaccharides (Galβ1-4[Fucα1-3]GlcNAcβ). These motifs were abundant in the isthmus and were required for normal sperm–oviduct binding. Our goal in the current experiments was to further delineate structural characteristics of glycans that bind sperm from multiple species. A specialized array enriched for more complex N-linked glycans was used. Sperm from mice, horses, pigs, and dogs bound glycans containing multiantennary motifs, including those with bisecting GlcNAc (a central branch of the N-glycan), as well as glycans with Gal(β1-4)GlcNAc at the termini. We also observed species-restricted binding. Murine, equine, and porcine sperm bound to terminal fucosylated motifs, including Fuc(α1-3)GlcNAc and Fuc(α1-6)GlcNAc, whereas canine sperm showed overall lower binding to fucosylated glycans. Although both are negatively charged, Neu5Ac(α2-3)Gal bound abundantly to dog and horse sperm, while the structural isomer Neu5Ac(α2-6)Gal bound more selectively to porcine sperm. Motifs bearing Lewis X bound abundantly to murine and porcine sperm but not equine or canine sperm. In contrast, glycans ending in LacNAc (N-acetyllactosamine) bound porcine sperm, as well as mouse, stallion, and dog sperm. In additional cross-species testing, ram semen showed binding to α2,3 sialosides, α1-4 galactosides, β1-4 galactosides and glycans bearing Lewis X trisaccharides, whereas no glycan-sperm binding was detected for human, domestic cat, or rooster sperm. These differences in sperm binding to glycans may help explain how species became reproductively isolated during speciation. These results also have implications for sperm binding to the zona pellucida during fertilization, because some of these glycans are components of both the oviduct and the zona pellucida. This research was supported by USDA.

Submission ID#2359033

Neonatal Estradiol Treatment as a Non-Surgical Strategy for Male Rabbit Sterilization

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Abstract Text

Rabbits are widely used as companion animals and research models. Male rabbits reach sexual maturity at 2-4 months of age, accompanied by androgen-driven behaviors such as aggression, territoriality, and mounting, which often necessitates early sex separation or single housing. These practices negatively affect animal welfare and increase housing demands. Although surgical castration is commonly used in other companion animal species to suppress reproductive function and associated behaviors, rabbits face comparatively higher anesthetic risk, highlighting the need for safe and effective non-surgical strategies for male reproductive management. Male reproductive system development is highly sensitive to early hormonal environments. Neonatal estrogen exposure is known to inhibit reproductive maturation in rodents, but whether this approach can induce long-term suppression of pubertal development in rabbits has not been established. We hypothesized that neonatal elevation of circulating estrogen levels is sufficient to disrupt testicular descent and pubertal progression in male rabbits.

In this ongoing longitudinal study, male rabbits received a single subcutaneous estradiol (E2) implant containing either 0.5 or 1.0 mg E2 on postnatal day (PND) 5. The implant was designed to release E2 over approximately two weeks, providing a transient elevation of circulating estrogen levels. Control animals received no treatment. Animals were monitored through early adulthood (PND 95) to assess somatic growth and pubertal progression, with testicular descent used as an external indicator of sexual maturation. Body weight was measured periodically to monitor growth. Control males exhibited testicular descent within the expected pubertal window (PND 65-75), with 60% descending by PND 65, 20% by PND 70, and the remaining 20% between PND 73 and 75. In contrast, neither the 0.5 mg nor the 1.0 mg E2-treated groups showed evidence of testicular descent by PND 95, indicating a marked disruption of pubertal progression. The comparable outcomes in each treatment group suggest that both E2 doses exceed the minimal threshold required to induce this effect. No statistically significant differences in body weight were detected between control and E2-treated animals at PND 90 (control: 2.46 ± 0.12 kg; E2 0.5 mg: 2.06 ± 0.24 kg; E2 1.0 mg: 2.28 ± 0.21 kg). A subset of E2-treated males exhibited enlargement of external genitalia during early development, consistent with heightened sensitivity of peripheral tissues to estrogen treatment. The duration, reversibility, and functional significance of these responses are currently under investigation.

Together, these findings demonstrate that brief neonatal estradiol exposure can durably disrupt testicular descent without impairing overall growth. Ongoing and future studies will directly assess fertility, testicular histopathology, and long-term reproductive function, as well as systematically evaluating lower estradiol doses to define the minimal effective exposure required for sustained reproductive suppression. This work establishes a foundation for developing a potential non-surgical strategy for male rabbit reproductive management and provides a controlled model for investigating the long-term consequences of transient neonatal hormonal intervention.

Submission ID#2357324

Treatment With BGP-15 Attenuates Collagen Deposition, Promotes Angiogenesis, and Improves Follicular Survival of Murine Ovaries After Subcutaneous Transplantation

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Abstract Text

Ovarian tissue transplantation (OTT) is a vital strategy for fertility preservation in cancer patients, but its effectiveness is severely limited by post-graft follicular loss, mainly caused by ischemia/hypoxia, atresia, and massive follicular activation, in addition to consequent oxidative stress and fibrosis, which compromise ovarian biomechanics and microenvironment, limiting follicular growth. BGP-15, a stress-response modulator and mitochondrial protector, has previously been shown to reduce oxidative stress, preserve mitochondrial integrity, and attenuate ovarian fibrosis in aged models. The combination of mitochondrial protection and control of fibrosis and neovascularization is central to graft survival. This preclinical study evaluated the efficacy of BGP-15 as a follicular growth stimulator and collagen deposition inhibitor, aiming to optimize graft quality in a murine model of ovarian autotransplantation. The study used C57Bl6J mice for ex vivo and in vivo assays. For *ex vivo* experiments, neonatal ovaries were treated with different concentrations of BGP-15. The 0.001 µM dose, considered the most effective for follicular activation, was selected for gene expression analysis by qRT-PCR (*Sirt1*, *Sirt3*, *Nampt*, *Foxo3a*, *KitL*, *Pten*, *Amh* genes). In vivo, adult mice undergoing subcutaneous ovarian autotransplantation received 10 mg/kg of BGP-15 (i.p.) daily for 5 days. Results were evaluated on days 6 and 22 post-transplant, focusing on follicular quantification, collagen deposition (Picrosirius), neoangiogenesis (CD31), macrophage infiltration (F4/80), and biometric parameters, including the resumption of the estrous cycle. Treatment with 0.001 µM of BGP-15 induced follicular growth, demonstrated by a reduction in the percentage of primordial follicles ($p< 0.0001$) and an increase in the percentage of growing follicles. There was an upregulation of mitochondrial activation and survival genes, including *Sirt1* ($p< 0.0001$), *Sirt3* ($p=0.028$), *Nampt* ($p=0.002$), and *Pten* ($p=0.012$), with downregulation of *Amh* ($p=0.031$). *In vivo* treatment with BGP-15 resulted in a significantly better follicular outcome on day 22, with an increase in the number of healthy follicles (transitional $p=0.0206$; primary $p=0.0125$; secondary $p=0.0428$) and a reduction in atretic follicles ($p=0.0381$). On day 6, there was an increase in transitional ($p=0.0134$) and primary ($p=0.0148$) follicles. The first few days post-transplant proved crucial for follicular survival, with a reduction in follicle count and a progressive increase in collagen deposition from the second day onwards. Animals treated with BGP-15 showed a reduction in the number of F4/80+ macrophages (capsule and ovary $p< 0.0001$) and a decrease in collagen deposition (PSR positive area, ovary $p=0.0465$; capsule $p=0.0344$). BGP-15 increased the density of new blood vessels (CD31+, $p< 0.0001$) in the transplanted ovary. In addition, the treatment was associated with a resumption of the estrous cycle ($p=0.0458$), indicating early restoration of endocrine function. In summary, BGP-15 has proven to be a promising agent for improving the outcomes of ovarian tissue transplantation. Its protective action is multifaceted: it induces follicular activation in vitro by regulating mitochondrial genes, and in vivo it promotes follicular growth and post-transplant survival, while reducing atresia, fibrosis, and inflammatory macrophage infiltration and stimulating neovascularization. These unprecedented findings position BGP-15 as a potential therapeutic candidate for future oncofertility applications.

Submission ID#2360550

Vitrified Swine Oocytes have Ionic Imbalances Leading to Premature Activation

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Abstract Text

The long-term and reliable cryogenic preservation of gametes is essential for supporting animal agriculture and biomedical research. However, the developmental competence of swine oocytes dramatically decreases post-vitrification, and mechanistic insights behind the damage are not clear. This study aimed to characterize how vitrification influences oocyte biology and leads to diminished developmental ability. Groups of 25-50 mature swine oocytes were vitrified in microdroplets of propylene and ethylene glycol (17.5% v/v each) on the stage of a Cryolock. The oocytes were thawed and rehydrated in media containing serially reduced sucrose concentrations. Following thawing, 82% (n=463) of oocytes remained viable, as determined by fluorescein diacetate staining. Despite their viability, the vitrified oocytes showed significantly reduced developmental competence following in vitro fertilization and culture (77% vs. 56% for embryo cleavage and 42% vs. 8% for day 7 blastocyst development between control and vitrified oocytes, respectively; $P< 0.001$, n=582). To identify mechanisms contributing to the poor embryo development, we investigated biological signatures of premature oocyte activation following exposure to cryoprotective agents (CPA) and vitrification. Staining of DNA and nuclear lamina at approximately 22-24 hours post-insemination revealed that CPA exposure and vitrification reduce the incidence of fertilization and induce spontaneous activation in the absence of a fertilizing spermatozoon (75% vs. 47% vs. 39% fertilization and 0% vs. 13% vs. 29% spontaneous activation for control, CPA treated, and vitrified oocytes, respectively; $P< 0.001$, n=229). These data are supported by a progressive loss of cortical granules and an increase in zona pellucida density in response to CPA exposure and vitrification ($P< 0.001$ with n=166 for cortical granule staining with Lectin PNA, and $P< 0.05$ with n=186 for zona dissolution assay using 0.1% pronase solution). The levels of calcium and zinc were monitored in these oocytes because changes in the ionic equilibrium of calcium and zinc are critical for meiotic resumption. Fixed-time imaging revealed increased zinc fluorescence following CPA exposure and vitrification, though calcium levels were unchanged ($P< 0.001$ with n=193 for zinc staining with FluoZin-3, AM and $P=0.51$ with n=193 for calcium staining with Fluo-4, AM). Given the transient nature of calcium signaling, we employed time-lapse fluorescence imaging during exposure to CPAs at concentrations used for equilibration (2% v/v; eCPAs) and vitrification (17.5% v/v; vCPAs). Using this approach, we observed a gradual increase in intracellular calcium content from the baseline fluorescence values during eCPAs, followed by a pronounced spike during exposure to vCPAs, indicating initiation of the oocyte activation cascade ($P< 0.05$ during eCPAs and $P< 0.0001$ during vCPAs, n=141). The same approach was used to better understand zinc dynamics during the vitrification process. We observed a gradual decrease in zinc fluorescence from the baseline values during eCPAs exposure, followed by an upward spike during exposure to the vCPAs ($P< 0.05$ during eCPAs and $P< 0.01$ during vCPAs, n=75). Release of zinc into the extracellular space, or zinc sparking, was observed in a subset of oocytes during exposure to CPAs (5.7% during CPAs vs. 0% for controls, $P< 0.05$, n=75). These findings are consistent with induction of the signaling cascade that leads to premature oocyte activation. Altogether, these data show that the vitrification process initiates the oocyte activation cascade prior to fertilization, leading to diminished developmental competence post-thaw in swine oocytes.

Submission ID#2358963

The Wild West: Contraception in Model Organisms and Conservation

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Abstract Text

Effective management of wildlife and overabundant species is becoming increasingly necessary throughout the world where wildlife and humans are increasingly competing for resources. Managing these species that are simultaneously abundant, protected, competing for resources, and in conflict with some yet beloved by others is a formidable challenge. At present, some of the most effective methods of wildlife population control are achieved using lethal methods, including poisons, that are also becoming more highly regulated and increasingly controversial. The use of fertility control as a tool to aid in wildlife management strategies is considered to have numerous benefits and has attracted substantial attention. The greatest benefits from the use of wildlife fertility control will be realized when it is used in integrated, cooperative and thoughtful management programs. The challenges associated with the management and use of fertility control in wildlife are not only technical in nature, but also involve regulatory, social, political and cultural aspects.

In the United States, there are two different contraceptive vaccines/immunocontraceptives that are registered by the Environmental Protection Agency for use in wildlife and free-roaming populations; namely, ZonaStat and GonaCon. Both vaccines have been shown to be effective in suppressing fertility in individual animals of several species and have also been used successfully for the management of isolated populations of deer and horses. While the use of these vaccines and other methods of fertility control has provided important evidence in support of this approach for wildlife management, it has also highlighted the need for improved methods that cause long-term infertility or permanent sterility, and the need for more effective methods of delivery.

Mechanisms of Early Ovarian Development

Wednesday, July 21 1:30 PM – 3:00 PM

Submission ID#2411923

How Do Oocytes Emerge from Germline Cysts? New Insights from Live Imaging

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Abstract Text

In many species, gametogenesis involves the formation of germline cysts – clusters of interconnected germ cells that arise through mitotic divisions with incomplete cytokinesis. In the mouse, oocytes arise from these germline cysts in a process known as cyst breakdown, during which oocytes undergo an increase in volume and organelle content while other germ cells in the cyst undergo cell death.
Several models have been proposed to explain how oocytes become enriched in volume and organelles, including a nursing mechanism similar to that observed during *Drosophila* oogenesis, cell fusion, and phagocytosis. Ultimately, however, understanding how oocyte enrichment occurs requires direct visualization of cytoplasmic dynamics within cysts.
Here, we use a combination of genetically encoded fluorescent reporters and high-resolution live imaging to directly visualize cyst breakdown and to track the dynamics of cytoplasm, organelles, and key oocyte factors within individual cysts. Using this novel approach, we test previously proposed models of mammalian oocyte enrichment and individualization. We find that mitochondrial transfer through intercellular connections is limited, whereas cytoplasmic proteins can be extensively shared between cyst cells. I will present our work in characterizing this sharing by systematically tracking key meiotic and oocyte specification factors through intercellular connections.
These findings provide new insight into the long-standing question of why oocytes develop within germline cysts and advance our understanding of the mechanisms that govern the establishment of the mammalian ovarian reserve.

Submission ID#2354970

Lactocrine Deficiency at Birth Alters the Post Natal Day 14 Porcine Ovarian Transcriptome

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Abstract Text

Lactocrine signaling refers to the postnatal transmission of milk-borne bioactive factors from mother to offspring, primarily through colostrum (first milk). This process is important for neonatal development with the potential for long-term consequences in adults. Colostrum intake in neonatal piglets can be quantified using the serum immunoglobulin immunocrit (iCrit) assay. Colostrum deficiency, indicated by low neonatal iCrit values, alters endometrial gene expression, delays uterine gland development in neonatal pigs, and increases age at puberty. Moreover, adult gilts that experienced neonatal lactocrine deficiency exhibit reduced litter size. Because litter size in pigs is influenced by both ovarian and uterine function, understanding the impact of lactocrine deficiency at birth on ovarian development is critical. The objective of this study was to characterize the ovarian transcriptomes of gilts with high (12.26% ± 0.71; n = 10) and low (1.95% ± 0.37; n = 12) iCrit values measured on postnatal day (PND) 1. High- and low- iCrit gilts were paired within litter. Ovaries were collected on PND 14, total RNA was extracted, and Illumina RNA sequencing was performed. A stringent bioinformatics pipeline was applied to identify and characterize both previously annotated transcripts in the*Sscrofa* 11.1 reference genome and novel transcripts expressed in the ovaries. A total of 54,525 expressed transcripts were identified, including 51,618 protein coding transcripts (30,858 annotated and 20,760 novel) and 2,907 noncoding transcripts (2,540 annotated and 367 novel). Transcript level differential expression analysis identified 724 transcripts that differed between high- and low- iCrit gilts at FDR-adjusted *P*-value ≤ 0.05. Of these, 322 were previously annotated, while 398 were novel protein coding transcripts, and four were novel noncoding transcripts. The expressed transcripts corresponded to 17,862 genes. To obtain gene level expression data, the raw transcript *P*-values were aggregated for each gene locus. Sixty differentially expressed genes were identified at FDR-adjusted *P*-value ≤ 0.05, including 57 previously annotated genes and three novel genes. Among these, 46 genes were more highly expressed in low iCrit gilts, whereas 14 were more highly expressed in high iCrit gilts. Genes highly expressed in low iCrit gilts included those involved in oxidative stress responses (*GPX6*, *CYBA*, *SPATA18*) and immune regulation (*RFX1*, *TRIM29*). Because colostrum is rich in antioxidants that help neonatal piglets adapt from the intrauterine to extrauterine environment, the elevated expression of these genes may reflect a compensatory ovarian response to putative oxidative stress in gilts that experienced lactocrine deficiency at PND 1. Additionally, lactocrine-deficient gilts exhibited increased expression of *LHB*, involved in gonadotropin synthesis and *PLA2G6*, involved in prostaglandin synthesis, suggesting altered endocrine signaling within the ovary. Collectively, these findings indicate that lactocrine deficiency on the first day of postnatal life alters porcine ovarian gene expression at PND 14, potentially contributing to disrupted ovarian development and reduced reproductive performance in adulthood.

Submission ID#2361127

Temporal Modulation of Immune Mechanisms During Ovarian Development

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Abstract Text

The establishment of the ovarian reserve of follicles, which is mostly non-renewing, occurs during fetal development in cattle, and the success of this process is a critical determinant of fertility. Formation of the ovarian reserve is not well understood, especially in livestock. The goal of this study was to improve understanding of biological pathways that drive ovarian development. For all experiments, fetuses were collected from a slaughterhouse, and fetal age was determined based on crown-rump length. Ovaries were divided into five age groups, 70 (range: 62-75), 90 (89-93), 120 (120-122), 150 (150-151), and 190 (179-198) days gestation (n=6/group). In cattle, gametes are in germ cell cysts and ovigerous cords on day 70, formation of primordial follicles begins around day 90, follicle activation begins before day 150, and secondary follicles form around day 190. RNA was extracted and sequenced. DEseq2 was used to analyze pairwise comparisons between consecutive age groups. Between days 70 and 90, 5,668 mRNAs changed, with 4,117 that increased and 1,551 that decreased. Between days 90 and 120, 2,605 mRNAs changed with 1,849 increasing and 756 decreasing. From 120 days to 150 days, 4,755 mRNAs changed with 1,794 increasing and 2,961 decreasing. Finally, between days 150 and 190, 1,016 mRNAs changed with 287 increasing and 729 decreasing (padj< 0.05, log2fc >|0.5|). Gene ontology (GO) analysis revealed that transcripts related to meiosis increased between days 70 and 90 and decreased between days 120 and 190, as expected. In addition, inflammatory response, including GO terms such as activation of innate immune response, leukocyte chemotaxis, and positive regulation of cytokine production, were upregulated between days 70 and 90, 90 and 120, and 120 and 150. This suggests that the immune microenvironment of the ovary may regulate developmental changes at these times, including follicular assembly. Therefore, we evaluated total immune cells (CD45+) in the ovary during development. Flow cytometry detected CD45+ cells in the ovary (average=3.4% of total cells) but the percentage of CD45+ cells did not change relative to gestational age (87-93 days, 119-122 days, 146-151 days, and 170-204 days, p >0.05). Nevertheless, preliminary histological immunolocalization indicated that CD45+ cells co-localize with oocytes and ovigerous cords during ovarian reserve establishment, suggesting functional interaction. In addition, we used the MILLIPLEX Bovine Cytokine/Chemokine Panel to evaluate ovarian cytokine abundance across development. IL-1a, IL-36RA, and MCP-1 (CCL2) tended to decrease between day 90 and day 190 (p=0.09 for IL-1a, p=0.07 for IL-36RA, p=0.06 for MCP-1), and MIP-1a (CCL3) tended to decrease between days 120 and 190 (p=0.07). IL-1a, MCP-1, and MIP-1a recruit immune cells. Finally, using the RNAseq dataset, we evaluated the expression of transcripts encoding markers for immune cell subtypes. *CD14,* expressed uniquely on monocytes and macrophages, and *CD8B*, expressed uniquely on cytotoxic T cells, increased between 70 and 90 days. Between days 120 and 150, *CD4*, a marker for helper T cells declined. In summary, inflammatory response was upregulated in the ovary during ovarian reserve establishment. Immune (CD45+) cells were detected in the developing ovary and colocalized with oocytes, suggesting functional interaction. Changes in chemotactic cytokines and markers of macrophages and T cells suggest regulatory roles for the immune system in the establishment of the ovarian reserve.

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Submission ID#2361193

Activation of the cGAS-STING Pathway in Mouse Early Secondary Follicles Induces an Inflammatory Signature and Compromises Follicle Development

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Abstract Text

The ovarian follicle, consisting of an oocyte and surrounding somatic cells, is the non-renewable functional unit of the ovary. Being a finite resource, follicles require tight developmental control to prioritize activation, development, and fertilization of high-quality oocytes while simultaneously eliminating poor-quality oocytes. DNA damage repair, pattern recognition receptors, and atresia pathways are known surveillance mechanism within the follicle. However, additional mechanisms must exist to respond to other challenges which can compromise gamete quality. The cGAS-STING pathway is a mediator of the innate immune response activated by the presence of cytosolic double-stranded DNA (dsDNA), resulting in the production of Type I interferons and pro-inflammatory cytokines. 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 determine if the machinery for the pathway was present in follicles, we examined the expression of cGAS and STING in ovarian follicles. RNA in situ hybridization utilizing whole ovarian sections demonstrated that *Cgas* transcript expression was localized to the oocyte and *Sting1* transcript expression was enriched in granulosa and theca cells. This localization was confirmed at the protein level through immunocytochemistry and western blots. We also observed a significant increase in cGAS expression in the primary to secondary follicle transition. Follicles were isolated from mice and were treated short-term with Q-VD-OPH & ABT-737 or a vehicle to induce release of cytosolic mtDNA to activate the cGAS-STING pathway. After confirming cell viability, TEM was used to quantify mitochondrial membrane rupture. Western blots were utilized to confirm pathway activation. Bulk RNA-Seq was performed to evaluate an inflammatory response. An ex vivo encapsulated follicle growth assay was used to determine how treatment impacts follicle and oocyte outcomes. Short-term Q-VD-OPH & ABT-737 treatment induced rupture of mitochondrial membranes with increased abnormal morphology and decreased size in both oocytes and granulosa cells relative to vehicle-controls. Short-term treatment and mitochondrial membrane rupture activated the cGAS-STING pathway confirmed through increased phospho-STING, -IRF3, and -TBK1 expression relative to controls. Wild-type treated follicles produce a Type I interferon-dependent inflammatory response that was not recapitulated in treated *Cgas* KO follicles. RNA and protein localization of subunits of the Type I Interferon Receptor was predominantly localized to the somatic cells of the follicle. Utilizing an ex vivo encapsulated follicle growth assay, short- and long-term treatment of cultured wild-type follicles have decreased follicle survival and growth. This was associated with increased atretic oocytes relative to controls. GV oocytes collected from surviving treated follicles on the terminal day of culture were smaller than GV oocytes from vehicle-treated follicles. Additionally, short- and long-term culture with Q-VD-OPH & ABT-737 negatively impacted follicle rupture and oocyte maturation. Follicles cultured from *Cgas* knockout animals in the presence of Q-VD-OPH & ABT-737 had increased survival relative to wild-type controls, indicating specificity of these compounds to the cGAS-STING pathway. These findings provide novel evidence that the cGAS-STING pathway functions as a surveillance mechanism in preantral follicles to moderate oocyte quality. Future studies will investigate how reproductive aging impacts the responsiveness and function of the cGAS-STING pathway in preantral follicles.

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Submission ID#2361197

KIT and Insulin Signaling in Primordial Follicle Formation

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Abstract Text

The ovarian reserve, essential for female fertility, is composed of primordial follicles, each consisting of an oocyte surrounded by pre-granulosa cells. Disruption in ovarian reserve formation during perinatal development may lead to conditions such as Primary Ovarian Insufficiency (POI). During development, primordial germ cells migrate to the gonad and become oogonia, which divide mitotically with incomplete cytokinesis to form germ cell cysts. These oogonia enter meiosis, becoming oocytes that arrest in prophase I. The cysts then break down, allowing individual oocytes to be encased by pre-granulosa cells to form primordial follicles. KIT ligand (KITL) signaling through the receptor tyrosine kinase KIT is a crucial regulator of cyst breakdown and primordial follicle formation. However, targets downstream of KIT signaling are unknown. In addition, previous studies have shown that insulin is vital for promoting later follicle development and works additively with KITL, but its function during cyst breakdown and primordial follicle formation has yielded conflicting results. Recent research using a gestational diabetes mouse model revealed that fetal ovaries exhibited elevated glucose and insulin levels as well as increased primordial follicle numbers, though the individual contributions of glucose versus insulin remained undetermined. The goals of this project are to identify targets downstream of KIT signaling involved in primordial follicle formation and to investigate the role of insulin. We blocked KIT signaling in ovary organ culture during follicle formation and used RNA-seq to determine the differential expression profiles of ovaries in the presence and absence of KIT activity. One group of genes downregulated when KIT signaling was blocked were Mast cell specific genes which suggests that Mast cells may play a role in primordial follicle formation. Using immunocytochemistry, we found the insulin receptor expressed in oocytes. Initial studies investigating effects of insulin in ovary suggest that insulin promotes primordial follicle formation and overall ovarian growth. Our work will provide insight into how the ovarian reserve is established and help to identify novel molecular targets for the development of treatments for reproductive disorders such as primary ovarian insufficiency.

Cross-Species Perspectives on Male Fertility

Wednesday, July 21 1:30 PM – 3:00 PM

Submission ID#2361345

Bovine Sperm With Abnormal Head Morphology Exhibit Increased Histone Retention

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Abstract Text

During mammalian spermatogenesis, nearly all histones within germ cell chromatin are evicted and replaced with protamines. This process results in a dramatic change in chromatin condensation, with only 1-15% of histones retained in sperm, depending upon species. Histones can be modified with post-translational modifications that are transmitted as epigenetic information to the embryo. Aberrations to the histone eviction process can result in increased histone retention, which has been linked to impaired sperm function, abnormal sperm morphology, poor embryo development and/or pregnancy loss in multiple mammalian species, including humans. Clinically, male infertility is identified by visual assessment of sperm morphology and motility, without consideration of molecular or epigenetic factors. Similarly, the bovine industry employs a breeding soundness exam (BSE), which utilizes morphology and motility parameters of sperm to determine presumed fertility. However, a significant issue facing the cattle industry is pregnancy loss occurring from sires that pass BSEs, suggesting that other factors, including epigenetic regulation through paternal histones may be involved. The goal of this study was to investigate potential links between histone retention and bovine sperm morphology. Angus bulls (n=18) were subjected to industry standard BSEs, including visual assessment of semen for morphology and motility. A portion of the ejaculate was fixed and stained with Eosin-Nigrosin to assess morphology, and the remaining ejaculate was snap frozen. Bulls were classified as BSE passed (BSE-PS, n=12) or failed (BSE-FL, n=6) based on Society for Theriogenology defined criteria (≥30% motility, ≥70% normal morphology). Snap frozen ejaculates were washed with PBS to remove seminal fluids and treated with somatic cell lysis buffer (0.1% SDS, 0.5% Triton-X-100). Histones were subsequently extracted from 10^8 cells/animal via sulfuric acid extraction and trichloroacetic acid precipitation. Histone extracts were equally loaded on protein gels and assessed for the abundance of H2A, H2B, H3, or H4 via western blot and protein band intensity was quantified (ImageJ). Initial analysis identified increased histone retention for H2B BSE-FL bulls ($p< 0.05$; fold change (FC)=2.55), suggesting potential epigenetic changes to BSE-FL sperm. Given the multiple sperm parameters assessed with BSE analysis, we next assessed changes in histone retention in sperm with specific morphological defects. Samples from Hereford (n=7) and Angus bulls (n=3) were classified as having normal morphology (n=3), >30% abnormal tail morphology (n=3), or >30% pyriform head defects (n=4). Sperm with abnormal head morphology exhibited increased retention of all core histones when compared to normal sperm and abnormal tail morphologies ($p< 0.05$; H3 FC=12.50). Excitingly, these findings mirror previous findings of increased histone retention previously identified in abnormal human sperm and mouse sperm. Furthermore, 17% of bulls with normal morphology exhibited increased histone retention, suggesting alterations to sperm chromatin in morphologically normal sperm. Additionally, head area from fixed and Eosin-Nigrosin stained sperm were measured (ImageJ) and compared across control, tail, and head abnormalities. Unexpectedly, sperm with abnormal head morphology exhibited reduced area compared to normal and tail defect sperm ($p< 0.05$; abnormal head: 32.63±0.248μm^2; normal: 36.1±0.277μm^2), highlighting the need for additional study to understand the association between sperm chromatin and morphology. This study demonstrates for the first time increased histone retention in morphologically abnormal bovine sperm, a phenomenon that is conserved in humans and mice. Furthermore, this data supports the theory that histone retention may be an overlooked contributor to impaired fertility and unexplained pregnancy loss across mammalian species.

Submission ID#2358895

Lactocrine Programming by Early-Life Colostrum Intake Shapes Testicular Development in Neonatal Boars

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Abstract Text

Boar fertility is a critical driver of swine reproductive efficiency. Extensive testis development occurs during neonatal life, a highly plastic period that overlaps temporally with nursing. Previous work reported a strong, positive correlation ($r = 0.8539$) between neonatal colostrum intake and total sperm per ejaculate in adulthood; boars with high colostrum intake produced 17 billion more sperm (15% increase) than low-intake boars. However, the mechanisms linking early-life colostrum intake with subsequent sperm production remain unexplored. The objective was to determine the effect of differential colostrum intake on neonatal testis development in boars. Colostrum consumption was assessed on postnatal day (PND) 1 via the immunoglobulin immunocrit assay (iCrit). Boars with high (12 ± 0.5%; n = 8) or low (4 ± 0.5%; n = 8) iCrit levels were identified ($P < 0.0001$). Paired high- and low-iCrit littermates with similar birth weights (1.5 ± 0.1 kg; $P = 0.34$) were selected. On PND 3, boars were weighed and castrated; testes were weighed and snap-frozen or fixed for downstream analyses. Testis samples were subjected to protein extraction and label-free proteomics. On a subset of animals (n = 6/treatment), histological sections were prepared to assess testicular architecture, and immunofluorescence was performed to quantify Sertoli and germ cell numbers. Morphometric and immunofluorescence data were analyzed by ANOVA with boar as the experimental unit. For bioinformatics, proteins were considered differentially abundant at a Benjamini–Hochberg–adjusted $P < 0.05$ and ≥ 2-fold change (up or down). Body and testis weight did not differ by iCrit status ($P > 0.10$). Histological analysis revealed that Leydig cell area was increased in high- versus low-iCrit boars ($P < 0.0001$); likewise, interstitial area tended to be increased in high-iCrit boars ($P = 0.0940$). These findings suggest greater steroidogenic capacity in high-iCrit testes. Total tubular area tended to be reduced in high- versus low-iCrit boars ($P = 0.0952$), which likely reflects fewer seminiferous tubules per field ($P = 0.04$), given the area of individual seminiferous tubules did not differ by iCrit status ($P = 0.36$). Sertoli cell counts did not differ by iCrit status ($P > 0.10$). However, germ cell number per field and per seminiferous tubule were greater in high- versus low-iCrit boars (49% and 65%, respectively; $P \leq 0.0024$). These data indicate lower seminiferous tubule density but greater germ cell abundance in high-iCrit neonates, consistent with enhanced early germ cell establishment. Regarding proteomics, 5,068 testicular proteins were identified and 498 (10%) differed by iCrit status; 228 proteins were upregulated and 270 were downregulated in high- versus low-iCrit testes. Androgen receptor protein abundance was decreased in high- versus low-iCrit boar testes; whereas both LHCGR and HSD17B6 were upregulated, suggesting effects on LH-mediated steroidogenesis and androgen signaling. Germ cell development–associated proteins TDRD1 and UTF1 were downregulated in high-iCrit testes. Several germ cell–associated proteins were increased in testes from high-iCrit boars, including PIWIL2, YTHDC2, SPAG7, and USP9Y. Proteins downregulated in high-iCrit testes were associated with ubiquitin-mediated protein turnover (e.g., UCHL3), lysosomal processing (e.g., CTSH), immune signaling (e.g., REL), and chromatin regulation (e.g., SETD1A). Overall, these data indicate that colostrum intake influences neonatal testicular development, particularly Leydig and germ cells. Greater germ cell abundance within seminiferous tubules of high-iCrit neonates may underlie greater sperm production in adulthood. The USDA is an EEO provider.

Submission ID#2360848

Sperm Aggresomes Program Early Embryonic Stress Responses and Developmental Competence

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Abstract Text

Pregnancy loss in dairy cattle has been largely attributed to maternal environment, female fertility phenotype, and genetic background. More recently, attention has shifted toward paternal contributions to early embryonic development and pregnancy establishment. Although sires differ markedly in their impact on pregnancy outcomes, the molecular mechanisms underlying these differences remain poorly defined. Conventional semen quality parameters, including motility, concentration, and morphology, incompletely explain fertility divergence among sires, highlighting the need to identify paternal molecular determinants that influence embryo competence and early pregnancy survival. A deeper mechanistic understanding of paternal contributions from fertilization through blastocyst formation is essential to advance strategies aimed at reducing early pregnancy loss.
Beyond delivery of the paternal genome, sperm transmit diverse molecular cargo that can influence early embryogenesis. Aggresomes (AGG), cytoplasmic inclusions composed of misfolded and ubiquitinated proteins, have been described in mammalian gametes, yet their functional significance in reproduction is unknown. Here, we tested the hypothesis that sperm AGG content represents a paternal determinant of embryo developmental competence. Using image-based flow cytometry, sperm from 32 Holstein sires were quantified and stratified into low-, moderate-, and high-AGG groups. AGG abundance was independent of sire age and did not affect in vitro capacitation or acrosome remodeling, indicating that AGG content is not reflected by conventional functional semen parameters.
In contrast, marked differences were observed at the level of embryo development. Embryos generated by in vitro fertilization using sperm from high-AGG sires exhibited reduced blastocyst formation, delayed cleavage kinetics, and increased developmental arrest at the 4–6 cell stage compared with embryos derived from low-AGG sires. Subcellular localization of AGG to the sperm head was particularly detrimental and strongly associated with impaired blastocyst development. Embryos from high-AGG sires accumulated higher levels of AGG during early development, displayed elevated reactive oxygen species, and exhibited altered mitophagy dynamics, suggesting disrupted protein quality control and mitochondrial homeostasis. Pharmacological attenuation of endoplasmic reticulum stress using tauroursodeoxycholic acid partially improved early cleavage but failed to rescue blastocyst development, indicating that AGG-associated defects are not fully reversible by acute stress modulation.
Importantly, under in vivo conditions, embryos generated from high-AGG sires exhibited reduced transferable quality relative to embryos from low-AGG sires, demonstrating translational relevance to embryo transfer programs.
Collectively, these findings identify sperm aggresome burden as a previously unrecognized paternal determinant of embryo quality and developmental competence. Our data demonstrate that misfolded protein aggregates can be transmitted from sperm to the embryo, perturb redox balance and mitochondrial quality control pathways, and compromise developmental progression. This work reveals a novel molecular mechanism underlying sire-dependent fertility differences and supports the incorporation of protein quality control metrics into advanced molecular screening strategies for sire selection and fertility prediction.

Submission ID#2360650

Identification of Multiple Gene Variants Underlying Bovine Stump Tail Spermatozoa Defect

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Abstract Text

Stump-tail defect is a rare, heritable sperm morphological abnormality characterized by almost complete absence of the sperm midpiece and tail in most ejaculated spermatozoa, leaving only a residual stump-like remnant. It precludes sperm motility and contributes to male infertility in humans and reduced reproductive success in livestock. This non-recoverable condition is considered to be inherited through homozygous recessive genes. Identification of the heritable molecular factors of sperm tail malformation may therefore enhance the reproductive efficiency and genetic selection in animal breeding programs. To further define the genetic causes of the stump tail defect, an infertile, asthenozoospermic bull with 100% stump tail spermatozoa was genome sequenced and found to be homozygous for recessive mutations in five genes- *DCDC2C, MICAL1, CEP152, KIAA0586, and POC1A*, which are predicted to result in missense variants of corresponding proteins. Evolutionarily, these proteins are conserved and have well-established functions in sperm microtubulome organization, cytoskeleton regulation, centriolar duplication, centriolar maintenance, and ciliogenesis, respectively. However, their roles in spermatogenesis remain largely unexplored. Using a high-throughput phenotyping pipeline, which includes epifluorescence microscopy, transmission electron microscopy, image-based flow cytometry (IBFC), and proteomic analysis, aberrant localization patterns of these candidate proteins were identified in stump tail spermatozoa and spermatids of the affected bull compared to those of wild-type bulls. Moreover, disrupted spermatogenesis with loss of normal germ cell organization in affected seminiferous tubules and altered localization of microtubules and microtubule-associated proteins KATNAL2, EML5, and EML4 were observed in affected spermatozoa. Adding to the complexity of genomic influences on sperm quality, three of the five target genes in this study carry alternative polyadenylation (APA) sites, opening a new line of investigation into alternative transcripts in wild-type and mutant sires. Collectively, this study will advance our understanding of the molecular mechanisms of sperm tail development, providing further applications in diagnosing male infertility in animal models and humans, assisting in sire selection for artificial insemination service, and improving reproductive efficiency in livestock. 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.

Submission ID#2360845

Investigating the Effects of Cryopreservation Methods on Avian Germline Stem Cells from Adult White Leghorn Chicken Testicular Tissue

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Abstract Text

Gonadal germline stem cells (GSCs) are a crucial resource in conservation reproductive science due to the unique ability of germ cells to pass genetic material to the next generation. Cryopreservation of GSCs provides the opportunity to rescue genetic material of deceased individuals, thus guarding against complete diversity loss in critical populations. While many studies have been conducted on the cryopreservation of primordial germ cells (PGCs) harvested from young or embryonic birds, there is a major gap in research on the cryopreservation of adult avian testicular tissue. Harvesting testicular tissue from embryonic or juvenile birds in endangered populations is not ideal; therefore, it is essential to develop an effective cryopreservation protocol for adult tissue to maximize the recovery of viable GSCs. In this study, several cryopreservation techniques were tested on testicular tissue from adult White Leghorn chicken (*Gallus gallus*), comparing the effects of different cryopreservation solutions and different freezing techniques to find the best cryoprotectant-method combination for the most viable GSCs. The two freezing methods evaluated were the CoolCell system with a −1 °C/min controlled-rate freezing container and the CryoMed Programmable Freezer. Within each freezing method, seven cryoprotective solutions were tested, including the proprietary Stem-CellBanker, PBS +8% FBS +2% chicken serum-based media with 5%, 8%, or 10% DMSO and with or without 0.3M Trehalose. After a minimum of two weeks liquid nitrogen storage, tissue was thawed and enzymatically dissociated. Viability percentages were confirmed by propidium iodide staining, and immunocytochemical labeling with anti–SSEA-1, SSEA-3, and SSEA-4 antibodies was analyzed by flow cytometry to quantify germline stem cells and assess their preservation after cryopreservation. While no significant difference was found in the cell viability between treatments ($p > 0.05$), immunocytochemistry results show significant differences between cryopreservation treatments from SSEA-1, -3, and -4 ($p < 0.05$). These results give great insight into how cryopreservation affects adult avian tissue, which will aid significantly in the effort to preserve genetic material of threatened avian species worldwide.

Translational Outcomes Through Machine Learning

Wednesday, July 21 1:30 PM – 3:00 PM

Submission ID#2360683

Distinct Proteomic Profiles in Non-Capacitated and Capacitated Boar Sperm Linked to Pregnancy Rate and Litter Size

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Abstract Text

Pregnancy rate (PR) and litter size (LS) are widely used as indicators of boar fertility, reflecting sperm fertilization and early embryonic competency. Notably, these outcomes tend to vary more across males than females. Biomarker discovery efforts in male reproduction have expanded rapidly with advances in high-throughput detection methods such as flow cytometry and proteomics. However, most boar proteomic studies focus on a single fertility outcome without examining functional changes across non-capacitated (NC) and capacitated (C) states. Given that capacitation is the final maturation step required for fertilization competence, integrating PR and LS across NC and C states within a single dataset provides a more comprehensive understanding of sperm functional physiology. In this study, semen from 22 boars was examined based on industry field performance records, with high PR defined as > 92% and low as < 83%, high LS defined as > 13.44 total born and low as < 13.43 total born. Three biological replicates were included per boar with a minimum of 7 sows inseminated per replicate. Sperm proteins were extracted and digested using phenol-based filter-aided sample preparation and analyzed by LC-MS/MS. Quantification was performed in Spectronaut. Gene ontology (GO) enrichment of significant proteins was conducted in R using clusterProfiler with a Benjamini–Hochberg FDR cutoff of 0.05 and $\log_2$FC ±2. A total of 527 proteins were identified across comparisons. Gene ontology (GO) enrichment analysis revealed shared signatures associated with reproductive performance. In NC sperm, both low PR and low LS enriched biological processes (BP) terms including carboxylic acid metabolic process, oxoacid metabolic process, generation of precursor metabolites and energy, and oxidation–reduction process. Corresponding cellular component (CC) terms included mitochondrial respiratory chain complex and oxidoreductase complex, supported by molecular function (MF) enrichment for oxidoreductase activity. Together these enrichments indicate elevated baseline mitochondrial oxidative metabolism. In contrast, high fertility phenotypes demonstrated greater functional specialization. High PR NC sperm were enriched for BP terms such as protein folding, protein maturation, and vesicle-mediated transport, with CC enrichment of endosomal and multivesicular body components, suggesting enriched proteostasis and membrane organization prior to sperm capacitation. High LS NC sperm showed BP enrichment for chromatin organization, nucleosome assembly, and DNA packaging. CC terms included nucleosome and chromatin complex, implicating chromatin integrity and potential developmental competence. After sperm capacitation, both the high PR and LS sperm showed similar functionality with shared enrichment for fertilization, single fertilization, proteolysis, and protein catabolic processes, reflecting activation of fertilization-associated pathways. Conversely, both low PR and LS sperm shared enrichment for protein transport, ribosomal subunit assembly, and small GTPase-mediated signal transduction, suggesting that these cells are stalled in reorganization and signaling processes rather than being primed for fertilization. Collectively, GO enrichment reveals shared and diverging sperm proteomic signatures that reflect reproductive performance. Integrating multiple fertility outcomes within a single proteomic framework provides deeper insight into sperm functional states and advances the development of biologically informative biomarkers to enhance reproductive efficiency in swine production systems.

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Submission ID#2360158

Integrating Single-Oocyte and Spatial Transcriptomics to Investigate Correlation Between Oocyte Gene Expression and Follicle Stage in the Human Ovary

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Abstract Text

Ovarian follicle activation is the process in which the germ cell and its surrounding support cells transition from a dormant, primordial state into a growing follicle, marking the crucial first step towards the production of a mature, fertilizable egg. Understanding this process, including the transcriptional changes that occur within the oocyte during activation, has wide implications for preserving the ovarian reserve for fertility and slowing ovarian aging. Here, we integrated single-oocyte RNA sequencing with high-resolution spatial transcriptomics to investigate the relationship between oocyte transcriptional state with follicle morphology across primordial, transitioning, and primary stages. Single oocyte sequencing was performed on 133 individual oocytes isolated from ovarian tissue from nine reproductive-age donors (ages 16-30). Unsupervised analysis identified two interconnected clusters (C1 and C2). Gene Ontology analysis revealed enrichment of cell cycle and meiotic processes in C1 as opposed to increased expression of genes associated with protein translation, metabolic activity, and extracellular matrix organization in C2. Comparison with published datasets of early human follicles indicated that C1 exhibited characteristics of a more dormant, primordial-like state, while C2 corresponded to a more active, primary-like state. To relate oocyte transcriptional state to follicular morphology, we generated Xenium spatial transcriptomic datasets for ovarian tissue from six donors (ages 18-30). A customized gene panel was created, augmenting the Xenium Prime 5K assay with 100 candidate markers derived from our differential expression analysis and literature review. Following rigorous histology-based quality checks, including oocyte segmentation accuracy, follicle health, and stage assignment, 242 high-quality oocytes were retained for analysis. Ongoing studies are evaluating whether individual oocyte transcriptional signatures predict morphological stage and testing the concordance of activation states across sequencing modalities. By synthesizing single-cell transcriptomics with imaging-based spatial sequencing that preserves tissue architecture, this work aims to define the molecular landscape of early human follicle activation at unprecedented resolution. Together, these datasets provide a powerful platform for identifying biomarkers of follicle recruitment and enabling future strategies to preserve the ovarian reserve, extend reproductive lifespan, and improve fertility outcomes.

Submission ID#2360255

IsomiR Profiles in Bovine Granulosa Cells Distinguish Dominant Preovulatory from Atresia-Associated Subordinate Follicles

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Abstract Text

Follicular atresia is a non-inflammatory process of degeneration and resorption of bovine ovarian follicles driven by granulosa cells apoptosis or necrosis and impaired oocyte competence. MicroRNAs (miRNAs) are important post-transcriptional regulators of these processes. Beyond canonical miRNAs, variants generated by alternative cleavage, nucleotide additions/removal at the 5´/3´ ends, or internal sequence differences give rise to isoforms termed isomiRs. Because small sequence shifts can modify seed pairing, isomiRs may direct target recognition differently than their canonical counterparts; they can also differ in half-life and exhibit distinct loading preferences for argonaute proteins. Despite these features, the contribution of isomiRs to follicular atresia remains poorly characterized. Here, follicular status is used as a biological model of contrasting atresia-related states, with dominant preovulatory follicles as a non-atretic reference and subordinate anovulatory follicles as an atresia-associated condition. Public small RNA-seq data from bovine granulosa cells (Bos taurus) were obtained from NCBI GEO (GSE56002), including dominant preovulatory follicles (n = 3) and subordinate anovulatory follicles (n = 3). After quality control (FastQC/MultiQC) and adapter trimming (Cutadapt), reads were mapped, and a miRNA/isomiR count matrix was generated with miRge3.0. Across replicates, the distribution of isomiRs among the most abundant miRNAs was consistent, and it differed between follicular groups. Global separation between groups was evaluated by principal component analysis (PCA) using logCPM (TMM) values for isomiRs filtered with filterByExpr. Dominant and subordinate follicles separated clearly (PC1 = 82.6%). Differential expression analysis with edgeR identified 498 differentially expressed isomiRs (FDR < 0.05, p-value ≤ 0.023, and |log2FC|≥1), where 180 were upregulated in subordinate follicles, and 318 were upregulated in dominant preovulatory follicles, indicating a predominance of increased isomiRs in the dominant state. To assess changes in the relative usage of isoforms within each pre-miRNA, differential isoform usage (DIU) analysis was performed with DRIMSeq, which models multivariate counts per isomiR using a Dirichlet-multinomial distribution and tests changes in usage proportions between conditions. Events with |Δproportion| ≥ 0.15 and FDR < 0.05 were prioritized, yielding 16 pre-miRNAs with significant DIU and 25 associated isomiRs (1–2 isomiRs per pre-miRNA). PCA based on the isoform-usage profile again separated dominant from subordinate follicles (PC1 = 77.5%), supporting coordinated isoform remodeling across precursors. Sample-level metrics were calculated to quantify isomiR usage, the fraction of isomiR reads per pre-miRNA, and a global usage score. In both metrics, dominant follicles showed a higher isomiR usage than subordinate follicles. Per pre-miRNA, the isomiR fraction was higher in dominant follicles (72.3%, IQR 1.1) than in subordinate follicles (51.2%, IQR 6.8). Consistently, the global score was also higher in dominant follicles (59.0%) than in subordinate follicles (27.6%) (95% bootstrap CI: 20.8–36.0 pp). Overall, comparisons between dominant and subordinates reveal regulation at the isomiR level through changes in abundance: 498 isomiRs were enriched in the dominant state, and DIU was observed in 16 pre-miRNAs, which likewise favor this follicular state. Together, these results provide initial evidence in a bovine model that isomiR profiles and usage are associated with follicular state and support isoform-specific regulation linked to atresia-related conditions.

Submission ID#2361068

A Multi-Biomarker Label-Free Framework for Boar Sperm Fertility Diagnostics

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Abstract Text

Functional sperm diagnostics remain constrained by reliance on fluorescent probes, which limit throughput, increase cost, and introduce assay-dependent variability. Our recent work demonstrated that convolutional neural networks (CNNs) trained on brightfield images collected using image-based flow cytometry (IBFC) can predict propidium iodide (PI)-defined plasma membrane integrity in a label-free manner across mammalian species (boar, non-human primate, and human). In boar sperm, label-free PI models trained on large-scale datasets (500,000 images total; 250,000 per class, PI-positive and PI-negative) achieved F1 scores of 97.0% and predicted unstained populations with a mean absolute error (MAE) of 1.07 and an R^2 of 0.97 relative to stained ground truth. Models trained in human (500,000 images total) and bonobo (50,000 images total; limited availability) showed lower baseline performance (92.0% and 86.8%, respectively), but transfer learning (TL) from boar substantially improved classification in both species to 93.7% and 94.4%. Sequential TLfrom boar to bonobo to human further increased the human F1 score to 94.9%. Prediction error with the TL human model resulted in a 0.77 MAE and an R^2 of 0.99, with the sequential boar-to-bonobo-to-human transfer producing the highest human performance. These findings demonstrate that conserved micro-morphological features in brightfield images reflect sperm membrane state across mammals and support a scalable, cross-species label-free sperm health diagnostic framework. Next, we examined the feasibility of additional label-free biomarkers in boar using 10,000 images total. For PI under this reduced training size, F1 = 88.6%. Label-free inference of the additional biomarkers achieved robust performance across multiple functional biomarkers relative to fluorescence-annotated ground truth, including mitochondrial membrane potential (JC-1; F1 = 84.6%), ΔΨm-linked mitochondrial functional status (MitoTracker Deep Red CMXRos; F1 = 89.5%), oxidative stress (CellROX Orange; F1 = 90.1%), acrosomal remodeling or defects (lectin PNA; F1 = 95.6%), and defect-associated tail membrane glycan remodeling (lectin LCA; F1 = 94.0%). To evaluate cross-species scalability within this framework, human lectin PSA (functional analog to PNA in boar) models trained de novo achieved an F1 of 87.0% with 10,000 total. When initialized using pretrained boar label-free PNA networks, TL improved human classification performance to an F1 of 92.2%, indicating that structural signatures of sperm functional state learned in boar sperm can be translated to human samples. This cross-species transfer supports development of a unified architecture in which foundational models trained in boar can accelerate label-free functional diagnostics in humans. Brightfield-derived morphology enables simultaneous structural assessment alongside functional biomarker inference, allowing estimation of the proportion of sperm that are both morphologically normal and physiologically competent. Inferring membrane integrity, mitochondrial status, oxidative stress, and acrosomal state from a single brightfield acquisition reduces reliance on fluorescent labeling while preserving multi-parameter functional insight. Such a platform has direct applications in livestock breeding, clinical andrology, and conservation biology and provides a scalable basis for AI-driven assessment of sperm fertilizing potential across mammalian species. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2022-67015-36298 (K.K.) from the US Department of Agriculture's National Institute of Food and Agriculture and the National Pork Board (I.S. & K.K.)

Submission ID#2365161

CoRSIVs – A Powerful New Tool to Study Epigenetic Variation in Reproduction

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Abstract Text

Epigenome-wide association studies (EWAS) aim to advance our understanding of the causes and consequences of interindividual epigenetic variation by profiling DNA methylation at subsets of the 30M methylation sites (CpG sites) in the human genome. To improve the power of EWAS, we discovered and validated correlated regions of systemic interindividual epigenetic variation (CoRSIVs) in the human genome. These are genomic regions (median size 200 bp) each containing at least 5 CpG sites. Systemic (i.e. not tissue-specific) interindividual variation at CoRSIVs results from these methylation states being established in the cleavage-stage embryo then maintained during subsequent organogenesis and differentiation. Because of their systemic nature, DNA methylation measurements in blood provide information about epigenetic regulation throughout the body. Moreover, establishment of CoRSIV methylation is influenced by both genetic variation and periconceptional environment, implicating them in the developmental origins of disease. I will summarize our recent data on an expanded multi-racial CoRSIV screen (CoRSIVs 2.0) including both Black and White Americans, as well as extensive data demonstrating the sensitivity of CoRSIV methylation to periconceptional environment, particularly in the context of medically assisted reproduction. I will share an analysis of CoRSIV methylation in the sperm and blood of oligozoospermic males vs. fertile controls, indicating that CoRSIV methylation in blood may be useful in the diagnosis of male infertility. Lastly, I will describe our discovery of CoRSIVs in dairy cattle, which both points to potential opportunities to optimize agricultural outcomes based on epigenetic variation and suggests that CoRSIVs likely exist in all mammals.

Environmental Impacts on Male Reproductive Health

Wednesday, July 21 1:30 PM – 3:00 PM

Submission ID#2361198

Integrative Analysis of Cellular Stress Response in Male Fertility and Beyond

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Abstract Text

Infertility affects one in six couples in the United States, with male factor infertility contributing to ~50% of cases. In a significant fraction of these men, the rationale for low sperm quality and quantity are unknown or couples have difficulty conceiving despite normal sperm parameters and absence of female contributing factors. Mounting evidence suggests that such idiopathic infertility or subfertility correlate with environmental stressors and suboptimal responses, but the molecular and cellular mechanisms remain incompletely understood. Here, we use a suite of contemporary technologies, including single-cell and spatial omics, high-resolution microscopy, epigenomics and nascent RNA sequencing to dissect the impact of environmental stress on the mouse testis and molecular mechanisms that drive appropriate cellular stress responses. Notably, heat stress (e.g. from occupational hazard, recreational practices, intense febrility and global rises in temperature) degenerates the testis and impairs spermatogenesis, but drives dramatic induction of cytoprotective gene expression in surviving cells. We find that stress-responsive gene regulatory programs are intracellularly organized within nano-to-micron scale stress-induced transcription factor condensates, whose sustenance can be explained by a principle in physical chemistry called liquid-liquid phase separation. Alterations in condensate state, affects transcriptional responses, and consequently, cell fitness. Overall, our work seeks to provide molecular rationales for sub/infertility that arises during stressful environmental exposures and identify paths that help overcome these deleterious effects.

Submission ID#2360387

Effects of *In Utero* Heat Stress and Genomic Selection for Heat Tolerance on Semen Quality and Sperm Physiology in Boars

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Abstract Text

In utero heat stress (IUHS) is associated with reduced semen quality in boars. Specifically, IUHS boars exhibit a 23% decrease in total sperm production, a 74% increase in tail abnormalities, and alterations in sperm kinematics. However, effects of IUHS on sperm physiology and capacitation have not been investigated. Further, genomic selection for greater heat tolerance may offer a practical solution to safeguard against the negative reproductive consequences of IUHS. The objective of this study was to evaluate semen quality and sperm health in boars gestated under IUHS or *in utero* thermoneutral (IUTN) conditions whose parents were genomically selected for greater heat tolerance (TOL) or sensitivity (SEN). Pregnant crossbred (Landrace x Large White) dams were subjected to cyclic heat stress (26–36°C) or thermoneutral (17–20°C) conditions between gestational days 6–70. Male offspring were categorized as IUHS+SEN (n = 8), IUHS+TOL (n = 8), IUTN+SEN (n = 9), or IUTN+TOL (n = 6). Semen was collected weekly for 10 weeks, beginning at a similar age (202 ± 15.8 days). Ejaculates were analyzed using computer-assisted sperm analysis (CASA). During the final 3 weeks of the collection period, one ejaculate per boar (n = 10–11 boars/week across treatments) was analyzed by image-based flow cytometry (IBFC) to assess plasma membrane integrity (propidium iodide), acrosomal status (PNA-AF647), zinc localization (FluoZin-3 AM; biomarker of capacitation), and oxidative stress (CellROX Orange) pre- and post-capacitation; Δ was calculated as the difference between pre- and post-capacitation values. Data were analyzed using PROC MIXED in SAS, with boar as the experimental unit. For CASA data, treatment, time, and the treatment x time interaction were considered fixed effects in the initial model, with time as a repeated measure and semen collector, CASA evaluator, and time between collections included as covariates when necessary. Time and the treatment x time interaction were not significant and were removed from the model; therefore, only treatment main effects are reported. For IBFC data, treatment was the fixed effect, with collection week and semen collector included as a covariate when appropriate. There was an overall treatment effect for progressive motility (P=0.0134) and slow sperm (P=0.0159). Specifically, within the TOL genotype, IUHS boars had a greater number of slow sperm compared with IUTN boars (P=0.0413); but overall, IUHS+TOL boars tended to have a greater proportion of slow sperm than IUHS+SEN boars (P=0.0608). Moreover, IUHS+SEN boars tended to have an increased number of sperm with normal morphology versus IUHS+TOL boars (P=0.1023). The percentage of sperm with the distal midpiece reflex (DMR; tail defect associated with total number born) differed between treatment groups (P=0.0025). IUHS+TOL boars experienced an 8-fold greater percentage of DMR than IUTN+TOL boars (P=0.0108). Further, in IUHS boars, the TOL genotype had a greater number of sperm with DMR than the SEN genotype (P=0.0205). In addition, sperm from IUHS+SEN boars had an altered head shape compared with IUTN+SEN boars (P=0.0358). For IUHS males, TOL boars tended to have a reduced head elongation score compared with SEN boars (P=0.0592). Based on Δ, IUHS+TOL animals had a lower proportion of sperm that underwent the acrosome reaction compared with IUTN+TOL boars (P=0.0211). Overall, these data suggest that IUHS compromises aspects of porcine sperm structure, motility, and ability to achieve a fertilization-competent (capacitated) state, with several effects dependent on heat tolerance genotype. The USDA is an equal opportunity employer.

Submission ID#2361131

Development of Penile Organoids to Screen for Hypospadias-Inducing Compounds

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Abstract Text

Exposure to environmental toxicants during critical developmental windows has been linked to congenital abnormalities of the male reproductive system. Birth defects of the penis are among the most common malformations in the world. Children with congenital birth defects often require surgical correction, which is associated with a high risk of complications. Although common, ~70% of penile malformations have no clear cause. A mixture of androgen signalling deficiency, maternal exposure to estrogenic compounds, and environmental toxic chemicals leads to disruptions in penis formation and function. Here we develop and validate a penis organoid protocol that can be used to study the impacts of environmental chemicals on penis signalling pathways. To determine if penis organoids can be established, we collected penises from CD1 mice during the critical window of penis formation (embryonic day (E) 16.5). Penises were disassociated using enzymatic cocktails and plated in 96-well U-bottom plates. By 12 hrs, organoid formation was already observed. To identify whether there were representative penis cell populations in the organoids, we used immunofluorescence against E-cadherin, vimentin, NR2F2, AKAP12, FOXA1, FOXF1, TFAP2A, SMA-alpha, and AR. We found that the organoids had distinct cell populations, including epithelial cells (E-cadherin), mesenchymal cells (vimentin), NR2F2 (external preputial marker), FOXA1 (urethra), FOXF1 (periurethra), SMA-alpha (myofibroblast), AKAP12 (external prepuce marker), in developing penis organoids. These data suggest 3D penis organoids maintain multilineage niches and mimic in vivo tissue-like characteristics through marker expression. To determine if the organoids required androgen signalling, we cultured the organoids with either 10^{-8}M dihydrotestosterone (DHT) or a vehicle control (DMSO). By five days of culture, DHT organoids were significantly bigger (400.7±17.3) in size than the DMSO (317.7±17.3) organoids. By 20 days, DHT organoids continued to grow (439.5±16.7), while the DMSO (284.4±16.7) organoids shrunk in size, suggesting that the size of the organoid is dependent on androgen signalling. To determine whether the penile organoids can be used to evaluate potential hypospadias-inducing compounds, we exposed the organoids to compounds found in hair dyes (m-Aminophenol, 10^{-6}M; p-Phenylenediamine, 10^{-6}M; Resorcinol, 0.002M), cosmetic facial creams (Octinoxate, 5×10^{-6}M), and herbicides (Oxyfluorfen, 2×10^{-5}M). By day 20, organoids grown in DHT were significantly larger (439.5±16.7) than those subjected to m-Aminophenol (410.5±28.1), p-Phenylenediamine (387.3±30.4), Oxyfluorfen (390.9±24.4) treatment ($p < 0.05$). Resorcinol treatment showed a decrease in organoid size (215.7±11.5) as compared to DHT (400.7±17.3) on day 5 ($P < 0.05$). Chemical exposure elicited decreased proliferation and increased cell death, which were evaluated by ki67 and tunel marker respectively. Overall, these 3D penis organoids have similar cell populations to the in-vivo penis, are androgen responsive, and can be used for toxicant screening. In the toxicant screen we have identified chemicals in cosmetics and herbicides that may negatively impact penis development. These penile organoid models provide precise tools for toxicant screening and will help reduce reliance on animal models.

Submission ID#2360361

Gut Microbial-Related Metabolites Modulate Steroidogenesis in Male Mice

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Abstract Text

The gut microbiome plays a critical role in sex steroid homeostasis and reproductive function through mechanisms such as steroid deconjugation. At the same time, changes in sex hormone status can influence gut microbial composition and the profile of microbial-related metabolites. Our previous work identified three microbial-host co-metabolites that respond to loss and gain of testosterone: hippuric acid (HA), 3-indolepropionic acid (IPA), and 3-(4-hydroxyphenyl) propionic acid (HPPA). However, the function and impact of these metabolites on the steroidogenesis of the hosts remain unknown.

The objective of this study was to examine the effect of HA, IPA, and HPPA on steroidogenesis and reproductive parameters in male mice. To achieve this, we used the following methods.

Eight-week-old male C57BL/6J mice were fed a 35% kcal fat diet for 6 weeks before being randomly assigned to 4 treatment groups (n=9-10/group): vehicle control (CON), HA, IPA, and HPPA. All solutions were prepared in 5% DMSO and delivered via subcutaneous injection. During the first week of treatment, metabolites were injected daily (60 mM for 3 days, followed by 25 mM for 4 days). For the subsequent three weeks, metabolites were administered twice weekly at 12.5 mM. Expression of steroidogenesis-related genes was assessed in testes by RT-qPCR, including steroidogenic acute regulatory protein (*Star*), cytochrome P450 family 11 subfamily A member 1 (*Cyp11a1*), cytochrome P450 family 17 subfamily A member 1 (*Cyp17a1*), 3β hydroxysteroid dehydrogenase (*Hsd3b1*), 17β hydroxysteroid dehydrogenase (*Hsd17b1*), aromatase (*Cyp19a1*), follicle stimulating hormone receptor (*Fshr*), and luteinizing hormone receptor (*Lhcgr*). Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method relative to the mean of the CON group. Testicular testosterone and dihydrotestosterone (DHT) concentrations were measured by ELISA.

Testosterone levels, testicular weight, and gonadal fat mass were not significantly different between each metabolite and CON. The IPA group exhibited a significantly higher DHT concentration compared with CON, along with a trend toward increased *Cyp17a1* expression and a significant decrease in *Cyp19a1* expression (fold change=1.43 and 0.82, respectively). These findings suggest that IPA may enhance steroidogenesis by promoting the production of androgen precursors (*Cyp17a1*) while reducing the subsequent aromatization (*Cyp19a1*). Although HA and HPPA did not impact DHT levels, both metabolites affected the expression of genes involved in steroidogenesis. The HA significantly reduced expression of *Cyp11a1* and *Cyp19a1* (fold change=0.65 and 0.81, respectively), indicating a potential decrease in the conversion of cholesterol to pregnenolone (*Cyp11a1*) and reduced estrogen synthesis (*Cyp19a1*). The HPPA group also exhibited significantly lower *Cyp11a1* expression (fold change=0.65).

Overall, these results suggest that IPA may increase DHT levels by altering steroidogenic pathways. Although the concentrations of HA and HPPA used in the current study were sufficient to modulate the expression of steroidogenic genes, they may not have been high enough to significantly impact DHT production. Together, these findings and our prior work suggest a bidirectional relationship between these microbial-host co-metabolites and testicular steroidogenesis. Further mechanistic studies are needed to clarify the functional roles of these metabolites and to support the development of therapeutics for sex hormone-related disorders.

Submission ID#2360758

Enriched Disruption of the Sperm Methylome at Transcription Factor Binding Sites and Genes Related to Early Development in Obese, Inactive Hispanic Men

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Abstract Text

Couples planning to have children often prioritize healthy lifestyle choices of the mother-to-be but rarely consider the father-to-be's lifestyle choices. The National Center for Male Reproductive Epigenomics is part of the NICHD-sponsored National Centers for Translational Research in Reproduction & Infertility (NCTRI) network, with a particular emphasis on three primary experimental questions: 1) Are obesity and a sedentary lifestyle associated with abnormalities in epigenetic programming (epimutations) and dysregulated gene expression in a male's sperm or somatic cells? 2) Can reversal of adverse lifestyle choices correct epigenetic and gene expression defects in a male's sperm or somatic cells? and 3) Do epimutations or dysregulated genes inherited from the sperm persist in a male's offspring, or are they corrected by generational epigenetic reprogramming? Here we show evidence for the impact of obesity and a sedentary lifestyle on the sperm epigenome in a study group of adult Hispanic men aged 18-40. Based on recruitment criteria, 18 men categorized as "lean and active" (LA) and 55 men categorized as "obese and inactive" (OI) were enrolled in our study. Analysis of sperm samples recovered from these men detected >16,000 differentially methylated cytosines (DMCs; as denoted by raw p-value < 0.05) and 10 differentially methylated regions (DMRs, adjusted p-value < 0.05) which distinguished OI men from LA men. The DMCs were distributed throughout the genome but were highly enriched at transcription factor binding sites related to early organismal development while DMRs were significantly enriched at ENCODE catalogued cis-regulatory elements (CREs) including promoter-like signatures, proximal and distal enhancers, and repeat elements. Finally, gene ontology (GO) enrichment analysis showed an elevated occurrence of DMCs at genes involved in biological processes including protein binding and transcriptional regulation as well as organismal development. This data is now being integrated with gene expression data from the same samples (assessed by the Yan lab) to correlate epimutations with dysregulated gene expression. Following enrollment in our study, the OI men were placed in one of four follow-up groups: 1) no intervention; 2) diet intervention; 3) exercise intervention; or 4) diet and exercise intervention. Sperm (and somatic cell) samples from these men recovered at 12 weeks post-intervention have now been collected and are undergoing analysis. Consideration of potential mechanisms that may or may not be involved in any intervention-induced reversal of sperm epimutations based on changes in diet or exercise habits will be discussed.

The Evolving Science of the Female Reproductive Tract

Wednesday, July 21 1:30 PM – 3:00 PM

Submission ID#2361261

WT1 Couples Progesterone Signaling Matrix Homeostasis to Prevent Fibrotic Uterine Aging and Preserve Fertility

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Abstract Text

Wilms' Tumor 1 (WT1) is a zinc-finger transcription factor highly expressed in endometrial stromal cells, yet its role in uterine function and reproductive aging remains unclear. Here, we identify WT1 as a direct downstream effector of progesterone receptor (PGR) signaling in the uterus. Integrated mining of PGR ChIP-seq and RNA-seq datasets, followed by qPCR and immunohistochemical validation, demonstrated that WT1 is an immediate PGR target gene. Blunted PGR signaling is a hallmark of uterine reproductive aging. Consistent with this paradigm, WT1 expression was markedly reduced in naturally aged uteri, *Sirt1*-deficient (genetically aged) uteri, and *Pgr* knockout (PRKO) uteri, implicating WT1 loss as a feature of progesterone resistance. To establish causality, we generated uterine-specific Wt1 conditional mutant mice ($Pgr^{cre/+}Wt1^{f/f}$, hereafter $Wt1^{\Delta/\Delta}$) that produce a truncated WT1 protein lacking the second and third DNA-binding zinc-figure domains. $Wt1^{\Delta/\Delta}$ uteri exhibited premature and persistent collagen accumulation as early as postnatal day 28, preceding suboptimal and defective decidualization. Bulk RNA-seq of isolated luminal epithelial and stromal compartments at gestational day (GD) 3.5, together with single-cell RNA-seq at GD 4.5, revealed widespread dysregulation of ECM remodeling pathways and collagen-associated gene networks, accompanied by disrupted epithelial-stromal communication. Functionally, $Wt1^{\Delta/\Delta}$ females were completely infertile ($P< 0.001$), whereas Wt1 haploinsufficiency ($Wt1^{\Delta/+}$) caused severe subfertility characterized by significant reductions in pups born per litter (3.4±0.5 vs. 6.0±0.4) and surviving pups at weaning (1.0±0.5 vs. 5.6±0.4) compared with $Wt1^{f/+}$ controls, along with increased stillbirth and early neonatal mortality ($P< 0.05$). Ovarian function remained intact, as evidenced by comparable blastocyst numbers at GD 3.5 and serum P4 levels at GD 4.5; however, fewer embryos reached the uterus in $Wt1^{\Delta/\Delta}$ females, suggesting an additional oviductal contribution. Implantation was profoundly impaired: blastocysts attached to the luminal epithelium but failed to invade and initiate decidualization, resulting in delayed implantation, defective decidualization, and embryonic resorption. Molecular analyses demonstrated dysregulated uterine hormonal responsiveness, including elevated epithelial ESR1 expression, reduced HAND2 expression, impaired induction of PTGS2 at implantation sites, and defective activation of decidual gene programs. Artificial decidualization assays confirmed a severe impairment in stromal decidualization in $Wt1^{\Delta/\Delta}$ uteri. Collectively, our findings establish WT1 as a critical mediator of PGR signaling that restrains fibrotic ECM remodeling to preserve uterine receptivity. Loss of WT1 accelerates collagen deposition, disrupts epithelial-stromal crosstalk, and compromises decidualization, therebymechanistically linking progesterone resistance to uterine aging and fertility decline.

Submission ID#2347522

A Transvaginal Rat Model for Intrauterine Perfusion and Endometrial Injury without Laparotomy: Establishment and Application

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Abstract Text

Rodent models are commonly employed in uterine-related experimental research, where intrauterine perfusion and curettage procedures represent critical steps. Conventional approaches predominantly rely on laparotomy or dorsal incisions to expose the uterus, which not only increase animal trauma and postoperative complication risks but also heighten operational complexity and may induce abdominal adhesions, thereby failing to accommodate the requirements for repeated intrauterine interventions. To address these limitations, we developed a transvaginal approach for rat uterine cavity perfusion and injury modeling, utilizing only routine surgical instruments to achieve convenient intrauterine operations and endometrial sampling without laparotomy.

Female Sprague-Dawley (SD) rats were anesthetized and positioned in the supine posture. A nasal speculum was utilized as a vaginal dilator to expose the cervix. Subsequently, a blunt-tipped long needle was employed to progressively dilate the cervical os, facilitating uterine cavity perfusion. For intrauterine injury or curettage, approximately 500 μL of normal saline was initially injected to distend the uterine cavity, followed by further cervical dilation and endometrial scraping using a cytobrush. The scraped material was aspirated via the blunt-tipped needle for subsequent analysis. Rats were randomly allocated into an endometrial injury group (undergoing consecutive curettage three times), a saline perfusion group, and a blank control group.

This method successfully accomplished precise bilateral uterine cavity perfusion and endometrial injury in rats via the transvaginal route. Hematoxylin-eosin (HE) staining revealed evident defects in the endometrial epithelial layer and vascular disruption in the injury group; Masson's trichrome staining demonstrated significant collagen fiber deposition, intrauterine adhesions, and cavity stenosis. Fertility in the injury group rats was markedly reduced, whereas no endometrial damage, fibrosis, or adhesions were observed in the saline perfusion and blank groups, with normal fertility preserved.

The transvaginal intrauterine operation method proposed in this study provides an efficient, minimally invasive alternative for rat uterine-related experiments. This approach circumvents the complexity and animal suffering associated with laparotomy, enhances experimental efficiency, and more authentically simulates clinical curettage procedures, thereby offering a reliable tool for constructing disease models such as intrauterine adhesions.

Submission ID#2348599

From Mouse to Human: A Multimodal View of Oviduct Architecture and Transport Physiology

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Abstract Text

Transport of eggs and embryos through the oviduct is essential for reproduction, and errors in tubal transport can lead to conditions such as tubal factor infertility and ectopic pregnancy. Despite the importance of tubal dynamics, the functional mechanisms governing the oviduct remain poorly defined and are largely inferred from animal models, which recapitulate human function to an unknown extent.

To address this gap, we conducted comparative multimodal imaging analyses of mouse and human oviducts to identify conserved and divergent features. In excised mouse oviducts, we used micro-computed tomography (microCT) to quantify mucosal fold geometry and luminal tortuosity, revealing region-specific architectural differences. These spatial patterns align with prior intravital findings from the Larina Lab showing that oviductal functions are regionally distinct, though the precise roles of these unique dynamics remain elusive. Using intravital optical coherence tomography (OCT), we further quantified ciliary activity *in vivo* in mouse oviducts, measuring an average cilia beat frequency of 5-15 Hz in the ampulla and a quantifiable, visualizable coordinated pattern of cilia metachronal wave propagation. Together, these datasets establish a mechanistic framework in which regional structure and ciliary dynamics jointly regulate transport.

Direct visualization of human oviductal dynamics *in vivo* is not possible, but by applying imaging approaches analogous to those used in mice to study function, we can enable strong cross-species comparison and yield biological insights. We first employed high-resolution microCT imaging of intact fresh excised human oviducts to uncover unexpectedly complex mucosal fold architecture with pronounced regional variation, including large ranges of curvature and luminal areas across the ampulla. These data challenge the traditional view of a simple, straight human oviduct and reveal notable parallels to mice. Using *ex vivo* OCT imaging of fresh excised, lumen-exposed human oviducts, we quantified cilia dynamics, demonstrating coordinated progression of the cilia metachronal wave and an average cilia beat frequency of 4 Hz across 22 samples, which is broadly comparable to but slightly slower than in mice.

Together, this highly innovative cross-species analysis of oviduct structural and functional imaging provides a framework for understanding human tubal transport and evaluating the extent to which mouse recapitulate human oviductal dynamics. By leveraging mouse functional accessibility alongside human structural and ciliary data, we identify conserved principles of oviduct physiology across species and emerging human-specific features.

Submission ID#2358145

Endometriosis-Associated Peritoneal Fluid Increases MUC1 Expression and Secretory Cell Density, Disrupting the Morphology of Fallopian Tube Epithelial Cells

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Abstract Text

Endometriosis affects approximately 10% of reproductive-age women worldwide and is present in around 50% of women experiencing infertility. Endometriosis can diminish effective oocyte/embryo transport due to tubal obstructions and reduce the ciliary beating frequency of fallopian tube epithelial cells (FTECs), leading to infertility. However, the impact of peritoneal fluid (PF) from endometriosis patients on development and function of FTECs remain unexplored. We hypothesize that PF exposure disrupts FTEC function and contributes to infertility. Here, we studied the effect of PF from women with endometriosis (n=2, from stage I and IV) on ovine derived FTECs in comparison to unexposed controls. PF was obtained after informed consent from women aged 18-45 undergoing laparoscopy for removal of endometriosis lesions or unrelated benign conditions (Monash Health HREC 20-0000-159A), centrifuged at 4 °C for 5 minutes at 300 g to remove cells and frozen at -80 °C until use. Only PF that had not been diluted (neat) during surgery was used in the experiments. Sheep cells were obtained from an abattoir, without information on cycle stage. Since the rate of ciliogenesis declines with passaging and is higher in primary cells than in cell lines, oviducts were dissected within 4h of collection for the isolation of primary FTECs. Isolated tissue was enzymatically dissociated and the cells were cryopreserved and used within one month. FTECs were seeded onto collagen-coated 96-well plates and treated with PF once 80% confluency was reached. Treatment lasted 12h using culture media supplemented with 25% or 50% PF in endometriosis cases and with 50% PF in patient controls. The control group was cultured in DMEM/F12 media only with no PF present. Initial cellular morphology was assessed using brightfield imaging. Cellular phenotypes and proliferation were analysed by immunofluorescence using acetylated alpha tubulin stain for cilia, PAX8 for secretory and Ki-67 for proliferating cells. qRT-PCR was used to quantify MUC1 expression. Statistical analyses were performed using non-parametric ANOVA tests with post-hoc comparisons. Results showed that PF from endometriosis patients disrupted the typical cobblestone morphology observed in controls. PF from control patients did not significantly affect FTEC morphology, confirming that any changes observed were the result of factors present in PF originating from endometriosis. Secretory cell density was significantly increased following treatment with 25% PF ($p=0.011$), while no notable changes were observed in ciliation density or the number of proliferating cells. Interestingly, a dose response was identified, with 25% PF exerting stronger effects on secretory cell density than 50% PF. MUC1 expression showed an increase at 25% PF exposure, consistent with the trend for greatest effect on secretory cells. The results obtained so far are promising, however this was carried out using low sample numbers of PF and variability is expected amongst patients. Hence, to achieve significance, ongoing experiments are underway to ensure sufficient sample size. Our results point to a novel effect on FTECs upon exposure to endometriosis PF. This effect could underlie mechanisms involved in impeding transport in the fallopian tube for embryo, ovum or sperm resulting from endometriosis. The role of MUC1 in ectopic pregnancies end embryo attachment has been previously explored, however the relationship that endometriosis PF could play in this adds a new layer worth exploring. This adds to our understanding of the mechanisms that drive MUC1, potentially opening new avenues for better understanding and treatment of endometriosis-related infertility.

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In vivo CRISPR-based functional genomics identifies novel genetic drivers of complex Müllerian anomalies

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Abstract Text

Müllerian anomalies (MAs) are congenital disorders arising from abnormal development of the embryonic Müllerian ducts, the paired structures that give rise to the oviducts, uterus, cervix, and upper vagina. MAs display marked phenotypic heterogeneity, ranging from complete uterovaginal agenesis to subtle uterine malformations. Severe forms include complex conditions such as Mayer–Rokitansky–Küster–Hauser (MRKH) syndrome and obstructed hemivagina with ipsilateral renal anomaly (OHVIRA) syndrome. MAs are frequently associated with renal, skeletal, and cardiovascular defects, substantially affecting reproductive, physical, and psychological health. Despite their clinical impact, the molecular mechanisms underlying MAs remain poorly understood.

Progress in the field has been limited by a disconnect between clinical genetics and functional developmental biology. Animal studies have identified pathways critical for reproductive tract formation, yet their direct relevance to human MAs is often unclear. Conversely, human genetic studies have generated candidate gene lists without in vivo validation of pathogenicity. Bridging this gap is essential to establish causal mechanisms.

We performed whole-exome sequencing in 27 patients with MRKH or OHVIRA syndrome and their unaffected family members. Both de novo and inherited variants were identified in known and novel candidate genes, including GREB1L, HNF1B, KIF14, FAT4, and MDC1. To functionally interrogate candidate variants, we selected four candidate genes: FAT4 (a planar cell polarity gene), MDC1 (a mediator of the DNA damage response), KIF14 (a mitotic factor required for cytokinesis), and MMP14 (an extracellular matrix factor). We then used in vivo CRISPR-based genome editing in the F0 generation. For Fat4 and Mdc1, patient-specific variants (FAT4: S1655N + T2032A; MDC1: E336X) were introduced via homology-directed repair. Kif14 and Mmp14 were disrupted using non-homologous end joining–mediated gene inactivation.

Mutation of each of the four genes resulted in Müllerian anomalies. Fat4 mutants exhibited Müllerian duct hypoplasia and cervical canal dysplasia, consistent with disrupted cell polarity affecting tissue organization and growth. Mdc1 mutants developed uterine horns approximately 50% shorter than controls, accompanied by cervical and vaginal dysplasia, suggesting impaired progenitor cell expansion and genomic stability during duct elongation. Kif14 and Mmp14 mutants showed Müllerian duct hypoplasia with variable severity, implicating defects in coordinated cell division and epithelial–mesenchymal interactions critical for duct morphogenesis and branching. In all models, expression of key epithelial and stromal markers was disrupted, supporting altered tissue patterning and differentiation. Importantly, all four mutations were also associated with renal anomalies, underscoring shared developmental mechanisms between the reproductive and urinary systems. These findings point to convergent disruption of pathways regulating epithelial organization, cell cycle regulation, and extracellular matrix dynamics—core processes required for both Müllerian duct and metanephric kidney development. Notably, the combined phenotype observed in Fat4 mutants – Müllerian hypoplasia with unilateral multicystic kidney dysplasia – represents a novel mouse model of OHVIRA syndrome.

This study represents the first systematic in vivo validation of multiple patient-derived MA candidate genes and provides direct functional evidence linking these genes to Müllerian and renal development. By integrating human genomics with rapid CRISPR-based modeling, our work advances mechanistic understanding of MA pathogenesis and lays the groundwork for improved molecular diagnosis, genetic counseling, and clinical management of these complex congenital disorders.

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GATA factor Pannier regulates sperm storage in female *Drosophila*

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Abstract Text

GATA-family transcription factors play essential and conserved roles in the formation of multiple organ systems including heart, immune system, and sensory organs; however, their roles in adult organ physiology have been sparsely studied. In this study, we found that *Drosophila pannier* (*pnr*) is specifically enriched in secretory cells of adult spermathecae and parovaria, two types of glandular organs in the female reproductive tract. Previous study has showed that secretions from these glands are essential for sperm storage in the spermathecae. Interestingly, we found that *pnr* knockdown in adult secretory cells of these glands leads to severe sperm storage defect in spermathecae. In addition, *pnr*-knockdown secretory cells in spermathecae show progressive hypertrophy and enlarged apical secretory cavity, which temporally stores secreted products and transports them into the central lumen through a cuticular end-apparatus and canal. The enlargement of secretory cavity is not due to the physical blockage of end-apparatus and canal according to electron micrography. All these results lead us to propose that Pnr regulates the composition of secreted products to ensure proper transferring of these products into the spermathecal lumen for attracting sperm. Furthermore, Pnr's role in spermathecae can be replaced by human GATA4 and GATA6, which manifests the conservation of GATA factors in regulating reproductive physiology. Our study demonstrated a novel role for Pnr in regulating secretions for sperm storage in Drosophila, and such a role may be conserved in mammals.