

ABSTRACT POSTERS



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Poster Session A

Griffin Hall

Monday, July 20,

8:00 AM – 9:45 AM

Developing an *in vitro* Culture System for Adult Avian Germline Stem Cells for Genetic Rescue

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Abstract Text

Over 70% of native Hawaiian forest bird species are experiencing severe population declines driven by habitat loss, climate instability, and the spread of mosquito-borne avian malaria. Although traditional conservation strategies including habitat protection, breeding programs, and population management remain essential, these approaches become increasingly limited as populations approach extinction and genetic diversity is irreversibly lost. As a result, innovative strategies that prioritize the preservation and recovery of genetic material are urgently needed. Germline preservation represents a promising yet underexplored avenue for avian conservation. In particular, the isolation and *in vitro* culture of post-mortem adult gonadal germline stem cells (GSCs) offer a novel opportunity to conserve genetic diversity from individuals that would otherwise be lost. Unlike embryonic primordial germ cells (PGCs), adult-derived GSCs can be recovered opportunistically from deceased animals, making this approach especially valuable for critically endangered species with limited reproductive potential. However, the development of robust and reproducible culture systems for adult avian GSCs remains a significant technical barrier to broader application. This study aims to establish and evaluate foundational protocols for adult avian GSC conservation using chicken, quail, and zebra finch models. Our objectives focus on developing *in vitro* culture conditions that support the survival, proliferation, and long-term maintenance of adult GSCs. Adult GSCs demonstrate reduced viability following tissue dissociation, increased sensitivity to *in vitro* conditions, and variability in cell surface markers compared to embryonic PGCs. Ongoing research in embryonic-derived zebra finch PGC cultures have shown success for up to 10 days, and up to 150 days in similarly derived chicken PGCs, as demonstrated by Biegler, M. et al. (2025). These cultured cells were in turn able to be reintroduced to host zebra finch gonads and contribute to transgenic healthy offspring, offering new possibilities in conservation and genetic rescue for hopeful application in species beyond. To address the disparities between PGCs and GSCs, we are testing dissociation techniques and systematically evaluating media composition and temperature parameters. Established PGC growth media were tested alongside alternative formulations, including Media 199 (M199), Dulbecco's Modified Eagle Medium (DMEM), KnockOut DMEM, and Advanced RPMI, at 37 °C and 40 °C. We are also assessing new enzymatic dissociation reagents, including papain and collagenase-hyaluronidase, to improve cell recovery and viability. Although adult GSC cultures have been hindered by contamination and variability in dissociation outcomes, preliminary results using papain demonstrated encouraging post-recovery viability and increased germ cell density. Early findings further suggest that 37 °C may provide a more favorable *in vitro* environment for chicken germ cell maintenance than the avian physiological temperature of 40 °C. Continued optimization of enzymatic protocols, sterile technique, and species-specific media formulations will be critical to improving culture stability and long-term expansion. Collectively, this work establishes groundwork for the development of germline-based conservation tools. By overcoming current culture limitations, adult GSC isolation and expansion may become a transformative strategy for genetic rescue and assisted reproductive technologies.

Utilizing the Xenograft Model to Assess Progestogen Effects on the Endometrium

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Abstract Text

The endometrium is a dynamic tissue cyclically transforming under the influence of ovarian sex-steroids. Hormonal contraception, while highly effective can cause changes in bleeding known as Contraceptive Induced Menstrual Changes (CIMC) which can include irregular, prolonged, or unpredictable bleeding and are the leading cause of contraceptive discontinuation worldwide. Despite their clinical significance, the cellular and tissue-level mechanisms driving CIMCs remain poorly defined, in part because few experimental models capture the architectural and hormonal complexity of the human endometrium. The endometrial xenograft (EnMX) mouse model, widely utilized in studies of endometriosis, presents an opportunity to investigate the effect of contraceptive hormones directly on vascular and tissue biology. In this pilot study, we characterize the response of the EnMX model to exogenous ovarian steroids to establish its utility for our future CIMC investigations. EnMXs were created by grafting 4-freshly harvested rhesus macaque endometrial tissue piece fragments (0.5 cm x 0.4w x 0.2h) into the dorsal subcutaneous space of 10 severe combined immunocompromised (SCID) mice (N = 4 grafts/mouse). Donor tissue was obtained from two rhesus during routine weekly necropsies, one animal in the proliferative phase, one in the secretory phase of the menstrual cycle. To recapitulate steroid hormone shifts of the menstrual cycle as well as ensure EnMX viability under prolonged exogenous hormone exposure, we sequentially treated mice with estradiol (E2, 0.05mg/mouse) followed by different progesterone (P4, 5 mg/mouse) regimens. Each hormonal treatment was delivered via subcutaneous pellets. All EnMX mice were initially treated with unopposed E2 for 15 or 28 days followed by P4 for 14 (n=2), 28 (n=2), or 42 (n=3) days. Control mice (n=3) received E2 alone. In total, 34/40 (85%) EnMXs were successfully collected with 30 EnMXs utilized for histology and the remaining for exploratory endpoints. Histological evaluations found all (30/30, 100%) retrieved grafts were vascularized and viable. Total area of necrotic regions increased as graft time persisted. Decidualization, spiral artery architecture, and epithelial-stromal compartmentalization were observed in all P4 treated groups. With increasing duration of P4 exposure, decidualization increased whilst the E2 control group showed little to no decidualization. In addition, the number of identifiable spiral arteries decreased with increasing P4 exposure. By immunohistochemistry (IHC), all treatment groups retained hormone receptor expression (Era and PGR) and exhibited comparable Ki67 staining. With P4 treatment, glandular PGR expression was markedly reduced or undetectable while stromal PGR expression was retained. Overall, our study supports the EnMX mouse model as a viable experimental platform for prolonged exogenous hormone exposure and that P4 exposure in the model tracks established effects on a primate endometrium. Grafts retained hormone sensitivity and response to P4 with P4 inducing decidualization and selectively diminishing glandular epithelial PGR expression. Finally, presence of a functional, hormonally responsive endometrial vasculature indicates that endpoints directly related to vascular remodeling and bleeding are feasible in the EnMX system. Above all, this model system will allow experimental designs and investigations that are not feasible in humans or monkeys. Future studies will further extend the model to progesterone withdrawal protocols as well as employ additional perturbation strategies to define the cellular and tissue level mechanisms underlying CIMC.

IVP affects the IGF system in a tissue-specific manner in bovine fetuses

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Abstract Text

In vitro production (IVP) of embryos promotes rapid cattle genetic improvement. Nevertheless, challenges related to IVP offspring phenotypes, health, and performance remain unsolved. On average, IVP results in heavier offspring. In addition, our results show that weight of day 105 IVP fetuses is more variable compared to AI-fetuses (25.94% vs. 11.93%, $p=0.0214$). We demonstrated that this resulted from epigenetic alterations affecting the regulation/expression of imprinted and non-imprinted genes. The insulin-like growth factor 2 receptor (IGF2R), a maternally-expressed gene, is downregulated in overgrowth because of IVP. IGF2R facilitates IGF2 (a paternally-expressed fetal growth factor) degradation. IGF2 signals via IGF1R and insulin receptor A stimulating the PI3K-AKT pathway. Here, we compared in liver, muscle, and placenta the expression of genes in the IGF system (by RT-PCR) and in liver and muscle the abundance of proteins (by Western Blot) of the IGF system and the PI3K-AKT-mTOR pathway of day 105 fetuses. Samples were AI: $n=11$, weight average = 466g (388-550); 4 females/7 males) and IVP: $n=29$, weight average = 543.03g (318-1080); 10 females/19 males). Additionally, we assessed how protein abundance contributes to fetal weight variability. IVP did not affect the expression of *IGF2*, *IGF1*, *IGF1R*, *IR-A* in any tissue, but in muscle reduced expression of *IGF2R* (-3.52 vs -2.74, $p=0.01$) and *IGFBP4* (-2.71 vs -2.26, $p=0.07$). In liver, IVP fetuses had more IGF2 (0.40 vs 0.82, $p=0.055$) than AI fetuses, while in muscle, it was reduced (0.57 vs 0.39, $p<0.05$). IVP did not affect IGF1R or IGF2R abundance in any tissue. IVP increased total (0.30 vs 0.75, $p<0.05$) and phosphorylated p110 alpha (0.63 vs 1.30, $p<0.05$) in liver. In muscle, IVP increased phosphorylated p110 alpha (1.17 vs 1.47, $p<0.05$), and its ratio (1.81 vs 2.56, $p<0.05$). Liver AKT total (0.57 vs 0.77, $p<0.05$) and phosphorylated (0.70 vs 1.06, $p<0.05$) were higher in IVP fetuses; with a similar effect in muscle (AKT total: 0.52 vs 0.86, $p<0.05$; AKT phosphorylated: 0.81 vs 1.26, $p<0.05$). IVP increased total mTOR in liver (0.78 vs 1.38, $p<0.05$) but reduced the phosphorylated mTOR ratio (2.68 vs 1.35, $p<0.05$). In muscle of IVP fetuses, phosphorylated mTOR (1.23 vs 1.94, $p<0.05$) and its ratio (0.44 vs 0.87, $p<0.05$) were increased. Abundance of IGF2 and IGF2R in liver and muscle, p110 total in liver, phosphorylated p110 alpha, and total mTOR in muscle, differed between IVP overgrown fetuses (>550 g) and the lighter counterpart (<550 g). In AI fetuses, only liver IGF2 was associated with weight ($\beta = 77.72$, $R^2 = 0.45$, $p<0.05$). In IVP fetuses, liver IGF2R and phosphorylated mTOR explained 74.49% ($\beta = -268.71$, $p<0.05$) and 39.86% ($\beta = 242.75$, $p=0.068$) of fetal weight variability, respectively. In muscle, phosphorylated p110 alpha ($\beta = 265.05$, $R^2 = 0.444$, $p<0.05$), mTOR total ($\beta = 45.18$, $R^2 = 0.220$, $p<0.05$), IGF2R ($\beta = -83.71$, $R^2 = 0.161$, $p<0.05$), phosphorylated mTOR ($\beta = 58.34$, $R^2 = 0.139$, $p=0.05$) and IGF2 ($\beta = -460.88$, $R^2 = 0.12$, $p=0.062$) were associated with fetal weight variability. These results suggest that IVP affects IGF-PI3K-AKT-mTOR signaling in a tissue-specific manner and alters the relation of some proteins with fetal weight. This helps explain the increased variability in weight of IVP-derived fetuses. We are designing a machine learning model to predict fetal weight in a sex-specific manner.

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.

Cryoprotective Mechanisms of Melatonin in Saanen Buck Semen: A Multifaceted Antioxidant Strategy

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Abstract Text

Cryopreservation of buck semen is commonly associated with oxidative stress, which leads to structural and functional damage to spermatozoa. Melatonin (MLT), a potent lipophilic and hydrophilic antioxidant, has been proposed as a cryoprotective additive to semen extenders.

This study aimed to investigate the effects of different concentrations of MLT on post-thaw sperm quality in Saanen bucks and to explore the underlying oxidative stress-related mechanisms. Six male Saanen bucks were used in this study. Ejaculates were extended with OviXcell semen extender containing varying concentrations of MLT, and post-thaw sperm quality were assessed for sperm motility, membrane integrity, acrosome status, DNA integrity, and apoptosis. Oxidative stress markers, including malondialdehyde (MDA) levels, reactive oxygen species (ROS) generation, and the activities of superoxide dismutase (SOD) and catalase (CAT), were determined in frozen-thawed spermatozoa. The findings showed that low concentrations of MLT significantly improved sperm motility and membrane integrity ($P < 0.05$). In contrast, high concentrations of MLT significantly enhanced acrosome integrity, DNA protection, and reducing apoptosis ($P < 0.05$).

These results suggest a dual, concentration-dependent role of MLT: low doses stimulate sperm metabolic activity, whereas high doses provide strong antioxidant protection. The findings have practical implications for optimizing MLT concentrations in semen extenders to enhance the cryopreservation outcomes of Saanen bucks' semen.

A Multiscale Integration of In Vitro and In Vivo Ovarian and Endocrine Dynamics, via Computational Modeling, to Predict Toxicant Effects on Female Reproduction

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Abstract Text

Most knowledge of ovarian toxicology comes from rodent studies, though rodent responses do not always predict human risk. While nonhuman primates are perhaps the best animal model for humans, studies are constrained by cost, shortages, and complex welfare requirements. To address these limitations, our NIEHS-funded project, In Vitro to In Vivo Extrapolation of Toxicant Effects on Ovarian Function, advances New Approach Methodologies (NAMs) by developing in vitro assays and in silico, computational models to predict in vivo effects from in vitro data. Ultimately, we aim to enable robust in vitro to in vivo extrapolation (IVIVE) and cross-species extrapolation, though we currently require in vivo data for model development and evaluation. This NAM framework leverages mechanistic biology and predictive modeling to generate human-relevant ovarian toxicity insights while reducing our reliance on animal experimentation.

We are developing biologically based, multiscale computational models using harmonized in vitro and in vivo data from CD-1 mice (*Mus musculus*) and rhesus macaques (*Macaca mulatta*) exposed to a representative toxicant, dibutyl phthalate (DBP), which has known human exposure and reproductive effects. By aligning endpoints across species and experimental systems, these models will simulate follicle maturation under normal and chemically perturbed conditions. Initial efforts focus on proof-of-concept IVIVE and cross-species extrapolation models, establishing a scalable framework for predicting ovarian toxicity and ultimately informing human fertility risk assessment.

This presentation highlights our progress toward this NAM framework. Experimental data include in vitro mouse follicle cultures that quantify follicle growth dynamics and functional endpoints across folliculogenesis stages. These data have been used to calibrate a tissue-scale computational model describing follicle development from the multilayer stage through the antral stage, enabling simulation of growth trajectories as well as cell type-specific steroid hormone synthesis under normal conditions. In parallel, we developed a whole-body physiologically based kinetic model that includes the ovary and key reproductive hormones in the hypothalamic-pituitary-ovarian axis providing a systems-level representation of DBP and hormone distribution and regulation.

Together, these components establish the foundation for integrating follicle-scale biology with organism-level hormonal dynamics and advances this integrated computational platform as an in silico NAM tool to support IVIVE, cross-species extrapolation, and reduced reliance on traditional animal testing. (Supported by NIEHS Grant R01ES034690-02)

Longitudinal Transcriptomic Analysis of the Equine Amniotic Membrane During Normal Gestation

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Abstract Text

The amniotic membrane in horses is the main barrier to the fluid-filled amniotic compartment and provides physical protection to the fetus against mechanical trauma. Recent evidence suggests additional roles, including responsiveness to placentitis and active contribution to preterm birth and abortion. However, whether the amniotic membrane contributes to fetal development and parturition during normal gestation remains unknown. Thus, our objective was to characterize gene-expression dynamics in the equine amniotic membrane across gestation, providing insights into its potential roles in fetal development and parturition.

Healthy pregnant mares were euthanized at mid-gestation [4 (n = 4) and 6 (n = 4) months] and late gestation [9 (n = 6) and 10 (n = 4) months]. Amniotic membrane samples were collected at each time point and stored in RNeasy Lysis Buffer (Qiagen; #AM7021) until RNA isolation. Following RNA extraction using commercial kits, next-generation RNA sequencing was performed (NovaSeq 6000; 150 bp paired-end). Reads were mapped to the equine reference genome (EquCab3.0), and gene-level assembly was conducted using an HISAT2/StringTie2 workflow. Gene-level count matrices were generated using prepDE.py, and raw counts were imported into R (v4.4.2). Data were filtered for zero counts, normalized using the trimmed mean of M-values (TMM) method, and transformed to log2 counts per million (log2CPM).

Differential gene-expression analysis (edgeR v4.7.4) and dynamic expression modeling (EBSeq-HMM v1.38.0) revealed major transcriptional shifts across gestation. Functional annotation was performed using QIAGEN Ingenuity Pathway Analysis (IPA; v01.23.01) and g:Profiler (accessed 3 October 2025). Differential expression analysis identified 1,580 genes ($|\log_2FC| > 1$; FDR < 0.05) that were significantly altered during late gestation compared with mid gestation. Additionally, EBSeq-HMM identified 3,970 genes across eight dynamic expression trajectories (e.g., Up-Up-Up, Up-Up-Down).

Functional annotation of differentially expressed genes revealed 438 significantly enriched pathways. Pathways activated during mid-gestation were primarily associated with cellular proliferation, tissue and extracellular matrix remodeling, metabolic adaptation, cell-cycle regulation, DNA replication, cytoskeletal organization, and growth factor-, metabolic-, and hormone-mediated signaling. In contrast, pathways activated during late gestation were predominantly related to inflammation and immune regulation, including cytokine signaling, immune-cell activation, pathogen sensing (TLRs, NOD-like receptors, interferons), oxidative stress, and inflammatory cell death. Upstream regulator analysis identified transforming growth factor beta 1 (TGFB1) as the most strongly activated regulator associated with inflammatory modulation of the amniotic membrane as parturition approaches.

Dynamic gene-expression analysis further demonstrated a predominantly proliferative transcriptional profile during mid-gestation, characterized by genes involved in extracellular matrix interactions, cell-cycle regulation, and cell proliferation, which were progressively downregulated toward parturition. Overall, these findings reveal a gestational shift in the amniotic membrane from a proliferative and tolerogenic state to a more inflammatory profile as parturition approaches. Collectively, our results demonstrate that the equine amniotic membrane plays an active role in immunomodulation, solute transport, fetal-maternal signaling, and regulation of tissue proliferation and structural remodeling throughout gestation.

A Multi-Cohort Evaluation of Sperm DNA Methylation Signatures Associated with Semen Quality

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Abstract Text

Sperm DNA methylation plays a crucial role in the regulation of spermatogenesis. However, its association with general semen parameters routinely used in determining male fertility in a clinical setting remains relatively unknown. The aim of this study was to investigate the associations of sperm DNA methylation patterns and general semen quality parameters across general population and clinical in vitro fertilization (IVF) cohorts. This study included a total of 768 men drawn from three cohorts: a general population cohort: the Longitudinal Investigation of Fertility and the Environment (LIFE) study ($n=379$), and two clinical cohorts: the Sperm Environmental Epigenetics and Development Study (SEEDS) ($n=173$), and the Environment and Reproductive Health (EARTH) study ($n=216$). Semen parameters (volume, concentration, total motility, normal morphology, and total sperm count) were assessed according to the World Health Organization sperm processing manual. Sperm DNA methylation was measured using the Illumina EPIC Beadchip array (v1). Differentially methylated CpGs were identified using the *CpGassoc* R package, meta-analyzed using METAL, and subsequently used as input variables for identifying differentially methylated regions with the *ipDMR* pipeline. Results for differentially methylated regions were corrected by Holm-Bonferroni adjustment with $p < 0.05$ as the significance threshold. Metascape was used to identify relevant biological pathways linked with these regions. Most participants were over 30 years of age, had a BMI greater than 25, and were White non-smokers. Men recruited in the clinical cohorts had a higher proportion of semen characteristics below the WHO 2010 5th percentile values compared with those in the general population cohort. Except semen volume, all semen parameters were significantly associated with sperm DNA methylation patterns in the three cohorts. Notably, numerous differentially methylated regions were common across all three cohorts for sperm concentration (299 regions), motility (60 regions), morphology (544 regions) and total sperm count (176 regions). These shared regions were enriched in genes associated with spermatogenesis, male gamete generation, male meiotic nuclear division, and actin cytoskeleton mobilization. These findings reveal that sperm DNA methylation patterns are consistently associated with semen quality across multiple cohorts, suggesting an epigenetic contribution to male fertility impairments in both the general population and men seeking fertility care. Further studies are needed to examine the mechanisms through which differential methylation impacts semen quality.

Improving Oocyte Quality Through an Engineered Biomimetic Follicular Microenvironment

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Abstract Text

Folliculogenesis is a dynamic process in which follicular somatic cells support the development of a mature oocyte capable of meiotic maturation, fertilization, and early preimplantation embryo development. Thus, the follicle plays a critical role in determining the developmental competence and overall quality of the oocyte. Recapitulating folliculogenesis *in vitro* has important applications in the study of ovulation biology, drug screening, and fertility preservation. Encapsulated *in vitro* follicle growth (eIVFG) using alginate-based hydrogels is a powerful and widely used system in which uniform forces are exerted from all directions on the follicle throughout development. Although the eIVFG assay yields a high percentage (60-80%) of mature eggs, the developmental potential of the resulting gametes is low relative to *in vivo*, indicating room for further culture optimization. To more accurately mimic the highly dynamic and mechanical heterogeneous follicular microenvironment, a custom scaffold-free culture platform was developed using precise agarose microwells, providing non-uniform mechanical support to the follicle. This platform has shown comparable hormone production and viability relative to eIVFG systems, improved follicle growth and ovulation rates, and is a less technically demanding method that allows for time-lapse imaging. However, the original microwell design provides limited mechanical support and has only one contact point with the follicle at the bottom. In this work we sought to improve the mechanical stimulation provided by modulating the microwell geometry. Additionally, because optimal ovulation time is size dependent, we will test if time-lapse imaging can improve ovulation induction timing and subsequently improve gamete quality.

We developed conical microwells with 90-, 60-, and 30-degree angles to provide a distributed ring of support around the follicle. The 90-degree microwell offers minimal additional support to the follicle compared to the original design, with 60 and 30 degrees incrementally providing more support. Master molds were designed using computer-aided design software and 3D resin-printed at 25 μm resolution. These master molds were coated in a 4.2 μm -thick layer of Parylene-C to aid in mold manufacturing, then used to make a polydimethylsiloxane (PDMS) negative, followed by silicone molds that cast the final agarose molds. The use of PDMS was required to accurately recreate the complex microwell design, as it separates cleanly from the subsequent silicone mold compared to other silicones. Microwell depth was designed to be constant between versions, and a final design was reached after 4 iterations. The V4 mold reduced printing artifacts and unwanted follicle movement between microwells, and improved visibility of microwells during follicle seeding.

For experiments, 10 follicles were placed in one 1.5% agarose mold and were cultured with 750 μL of media for 8 days before ovulation was induced. Throughout the entire culture, the well was autonomously imaged every 30 minutes for time-lapse monitoring. Qualitative and quantitative assessments included measuring follicle survival and diameter, ovulation rate, oocyte maturation, and estradiol production. Ovulation was only induced once a follicle had reached the terminal diameter of 350-400 μm , and MII eggs were then collected to assess spindle morphology and chromosome alignment.

Future directions include utilizing optimized timing for ovulation based on time-lapse data, adapting hydrogel rigidity, and assessing embryo quality post-ex *vivo* folliculogenesis. The resulting physiomic platform combines the flexibility of 3D printing with optimized timing kinetics, providing important insights into ovarian biology to improve oocyte quality.

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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.

MiR-10b-5p in Spent Culture Media as a Potential Biomarker in Assessing Mouse Embryo Development and Implantation

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Abstract Text

MicroRNAs (miRNAs) are a class of small non-coding RNAs that play crucial roles in regulating gene expression by binding to target messenger RNAs (mRNAs) and modulating transcriptional and translational processes. It has been suggested that miRNA profiling in spent culture media (SCM) provides a non-invasive method for assessing embryo quality. This study aimed to profile miRNAs in the SCM of mouse morphologically good- and poor-quality embryos (GQEs and PQEs) by small RNA sequencing, and to evaluate the potential of specific miRNAs as biomarkers for embryo quality determination. Small RNA sequencing was performed to compare miRNA profiles in SCM from GQEs and PQEs, followed by RT-qPCR validation of candidate miRNAs. Functional analyses of targeted miRNAs in vitro on mouse embryo development by electroporation of miRNA inhibitor, and in vivo on embryo implantation by transcervical injection of miRNA inhibitor. The expression of endometrial receptivity markers was examined by RT-qPCR and Western blotting, and functions by spheroid-endometrial cells attachment assay. It was found that three differentially expressed miRNAs (miR-10b-5p, miR-322-3p, miR-467c-5p) were identified from single or group cultured SCM via small RNA sequencing. Further qRT-PCR validation and expression analysis revealed that miR-10b-5p levels were low at the early cleavage stage embryo but increased significantly at the blastocyst stage. Knockdown of miR-10b-5p in 2-cell mouse embryos markedly reduced blastocyst formation in vitro and implantation sites in vivo. In human endometrial Ishikawa cells, downregulation of miR-10b-5p decreased BeWo spheroid attachment and suppressed the receptivity marker integrin $\beta 3$ (ITGB3) expression. Taken together, miR-10b-5p in SCM correlates with embryo quality, its downregulation impairs blastocyst development and endometrial receptivity. MiR-10b-5p has potential as a biomarker for embryo development and implantation.

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Optimizing Oligopaint DNA FISH for Human Ovarian Tissue and Somatic Cells

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Abstract Text

Turner syndrome (TS) is genetic disorder caused by full or partial monosomy of the X chromosome in females. The impact of TS on pubertal health and fertility is significant, with only 10-20% of TS patients achieving spontaneous menarche or unassisted conception. While chromosomal aneuploidies are likely to alter gene expression and impact chromosome segregation events, how 10-20% of women with TS still have normal ovarian function is unclear. One possibility is cell-type or tissue-specific mosaicism, where some or all cells in the ovary have normal karyotypes, allowing for normal ovary function. To investigate this possibility, we will karyotype X chromosome aneuploidies in human ovarian tissue samples from TS and control patients using Oligopaint DNA FISH. Parallel analysis of various somatic tissues from the same individuals will also be conducted, enabling us to assess whether certain tissues more accurately reflect ovarian karyotypes than peripheral blood samples, which are commonly used in clinical diagnostics.

A key challenge for these analyses is that our ovarian and somatic samples were prepared using diverse fixatives, some of which have not previously been used with Oligopaint FISH. Therefore, protocol optimization is required prior to analysis.

Our preliminary results indicate that PFA-fixed urine, buccal, and ovarian tissues yield robust FISH signals without modification of published protocols. In contrast, ovarian tissue fixed in modified Davidson's fluid (mDF) or neutral buffered formalin (NBF) require protocol modifications, such as secondary 4% PFA fixation or the addition of a protein digestion. Buccal and urine samples fixed in 3:1 methanol:acetic acid also require similar protocol optimizations.

Optimizing these FISH protocols is critical for accurate karyotype analysis of TS patient samples. Understanding how karyotype variation influences fertility in TS patients will provide broader insights into the relationship between aneuploidy, mosaicism, and ovarian function, with broader implications for improving our understanding of reproduction and fertility in the human population.

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.

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 (2 μ l/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 $\pm$ 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/MI). 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 ubiquitin-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.

A Hormone-Responsive Porcine Endometrial Organoid Platform for Studying Early Pregnancy

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Abstract Text

The endometrium, composed of luminal (LE) and glandular (GE) epithelial cells and stromal cells, plays a central role in integrating maternal hormonal signals and conceptus-derived cues to establish and sustain pregnancy in mammals. While endometrial epithelial organoids (EEOs) have advanced mechanistic studies in humans and mice. A comparable model in pigs has been lacking, limiting opportunities to investigate peri-implantation events in this agriculturally and biomedically relevant species. Here, we report the first establishment of porcine endometrial epithelial organoids from postnatal day-10 uteri. Uterine horns were harvested from Day 10 female piglets, the endometrium epithelial (EECs) and stromal (ESCs) cells were isolated by enzymatically digesting with trypsin, collagenase IV and Dispase II. EECs were encapsulated in Matrigel with a stem-cell-favoring medium to form spheroids. EEOs and ESCs were fixed in 4% paraformaldehyde for histological analysis. Once formed, EEOs were treated with 10 nM 17 β -estradiol (E2) for two days, followed by a combination of 10 nM E2, 1 μ M medroxyprogesterone acetate (MPA), and 200 μ M dibutyryl cyclic-AMP (cAMP), termed EPC, for another six days. Total RNA was extracted for qPCR and bulk RNA sequencing, with statistical significance determined using one-way ANOVA (qPCR: $P < 0.05$; RNA-seq: fold change > 1.5 and adjusted $P < 0.05$). The pEEOs demonstrated long-term viability (> 10 passages) and maintained epithelial identity, confirmed by cytokeratin 8 expression and lack of mesenchymal contamination (vimentin-negative) by qPCR and immunofluorescence. When aligned to published sequencing data (GEO accession: GSE123265), a total of 1,769 commonly increased genes were found in both luminal epithelial (LE) and EEOs compared to endometrium stromal cells, such as epithelial cell adhesion molecule (*EPCAM*), keratins (*KRTs*), integrins (*ITGAs*), and fibroblast growth factor receptors (*FGFRs*) that are spatial-specifically expressed in LE, demonstrating their physiological relevance. Importantly, pEEOs displayed robust hormone responsiveness: estradiol (E2) alone or EPC treatment significantly induced expression of steroid-responsive genes critical for endometrial receptivity (*PGR*, *HSD17B2*, *OLFM4*, *ESR2*, *SPP1*; $P < 0.05$). Bulk RNA-seq analysis revealed that E2 alone treated pEEOs (two days) significantly activated ESR1 signaling ($P < 0.05$, z-score = 4.13). EPC-treated pEEOs (E2 two days followed by EPC 6 days) exhibited a distinct transcriptomic landscape, with 942 genes upregulated and 1124 genes downregulated relative to vehicle controls (adjusted $P < 0.05$, Fold change > 1.5 or < -1.5). Ingenuity pathway analysis (IPA) highlighted activated pathways central to early pregnancy establishment, including signaling by FGFR1, BMP signaling pathway, signaling by EGFR, metabolism of polyamines ($P < 0.05$, z-score > 1.2). Upstream analysis revealed that fundamental transcription regulators such as PGR, SIRT1, PTEN, DNMT3A, HOXA7, and EIF4EBP1 were actively triggered in EPC treated pEEOs ($P < 0.05$, z-score > 1.2). Collectively, this work provides the first validated porcine endometrial epithelial organoid system, offering a powerful new platform for investigating endometrial biology conceptus-uterus interactions, and mechanisms regulating early pregnancy in pigs. This model holds broad potential for advancing both reproductive biology research and the improvement of swine reproductive efficiency.

Baboon Endometrial Organoids as a Translational Model for Assessing Cisplatin-Induced Endometrial Toxicity

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Abstract Text

Chemotherapy-induced reproductive toxicity remains a major clinical concern, particularly regarding endometrial integrity and function. However, physiologically relevant in vitro models for evaluating uterine-specific adverse effects are limited. In this study, for the first time, we demonstrate the utility of baboon endometrial organoids as a robust and translational model for assessing the endometrial toxicity of the widely used chemotherapeutic agent cisplatin. Owing to the close anatomical, hormonal, and molecular similarities between baboon and human endometrium, this organoid system recapitulates epithelial–stromal interactions and hormone responsiveness. Endometrial tissues were sampled from 5 baboons under general anesthesia and following our recent protocols (Saadeldin et al. 2025; PMID: 40710304 & Saadeldin et al. 2024; PMID: 38112579).

Cisplatin exposure induced pronounced alterations in endometrial cellular morphology, proliferation, angiogenic signaling, and hormonal responsiveness, as revealed by comprehensive immunofluorescence (IF) and protein expression analyses. IF staining demonstrated disrupted epithelial integrity and differentiation, evidenced by altered mucin-1 localization and significant reduction in cellular proliferation was confirmed by decreased Ki-67 labeling. Furthermore, cisplatin markedly impaired angiogenic signaling, as reflected by altered expression of VEGFA and its receptors VEGFR1 and VEGFR2, suggesting compromised vascular support within the endometrial microenvironment. Hormone receptor signaling was also affected, with notable changes in progesterone receptor (PR) and estrogen receptor (ER) expression, highlighting the endocrine-disrupting potential of cisplatin at the uterine level.

Western blot and immunofluorescence analyses corroborated these findings, revealing dysregulation of mucin-1, activation of apoptotic pathways through increased caspase expression, and altered DNA damage response as indicated by DDB1 modulation, as well as reduced Ki-67 as a marker for reduced proliferation.

Collectively, these findings establish baboon endometrial organoids as a sensitive and physiologically relevant platform for mechanistic evaluation of chemotherapy-induced endometrial toxicity. This model offers significant potential for preclinical drug screening and fertility-preservation research, reducing reliance on in vivo studies while enhancing translational relevance.

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Quiet Origins of Metabolic Fate: Vitamin B6 Deficiency during Pregnancy Impairs Pancreatic Beta-Cell Development and Glucose Metabolism in the Male Offspring

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Abstract Text

Type 2 diabetes (T2D) is a chronic metabolic disorder characterized by high blood glucose levels resulting from impaired insulin production or action. By 2050, around 800 million individuals worldwide are projected to have T2D. Decades of epidemiological and animal research show that imbalances in intrauterine nutrition influences the offspring's risk for developing T2D. These studies indicate that poor intrauterine nutritional environment developmentally reprograms the offspring's insulin-producing pancreatic beta-cells and glucose metabolism increasing risk for T2D. Vitamin B6 deficiency is one of the most common nutritional deficiencies affecting the US population, especially women of reproductive age. In pregnant women, plasma levels of vitamin B6 decline by 30% in the second trimester compared to non-pregnant levels, reaching the lowest levels in the third trimester. Cord blood and infant plasma levels of vitamin B6 correlate with maternal levels during pregnancy, indicating that vitamin B6 reserves acquired by the offspring gestationally is essential for prenatal and postnatal adequacy. Although vitamin B6 supplementation during pregnancy is common, factors including genetics, poor diet, malabsorption, alcohol use, endocrine-disrupting chemical exposure and low socioeconomic status influence vitamin B6 levels. Vitamin B6 is a critical cofactor for tryptophan catabolism, a pathway upregulated in perinatal beta-cells both in humans and mice. Serotonin, a tryptophan catabolite, directly promotes the rapid proliferation of perinatal beta-cells crucial for setting the baseline of beta-cell mass during adulthood. Nicotinamide dinucleotide (NAD), another tryptophan catabolite, is a key regulator of mitochondrial metabolism and redox balance which are crucial for perinatal beta-cell proliferation. Although pregnancy is a particularly vulnerable period for vitamin B6 deficiency, the effects of intrauterine vitamin B6 deficiency on perinatal beta-cell proliferation, beta-cell mass and long-term metabolic health remain unknown. In this study, utilizing custom diets, we induced *in-utero* vitamin B6 deficiency in mice at a level relevant to human populations. We observed that *in-utero* vitamin B6 deficiency is associated with intrauterine growth restriction in male offspring. Glucose metabolic tests in 16-week-old adult offspring revealed worsened glucose tolerance and lower insulin secretion in males. In the perinatal pancreas of males exposed to *in-utero* vitamin B6 deficiency, we observed lower beta-cell proliferation and mass via immunostaining. Moreover, *in-utero* vitamin B6 deficiency lowered tryptophan catabolite, NAD, in the perinatal pancreas and elevated the mitochondrial metabolite succinate, an indication of mitochondrial dysfunction and increased oxidative stress. Gene expression analysis in the male perinatal pancreas revealed lower expression of cell cycle regulators *Foxm1*, *Ccnb1*, *Ccnb2* and key beta-cell identity and function related genes *Pdx-1* and *Glut2*. Overall, our findings reveal for the first time that intrauterine vitamin B6 deficiency impairs perinatal beta-cell proliferation, beta-cell mass and long-term glucose metabolism, potentially through the cumulative effect of altered tryptophan catabolism, mitochondrial function and lowered expression of genes crucial for beta-cell identity and function. This research helps in elucidating how intrauterine nutrition influences the future risk of T2D, helping develop early preventative interventions that improve long-term health outcomes in the offspring. Our focus on nutritional levels relevant to humans will support health improvement in populations at disproportionate risk of nutritional deficiencies and T2D.

Development of a Rhesus Monkey (*Macaca mulatta*) Whole-Body Physiologically Based Kinetic (PBK) Model for Dibutyl Phthalate-Induced Reproductive Effects Using Molecular Docking

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Abstract Text

Environmental phthalates remain a major concern for female reproductive health, yet the mechanistic basis of their ovarian toxicity in primates is poorly understood. Dibutyl phthalate (DBP) and its active metabolite monobutyl phthalate (MBP) have been associated with disrupted folliculogenesis and impaired fertility in rodents, but comparable data in nonhuman primates are limited. As part of an NIH Virtual Consortium for Translational/Transdisciplinary Environmental Research (ViCTER) project, our goal is to develop multiscale computational tools to predict the effects of ovarian toxicants across species. Here, we evaluate molecular initiating events for DBP and MBP toxicity and develop a physiologically based kinetic (PBK) model to study ovarian toxicity in the female rhesus monkey (*Macaca mulatta*).

We first investigated whether DBP or MBP directly interacts with key nuclear receptors and steroidogenic enzymes involved in ovarian follicle maturation. Using molecular docking simulations, we assessed binding affinity for targets such as estrogen receptors (ER α /ER β), aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptor (PPAR) isoforms, and enzymes involved in the steroidogenesis pathway, such as aromatase (CYP19A1), 3 β -HSD, 17 β -HSD, and CYP11A1. Docking was performed using Autodock Vina with validated receptor and chemical structures and standardized ligand preparation. Across all targets, neither DBP nor MBP showed strong binding interactions. Predicted affinities were weak and did not support a receptor-mediated mechanism of action. Further docking will be conducted with additional key proteins that may be relevant to ovarian toxicity as we explore whether a receptor-mediated mechanism exists.

In addition to investigating a clear molecular initiating event, there is a need to evaluate DBP and MBP toxicity at the whole-organism level, where distribution, metabolism, and endocrine feedback loops affect ovarian toxicity. To address this, we initiated development of a whole-body

PBK model for the female rhesus monkey in COPASI, harmonized with parallel mouse models, and designed to integrate with a follicle-level computational module. The PBK model includes compartments for brain, gut, liver, ovary, kidney, arterial and venous blood, and “other tissues,” parameterized using macaque-specific anatomical and physiological data. DBP absorption, DBP metabolism to MBP, systemic distribution, renal clearance, and steroid hormone concentrations are represented using mass balance differential equations. The model was informed by available primate and rodent data, with explicit assumptions and allometric scaling used where macaque-specific values were unavailable.

This PBK model will be calibrated using in vivo and in vitro measurements of circulating DBP/MBP concentrations and steroid hormone levels. Once validated, the model will support in vitro-to-in vivo extrapolation (IVIVE) of in vitro follicle culture data and enable cross-species extrapolation between mouse and macaque. This integrated modeling framework will ultimately provide a mechanistic basis for predicting human-relevant ovarian toxicity.

Characterization of Per- and Polyfluoroalkyl Substances (PFAS) in Human Follicular Fluid from an Assisted Reproduction Cohort in Omaha, Nebraska.

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Abstract Text

Per- and polyfluoroalkyl substances (PFAS) are persistent environmental contaminants increasingly associated with adverse reproductive health outcomes; however, limited information exists regarding PFAS accumulation within the ovarian follicular microenvironment. Our previous studies demonstrated that PFOS, PFOA, and PFHxS are detectable in human follicular fluid and that exposure of primary human granulosa cells to this environmentally relevant PFAS mixture alters granulosa cell proliferation, steroidogenesis, and transcriptomic profiles. Building upon these findings, the objective of this study was to characterize concentrations of legacy and emerging PFAS compounds in human follicular fluid samples collected from patients undergoing assisted reproductive procedures in Omaha, Nebraska. The patient cohort ranged in age from 15-45 years, with an average age of 34 years. Body mass index (BMI) ranged from 18.36-50.12, with a mean BMI of 30.03. The study population was predominantly White (72.2%), followed by Asian (6.5%), Hispanic (6.5%), and Black (4.3%) participants; 10.9% of patients had unknown or unreported race. Most patients resided in Nebraska (84.6%), with additional representation from Iowa (7.2%) and smaller numbers from Missouri, Texas, Arizona, Pennsylvania, California, and Kansas. Follicular fluid samples (n = 138) were analyzed via LC/MS/MS for a panel of 45 legacy and emerging PFAS compounds at Eurofins Environmental Testing in Sacramento, California utilizing the EPA method 1633. Among the PFAS compounds analyzed in follicular fluid samples, PFOS demonstrated the highest detection frequency, identified in 138/138 samples (100%), followed by PFHxS in 135/138 samples (98%), PFOA in 105/138 samples (76%), and PFNA in 98/138 samples (71%). PFOS also exhibited the highest overall concentrations, with levels ranging from 0.091-3.8 ng/mL and a median concentration of 0.645 ng/mL. PFHxS concentrations ranged from 0.054-5 ng/mL, with a median of 0.22 ng/mL. PFOA concentrations ranged from 0.14-2.6 ng/mL, with a median concentration of 0.29 ng/mL, while PFNA was detected at lower concentrations ranging from 0.051-0.490 ng/mL and a median of 0.0895 ng/mL. Mean concentrations $\pm$ SEM were 0.368 $\pm$ 0.029 ng/mL for PFOA, 0.783 $\pm$ 0.042 ng/mL for PFOS, 0.324 $\pm$ 0.041 ng/mL for PFHxS, and 0.114 $\pm$ 0.007 ng/mL for PFNA. Additional PFAS compounds detected included PFDA, PFUnA, NMeFOSAA, PFDS, PFHpA, PFTrDA, NEtFOSAA, 9Cl-PF3ONS, PFPeS, PFBS, PFBA, and HFPO-DA. Overall, these findings demonstrate ubiquitous PFAS exposure within human follicular fluid samples from a Midwestern assisted reproduction cohort. The detection of multiple PFAS compounds within the ovarian follicular microenvironment highlights the need for future studies investigating relationships between PFAS exposure and ovarian function, oocyte quality, and fertility outcomes.

Co-Exposure to High Temperature and Mono(2-ethylhexyl) Phthalate Disrupts Mouse Antral Follicle Function *In Vitro*

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Abstract Text

Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer used in common consumer goods that is rapidly metabolized upon ingestion to its bioactive metabolite, mono(2-ethylhexyl) phthalate (MEHP). DEHP and MEHP are endocrine-disrupting chemicals that have been shown to target the ovary and disrupt follicle development and steroidogenesis. Due to the extensive use of DEHP in consumer goods, humans are ubiquitously exposed and therefore vulnerable to endocrine and reproductive dysfunction by MEHP. Simultaneously, humans are ubiquitously exposed to non-chemical stressors like rising global temperatures, and studies have shown that exposure to high temperatures can impair female reproductive health. Given the unavoidable exposure to both increasing heat and DEHP, this study used an *in vitro* follicle culture system to test the hypothesis that co-exposure to high temperature and MEHP will result in greater disruption of antral follicle function than either stressor alone. Antral follicles were isolated from CD-1 mice and cultured for 24-96hr in media containing follicle-stimulating hormone at a control temperature (CT; 37°C) or high temperature (HT; 42°C). Follicles were treated with vehicle control (dimethyl sulfoxide; DMSO) or MEHP (0.2-20µg/ml) in both temperature groups for 24-96hr. Separate incubators were used for the CT and HT treatment groups, in which all HT treatments occurred for 8hr per 24hr period to model daytime, occupational exposure to high temperatures. Areas of investigation included follicle growth, sex steroid hormone production, and expression of genes involved in cell cycle progression, steroidogenesis, apoptosis, and oxidative stress response. Data were analyzed via two-way ANOVA ($n=3-7$ with 5-10 follicles/group/replicate; $p \leq 0.05$). At 24hr, *Ccnd2*, *Hsd17b1*, and *Cyp19a1* mRNA levels were decreased by HT+MEHP compared to CT+DMSO. Additionally, *Cdkn1a* and *Fshr* mRNA levels were increased by HT+DMSO and HT+MEHP compared to CT+DMSO. At 48hr, follicle growth was decreased by HT+MEHP compared to CT+DMSO. Additionally, *Casp8* mRNA levels were decreased and *Cdkn1a* mRNA levels were increased by HT+DMSO compared to CT+DMSO. At 72hr, follicle growth, mRNA levels of *Ccnb1*, *Cyp11a1*, and *Cyp19a1*, and estrone and estradiol levels were decreased by HT+DMSO and HT+MEHP compared to CT+DMSO. At 96hr, follicle growth was decreased by HT+DMSO and HT+MEHP, and estrone and estradiol levels were decreased by CT+MEHP, HT+DMSO, and HT+MEHP compared to CT+DMSO. These results demonstrate that while MEHP exposure alone resulted in decreased estradiol synthesis by 96hr, HT exposure alone and HT+MEHP co-exposure dysregulated cell cycle and steroidogenic gene expression at earlier timepoints leading to both inhibited antral follicle growth and estradiol synthesis over time. Further, several defects in cell cycle regulation, follicle growth, and steroidogenesis occurred by HT+MEHP co-exposure but not by individual exposures, indicating that HT and MEHP may exert additive effects. Additionally, significant interactions were identified between HT and MEHP in analysis of *Fshr* mRNA levels (24hr) and estradiol levels (96hr). Specifically, *Fshr* mRNA levels were increased by HT regardless of MEHP, but the highest dose of MEHP blunted the HT effect. Further, while estradiol levels were decreased by all treatment groups, there was an effect of MEHP in CT conditions but not in HT conditions. These interactions indicate that the effects of MEHP were temperature-dependent. Thus, exposure to ubiquitous chemical and non-chemical stressors like MEHP and HT disrupts critical ovarian processes and may exert additive effects or interact to exert antagonistic effects when combined. Supported by R01ES033767, UL1TR001998, and P30ES026529.

Intrauterine Growth Restriction and Fetal Resistance to Porcine Reproductive and Respiratory Syndrome Virus

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Abstract Text

Intrauterine growth restricted (IUGR) pigs are born weak and experience exceptionally high preweaning mortality compared to non-IUGR littermates. Surprisingly, this undesirable IUGR phenotype has previously been correlated with in utero resistance to porcine reproductive and respiratory syndrome virus (PRRSV) infection. To investigate the complex relationship between uterine crowding, IUGR and in utero PRRSV resistance, we combined classic surgical models capable of producing biological extremes in porcine uterine crowding with a well-established reproductive PRRSV challenge model. First, eighteen gilts were randomly assigned to one of two surgical interventions. A unilateral oviductal ligation (UOL) was used to prevent fertilization of oocytes originating from the ipsilateral ovary, decreasing fetal crowding by ~50% through a reduction in litter size. In opposition, a unilateral hysterectomy-ovariectomy (UHO) was used to reduce uterine space by ~50%, while compensation in the contralateral ovary maintained initial conceptus number. Following a minimum 21-day recovery period, gilts were synchronized, bred via artificial insemination and monitored for return to estrus and general health. On gestation day 85 of 114, the pregnant gilts were challenged with a type 2 PRRSV strain, NVSL 97-7895, and confirmed viremic 7 days after. At 21 days post-inoculation, gilts were euthanized to allow for in-depth phenotyping, including collection of fetal preservation status, brain (BRN) and liver (LVR) weights. Individual samples of fetal serum and thymus were collected for quantification of viral load using a strain-specific qPCR assay. Litter size and fetal preservation status were not significantly different between surgery groups. Apparent early embryonic loss was significantly higher in the UHO group ($P < 0.01$), while apparent fetal loss was not significantly different between groups, suggesting that uterine crowding has a greater impact on early embryonic mortality. Fetuses from the UHO group exhibited lower body and organ weights ($P < 0.05$). However, the brain-to-liver weight ratio (BRN:LVR) did not significantly increase in the UHO group compared to the UOL group, possibly due to partial alleviation of early uterine crowding through increased embryonic loss. Serum and thymic viral loads did not differ significantly between UOL and UHO groups. When considered together with fetal preservation status (viable or meconiumstained), UOL litters contained a greater proportion of viable fetuses with lower viral loads, whereas UHO litters had a higher proportion of meconium-stained fetuses with higher viral loads ($P < 0.05$). These results suggest that uterine crowding exacerbates adverse fetal outcomes following maternal PRRSV infection. To further investigate the relationship between IUGR and PRRSV resistance, fetuses representing the highest and lowest 10% of BRN:LVR values were selected, regardless of surgical group, to represent biological extremes in IUGR and non-IUGR, respectively. IUGR fetuses exhibited significantly lower viremia and thymic viral loads than non-IUGR fetuses ($P < 0.01$), suggesting that IUGR confers protection against PRRSV infection. In conclusion, uterine crowding increased early embryonic loss and aggravated fetal outcomes following maternal PRRSV infection. IUGR fetuses were more resistant to PRRSV infection than their non-IUGR counterparts. Future research is required to identify the biological factors responsible for PRRSV resistance in IUGR fetuses. This work is supported by the Foundational and Applied Science Program, project award no. 2023-6701545223, from the U.S. Department of Agriculture's National Institute of Food and Agriculture.

Kaempferol Partially Mitigates PFDA-Induced Disruption of Estrous Cyclicity and Ovarian Steroidogenic Regulation in Female Mice

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Abstract Text

Perfluorodecanoic acid (PFDA), a long-chain per- and polyfluoroalkyl substance, has been associated with endocrine disruption and reproductive toxicity. However, its effects on ovarian endocrine function and the potential protective role of dietary flavonoids remain incompletely understood. Kaempferol is a naturally occurring flavonoid with antioxidant and endocrine-modulatory properties. In this study, we tested the hypothesis that kaempferol attenuates PFDA-induced disruption of reproductive endocrine homeostasis in female mice. After acclimation, female CD-1 mice, 11 weeks of age, were randomly assigned to six experimental groups: control, kaempferol 25 mg/kg, kaempferol 50 mg/kg, PFDA 2.5 mg/kg, kaempferol 25 mg/kg plus PFDA, and kaempferol 50 mg/kg plus PFDA. Kaempferol was administered once daily from day 1 to day 17, including a 3-day pretreatment phase. PFDA was administered from day 4 to day 17, approximately 4 h after the daily kaempferol dose in the kaempferol plus PFDA groups. Estrous cyclicity was monitored by vaginal cytology, and mice were euthanized at diestrus for serum hormone analysis, organ-weight assessment, and ovarian gene-expression analysis. Serum testosterone, estradiol, progesterone, follicle-stimulating hormone, and luteinizing hormone levels were measured. Ovarian expression of steroidogenesis-related genes, including *Cyp19a1*, *Hsd17b1*, *Hsd3b1*, *Cyp17a1*, *Star*, and *Cyp11a1*, was analyzed by quantitative PCR. Body weight remained relatively stable across groups, suggesting that the observed reproductive endocrine changes were not primarily driven by overt systemic weight loss. PFDA markedly increased liver weight, confirming a systemic biological response to PFDA exposure, whereas ovary, spleen, kidney, heart, and uterus weights showed no consistent major changes. Kaempferol pre- and co-administration, particularly at 50 mg/kg, partially reduced PFDA-associated liver enlargement but did not fully normalize liver weight. Vaginal cytology analysis showed that PFDA shifted estrous cycle distribution toward metestrus/diestrus, while kaempferol administration before and during PFDA exposure shifted the distribution toward a more control-like cyclic pattern. PFDA exposure altered circulating reproductive hormones, characterized by increased progesterone and follicle-stimulating hormone, with a downward trend in estradiol and no clear treatment-dependent change in luteinizing hormone. Kaempferol administration before and during PFDA exposure reduced PFDA-associated progesterone elevation and increased estradiol relative to PFDA, suggesting partial improvement of reproductive endocrine balance. Testosterone was increased in the kaempferol plus PFDA groups, suggesting that kaempferol may alter steroidogenic flux rather than simply normalize all circulating hormones. At the ovarian transcriptional level, PFDA reduced *Cyp19a1* and *Hsd17b1* expression while increasing *Hsd3b1* expression, suggesting impaired estrogen-associated steroidogenic regulation with a shift toward progesterone-associated steroidogenic activity. Kaempferol pre- and co-administration, particularly at 50 mg/kg, restored *Cyp19a1* and *Hsd17b1* expression toward control levels. In contrast, *Star*, *Cyp11a1*, and *Cyp17a1* showed less consistent treatment-dependent changes. Together, these findings suggest that PFDA disrupts reproductive endocrine homeostasis by altering estrous cyclicity, serum hormone balance, and ovarian steroidogenic gene expression. Kaempferol partially counteracts these effects, especially when administered before and during PFDA exposure, by improving estrous cycle distribution and restoring estrogen-associated steroidogenic markers. These data suggest that kaempferol may serve as a potential protective dietary flavonoid against PFDA-induced ovarian endocrine dysfunction.

Long-Term Exposure to Di-(2-ethylhexyl) Phthalate Through the Diet Alters Expression of Ovarian Function Genes and Aging Markers in Female Mice

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Abstract Text

Phthalates such as di-(2-ethylhexyl) phthalate (DEHP) are used as plasticizers in polyvinyl chloride plastics, flooring, food packaging, personal care products, medical tubing, plastic toys, and paints. Humans are continuously exposed to phthalates that are ubiquitously found in consumer products and the environment. Previous studies in our lab have shown that exposure to phthalates for up to 6 months has late-life consequences on female reproduction and may accelerate reproductive aging. However, limited data exist regarding the effects of long-term exposure (12 months) to phthalates on ovarian health and aging. Thus, the goal of this study was to determine whether long-term DEHP exposure affects markers of ovarian health and ovarian aging such as changes in ovarian follicle populations, expression of cell death, steroidogenesis, and telomere associated genes, ovarian fibrosis, and circulating bone morphogenic protein 4 (BMP4) levels. Adult female mice were exposed to vehicle control (corn oil) or DEHP at 0.15 ppm, 1.5 ppm, or 1500 ppm via the chow for 12 months. After dosing, the mice were euthanized, and some ovaries were collected and processed for histological evaluation of ovarian follicle numbers and fibrosis levels by Masson's trichrome staining. Other ovaries were used for quantitative polymerase chain reaction (qPCR) analysis of gene expression of cell death markers, steroidogenesis, and telomere associated factors. Sera were collected and subjected to enzyme-linked immunosorbent assays for BMP4 level quantification. Long-term exposure to DEHP at 1500 ppm borderline decreased the number of primary follicles compared to control, and DEHP at 0.15 and 1.5 ppm did not affect the numbers of primordial, primary, secondary, or antral follicles compared to control. When expression of cell death markers was analyzed, DEHP increased the expression of the pro-apoptotic gene Bcl2-associated X protein *Bax* (1.5 ppm), but it did not change the expression of the anti-apoptotic B-cell lymphoma 2 gene (*Bcl-2*) compared to control. Interestingly, DEHP exposure did not affect expression of the pro-apoptotic factor caspase 3 (*Casp3*) compared to control. When expression of steroidogenesis associated genes was analyzed, DEHP exposure did not affect expression of steroid acute regulatory protein (*Star*) at any tested concentration. Interestingly, DEHP exposure decreased cytochrome P450 19a1 (*Cyp19a1*) expression in the ovary at all tested concentrations compared to control. DEHP exposure (1.5 and 1500 ppm) also decreased follicle-stimulating hormone receptor (*Fshr*) expression in the ovary compared to control. However, DEHP exposure did not affect luteinizing hormone receptor (*Lhcgr*), androgen receptor (*Ar*), or estrogen receptor alpha (*Esr1*) expression in the ovary compared to control. When reproductive aging markers were examined, DEHP exposure (1.5 ppm) borderline increased the expression of the telomere associated gene *Trf2* compared to controls, but it did not change the expression of other telomere associated genes. DEHP exposure (0.15 and 1500 ppm) increased ovarian fibrosis compared to control. Finally, DEHP exposure (1500 ppm) borderline increased the circulating levels of BMP4 compared to control. Collectively, these results indicate that DEHP exposure decreased primary follicle numbers, ovarian *Fshr* expression, and ovarian *Cyp19a1* expression compared to control and that DEHP exposure affects markers of reproductive aging such as altered telomere associated genes, increased ovarian fibrosis, and increased BMP4 levels. Overall, these results indicate that DEHP exposure through the diet for 12 months alters some markers of ovarian health and reproductive aging in mice. Supported by R01 ES036194, R01 ES034112, and T32HD100075.

Deciphering the mechanism of maternal diabetes-induced congenital heart defects from RNA m⁶A modification

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Abstract Text

Congenital heart defects (CHDs) are the most common structural birth defect, occurring in 1-5% of live birth. Epidemiological studies have demonstrated that maternal diabetes is the major contributing factor for the development of CHDs. With the rapidly raised prevalence of diabetes among women of childbearing age, it's urgent to reveal the mechanism of maternal diabetes-induced CHDs, which would provide insight for preventing this disease. RNA m⁶A is a conserved and the most common post-transcriptional modification, and it has been recognized as related to cardiac development. However, the mechanism of RNA m⁶A on maternal diabetes-induced CHDs remains unknown. In this study, type 1 diabetes mellitus (DM) of C57BL/6J female mice was induced with streptozotocin (STZ), and DM model was constructed both in wild type and Wnt1-ZsGreen mice. The blood glucose levels ≥ 250 mg/dl were considered as diabetes. At E17.5, the blood glucose levels in pregnant DM mice were 284 ± 29.5 mg/dl. Histology analysis showed maternal diabetes-induced CHDs phenotypes including persistent truncus arteriosus (PTA), pulmonary subvalvular stenosis and ventricular septal defect (VSD). During early cardiogenesis, cardiac neural crest cell (CNCCs) originate from the dorsal neural tube and migrate to the developing heart where they are involved in the formation of septum, cardiac valves, and heart conduction system. To investigate the relationship between maternal diabetes-induced CHDs and m⁶A modification, we tested the RNA m⁶A expression in cNCCs at the initial stage of migration in Wnt1-ZsGreen mice. Immunofluorescence staining showed that hyperglycemia downregulated RNA methylation in CNCCs. Reduction of RNA methylation by conditional knockout of RNA methyltransferase 3 (Mettl3) caused full penetration of CHDs. Furthermore, RNA sequencing indicated that in O9-1 neural crest cells, hyperglycemia-upregulated genes were enriched in positive regulation of response to external stimulus, regulation of response to biotic stimulus and autophagy, while downregulated genes enriched in ossification, muscle tissue development and axonogenesis. Deletion of Mettl3 in O9-1 cells triggered innate immune response gene expression, especially enriched in Toll-like receptor (TLR) pathway. Then we verified the target genes through MeRIP-qPCR. We also tested the RNA stability of target genes with actinomycin D treatment. Result showed that deletion of Mettl3 in O9-1 cells would increase the stability of innate immune response genes. And tunnel staining indicated increased apoptosis in Mettl3 deleted O9-1 cells. In conclusion, this study revealed that maternal diabetes-induced suppression of RNA m⁶A modification of CNCCs is critically involved in CHDs formation, and TLR signaling related genes are the major targets of RNA m⁶A modification in CNCCs.

Single-Cell Transcriptomics Reveals That Early-Life Atrazine Exposure Reprograms Gene Expression in Leydig Cells of the Neonatal Boar Testis

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Abstract Text

The development of fertile boars is essential for efficient swine production. Early testis development is highly plastic, and perturbations during this period can compromise adult reproductive capacity. Atrazine is a widely used herbicide that frequently contaminates water sources in agricultural regions. Atrazine is also a potent endocrine-disrupting chemical associated with reproductive dysfunction across species, including swine. Recent data from our group indicate that 1) atrazine is detectable in pig drinking water and 2) early-life atrazine exposure (eATZ) during critical windows of testicular development (i.e., fetal and neonatal periods) markedly impairs endocrine function of the boar testis. For example, eATZ boars had reduced secretion of 10 gonadal steroids (androgens and estrogens; decreases of up to 67%), suggesting Leydig cell dysfunction. The objective of this study was to determine the molecular mechanisms that underlie Leydig cell dysfunction in eATZ boars. Pregnant littermate gilts drank water containing either an environmentally relevant dose of atrazine (20 µg/L) or vehicle control [0.002% (v/v) ethanol] *ad libitum* from 28 days of gestation through parturition (99.0 ± 0.9 days). Control and eATZ boars (n = 5/treatment) nursed their respective dams *ad libitum* prior to testis tissue collection on post-natal day 10. Samples were snap frozen for single-nuclei RNA-sequencing. Briefly, nuclei were isolated, processed with 10x Genomics technology, sequenced on an Illumina NovaSeq, and analyzed using the *Sscrofa11.1* genome. Classical Leydig cell markers (e.g., *STAR*, *CYP17A1*, *CYP11A1*) were utilized for cluster identification. A total of 79 differentially expressed genes (DEGs) were identified in Leydig cells from eATZ boars compared with controls (FDR-adjusted $P < 0.05$); 33 genes were down-regulated and 46 genes were up-regulated). Fourteen DEGs associated with Leydig cell proliferation and differentiation were identified, including *CDK6*, *LGR4*, and *MAP3K4*. Notably, *MAP3K4* is indispensable for normal testis differentiation in mice; thus, downregulation of *MAP3K4* may contribute to atrazine-induced defects in porcine testis development. In addition, thirteen DEGs were associated with lipid availability, steroidogenesis, and steroid signaling, including *LIPG* (lipid hydrolysis), *OSBPL1A* (intracellular cholesterol transport), *TSPOAP1* [supports StAR-mediated cholesterol transport (the rate-limiting step in steroidogenesis)], and *ADCY3* (generates cAMP for steroidogenic signaling). KEGG analysis revealed enrichment of lipid metabolism, hormone synthesis, and hormone signaling pathways, including arachidonic acid metabolism, steroid synthesis, and estrogen signaling ($q < 0.05$; pathway-level FDR adjustment). Furthermore, 10 DEGs were related to cellular stress and toxicant response pathways including *ATF3*, *DUOX2*, and *EPHX2*. Notably, *ATF3* is a stress-activated gene that was recently implicated in testicular ferroptosis and hypospadias. Its 4-fold upregulation in Leydig cells from eATZ boars suggests substantial cellular stress. Ultimately, these results provide novel insights into the mechanisms by which eATZ drives Leydig cell dysfunction in neonatal boars. These perturbations may alter the trajectory of testis development and compromise future fertility. Therefore, eATZ represents an emerging threat to the development of future breeding boars.

How Safe Is a Ketogenic Diet During Pregnancy? Effects of Maternal Ketogenic Diet on Birthweight and Long-Term Health in a Mouse Model

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Abstract Text

The increasing prevalence of ketogenic diets (KGD) as a nutritional approach for weight loss has extended to pregnant individuals, despite insufficient evidence on their impact on offspring health. Using a mouse model, we are developing a periconceptional model of nutritional ketosis to investigate the effect of maternal consumption of KGD during the periconceptional period on postnatal health of offspring. Experimental groups: (10 litters each): Three groups (C57/BL6) female mice at age of (3-4 weeks) were used in this study. Normal diet group (ND, mice were fed standard chow diet for 10 weeks), Ketogenic-diet group (KD, mice were fed standard chow diet for 6 weeks followed by 4 weeks of ketogenic-diet consisting of 67.4% fat, 0.6% carbohydrate, and 15.3% protein), and obesogenic-ketogenic-diet group (HF-KD, mice were fed 45% obesogenic high-fat-diet for 6 weeks followed by 4 weeks of ketogenic-diet). The control/ketogenic-diet group will help us to elucidate postnatal performance of offspring derived from non-obese mothers that switch to a ketogenic-diet during periconception, whereas the obesogenic/ketogenic group will be representative of obese mothers using ketogenic-diets to lose weight around the time of conception. After 10 weeks on respective diets, females were mated with C57/BL6 males. Vaginal plug detection defined embryonic day 0.5 (E0.5). Pregnant were maintained on the ketogenic-diets until E3.5, when they were switched to standard feeding for the remaining days of gestation. Offspring were weighed weekly from birth to week 27, systolic blood pressure (SBP) were recorded; with random effects regression statistical analysis used to assess independence of litter size and maternal origin. Metabolic analyses are currently in progress. The gestation period was notably prolonged in the KD, averaging 20.8 days, in contrast to the ND at 19.4 days and the HF-KD at 19.5 days. While the litter sizes did not differ significantly across the groups, the male: female ratio was significantly elevated in the KD (1.74) compared to the ND (1.3) and HF-KD (0.81). Offspring from KD (n= 63) and HF-KD (n= 68) groups when compared with ND controls (n=68), exhibited a significant reduction in birthweight ($p < 0.005$), independent of litter size. Notably, female offspring in the KD and HF-KD groups exhibited a marked decrease in birthweight compared to their ND counterparts (0.006 and 0.007, respectively). In contrast, no significant birthweight differences were detected among male offspring across the three groups. Female offspring from the HF-KD consistently showed lower body weight than those in the ND at weeks 11, 12, 13, 14, 17, 18, 22, and 24 ($p < 0.05$), and compared to the KD at weeks 11, 13, 14, 15, 17, 20, and 22 ($p < 0.05$). Conversely, male offspring from the KD demonstrated increased body weight relative to the ND at weeks 3, 4, and 5 ($p < 0.05$), and compared to the HF-KD at weeks 4, 5, 12, and 17 ($p < 0.05$). At week 9, SBP was elevated in both male and female KD offspring ($p < 0.005$) compared to ND males and HF-KD males. By week 21, however, both male and female offspring from the HF-KD exhibited a significant increase in SBP compared to those in the ND and KD groups. We conclude that maternal consumption of ketogenic diets during the periconceptional period alters sex ratios, dysregulates blood pressure, and results in divergent postnatal growth trajectories in male and female offspring.

Puberty-Associated Changes in Kiss1r, MC3R, and MC4R in the Ewe

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Abstract Text

The neural mechanisms that control the onset of puberty are not completely understood. In livestock, the onset of puberty relies on an increase in gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH). Kisspeptin, neurokinin B, and dynorphin neurons (KNDy) and their receptors, Kiss1r, NK3R, and KOR, respectively, are important regulators of puberty. Previous data from our laboratory has shown that KNDy neurons may be responding to other inputs, including the melanocortin system, that will affect the timing of puberty. Given this, we examined kisspeptin receptor (Kiss1r) mRNA expression in GnRH neurons as well as examining melanocortin 3 and 4 receptor (MC3R/MC4R) in kisspeptin neurons throughout pubertal development. Ewes were ovariectomized and a silastic estrogen implant was inserted two weeks before serial blood collection and sacrifice at 5 months (prepubertal), 8 months (peripubertal), and 10 months (postpubertal) of age. Hypothalamic tissue was perfused, obtained, and analyzed by RNAscope. We hypothesized that expression of Kiss1r mRNA in GnRH neurons, expression of MC3R/MC4R mRNA in kisspeptin neurons, and Kiss1r mRNA expression in the arcuate nucleus (ARC) would increase in association with puberty-related changes in LH secretion. We found that the percentage of GnRH neurons expressing Kiss1r increased throughout development. The percentage of ARC kisspeptin neurons expressing MC3R, but not MC4R, mRNA increased throughout development, but the area of MC3R, percent area of MC3R, and integrated density did not change. There were no significant changes in ARC Kiss1r throughout development. This work supports the idea that increased sensitivity to input by aMSH as well as responsiveness to kisspeptin input in GnRH neurons comprise part of the neurocircuitry involved in puberty.

Investigation of an Immune-Isolated Bovine Ovarian Xenograft for Restoration of Endocrine Function in Ovariectomized Mice

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Abstract Text

Anticancer therapies can induce gonadotoxicity, implicating ovarian dysfunction with downstream cardiovascular, cognitive, and reproductive consequences that are increasingly recognized as major quality-of-life concerns in the expanding population of female pediatric cancer survivors. Many of these systemic outcomes reflect disruption of hypothalamic–pituitary–gonadal (HPG) axis endocrine feedback: loss of native ovarian hormone production alters gonadotropin-releasing hormone (GnRH) signaling and is accompanied by dysregulated pituitary follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion. In prior work, our laboratory demonstrated that implantation of an immune-isolated human ovarian xenograft into ovariectomized immune-compromised mice restored estrous cyclicity and re-established endocrine feedback, as evidenced by circulating estradiol and FSH profiles across the 20-week study duration. However, donor-derived human tissue remains constrained by limited availability, and autologous ovarian tissue transplantation may carry risks of reintroducing malignant cells, particularly in hematologic cancers. A promising alternative to the scarce human donor tissue is bovine ovarian tissue due to the mono-ovulatory nature of cows. Bovine tissue exhibits cycle dynamics and folliculogenesis timelines comparable to humans, alongside conserved molecular pathways regulating follicle development, and shared expression of key genes in bovine and human fetal primordial germ cells. Thus, the goal of this project is to investigate whether an immune-isolated bovine ovarian graft restores endocrine function in an ovariectomized host, similar to human tissue. Published studies additionally suggest that vitrification minimally affects bovine tissue morphology, follicle density, and transcriptomic signature post-thawing, motivating its evaluation as a scalable graft source with cryopreservation compatibility for endocrine restoration.

Bovine ovarian cortical tissue was harvested, cryopreserved, and histologically assessed for follicle staging and stromal health prior to implantation. To enable graft immune isolation, ovarian tissue was encapsulated within a hydrogel construct consisting of a degradable polyethylene glycol core, designed to accommodate tissue expansion, and a photopolymerized non-degradable polyethylene glycol shell that permits diffusion of nutrients, oxygen, and hormones while precluding immune cell infiltration. Female NSG mice were bilaterally ovariectomized and implanted subcutaneously with either encapsulated bovine ovarian tissue (n=15) or non-encapsulated tissue (n=15) to assess whether tissue encapsulation affects endocrine function restoration for the 20-week study duration.

Restoration of endocrine function is being evaluated through daily vaginal cytology, serum collection for hormone analysis, and histological assessment of retrieved explants. Four-week explants have been collected, with additional retrievals planned at 10 and 20 weeks to assess follicle staging and explant stromal health over time. Preliminary vaginal cytology data indicates restored vaginal openings following a period of persistent diestrus in some ovariectomized mice implanted with both encapsulated and non-encapsulated bovine xenografts, suggesting a resumption of estrus activity. Cytology data will be collected for the remainder of the 20-week study duration in conjunction with blood serum and explants to assess resumption of estrus activity, hormone activity, explanted graft follicle staging and stromal health, and graft steroidogenic capacity in a murine model of premature ovarian insufficiency. This work is supported by R01HD105018.

Mechanisms by Which Estrous Cycles Influence Beige Fat Appearance

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Abstract Text

Across the female menstrual cycle, dynamic shifts in energy expenditure, insulin sensitivity, lipid metabolism, and basal body temperature occur in accordance with the rise and fall of estradiol. Estradiol affects multiple reproductive and non-reproductive tissues, including white adipose tissue (WAT). Many female-specific differences in WAT distribution and function are attributed to the effect of estrogens on WAT. Premenopausal women typically store fat in the more metabolically favorable subcutaneous WAT (sWAT) depot compared to men, and postmenopausal women, who tend to accumulate more central and visceral WAT. As such, there remains a critical need to understand how reproductive cycles affect adipose tissue dynamics to not only clarify sex-specific differences in energy balance and insulin sensitivity but also reduce the metabolic and cardiovascular disease risks that accompany the menopausal transition. My analysis of sWAT in wild-type mice during the mouse estrous cycle revealed morphological changes in adipocyte size, the presence of multilocular adipocytes, and alterations in beige fat gene expression around the time of ovulation. We find that these observations are conserved across mouse strains but not observed in mice of increased reproductive age. It is well understood that the amount of thermogenic beige adipocytes in sWAT corresponds with insulin sensitivity and protection against the comorbidities of obesity and cardiovascular disease. Therefore, we hypothesize that estrous cycles regulate beige adipocyte emergence and influence whole-body energy balance to promote ovulation. In support of this hypothesis, we find that mice lacking UCP1, the canonical beige fat marker, are sub-fertile with abnormal estrous cycles and smaller litter sizes. Additionally, our studies using the triple beta-adrenergic receptor knockout mice (*Adrb1*, *Adrb2*, *Adrb3*; ‘betaless’) demonstrate that the beta-adrenergic pathway is involved in the adipocyte dynamics we observe during the estrous cycle. Importantly, this research represents a crucial step towards detailing WAT responses to estrous cycles and defining why the loss of estrous cycling results in an elevated risk for metabolic disorders, including type 2 diabetes and cardiovascular disease.

Induction and Regulation of the Long Non-coding RNA, Gm12648, in the Ovulatory Process

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Abstract Text

Ovulation is a tightly coordinated process initiated by the luteinizing hormone (LH) surge. This event is driven by extensive transcriptional reprogramming mediated by multiple regulatory factors. Accumulating evidence has shown that long non-coding RNAs (lncRNAs, transcripts >200 nucleotides) are widely expressed in several tissues and have key roles in gene regulation by interacting with DNA, RNA, and proteins. However, the regulatory function of lncRNAs in the ovulatory process has not been explored. Recent studies from our lab have discovered that Gm12648, a 776-nucleotide lncRNA, is highly expressed in the mouse ovary, compared to other tissues taken from the same animal. We then examined the Gm12648 expression pattern in the whole mouse ovaries and in isolated granulosa cells, and cumulus oocyte complexes (COCs) at 0, 4, 8, 12, and 24h after human chorionic gonadotropin (hCG; LH analog) administration. We observed a slight induction of Gm12648 at 4h in the ovary, granulosa cells, and COCs. More specifically, in the ovary, Gm12648 was highest (245-fold) at 8h after hCG administration and remained elevated (130-fold) until 12h before declining at 24h. Similarly, in granulosa cells, Gm12648 was highest (160-fold) at 8h after hCG administration and remained elevated (110-fold) until 12h before declining at 24h. Notably, in COCs, Gm12648 expression increased to 145-fold at 8h after hCG and further elevated to 190-fold at 12h. To better understand the regulation of Gm12648 by LH/CG, granulosa cells were treated in vitro with inhibitors of the LH/CG signaling pathways for 4h. Our data revealed that hCG regulates Gm12648 expression through protein kinase A (PKA) signaling pathways in granulosa cells. Focusing on the potential role of Gm12648 in the ovulatory process, we knocked down the expression of Gm12648 with small interfering RNA (siRNA; specific to Gm12648) with or without hCG treatment for 8h in the mouse granulosa cells. As expected, hCG treatment increased the expression of Gm12648. Interestingly, the hCG-induced expression of Gm12648 was decreased by 87% with Gm12648 siRNA treatment as compared to the control (scrambled siRNA with hCG). Next, through the predicted interaction network by an RNA interactome database, we found putative interactions of Gm12648 with transcription factors such as Cebpa, Cebpb; CCAAT/enhancer-binding proteins, Runx2; a DNA-binding alpha subunit of core binding transcription factor complex, and Yin-Yang1 (Yy1); a multifaceted polycomb group gene involved in a variety of transcriptional regulatory roles. Besides transcription factors, Gm12648 could also possibly interact with ovarian proteins, mRNAs, small non-coding RNAs, and/or microRNAs. In summary, Gm12648 is induced in the ovary after the ovulatory surge of LH via the PKA signaling pathway. Gm12648 may act to regulate ovulatory transcription factors, possibly exerting its function through the interactions with those factors, culminating in successful ovulation. These findings provide new insights into the expression, regulation, and potential role of long non-coding RNAs in the ovulatory process.

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Chromosome 19 miRNA cluster (C19MC) Disruption via Deletion of Upstream Regulatory Region Impairs Preimplantation Development in Rhesus Macaque Embryos

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Abstract Text

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression. The chromosome 19 miRNA cluster (C19MC) is a placenta-specific miRNA cluster found only in primates and is hypothesized to play a key role in trophoblast development, differentiation, and function. To further define C19MC function during embryonic development, we perturbed C19MC expression in rhesus macaque embryos by CRISPR–Cas9–mediated deletion of differentially methylated region 1 (DMR1), the proposed regulatory region of this cluster. After fertilization and prior to the initial cleavage event, embryos were either (1) microinjected with a Cas9–gRNA ribonucleoprotein (RNP) targeting C19MC DMR1 or (2) poked with clean microinjection needles to serve as controls. Embryos were cultured and monitored through 10 days post-fertilization to evaluate preimplantation development. A subset of embryos underwent time-lapse imaging for morphokinetic assessment. End-stage development was categorized by the stage of arrest: at <16 cells, >16 cells, morula, early blastocyst, expanded blastocyst, hatching blastocyst, or hatched blastocyst. All cleaved RNP-injected embryos were collected for genotyping to determine editing status. For genotyping, whole-genome amplification was performed, followed by PCR amplification of a 1500-bp region upstream of C19MC containing DMR1. PCR products were analyzed by gel electrophoresis. Embryos that retained the 1500-bp wild-type size were classified as unedited, whereas embryos with an ~830-bp band consistent with an ~670-bp deletion were classified as edited. A subset of amplicons from edited embryos was validated by amplicon sequencing. In total, we obtained 45 sham controls, 33 injected but unedited embryos, and 11 successfully edited embryos. A significant proportion of edited embryos arrested at the morula stage compared to RNP-injected unedited and control embryos (Fisher's exact test with Monte Carlo estimation: edited vs control, $p = 0.00124$; edited vs unedited, $p = 0.03256$), and all 11/11 edited embryos arrested prior to blastocyst expansion. Together, these data indicate that disruption of the C19MC regulatory region via DMR1 deletion impairs developmental progression before blastocyst expansion, with a pronounced morula-stage arrest phenotype. Ongoing studies will define the timing of C19MC miRNA expression during rhesus macaque preimplantation development and assess how DMR1 deletion alters C19MC miRNA expression.

Functional Roles of SP1 in Bovine and Human Embryonic Genome Activation and Early Embryonic Development

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Abstract Text

Embryonic genome activation (EGA) initiates the embryonic developmental program and enables the transition toward the first lineage specification. However, the molecular characteristics of EGA and the transcription factors (TFs) that orchestrate this process remain poorly defined. Here, using single-cell RNA-seq in bovine embryos, we show that bovine major EGA initiates at the 8-cell stage and proceeds heterogeneously among blastomeres, with no obvious signs of lineage bias, indicating a regulated yet asynchronous wave of activation across individual blastomeres. Integrative analyses further reveal uncoupled patterns of protein accumulation relative to transcription and translation activation during bovine EGA. We next examined the function of SP1, a transcription factor activated during minor EGA, whose binding motifs are enriched in accessible chromatin during major EGA in both bovine and human embryos. Loss of SP1 resulted in developmental arrest at the morula stage in bovine embryos. Notably, although SP1 regulates a small subset of EGA genes, it is not an essential regulator of EGA in cattle. SP1 binds to thousands of genomic regions, the majority of which are located at promoters. Strikingly, SP1 appears to cooperate with distinct sets of transcription factors at promoters versus distal regulatory regions. At promoters, SP1 colocalizes with ubiquitously expressed factors including NFY and MAZ. In contrast, at distal regions, it colocalizes with lineage-specifying factors such as the trophectoderm regulator TFAP2C and OTX2. Multi-omics analyses demonstrate that SP1 promotes early lineage gene expression in bovine embryos by shaping local chromatin states. In sharp contrast, SP1 deficiency in human embryos led to impaired preimplantation development and failed EGA. Mechanistically, SP1 specifically regulates a panel of PRD-like transcription factors in human—but not bovine—embryos, including TPRX1/2 and DPRX, which have been functionally linked to human EGA. Moreover, SP1 activates several markers of human 8C-like cells, including DUXA and ZSCAN5B, but does not appear to regulate DUX4, suggesting that SP1 acts in parallel with other lineage-specific TFs to govern human EGA. Together, our study delineates the dynamic landscape of bovine EGA and uncovers evolutionarily conserved and species-specific functions of SP1 in controlling EGA and early embryonic development in mammals.

Mitochondrial DNA Quantity in the Bovine Oocytes and Preimplantation Embryos Derived from *In Vitro* Fertilization.

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Abstract Text

In vitro fertilization (IVF) is valuable technology of producing bovine embryos for embryo transfer (ET). However, the conception rate of bovine IVF embryos is lower than that of *in vivo* derived embryos, and improvement in the conception ability of bovine IVF embryos is necessary. Blastocyst embryos consist of the inner cell mass (ICM) and the trophoblast (TE), which develop to the fetus and the placenta after implantation, respectively. Among these lineages, the TE also mediates early embryo-maternal communication with the maternal endometrium and plays a central role in the implantation process. Recent studies have reported that mitochondrial DNA (mtDNA) quantity in the TE of human blastocysts may be associated with embryo implantation capacity. In addition, quantity of mtDNA in oocytes and early embryos has been suggested to correlate with fertilization and developmental potential. In early mammalian embryonic development, mtDNA replication does not occur until the blastocyst, and replication resumes exclusively in the TE at this stage. Thus, mitochondrial dynamics in the TE may be involved in implantation-related processes. However, most findings regarding the relationship between developmental ability and mtDNA quantity in bovine IVF embryos remains unclear. Therefore, this study focused on mtDNA as one factor involved in successful implantation, aiming to clarify the relationship between mtDNA quantity and early development in bovine IVF embryos. As a first experiment, mtDNA quantity in *in vitro* matured oocytes and IVF embryos at 1-cell, 2-cell, 4-cell, 8-cell, 16-cell, morula, and blastocyst stages were examined by Real-time PCR. mtDNA quantity increased in the blastocyst stage embryos compared to both IVM oocytes and the morula stage embryos ($P < 0.05$). Furthermore, to confirm mtDNA replication in the TE of the blastocyst, mitochondrial localization was examined using MitoTracker Green staining in the ICM and TE at the blastocyst stage. Mitochondrial fluorescent intensity showed significantly ($P < 0.01$) higher in the TE than that in the ICM. These results suggest that mtDNA replication occurs in a stage-dependent manner during preimplantation development of bovine IVF embryos. Furthermore, that mtDNA is involved in the function of the TE, which is related to the implantation potential of the bovine IVF embryos. Currently, we are examining the correlation between mtDNA quantity and gene expression status within individual bovine IVF embryos. Our results will provide valuable information into the relationship between mtDNA quantity and implantation potential in bovine IVF embryos.

Using Ultra-low Coverage Whole-Genome Sequencing from Preimplantation Genetic Testing to Identify Risk Genes for Embryo Aneuploidy

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Abstract Text

Aneuploidy frequently arises during human meiosis and is the primary cause of early miscarriage and *in vitro* fertilization (IVF) failure. Individuals undergoing IVF exhibit significant variability in aneuploidy rates, although the exact genetic causes of the variability in aneuploid egg production remain unclear. Preimplantation genetic testing for aneuploidy (PGT-A) using next-generation sequencing is a standard test for identifying and selecting IVF-derived euploid embryos. The wealth of embryo aneuploidy data and ultra-low coverage whole-genome sequencing (ulc-WGS) data from PGT-A has potential for discovering genes in parental genomes that are associated with aneuploidy risk in their embryos. Using ulc-WGS data from ~40,000 PGT-A biopsies, we imputed genotype likelihoods of genetic variants in embryo genomes. We then used the imputed variants and embryo aneuploidy calls to perform a genome-wide association study of aneuploidy incidence. We identified multiple loci that are significantly associated with meiotic aneuploidy risk and are conducting candidate gene analyses. Our work extended the research utility of PGT-A ulc-WGS data by allowing robust association testing and improved the understanding of the genetic contribution to maternal meiotic aneuploidy risk.

Actin-dependent Dynamic Reorganization of Endoplasmic Reticulum During Oocyte To Embryo Transition in *Xenopus laevis*

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Abstract Text

Abstract: The oocyte to embryo transition (OET) is one of the critical developmental events that transform a fully grown oocyte into an actively developing embryo. This transition is orchestrated through a series of tightly regulated events, including oocyte maturation, ovulation, fertilization and the subsequent activation of the zygotic genome. Oocyte maturation is accompanied by extensive cellular remodeling, including reorganization of cytoskeletal and organelle networks. Among these organelles, the endoplasmic reticulum (ER) undergoes dynamic reorganization that is essential for developmental process. However, the mechanism underlying this dynamic reorganization remains elusive. Here, we report that the endoplasmic reticulum remodels from a sheet-like structure to tubular networks during oocyte maturation in *Xenopus* in an F-actin-dependent manner. To understand the mechanism regulating ER remodeling, we performed comparative proteomic analysis to identify ER-associated F-actin regulators. We identified a significant increase in ER-associated Wasl, a Cdc42 effector that promotes Arp2/3 dependent F-actin nucleation. Overexpression of constitutively active Cdc42 converted ER sheets into a tubular ER network, whereas inhibition of F-actin, Wasl, or Arp2/3 disrupted ER remodeling. Together, these findings suggest that the Cdc42-Wasl-Arp2/3 cascade regulates ER remodeling during the OET.

Individual Variability of in vitro Fertilization and Embryogenesis in the Domestic Cat

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Abstract Text

As environmental pressures on ecosystems intensify, assisted reproductive technologies (ARTs) are becoming increasingly important tools for the conservation of threatened species. In vitro oocyte maturation (IVM) and fertilization (IVF) are central components of these efforts, and the domestic cat serves as a valuable model for developing ARTs for wild felids. However, rates of advanced embryonic development in felines remain low (~20%), highly variable, and difficult to predict based on oocyte morphology alone. Previous studies have shown that oocytes collected from young females during the breeding season achieve the highest developmental success, with minimal effects of estrous cycle stage. The goal of this study is to characterize variability in IVF success for individual queens when season, age, and estrous stage are controlled. High-quality oocytes from 18 follicular phase females (5 months-5 years) were harvested (n = 161) between the months of January and May. Oocytes were cultured at 38.5°C in commercially available human blastocyst media supplemented with 1 IU/mL luteinizing hormone and 1 IU/mL follicle-stimulating hormone for 26 hours, followed by IVF. Presumptive zygotes were rinsed and incubated for 6 days post-fertilization. Cells and embryos were fixed and imaged, with DAPI used to stain DNA. Formation of morulae and blastocysts (advanced embryonic stages) varied widely among females. We observed values ranging from 0% to 100% per individual. The distribution yielded an interquartile range of 45.8%, indicating substantial variability in success rates across age-, season-, and cycle-stage-matched females. Notably, this variability persisted even when oocytes were fertilized using sperm from the same male, supporting the conclusion that female-intrinsic factors are a major driver of developmental competence. For descriptive purposes, females were categorized as low (< 20%), medium (21–49%), or high (≥50%) responders based on the percentage of oocytes that developed advanced embryos, with 39%, 17%, and 44% of individuals falling into each category, respectively. These preliminary data suggest that individual females are a significant source of variation in IVF success rates. We therefore hypothesize that shared gene-expression profiles among high and low responders may predict developmental competence and inform optimization of culture conditions. Future studies will investigate gene expression in embryos and their corresponding cumulus cells to identify molecular predictors of developmental success.

Optimization of culture conditions and embryo selection improves pregnancy outcomes in the direct transfer of frozen-thawed bovine in vitro produced embryos.

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Abstract Text

Improving pregnancy outcomes for frozen-thawed in vitro produced (IVP) embryos remains a major challenge in bovine embryo transfer. IVP embryos have lower pregnancy rates and freeze resistance than in vivo-produced embryos (Hansen et al., 2020). No studies have systematically analyzed factors related to embryonic development and assessed their impact on pregnancy rates. In this study, we aimed to identify key factors affecting pregnancy rates after direct transfer of frozen IVP embryos and to establish a practical in vitro evaluation method. We focused on maturation/culture medium, blastocyst stage, day of development, and thawing temperature. In addition to in vitro evaluations, the study examined miscarriage rates after pregnancy and information on the resulting offspring.

[Materials and Methods]

Immature cumulus-oocyte complexes were collected from bovine ovaries obtained from a slaughterhouse and used for in vitro maturation, fertilization, and development. The resulting blastocysts were loaded into straws with a freezing solution composed primarily of ethylene glycol and sucrose, then slowly frozen using a programmable freezer. After storage in liquid nitrogen, they were thawed in a water bath. For in vitro evaluation, embryos were left to stand on a 38.8°C hot plate for 5 minutes after freezing and thawing, and their condition was assessed by culture in BO-IVC (IVF Bioscience). For the transfer experiment, direct transfers were performed into recipients with a good corpus luteum on the 7th day of estrus, and the conception rate was evaluated by pregnancy testing approximately 30 days after transfer.

Exp1: We investigated embryo culture assays that more simply and accurately reflect embryo transfer outcomes. IVP embryos after freeze-thawing were cultured in BO-IVC for 24 or 48 hours. The results showed that the hatching rate after 48 hours of culture closely mirrored pregnancy outcomes, suggesting its utility as a predictive indicator in vitro.

Exp2: We compared conventional media (Medium 199 and m-SOF) with the new simple media BO-IVM and BO-IVC (IVF Bioscience). Culture conditions significantly affected embryo freeze tolerance and the live birth rate: BO-IVM and BO-IVC improved blastocyst development, post-thaw survival, and pregnancy rates compared with conventional media. The incidence of large offspring syndrome was lower, and the live birth rate was higher when BO-IVM was used, suggesting that the maturation medium may influence fetal normality.

Exp3: We compared blastocyst stages (Blastocyst vs Expanded blastocyst) and embryo development days (Day 7 vs Day 8 after in vitro insemination). Embryo selection based on developmental stage and timing revealed that Day 7 expanded blastocysts had significantly higher post-thaw survival and pregnancy rates than Day 8 embryos or earlier-stage blastocysts.

Exp4: We also examined the thawing temperature of slow-frozen embryos. When embryos were thawed in water at 25, 30, and 35 °C, the optimal thawing temperature influenced outcomes, with 35 °C showing a tendency toward higher pregnancy rates.

Collectively, these findings demonstrate that integrating optimized culture systems with appropriate embryo selection criteria can significantly enhance the efficiency and reliability of direct transfer of frozen-thawed bovine IVP embryos. e IVP embryos.

Pregnancy-Induced Decidualization Alters Collagen Microarchitecture and Mechanical Response of Murine Endometrium

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Abstract Text

Approximately 20% of infertility cases lack a clear cause. Growing evidence points to the uterine microenvironment as a key source of dysfunction. The endometrium, an extracellular matrix (ECM) rich tissue, cyclically regenerates to support implantation and early pregnancy. This transformation, known as decidualization, is a hormonally driven process involving dramatic cellular changes, tissue thickening, and ECM remodeling. This study evaluates how healthy decidualization alters endometrial stiffness and collagen microarchitecture. We hypothesize that ECM remodeling increases collagen content and alignment, leading to higher endometrial stiffness. Eight-week-old female C57BL/6 mice ($n=6$) were mated and euthanized on pregnancy day 8. One uterine horn was cut along the antimesometrial (AM) pole, and the other along the mesometrial (M) pole. Samples were stained with fluorescent probes and indented under an upright confocal microscope using the weight of a 1 mm glass microsphere. 3D confocal stacks were acquired during and after indentation at decidualized implantation sites (IS) and non-decidualized inter-implantation sites (IIS). Indentation depth was quantified by comparing indented and non-indented stacks, and stiffness was estimated using the Hertz model. The mechanical anisotropy index (MAI) was estimated using the logarithm of the x-y displacement-decay ratio, using vertical displacements measured 555 microns from the indenter center in xz and yz planes. Collagen microstructure was evaluated using second-harmonic generation imaging, and custom Python code quantified fiber orientation (azimuth, elevation), thickness, and alignment (inverse azimuthal standard deviation). Effects of pole (AM vs. M) and location (IS vs. IIS) on stiffness, MAI, fiber alignment, average elevation angle, and fiber thickness were tested using two-way ANOVA with significance set at $p < 0.05$. Endometrial stiffness was significantly influenced by both pole and location, with stiffness peaking at the AM-IS. Post-hoc comparisons confirmed that AM-IS was significantly stiffer than both AM-IIS and M-IS (778.5 ± 296 vs. 321.1 ± 147.8 and 313.2 ± 146.5 Pa, respectively), indicating that stiffness increases with decidualization specifically at the AM-IS. Collagen fibers did not exhibit any preferred orientation at IIS (alignment index = 0.019 ± 0.001 for both AM and M). However, at the IS, collagen fibers were more aligned in the circumferential direction (0.023 ± 0.005 and 0.025 ± 0.005 , for AM and M, respectively). This increased structural anisotropy was consistent with greater mechanical anisotropy (MAI = 0.558 ± 0.271 (AM) and 0.483 ± 0.209 (M) vs. 0.144 ± 0.237 (AM) and 0.148 ± 0.175 (M) at the IS and IIS, respectively), indicating direction-dependent stiffness only in the decidualized endometrium. This suggests that decidualization not only increases stiffness but also reorganizes the ECM to produce anisotropic mechanical function. Elevation angle (from the myometrium to lumen) was higher at the AM pole regardless of location, suggesting that pole-specific collagen architecture persists even as decidualization remodels the tissue. In contrast, collagen volume fraction and fiber diameter did not differ significantly with pole or location. These non-significant results suggest that decidualization alters collagen organization more than bulk collagen content. This is the first study to link decidualization with changes in both endometrial stiffness and collagen microarchitecture. Our results show that decidualization alters both endometrial mechanics and collagen microstructure, including increased stiffness, greater mechanical anisotropy, and higher fiber alignment at decidualized sites. These changes in collagen organization and tissue-level mechanical function may be essential for supporting implantation and early pregnancy and may represent biomarkers of infertility linked to endometrial dysfunction. Future work will test this hypothesis by employing these methods to study the endometrium in mouse models of impaired decidualization.

Transcriptomic Profiling Reveals Central Drivers of Myometrial Activation in Equine Placentitis

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Abstract Text

Equine ascending placentitis is a significant reproductive disorder in mares characterized by inflammation of the placenta, most commonly resulting from ascending bacterial infection. It is a leading cause of abortion, stillbirth, and perinatal mortality in horses worldwide. This condition is orthologous to human chorioamnionitis, an inflammatory disorder of the fetal membranes that is strongly associated with infection-driven preterm birth. In both species, inflammation at the maternal–fetal interface disrupts gestational homeostasis and promotes premature activation of parturition pathways. A central event in placentitis-induced preterm labor is the loss of myometrial quiescence, which precipitates uterine activation and the onset of labor. However, the molecular mechanisms driving myometrial activation in horses remain poorly understood. To explore these mechanisms, we examined the transcriptome of the equine myometrium during ascending placentitis and compared it to gestationally matched normal pregnancy. In brief, myometrial samples were collected post-euthanasia from mares with induced placentitis around 290 days of gestation (placentitis group; n=6) and from un-inoculated mares (control group; n=6). RNA sequencing (RNA-Seq) was performed using the Illumina NovaSeq6000 platform, with raw reads processed using TrimGalore 0.4.3 before mapping to EquCab3.0 via STAR 2.4.3a. Mapped reads were quantified using Cufflinks 2.2.1 with NCBI annotation. Differentially expressed genes (DEGs) were identified using Cuffdiff 2.2.1, with a false discovery rate (FDR) < 0.05. Our analysis revealed 263 DEGs in the myometrium during placentitis compared to the control, including seven genes (APOBEC3Z1B, PLA2G2D, CXCL17, MMP8, MMP13, S100A8, and S100A9) that were uniquely upregulated (exclusively expressed) during placentitis. Gene groups related to connexins (GJC2, also known as connexin-47), matrix metalloproteinases (MMP1, MMP8, MMP13, and MMP25), prostaglandin signaling (PTGS2, PTGER3), calcium channel regulation (TRPM2), and calcium ion transport (RAMP2, CACNA1D, PRKCB, COR1A, and DEFB1) point to a clear pathway for myometrial activation during equine placentitis. Additionally, we identified several potential chemoattractants (CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL3, CXCL16, and CXCL17) in the myometrium during placentitis, a finding supported by significant leukocytic infiltration observed in myometrial samples from the placentitis group. Leukocytic infiltration is a well-known hallmark of myometrial activation. To identify potential upstream regulators of myometrial activation, we performed upstream regulator analysis using Ingenuity Pathway Analysis, which revealed 12 potential regulators: TLR2, STAT1, IRF7, SPI1, PTGS2, S100A8, S100A9, ICAM1, NRG1, HIF1A, PRKCB, and TYROBP. Immunolabeling of TLR2, PTGS2, S100A8, S100A9, and HIF1A showed significant positive correlation with their respective mRNA expression. Overall, this study enhances our understanding of key regulators and mechanisms of myometrial activation during equine placentitis, which may inform the development of therapies to prevent preterm labor and treat placentitis. Notably, our findings underscore the central role of TLR2 in initiating inflammatory signaling within the equine myometrium during placentitis, which drives the upregulation of contraction-associated transcripts such as PTGS2, PTGER3, MMP1, MMP8, and GJC2. Consequently, targeting TLR2 and its associated pathways—for example, through TLR antagonists—represents a promising therapeutic strategy to mitigate placentitis-induced preterm birth. Beyond elucidating the mechanisms of myometrial activation described in this study, our work establishes a foundation for generating new hypotheses and guiding future research directions.

Targeting Endometrial Cancer with Graphene Oxide–Zinc Sulfide (GO–ZnS) – Mediated Photothermal Therapy: Effects on Cell Viability and KLK6 Expression

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Abstract Text

Endometrial cancer, the most common malignancy of the female reproductive tract in developed countries, arises from the uterine epithelium and continues to increase in incidence, particularly among postmenopausal women aged 55–65. Disease progression is closely linked to dysregulation of the endometrial microenvironment, including proteolytic remodeling of the extracellular matrix. Kallikrein-related peptidase 6 (KLK6), a serine protease involved in tissue remodeling and cellular invasion, is overexpressed in endometrial carcinoma and is associated with tumor progression and altered endometrial function. However, a critical gap remains in understanding how emerging therapeutic strategies modulate KLK6 expression within the reproductive tissue context of endometrial cancer. Nanomaterials with strong near-infrared (NIR) absorption, including graphene-based nanomaterials, have demonstrated significant potential in cancer therapy. Photothermal therapy (PTT), a minimally invasive and highly selective modality, is emerging as an alternative strategy for treating tumors resistant to conventional therapies. Graphene oxide (GO)-based nanocomposites enhance photothermal conversion efficiency and therapeutic efficacy, yet their effects on proteases relevant to endometrial biology remain largely unexplored. This study evaluated the therapeutic efficacy of GO, ZnS, and GO–ZnS nanocomposites in combination with PTT in RL95-2 endometrial cancer cells while assessing modulation of KLK6 expression. We hypothesized that GO–ZnS-mediated PTT would significantly reduce cell viability and suppress KLK6 expression, thereby attenuating protease-driven invasive potential in endometrial cancer cells. RL95-2 cells were treated with GO, ZnS, GO–ZnS nanocomposites, or untreated controls for 24 and 48 hours, followed by NIR irradiation at 980 nm for 5 minutes (two cycles). Cell viability was assessed using MTT assay. KLK6 protein expression was quantified using Western blot analysis of cell lysates and ELISA of conditioned media to measure secreted KLK6. GO–ZnS nanocomposites combined with PTT produced the greatest reduction in cell viability compared to all other groups. This treatment was also associated with decreased KLK6 protein expression, suggesting reduced proteolytic activity linked to tumor invasion. These findings support GO–ZnS nanocomposites as an effective photothermal therapeutic platform and identify KLK6 as a potential biomarker of therapeutic response in endometrial cancer.

Elucidating the Direct Role of Tryptophan Catabolism in Mediating Immune Tolerance and Metabolic Adaptation During Pregnancy

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Abstract Text

The catabolism of tryptophan contributes to key processes essential for a healthy pregnancy including maternal-fetal immune tolerance. Tryptophan is degraded by the rate-limiting enzymes indoleamine 2,3-dioxygenases (IDO1 and IDO2) and tryptophan 2,3-dioxygenase (TDO). In mice, IDO1 is highly expressed in trophoblast giant cells within the placenta. IDO activity generates kynurenine metabolites that contribute to immune tolerance partly by promoting the differentiation of potent immunosuppressive cells, regulatory T cells (Tregs). Disruption of this pathway has been associated with pregnancy complications such as preeclampsia, intrauterine growth restriction (IUGR), and pregnancy loss in humans and animal models. Our lab has also previously demonstrated that IDO1 expression is susceptible to environmental exposure and reduced expression is associated with higher fetal resorption rates in mice. Despite these observations, our understanding of the specific physiological role of IDO1-dependent tryptophan catabolism in the placenta and its association with other tryptophan-degrading enzymes is limited. This project will elucidate how IDO1-mediated tryptophan catabolism contributes to immune tolerance and supports pregnancy. Using a global IDO1 knock out (KO) mouse model, we will use flow cytometry to investigate the effects of Ido1 ablation on maternal Treg populations by comparing immune cell profiles to wild-type mice. We will also determine the impact of global Ido1 deletion on the abundance of tryptophan metabolites and the expression of TDO and IDO2 in maternal and placental tissues using LC-HRMS, qPCR and Western blot analyses, respectively. This research project will also generate and employ a novel trophoblast cell-specific Ido1 knockout model to evaluate how placental IDO1 loss affects pregnancy outcomes, including fetal viability, placental development, and maternal preeclampsia-like symptoms. Together, these studies will be the first to systematically define the mechanistic role of placental IDO1 in promoting immune and metabolic adaptation during pregnancy. These findings will provide fundamental insights into how disruptions in tryptophan catabolism by genetic and environmental factors contribute to adverse pregnancy outcomes and inform approaches to promote favorable maternal-fetal health. (Research supported by: NIH R01 ES029469, NIH P30 ES001247, and University Research Award NIH T32)

SLC2A1 gene knockout reprograms human trophoblast bioenergetics and cell behavior

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Abstract Text

Glucose serves as a vital energy source for fetal growth and development, being transferred from maternal blood to fetus via the placenta. This transfer process involves the transportation of glucose through carriers known as GLUTs (glucose transporters). GLUT1, a subtype of the GLUT family encoded by *SLC2A1*, is predominantly located in the syncytiotrophoblast, the outer layer of the placenta that interfaced directly with maternal blood, where it functions as the primary transporter of glucose. Despite its importance, the precise mechanism by which *SLC2A1* influences trophoblast function is still not clearly defined. Using CRISPR/Cas9-mediated knockout of *SLC2A1* in HTR8/SVneo cells, we assessed its impact on metabolism, survival and invasiveness to elucidate its critical role in trophoblast function and placental development. *SLC2A1* deficiency in human trophoblast cells forced a shift from glycolysis to compensatory mitochondrial respiration. This metabolic reprogramming increased mitochondrial reactive oxygen species (ROS) production, causing severe oxidative stress. Elevated ROS at the ER-mitochondria junctions (MAM) triggered excessive mitochondrial calcium influx (Rhod-2 AM), further activating the unfolded protein response (UPR) through the PERK/IRE1 α /GRP78 pathway. Simultaneously, mTOR overactivation impaired autophagy flux, resulting in the accumulation of LC3-II and p62. These homeostatic failures suppressed PI3K/AKT/P70S6K signaling, significantly reducing cell motility and spheroid formation. In summary, *SLC2A1*-driven metabolic reprogramming is essential for maintaining the mitochondria–ER–autophagy axis, and its disruption suppresses trophoblast invasion, potentially serving as a key driver of placental dysfunction during early pregnancy.

The Trophectoderm of Elongating Conceptuses Relies on Non-Canonical TCA Cycle Metabolism to Supply Biosynthetic Precursors within the Physiologically Low-Oxygen Intrauterine Environment in Pigs

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Abstract Text

Porcine conceptuses elongate rapidly through extensive cellular proliferation, migration, and morphological change. Tricarboxylic acid (TCA) cycle flux provides biosynthetic precursors for synthesis of lipids, nucleotides, and amino acids. However, elongating conceptuses primarily rely on aerobic glycolysis; therefore, most of the glucose-derived carbons are diverted away from the TCA cycle. To maintain TCA cycle flux during aerobic glycolysis, porcine conceptuses utilize glutamine as an alternate carbon source. Glutamine is converted into α -ketoglutarate (α KG), which is then converted to succinate through oxidative reactions. However, during the peri-implantation period of pregnancy, the low-oxygen intrauterine environment slows electron transport chain (ETC) activity, resulting in the accumulation of NADH and the depletion of NAD^+ , stalling upstream oxidative TCA cycle reactions and reducing the generation of biosynthetic intermediates. This disruption of redox homeostasis provides a significant challenge for elongating conceptuses. There is biological precedence for the use of non-canonical metabolic strategies such as reverse TCA cycle reactions to maintain TCA cycle flux and redox balance in low-oxygen conditions, but these pathways have not been examined or identified in the porcine conceptus trophoctoderm. In the present study, conceptuses from Days 11, 13, 15, and 17 ($n=4/\text{Day}$) of pregnancy were collected and subjected to LC-MS/MS. After Day 11, concentrations of pyruvate, citrate, and succinate decreased ($p < 0.01$), while concentrations of lactate and fumarate increased ($p < 0.01$), indicating a metabolic shift in response to reduced availability of glucose-derived acetyl-CoA and possible disruption of cellular redox due to low oxygen availability. When porcine trophoctoderm cells (pTr2) were cultured with low oxygen concentrations (5% O_2 ; $p < 0.01$) or with antimycin to inhibit ETC function ($p < 0.05$), an increase in the NADH/NAD^+ ratio was detected, suggesting that low-oxygen levels within the intrauterine environment generate a reductive force capable of reversing TCA cycle flux. Three isozymes of isocitrate dehydrogenase (IDH) catalyze the interconversion of isocitrate into α KG. In contrast to IDH3, which only operates in the forward (oxidative) direction of the TCA cycle, IDH1 and IDH2 can catalyze the reaction in either the forward or reverse (reductive) direction. Using immunofluorescence, IDH1 and IDH3 were detected in conceptus trophoctoderm on Days 13, 15, and 20 of pregnancy ($n=3/\text{Day}$). ATP citrate synthase, which irreversibly catalyzes the reverse conversion of citrate into oxaloacetate and acetyl-CoA, was also detected in the trophoctoderm on Days 15 and 20 of pregnancy ($n=3/\text{Day}$). The presence of these enzymes indicates that porcine conceptuses are capable of reversing TCA cycle flux. Consistent with these findings, *ex vivo* cultures of conceptuses from Day 15 of pregnancy ($n=3$) demonstrated that antimycin-mediated inhibition of the ETC causes accumulation of succinate ($p < 0.001$), which is the end-product of the reverse TCA cycle in mammals. We also identified increases in the expression of mRNA for uncoupling proteins (UCP2 and UCP3, $p < 0.0001$) in the conceptus trophoctoderm on Days 13, 15, and 16 ($n=4/\text{Day}$) of pregnancy. The activity of these proteins may complement reverse TCA cycle flux by maintaining ETC flux, regenerating NAD^+ , and reducing the formation of reactive oxygen species. Collectively, these results indicate that porcine conceptuses likely utilize non-canonical TCA cycle activity to fulfill biosynthetic demands associated with rapid elongation and to prevent the generation of reactive oxygen species in challenging redox conditions. This work was supported by Agriculture and Food Research Initiative Competitive Grant no. 2020-67015-31136 from the USDA National Institute of Food and Agriculture.

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Validating an endometrial cytology approach to study uterine function of cows 15 days after artificial insemination without terminating the pregnancy

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Abstract Text

In cattle ~70% of pregnancies are lost from fertilization to the 2nd week of gestation and are partially a consequence of a non-receptive endometrium. Studies characterizing uterine function were strictly done with slaughtered pregnant cows at specific time-points or non-pregnant cows, which did not allow the association between the uterine function on the 2nd week of gestation with the pregnancy status on day 30 (i.e. more stable period). Thus, there is a need to establish a minimally invasive approach to study the uterine function on the 2nd week of gestation without affecting the pregnancy outcome and associate it with the pregnancy status on day 30. The hypothesis was that endometrial cytology (cytobrush) collected 15 days after artificial insemination does not affect the pregnancy outcome on day 30, and harvests exclusively maternal cells that express conceptus-stimulated genes. Multiparous *Bos taurus* cows (n = 247) were submitted to an estrous synchronization protocol followed by artificial insemination (AI; day 0; D0). On D15, cows were randomly assigned to one of the treatment groups: cytobrush (n=55) cows were submitted to a cytobrush collection of the uterine body, or control (n=192), cows were brought to the operational facilities but the cytobrush sample was not collected. The cytobrush collection was performed by coupling the brush to a conventional artificial insemination gun covered with a sheath. In the cervix, the brush was exposed and rotated five times to collect the uterine epithelial cells. After the collection the samples were immersed in PBS (phosphate-buffered saline), the supernatant was recovered to measure interferon-tau (IFNT) concentrations in the uterine luminal fluid (n=18; using an in-house ELISA assay), and the pellet with cells were used for real-time PCR analysis (n=43) for *ISG15* and *RSAD2*. For a subgroup of cows (n=21) a part of the pellet with cells was also submitted to immunofluorescence analysis for luminal epithelial cells marker (KRT18), glandular epithelial cells marker (FOXA2), and trophoblast cell marker (CDX2). Pregnancy diagnosis was conducted on D30 to compare pregnancy/AI between cytobrush and control cows. There was no effect of the cytobrush on the pregnancy/AI on D30 (P >0.1 control: 47.2% 26/55 vs. cytobrush: 44.7% 86/192). Cytobrush samples harvested on D15 from cows diagnosed as pregnant on D30 had a IFNT concentration 42% greater (8.01 ± 0.82 pg/mL) than cows diagnosed as non-pregnant (5.60 ± 0.63 pg/mL; P=0.030). However, there was no effect of pregnancy on D30 (P >0.1) on the expression of *ISG15* and *RSAD2* of samples collected on D15. Immunofluorescence analysis confirmed that ~99% of the cells harvest by the cytobrush were epithelial, of these 26% were glandular epithelial and the remaining luminal epithelial. No fluorescence signals were found for CDX2 marker. In conclusion, cytobrush is a reliable and safe technic to be used in future studies to understand the uterine function that predicts the pregnancy outcome on D30. The samples contain conceptus-secreted proteins such as IFNT but no evidence of expression of conceptus-stimulated genes were observed. The future steps are to look at the transcripts abundance of genes in other signaling pathways rather than the traditional IFNT. This work was supported by the SSR emerging investigator grant (2024-5).

Circadian Regulation of Endometrial Receptivity in a Sheep Organoid Model

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Abstract Text

Current protocols of embryo transfer in humans rely primarily on identifying the correct *day* of endometrial receptivity, without considering the potential influence of *within-day circadian timing* on implantation success. Given that endometrial physiology and hormonal rhythms follow circadian patterns, transfer decisions based solely on calendar days may miss biologically optimal windows that occur on an hourly timescale. This study tested whether endometrial receptivity is under circadian control using a physiologically relevant sheep endometrial organoid model mimicking the luteal phase. Ovine organoids were cultured long-term (45 days), hormonally primed with estradiol and progesterone mimicking the luteal phase, and exposed for 24 hours to vehicle (control), lipopolysaccharide (LPS; 100 ng/mL) to induce inflammatory circadian disruption, or melatonin (100 μ M) to synchronize circadian signaling. Following treatment, organoids were returned to treatment-free media and sampled every 6 hours for 30 hours. Expression of core circadian genes (*BMAL1*, *PER2*) and receptivity markers (*FST*, *KRT18*, *LAMB3*, *SPP1*) was quantified, and rhythmicity parameters were analyzed using the MetaCycle::meta2d R package. Control organoids exhibited intact ~23-hour clock gene rhythms, and all receptivity markers showed distinct circadian-like oscillations, demonstrating that endometrial readiness fluctuates predictably over the day rather than remaining constant. LPS disrupted this coordination, dampening *PER2* amplitude and desynchronizing receptivity markers, supporting a mechanism by which inflammatory conditions impair implantation. Melatonin restored robust, aligned rhythms across both clock and receptivity genes, producing higher amplitudes and synchronized peak times (high receptivity phase) that closely mirrored the *BMAL1* oscillation pattern. These findings reveal that endometrial receptivity is dynamically regulated across the circadian cycle and that inflammation uncouples this timing. Collectively, the data suggests that optimal embryo transfer may require consideration of hourly circadian phase, not just cycle day, and provide strong feasibility for circadian-aligned embryo transfer studies in humans.

Gene Regulatory Networks Directing Adenogenesis and Epithelial Differentiation in the Developing Uterus

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Abstract Text

The mouse uterus is not fully differentiated at birth and is comprised of an undifferentiated epithelium surrounded by mesenchyme. After only two weeks, the epithelium is specified into a uterine type luminal epithelium (LE) and the mesenchyme segregates into endometrial stroma and myometrium. Concurrently, adenogenesis is initiated by postnatal day (PND) 7 as the glandular epithelium (GE) lineage bifurcates from epithelial progenitor cells; the new GE buds elongate through the stroma to reach the inner circular layer of the myometrium. Forkhead box A2 (*Foxa2*) is expressed exclusively in the GE and is required for adenogenesis. In other organs, *Foxa2* has important roles in cell type specification by promoting chromatin accessibility for the binding of other lineage- and cell-specific factors and the establishment of unique gene regulatory networks (GRN). However, the essential cellular and molecular factors and pathways governing *Foxa2* regulation of GE morphogenesis are not well understood. *Foxa2* Venus Fusion (FVF) reporter mice express a Venus fusion protein under endogenous *Foxa2* control without effects on *Foxa2* function. In the developing and adult mouse uterus, FVF and *Foxa2* were co-localized only in the GE cells. The approach used here was to isolate epithelial cells from the uterus of PND12 and PND15 FVF mice using enzymatic digestion followed by positive selection with CD326 (EpCAM) microbeads. Fluorescence-activated cell sorting (FACS) was then used to separate populations of LE (FVF-) and GE (FVF+) cells. Bulk transcriptome (mRNA-seq) analysis found more differentially expressed genes (DEG) between GE and LE on PND15 (1,060; 396 increased, 664 decreased than PND12 (696; 173 increased, 523 decreased). Genes with exclusive or increased expression in GE cells were enriched in GO biological process terms (epithelium development, tissue morphogenesis), GO cellular component terms (bicellular tight junction, apical junction complex), and biological pathways (glutathione metabolism, retinoic acid biosynthesis). Single cell RNA-seq (scRNA-seq) was performed using the isolated epithelial cells and a 10X Genomics Chromium system. Seurat analysis of 9,443 epithelial cells identified 9 different cell populations. Four clusters were GE based on expression of GE marker genes (*Cxcl15*, *Foxa2*, *Gas6*, *Sox9*). Four clusters were LE based on expression of LE marker genes (*Tacstd2*, *Wnt7a*). The remaining cluster contained both LE and GE cells and expressed cell proliferation-associated genes (*Birc5*, *Ccna2*, *Mki67*, *Pcna*). Heterogeneity was observed among the GE cell clusters. For instance, *Foxa2* was predominantly expressed in two GE clusters as well as in the proliferative cell population, whereas *Cxcl15* was expressed across all GE clusters and the proliferative population. Trajectory analyses provided a framework for understanding epithelium lineage bifurcation and differentiation. A candidate set of transcription factors and GRNs were identified that potentially direct epithelium lineage specification and morphogenesis. These studies provide a foundation to understand the cellular and molecular mechanisms regulating epithelial morphogenesis in the developing uterus.

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Modeling Early Placental Dysfunction in Down Syndrome with Blastoids

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Abstract Text

Down Syndrome (T21) is the most common aneuploidy disorder in humans and is characterized by a full or partial trisomy of chromosome 21. Individuals with T21 display fusion defects within their placental syncytiotrophoblasts (STBs) that are the primary barrier between mother and fetus. However, failed STB differentiation has only been observed in primary cultures from mid-late gestation T21 placental tissue, and the non-renewable nature of this tissue and sample variation serve as barriers for studies that require precise genetic manipulation. To this end, we propose that induced pluripotent stem (iPS) cells may serve as a tractable and renewable cell source for studies on T21 trophoblast differentiation. Using iPS cells, we 1) characterize fusion defects in T21 vs. euploid trophoblast-like cells and 2) identify a novel mechanism in-part responsible for T21 fusion defects. During implantation, embryos differentiate a primitive STB that drives invasion into the endometrium. We generated embryo-like structures (blastoids) from isogenic iPS cells reset to a naïve pluripotent state from a donor chromosomally mosaic for T21 and implanted them into immortalized endometrial stromal cells. After 72h of implantation, the primitive STB of T21 produced significantly less human chorionic gonadotropin (hCG) and had a reduced fusion index ((number of DAPI+ nuclei - number of syncytial cells/ total nuclei in the implanting blastoid)) compared to blastoids from the isogenic euploid control line. This is remarkable since blastoids self-direct their differentiation of STB in the absence of small molecule inhibitors. We then expanded our analysis to 6 pairs of age- and sex- matched iPS cell lines and differentiated trophoblast-like cells with BMP4, a TGF β inhibitor (A83-01), and a fibroblast growth factor receptor inhibitor (PD173074, BAP) and demonstrate that with the exception of one line ($p=0.22$), T21 all trophoblast-like cells exhibit significantly reduced cell fusion compared to euploid controls following differentiation ($p<0.05$). We then performed bulk RNA-sequencing of iPS cell lines, both BAP-differentiated and undifferentiated, to further understand fusion defects in T21. Following DESeq2, we identified nuclear receptor interacting protein 1 (NRIP1), as a differentially expressed gene on chromosome 21 that is highly expressed in T21 cells and may be in-part responsible for fusion defects. To determine if trisomic NRIP1 is sufficient to reduce STB fusion, we used Cre-recombinase to flip-in a doxycycline-inducible third copy of NRIP1 in a euploid iPS cell line (NRIP1^{OE}). We then BAP-differentiated NRIP1^{OE} alongside a GFP control (NRIP1^{GFP}) with 100ng/mL doxycycline and measured baseline fusion and STB marker expression. NRIP1^{OE} lines, like T21 lines, displayed significantly reduced fusion ($p<0.01$) and expression of STB-related transcripts (*ERVW-1* $p<0.01$, *CGB* $p<0.001$) compared to NRIP1^{GFP}. These data provide strong evidence that NRIP1 contributes to physiological defects in T21 placentas and suggests that NRIP1 may serve as a potential target for therapeutic intervention. All cell lines were obtained following thorough patient consent. Additionally, blastoids peri-implantation culture was terminated after 72h in alignment with international guidelines. Blastoid experiments were funded by institutional start-up funds from the University of Colorado at Boulder and by the Anna and John J. Sie Foundation. All other experiments were funded through an NIH Fellowship 1F32HD116568-01. We also received substantial support for RNA-sequencing and iPS cell line reprogramming from the Linda Crnic Institute for Down Syndrome at the University of Colorado Anschutz Medical Campus. Finally, all blastoid experiments were approved by a special ethical oversight committee.

Estrogen Receptor alpha in Smooth Muscle Is Required for Pre-implantation Embryo Transport and Fertility

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Abstract Text

Successful embryo transport from the oviduct to the uterus is essential for establishing pregnancy and depends on well-coordinated smooth muscle contractions regulated by steroid hormones. While uterine contractility has been extensively studied during labor, its role during early pregnancy is poorly understood. This knowledge gap has important implications for in vitro fertilization (IVF), where high estrogen levels at embryo transfer are associated with increased uterine contractility and decreased implantation and pregnancy success. The objectives of this study were to investigate the roles of classical estrogen receptor alpha (ESR1) in reproductive tract smooth muscle cells (SMC) during early pregnancy. A mouse model, namely *Myh11^{Cre/+};Esr1^{fl/fl}* (SMC *Esr1^{d/d}*), with a deletion of ESR1 in the SMCs was used. ESR1 deletion in SMCs was confirmed by immunohistochemistry staining and led to severely impaired oviductal contraction frequency, resulting in complete pre-implantation embryo retention in the oviduct and sterility. We found that pre-implantation embryo development was not affected. Interestingly, deletion of the progesterone receptor (PGR) in SMCs did not impair embryo transport, highlighting a specific requirement for ESR1 in this process. To explore the underlying mechanisms, we performed single-cell RNA sequencing (scRNA-seq) on oviductal tissues from control and SMC *Esr1^{d/d}* mice at 3.0 days post coitus, a critical time point for embryo transport. scRNA-seq analysis revealed that ESR1 loss in SMCs disrupts cell-to-cell communication from smooth muscle to both secretory and ciliated epithelial cells. Signaling through the non-canonical WNT (ncWNT) pathway was markedly affected, suggesting that ESR1 regulates cell-to-cell interacting networks critical for coordinating oviductal function during embryo transport. In addition to defective oviductal contractility, uterine contraction frequency and contraction distance were also significantly reduced in SMC *Esr1^{d/d}* mice. As a result, when wild-type blastocysts were transferred into the uterus, implantation failed in SMC *Esr1^{d/d}* females, indicating that ESR1 in SMCs is also required for successful embryo implantation. Taken together, ESR1 is essential for coordinated oviductal and uterine smooth muscle activity, and its loss disrupts embryo transport, implantation, and SMC-epithelium communication networks. These findings position ESR1-dependent signaling as a mechanistic driver of early pregnancy success and suggest that defects in estrogen-regulated smooth muscle function may underlie certain forms of embryo retention, tubal infertility, or implantation failure. This work provides a framework for identifying smooth muscle-based therapeutic targets in reproductive disorders. Funding: R01 HD097087

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.

Extracellular Matrix-Driven Adhesion of Bovine Uterine Epithelial Cells Defines an In Vitro Model to Study Progesterone and Lactate-Mediated Uterine Remodeling

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Abstract Text

Successful embryo implantation requires precise coordination between the developing embryo and the maternal uterus, with the endometrial luminal epithelium serving as the initial site of interaction. This process is characterized by extensive epithelial remodeling, including changes in cell adhesion, polarity, and cytoskeletal organization. Extracellular matrix (ECM) composition and mechanical properties, such as stiffness, play critical roles in regulating these cellular processes; however, their contribution to uterine epithelial cell adhesion and response to hormonal and metabolic signals remains poorly understood. In dairy cattle, early embryonic loss remains a major challenge to reproductive efficiency, highlighting the need to understand better the mechanisms regulating uterine receptivity. The objective of this study was to establish an ECM-based platform to evaluate bovine uterine epithelial (BUTE) cell adhesion and identify optimal biochemical and mechanical substrates for investigating the effects of progesterone (P4) and lactate on uterine epithelial remodeling. Using a high-throughput ECM array system, BUTE cells were seeded at a density of 200,000 cells per array onto defined ECM conditions, including Collagen I (C1), Collagen IV (C4), Fibronectin (FN), Collagen I + Collagen IV, and Collagen I + Fibronectin. These ECM proteins were presented on hydrogels with defined stiffness levels of 1 kPa, 6 kPa, and 25 kPa to mimic varying mechanical properties of the uterine microenvironment. Cell attachment was evaluated at 24- and 48-hours post-seeding. In addition, Cells can be maintained on these arrays for up to 5 days while remaining morphologically healthy and highly viable. Our preliminary results demonstrate that both ECM composition and substrate stiffness influence BUTE cell adhesion after both 24 and 48 hours of culture. The pattern of attachment varied across stiffness conditions. At 1 kPa, FN supported the highest level of cell attachment, whereas at 6 kPa, C4 showed the greatest attachment among the ECM substrates tested. At 25 kPa, Fibronectin again supported the highest level of attachment. Overall, 25 kPa hydrogels promoted the greatest level of BUTE cell attachment compared to 1 kPa and 6 kPa substrates. Cell attachment was strongly dependent on both ECM composition and mechanical stiffness, indicating that BUTE cells respond differently to specific ECM environments under varying stiffness conditions. These findings suggest that FN and C4 may provide favorable substrates for BUTE cell adhesion depending on the mechanical properties of the microenvironment. Based on these findings, ongoing studies aim to investigate the effects of P4 and lactate on BUTE cell morphology, cytoskeletal remodeling, and cell-cell adhesion using the optimized ECM substrates identified in this study. Emerging evidence suggests that lactate functions as a signaling molecule that promotes uterine receptivity by modulating epithelial barrier integrity, cellular proliferation, and gene expression. However, how P4 and lactate modulate uterine receptivity remains largely unknown. To address this, BUTE cells will be treated with varying concentrations of P4 and lactate following attachment to optimal ECM substrates and stiffness conditions. Immunofluorescence analysis of E-cadherin and F-actin will be used to assess changes in cell-cell adhesion and cytoskeletal organization, respectively, while quantitative image analysis will enable detailed characterization of cell morphology and spatial distribution. In summary, this study establishes a physiologically relevant ECM-based platform incorporating both biochemical and mechanical factors to investigate uterine epithelial cell adhesion. These findings may provide critical insight into how ECM composition and stiffness shape cellular responses and support future mechanistic investigations into the regulation of uterine receptivity.

GP130 and STAT3 Play Distinct Roles in Uterine Luminal and Glandular Epithelia During Mouse Pregnancy

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Abstract Text

Background: During early pregnancy in mice, it has been reported that 17 β -estradiol (E2) secretion in the presence of progesterone (P4) stimulates leukemia inhibitory factor (LIF) production from the uterine glandular epithelium. In the luminal epithelium, LIF binding to LIFR promotes recruitment of GP130, a shared signal-transducing subunit of the receptor complex, thereby inducing STAT3 phosphorylation and initiating embryo implantation on Day 5 (vaginal plug = Day 1). However, the precise epithelial-specific roles of GP130 and STAT3 during implantation and subsequent pregnancy remain unclear since localization of GP130 and STAT3 is not completely merged. This study aimed to clarify their distinct functions in uterine luminal and glandular epithelia using conditional knockout mouse models.

Methods: To investigate the role of GP130, uterine epithelial (luminal and glandular)-specific or glandular epithelial-specific Gp130 knockout mice (Gp130-eKO and Gp130-gKO, respectively) were generated. Female knockout mice were mated with wild-type males to assess fertility. Uteri were collected on Days 4–6 and analyzed by immunohistochemistry (IHC), immunofluorescence (IF), and quantitative PCR (qPCR) to evaluate implantation and the expression of implantation- and decidualization-related factors (e.g., CDH1, PGR, and HAND2). To examine the function of STAT3, uterine epithelial-specific or glandular epithelial-specific Stat3 knockout mice (Stat3-eKO and Stat3-gKO, respectively) were also generated. Fertility was assessed, and uteri were collected on Days 4–8 for histological and molecular analyses.

Results: Gp130-eKO females exhibited complete infertility, with no implantation sites detected on Day 5 and no offspring obtained. Expression of P4-responsive genes (*Alox15* and *Areg*) was significantly reduced. IF analysis showed persistent CDH1 expression in the luminal epithelium surrounding the embryos, suggesting impaired epithelial remodeling associated with defective P4 signaling, which likely led to implantation failure. Gp130-gKO females were also infertile, exhibiting abnormal implantation on Day 6. In some cases, decreased CDH1 expression in the luminal epithelium and altered expression of decidualization markers (e.g., ALPL and HAND2) in the stroma were detected. Similarly, Stat3-eKO females showed implantation failure and complete infertility. IF and qPCR analyses revealed abnormal expression of P4 downstream factors (e.g., PGR, *Hand2*, and *Fgf1*), indicating that STAT3 regulates implantation, at least in part, via PGR-mediated signaling. In contrast, Stat3-gKO females exhibited implantation on Day 8 but failed to produce offspring. Reduced expression of decidualization markers (e.g., ALPL and Ki67) and PGR downstream factors was observed, although PGR expression itself was unchanged.

Conclusion: GP130 and STAT3 exert distinct epithelial-specific functions in the uterus during mouse pregnancy. While luminal epithelial GP130 and STAT3 are essential for embryo implantation, glandular epithelial GP130 and STAT3 contribute to post-implantation events and pregnancy maintenance. These findings highlight differential regulatory mechanisms in uterine epithelia that are critical for successful implantation and sustained pregnancy.

Bovine Pregnancy Specific Protein B Modulates the Endometrial Luminal Epithelial Transcriptome Differently than Interferon-tau Following *in Vitro* Challenge.

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Abstract Text

Interferon tau (IFNT) and pregnancy associated glycoproteins (PAGs), which include pregnancy specific protein B (PSPB), are proteins secreted by the trophoblast of the bovine placenta. Both PAGs and PSPB are detectable in maternal blood and have been widely used for pregnancy diagnostics in cattle. Numerous studies have demonstrated associations between PAG and PSPB concentrations and pregnancy outcome. While IFNT is implicated as a major signal in maternal recognition of pregnancy and modulation of the maternal immune environment; some evidence suggests that PAGs may also be involved in chemokine regulation, prostaglandin synthesis, peripheral blood leukocyte transforming growth factor beta signaling, and extracellular matrix remodeling necessary for placental adhesion and attachment to the endometrium. Despite this, the biological functions of PAGs remain largely unknown. Given this, the objective of the present experiment was to determine if PAGs promote immune tolerance by modulating the innate immune response of endometrial luminal epithelial (eLE) cells. We hypothesized that PAGs would induce transcriptomic changes associated with immune tolerance and remodeling necessary for pregnancy success in a manner different from IFNT. Multiparous nonlactating Holstein-Angus crossbred cows ($n = 3$; 545.5 ± 75.9 kg) were estrus synchronized using a 7-day Co-Synch + CIDR protocol. Ovarian structure sizes were measured at -10, -3, 0, and 4 days relative to ovulation using transrectal ultrasonography. At 4 days post-ovulation, eLE cells were collected using a cytobrush and plasma concentrations of progesterone were quantified. After collection, eLE cells were isolated and subsequently cultured for 48 hours in a T75 flask. After the 48-hour attachment period, eLE cells were passaged and cultured at 1.0×10^5 cells/mL in 24-well tissue culture plates for an additional 24 hours in triplicate. Following the 24-hour reattachment period, eLE cells were treated with either medium alone (CON), 500 pg/mL recombinant ovine IFNT (IFNT), 2 ng/mL PSPB (loPAG), or 20 ng/mL PSPB (hiPAG) for 24 h. Following treatment, RNA was extracted for bulk RNA-seq analysis for differentially expressed genes (DEGs) determination. Mean corpora lutea size and plasma progesterone concentration at 4 days post-ovulation were 16.6 ± 0.97 mm and 1.65 ± 0.31 ng/mL, respectively. Treatment of eLE cells with IFNT resulted in 501 DEGs, with 385 upregulated and 116 downregulated, compared to CON. Upregulated genes following IFNT treatment were annotated to biological processes relating to innate immunity, antiviral defense, apoptosis, and necrosis ($P < 0.001$) compared to CON. Downregulated genes following IFNT treatment were annotated to biological processes relating to transport, electron transport, respiratory chain, and cytolysis ($P < 0.001$) compared to CON. Surprisingly, there were no DEGs following either loPAG or hiPAG treatment compared to CON. Next, we investigated the DEGs between IFNT and PAGs. Treatment of eLE cells with hiPAG resulted in 381 DEGs with 80 upregulated and 301 downregulated compared to IFNT. Upregulated genes were annotated to mitochondrial respiratory chain complex I assembly, mitochondrial electron transport, phospholipid biosynthetic processes, microtubule cytoskeleton organization, and microtubule-based processes ($P < 0.001$) compared to IFNT. Downregulated genes were annotated to innate immunity, immunity, antiviral defense, apoptosis, and necrosis ($P < 0.001$) compared to IFNT. Collectively this work demonstrates that IFNT strongly affects eLE cell innate immune response, while PSPB may be affecting eLE cell cytoskeleton. Further studies are warranted to understand the relationship between IFNT and PAGs regarding immune cell mediated extracellular matrix remodeling necessary for placental attachment.

Defining the Role of ATG9 in Human Placental Development

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Abstract Text

The placenta is the first organ to develop during pregnancy and functional impairments can lead to pregnancy disorders. Soon after blastocyst implantation, the outer trophoblast layer differentiates into mononucleated cytotrophoblast (CTB) cells. As pregnancy progresses and the placenta develops, these CTBs fuse to become a multinucleated cell layer surrounding placental villi called the syncytiotrophoblast (STB). The STB is in direct contact with the maternal circulation allowing for oxygen, nutrients, and hormones to exchange between mother and fetus. Autophagy is a cellular pathway that clears protein aggregates and damaged organelles in response to stressors and cytosol reprogramming during cell differentiation. For this, the cell uses several autophagy related (ATG) genes to generate a unique organelle, the autophagosome, that fuses with lysosomes to degrade and recycle its components. Vesicles transporting ATG9A, the only transmembrane ATG protein, act as membrane seeds for the autophagosome expansion. ATG9A is ubiquitously expressed while the expression of ATG9B, paralog to ATG9A, is restricted to the placenta. The goal of this project is to delineate the role of ATG9A and ATG9B for human placenta-specific autophagy functions. Firstly, using available single nuclei RNAseq transcriptomic maps from human placenta and trophoblast organoids, ATG9A was found in majority of subtypes while ATG9B expression was found mostly in STB precursor cells. These findings were validated in primary human trophoblasts (PHT), which fuse spontaneously to form STB, BeWo placental cell line, which undergo fusion upon treatment with forskolin (FSK), and human trophoblast cells (TSCs) when differentiated to STBs. In all cases the ATG9B expression was significantly upregulated in fused syncytial cells compared to ATG9A gene expression profiles. *In situ* hybridization on third trimester placentas and BeWo cells showed higher expression of ATG9B transcript in STB nuclei compared to the ubiquitous cytosolic expression of ATG9A. RT-qPCR analysis also showed increased levels of ATG9B transcript after FSK treatment compared to ATG9A. To better dissect the functions of ATG9A and ATG9B in the context of placental cells, using CRISPR, we generated and validated a panel of ATG9A, ATG9B, ATG9A/B DKO cells in BeWo cells. We observed that ATG9A and ATG9B KO cells do not compensate for each other's role in placental cells. ATG9A KO BeWo cells also failed to show autophagic flux, unlike ATG9B KO cells. In terms of STB differentiation capacity, ATG9A KO BeWo cells failed to show cell-cell fusion, with diminished STB fusion markers, unlike ATG9B. To continue with the momentum of our findings and to pinpoint the exact role of ATG9B, bulk RNA-seq/proteomics in the single and double ATG9A/B KO BeWo cells will be conducted to identify possible regulatory pathways. Understanding the mechanisms of how autophagy impairment affects cargo degradation in the placenta will allow us to better understand the pathogenesis of pregnancy disorders associated with faulty development and pave a pathway towards clinical interventions.

Uterine-Trophoblast Interactions

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Abstract Text

The establishment and maintenance of pregnancy require well-orchestrated interactions between the uterus and placenta. Key players in this dialog are uterine decidual cells, which differentiate from stromal cells, and extravillous trophoblast (EVT) cells, which arise from trophoblast progenitor cells. EVT cells migrate into the uterus and transform it into an environment supportive of fetal development. Disruptions in decidualization are linked to abnormalities in EVT cell behavior and pregnancy diseases. Evidence supports the involvement of the uterine environment controlling EVT cell behavior; however, the nature of the communication between decidual cells and EVT cells is not well understood. The objective of this study was to determine potential interactions between decidual cells and EVT cells. Immortalized human uterine stromal cells (ATCC, CRL-4003, PMID: 14726435) were directed to differentiate into decidual cells when exposed to estradiol, medroxyprogesterone acetate, and dibutyryl cAMP. RT-qPCR confirmed induction of *PRL* and *IGFBP1* transcripts and differentiation to the decidual cell state. Bulk RNA-sequencing was performed on stromal cells versus decidual cells and analyzed using DESeq2 package in R. The analysis revealed 6,192 differentially expressed transcripts (3,307 down-regulated in the stromal cell versus decidual cell state and 2,885 up-regulated in the stromal cell versus the decidual cell state). The CellChat package in R was used to search for ligand-receptor pairs between differentially expressed uterine transcripts and transcripts associated with EVT cells derived from human trophoblast stem cells (PMID: 29249463, 37563143). This analysis revealed 108 ligand-receptor pairs between stromal cells and EVT cells and 249 ligand-receptor pairs between decidual cells and EVT cells. All ligand-receptor pairs received one of three bioinformatically determined annotations: secreted signaling, ECM-receptor, or cell-cell contact. Future work will include experimentally testing these ligand-receptor interactions. Supported by GM103418 (SLS), Lalor Foundation (SLS), HD116506 (SLS), NIH-LRP (SLS), HD020676, HD105734, HD112559, and the Donald C. Johnson Research Endowment Fund.

Insights into the Regulation of Placental Phosphate Availability Using a Novel Tissue Non-Specific Alkaline Phosphatase Deficient Sheep Model

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Abstract Text

Phosphate is essential for conceptus (embryo/fetus and associated extraembryonic membranes) development. However, regulation of phosphate availability during gestation is poorly understood. Tissue non-specific alkaline phosphatase (TNSALP), an enzyme that generates inorganic phosphate, is increasingly recognized as important during gestation. TNSALP expression has been reported in utero-placental tissues in several species, suggesting a conserved role in utero-placental phosphate regulation. This study examined the role of TNSALP in fetal and placental development, placental phosphate availability, and angiogenesis utilizing a novel TNSALP deficient sheep model. Control (wild-type; WT) ewes or ewes homozygous (Hom) for a loss of function mutation in ALPL (encoding TNSALP; c.1077 C >G. CRISPR/Cas9 generated) underwent oocyte collection followed by in vitro fertilization using WT or Hom semen. Embryos (WT or TNSALP deficient) were transferred into WT recipient ewes. Ewes were euthanized on day 126 of gestation. Maternal and fetal serum, and placental tissues (placentomes, caruncles, and cotyledons) were collected (n=7 controls, n=8 TNSALP). TNSALP activity and the concentrations of phosphate in serum and tissue homogenates were quantified using spectrophotometric assays. Expression of candidate mRNAs in placental tissues were quantified using quantitative polymerase chain reaction. Fetal TNSALP deficiency did not affect placental weight or length, total number of placentomes, fetal weight, or crown-rump length. TNSALP activity decreased in serum from TNSALP deficient fetuses ($P < 0.05$) compared to controls. There was no effect of fetal TNSALP deficiency on TNSALP activity in maternal serum. TNSALP deficiency did not affect the concentrations of phosphate in fetal and maternal serum, nor did it affect TNSALP activity and concentrations of phosphate in placentomes, caruncles, or cotyledons. Female fetuses had higher TNSALP activity in placentomes ($P < 0.01$), caruncles ($P < 0.05$), and cotyledons ($P = 0.055$) compared to males however, this sex effect was independent of fetal TNSALP deficiency. Fetal sex did not affect the concentrations of phosphate in placentomes, caruncles, or cotyledons. TNSALP activity tended to be higher in caruncles compared to cotyledons ($P = 0.08$) but there was no effect when comparing by fetal sex or genotype status. The concentrations of phosphate were higher in caruncles compared to cotyledons ($P < 0.001$), with greater concentrations of phosphate in caruncles compared to cotyledons in TNSALP deficient animals ($P < 0.05$) and controls ($P = 0.08$). TNSALP deficiency did not alter the expression of ALPL mRNA in placentomes, caruncles, or cotyledons. Similarly, fetal TNSALP deficiency did not alter the expression of XPR1, SLC20A1, and SLC20A2 (phosphate transporters) or VEGFA and HIF1A mRNAs (angiogenic regulators) in placentomes or cotyledons. Expression of SLC20A2 mRNA was greater ($P < 0.05$) and XPR1 mRNA was lower ($P = 0.09$) in caruncles from TNSALP deficient fetuses compared to controls. Expression of VEGFA and HIF1A mRNAs was greater in caruncles from TNSALP deficient fetuses compared to controls ($P < 0.05$). Although TNSALP deficiency reduced TNSALP activity in fetal serum, the concentration of phosphate in the placental tissues was unaffected. Altered expression of phosphate transporters in caruncles of TNSALP deficient fetuses suggests maternal compensation to maintain placental phosphate availability. Increased expression of angiogenic regulators further suggests that TNSALP deficiency alters placental function, potentially enhancing angiogenesis. Sex-specific differences in placental TNSALP activity suggests differential phosphate regulation and warrants further investigation. Collectively, these findings suggest a role for TNSALP in the regulation of placental phosphate availability and function. This project was supported by NIH grants T32GM154124, 1R21HD114076 and start-up funds from Pennsylvania State University.

Inflammation Influences Extracellular Vesicle Release in Trophoblast Stem Cells

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Abstract Text

The peri-implantation period of human pregnancy is a highly vulnerable stage of embryonic development. Impaired implantation and invasion during this window is a significant contributor to early pregnancy loss. In this study, we sought to characterize how inflammation during the peri-implantation period influences the release of extracellular vesicles (EVs). EVs are critical mediators of placental-uterine communication and modulate the maternal immune response, promote immune tolerance and support pregnancy. Importantly, EV release and cargo are shaped by environmental cues, including infection and inflammation. We hypothesized that the concentration and type of inflammatory stimuli dynamically regulate EV quantity, cargo, and function in trophoblast stem cells (TSCs). To test this hypothesis, we compared the effects of inflammation on undifferentiated TSCs and syncytiotrophoblast (STB) cells. Cells were treated with inflammatory cytokines representative of moderate (15 ng/mL TNF α , 20 ng/mL IFN γ), robust (50 ng/mL TNF α , 50 ng/mL IFN γ) or pathologic (250 U/mL IFN β) uterine inflammatory conditions. TSCs were treated for 48 hours after which cells and spent culture medium were collected for cell counting, RT-qPCR, ELISAs, and EV isolation. Cell counts revealed that all three inflammatory conditions significantly ($p < 0.01$) reduced cell proliferation. RT-qPCR demonstrated that all treatments significantly ($p < 0.05$) increased mRNA levels of pro-inflammatory cytokines and interferon stimulated genes (*IFITM1*, *IFITM2*, *IFITM3*, *CXCL10*, *IL6*, *IL8*, *ISG15*, and *ISG20*). ELISAs confirmed that moderate and robust treatments significantly ($p < 0.05$) increased secretion of IL6 and CXCL10 into culture medium. Nanoparticle tracking analysis showed that, after normalization to cell number, IFN β , moderate, and robust treatments significantly increased EV secretion by TSCs. STBs were subsequently exposed to the same inflammatory conditions. RT-qPCR and ELISA analyses indicated significant induction of inflammatory cytokines in all treatment groups. Total cellular protein was modestly reduced in IFN β and robust conditions, albeit not significantly ($p < 0.07$). After normalization to total protein, only the IFN β treatment group significantly ($p < 0.01$) increased EV secretion from STBs, suggesting that only inflammation indicative of a pathologic uterine environment selectively stimulates EV release in STBs. Collectively, these findings demonstrate that placental EV release is dynamically regulated by inflammatory cues and that STB EV release is less responsive to inflammatory stimuli than TSCs. This work provides novel insight into how inflammation shapes placental-uterine communication during implantation. Future studies will focus on characterizing EV cargo and immunomodulatory capacity of EVs isolated from the different inflammatory conditions.

Early Postnatal Estrogen Exposure Induces Hypospadias in Female Mice: Morphological and Molecular Insights

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Abstract Text

Hormone signaling plays an important role in organ morphogenesis and alterations in signaling cascades can cause birth defects. Hypospadias is a birth defect characterized by incomplete closure of the urethra. Hypospadias is mainly reported in males but is most likely underreported in females due to lack of awareness. Female hypospadias causes recurrent urinary tract infections, dyspareunia and secondary infertility. Although clinically relevant, the mechanisms and etiology of hypospadias in females are largely unknown. Previous studies suggest that altered hormone signaling contributes to abnormal urethral development in females. However, the cell populations and genetic pathways involved in abnormal urethra closure were not investigated. In this study we aim to investigate how early life estradiol exposure influences urethra closure in female mice.

To address this, female pups of C57BL/6J mice were administered daily subcutaneous injections of estradiol (8µg/gm body weight) from postnatal days (PND) 1 to 5. Control mice were exposed to vehicle control (ethanol in sesame oil). External genitalia were collected on PND 5, 15, and 40. To determine difference in female external genitalia morphology, we investigated whole mount and histological sections. We found that estradiol-treated pups developed severe hypospadias that started as early as PND5 and persisted through PND40. In all estradiol-treated pups, the urethra opened into the vaginal canal, while the control pups had separate urinary and vaginal canals. In hematoxylin and eosin stains we identified changes in cell population morphology. In controls, the cell population adjacent to the urethra, the peri-urethral mesenchyme, had a migratory phenotype with elongated nuclei, while the estradiol-treated peri-urethra cells were not elongated. Immunostaining showed that the periurethral mesenchyme had the highest ESR1 expression and urethral epithelium had lower but still detectable expressions. When compared with controls, this expression was lower in estrogen-treated pups. To better determine which genital cell populations were most disrupted by estradiol treatment we investigated single cell mRNA data at PND5. Cell populations between the two treatment groups were mostly conserved, however one cell population was present in the estradiol-treated pups and absent in controls. Upon validating this cell population, the estradiol-specific population was part of the peri-urethral mesenchyme, with 5866 differentially expressed genes. The peri-urethra of the estradiol-treated pups exhibited elevated expression of a common estrogen responsive gene, progesterone receptor (*Pgr*), immune-related genes (*Ccl7*, *Ccl11*, *Il6ra*), and retinoic acid metabolism (*Cyp26a1*, *Adh7*) while having lower gene expression in WNT signaling genes (*Wnt11*, *Apc2*, *Rspo2*, *Wnt2b*).

Taken together, these transcriptional alterations suggest that early life exposure to estradiol derailed normal urethral morphogenesis in female mice, likely by altering peri-urethral mesenchyme differentiation and function. These findings will improve our understanding of female urogenital malformations and how early hormonal disruptions cause birth defects.

Keywords: Hypospadias, estrogen, female urethra, morphogenesis.

Three Novel Candidate Imprinted Genes Identified in Early Murine Placenta

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Abstract Text

Past studies have revealed that imprinted expression is dependent on tissue and developmental stage. While 128 imprinted genes have been identified in mice, almost all were identified during or after mid-gestation, well after tissues have been specified. Based on a preliminary analysis of published whole genome DNA methylation datasets from sperm, oocytes, and pre-implantation embryos, we identified 306 potential differentially methylated regions (DMRs), suggesting that some imprinted genes or even domains remain to be identified. We therefore performed total RNA sequencing in reciprocal C57Bl/6J x CAST/EiJ embryos, yolk sacs, and placentas at embryonic day 8.5 (E8.5) to identify novel candidate imprinted genes. Reciprocal crosses enabled imprinted gene expression to be distinguished from strain-specific expression. After removing likely decidual contaminant genes, our analysis identified 28, 31, and 133 genes with parent-specific expression in embryo, yolk sac, and placenta, respectively. These include 27, 28, and 56 genes previously published to be imprinted. By integrating our RNA-seq results with our DMR analysis and published literature, we identified 3 genes in placenta with no prior reports of parent-specific expression and potential gametic DMRs: *Plcl1*, *Ppm1h*, and *Sorbs2*. Notably, additional sequencing data from E9.5, E10.5, E13.5, and E15.5 placenta suggested that the imprinted states of these genes may be temporally constrained. Investigations are ongoing to validate our results. This work was supported by NIH/NICHD 5R01HD109347 and The 25 Club to MRWM; and MWRI Postdoctoral Fellowship to CP.

Cynoglossum zeylanicum Lehm.: A Natural Modulator of Steroid Receptor Activity Linked to VEGF-C Upregulation in Early Gestation in Mice

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Abstract Text

Cynoglossum zeylanicum Lehm., a shrub native to the Eastern Himalayas and traditionally used in folk medicine by tribal communities in Arunachal Pradesh, has been suggested to possess profertile properties. This study investigates the modulatory effect of the crude root bark extract (CRBE) of this plant on female reproductive physiology, particularly during the periimplantation period (D4–D8) of murine gestation. Immunofluorescence, western blot, and qPCR analyses were employed to examine the expression pattern of Vascular Endothelial Growth Factor-C (VEGF-C), a key regulator of endometrial receptivity, decidualization, and angiogenesis. VEGF-C was localized in luminal and glandular epithelium, stromal and decidual cells, as well as trophoblast giant cells, indicating a role in post implantation and placental development. The present study critically evaluates the modulatory effects CRBE on ovarian steroid activity during early pregnancy in mice. When administered orally alongside a commercially available ovarian steroid antagonist with CRBE decidual cell reaction is evidenced with significantly enhanced VEGF-C expression in the luminal epithelium, stromal, and decidual cells. Histological analyses corroborated structural remodeling of the uterus in response to CRBE. Notably, CRBE reversed the inhibitory effects of exogenous steroid antagonists on VEGF-C expression, indicating steroid receptor-mediated modulation. This heightened expression correlated with pronounced decidualization and improved embryo implantation, suggesting that CRBE counteracts the suppressive effects of synthetic steroid antagonists. CRBE appears to stimulate endometrial proliferation and prepare the uterus for embryo reception, indicating its potential to modulate or override the effects of exogenous steroid inhibitors. This highlights the plant's promise as a natural modulator of female reproductive function, especially in the context of disrupted hormonal signaling. Keywords: *Cynoglossum zeylanicum*, decidualization, steroid antagonist, VEGF-C, endometrial proliferation.

Gestational Age-Dependent Changes in Circadian Clock Gene Expression in the Human Placenta

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Abstract Text

Emerging evidence from murine models suggests that the fetoplacental unit harbors an endogenous circadian oscillator, the rhythmicity of which emerges gradually and entrains to maternal endocrine cues. The developmental trajectory of the human placental clock, however, remains largely unexplored. We hypothesized that the expression of core circadian clock genes in the human placenta increases with gestational age, reflecting the gradual emergence of circadian regulatory capacity.

To test this, we examined clock gene expression in gestational-age-stratified human placental samples from our previously reported datasets: GSE309986 (n=22, 7-39 weeks); and GSE203507 (n=10, 30-39 weeks). All pregnancies were uncomplicated except for spontaneous idiopathic preterm births. Validation used paired first trimester (chorionic villus sampling) and term villous tissue samples from GSE247382 (n=23, 10-41 weeks). Clock transcripts queried included core activators (*BMAL1-2*, *CLOCK*, *NPAS2-3*), repressors (*PER1-3*, *CRY1-2*, *TIMELESS*), and auxiliary transcription factors (*NR1D1-2*, *RORA*, *DBP*). Correlation analyses were performed using the psych R package, differential abundance used DESeq2, and linear mixed models were fit using lmerSeq.

In the GSE309986 dataset, *CLOCK*, *BMAL2*, *NPAS2*, *NPAS3*, *NR1D1*, *NR1D2*, *DBP*, and *RORA* were positively correlated with gestational age and the placental maturation marker *ALPP* (PLAP), while *BMAL1* and *TIMELESS* were negatively correlated (all $p < 0.05$). In addition to gestational age, *TIMELESS* expression was also strongly correlated with cell cycle genes including *CDCA7*, *CDC20*, and *CDC25A* ($p < 0.05$). Except for *BMAL1* and *NPAS2*, these transcripts were differentially abundant in models adjusted for sex chromosome complement between early (< 20 weeks) and late gestation ($\text{FDR} < 0.05$). No clock transcripts differed significantly between preterm and term samples in the GSE203507 dataset, although *NR1D1* and *RORA* increased in the preterm vs. term subset of GSE309986 (n=12, 24-39 weeks; $\text{FDR} < 0.05$). In the independent GSE247382 validation dataset, linear mixed models accounting for the paired structure of these samples revealed gestational-age-dependent increases in *BMAL2*, *NPAS2*, *PER1*, *NR1D1*, and *NR1D2*, coupled with a reciprocal decline in *TIMELESS* ($\text{FDR} < 0.05$); these findings were concordant with parallel differential analysis using gestational age as a factor variable.

Our findings indicate that early human placental development is marked by a *TIMELESS*-dominant, cell-division-oriented milieu. With advancing gestation, core clock gene expression rises, most robustly for *BMAL2* (ARNTL2), *NR1D1* (Rev-Erb α), and *NR1D2* (Rev-Erb β), in a pattern consistent with the gradual emergence of canonical circadian regulation described in murine models. Together, these data suggest that the emergence of circadian regulatory capacity in the human placenta occurs as proliferative activity declines, with expression patterns stabilizing in the third trimester. That circadian regulation in the placenta is developmentally acquired rather than constitutive represents a previously underappreciated dimension of placental biology.

CD1a-Driven Th17 Inflammation Disrupts Female Reproductive Cyclicity and LH Regulation in a Novel Mouse Model

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Abstract Text

Women with Type 2 Diabetes (T2D) suffer reproductive dysfunction, yet we lack models to investigate underlying mechanisms. Female mice resist metabolic impairment in conventional diet induced obese (DIO) models, and critically, while human T2D is characterized by Th17 inflammation, mouse models develop Th1 responses. We hypothesized that CD1a, a lipid antigen presenting molecule unique to human Th17 responses, would amplify lipid reactive inflammation and trigger downstream reproductive and metabolic dysfunction. The results reveal a paradigm shift in understanding immune reproductive crosstalk. High fat diet (HFD; 45% kcal) and low-fat diet (LFD) CD1a mice were perpetually arrested in diestrus, unable to cycle regardless of diet. HFD CD1a mice showed 1.5-fold elevated luteinizing hormone (LH) during diestrus versus HFD wild type (WT) controls. Strikingly, even LFD CD1a mice maintained elevated LH. This demonstrates that CD1a-driven Th17 inflammation is sufficient to collapse reproductive cyclicity. In HFD CD1a mice, metabolic dysfunction emerges early and independent of weight. At just 6 weeks on HFD, CD1a females showed markedly elevated fasting glucose versus HFD WT controls, despite identical body weight. This vulnerability persisted through 22 weeks, with CD1a mice developing elevated insulin, HOMA IR, and severe glucose intolerance. By 24 weeks, HFD CD1a mice gained 5 grams more than HFD WT and showed a one-fold increase in fat to lean ratio. Histology revealed immune infiltration in both gonadal adipose and liver tissues. By introducing CD1a mediated lipid presentation, we have unmasked a lipid reactive Th17 pathway that directly regulates reproductive cyclicity and gonadotropin secretion. Our findings establish Th17 inflammation as a driver of reproductive axis disruption and provide a transformative platform for developing therapies for the millions of women worldwide facing reproductive and metabolic complications.

A Novel iPSC-Derived Macrophage System to Model Immune Interactions in Endometriosis

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Abstract Text

Endometriosis is a chronic disease and is defined as the presence of endometrial glands and stroma outside the uterine cavity, predominantly, in the pelvic compartment. Endometriosis is one of the most widely distributed female diseases that affects about 10% (190 million) of reproductive-age women and girls globally. The causes and mechanisms behind endometriosis are not well understood, making treatment and diagnostic options limited. We have established a new model system to study endometriosis, using induced pluripotent stem cells (iPSCs). Peripheral blood mononuclear cells were collected from women without and with diagnosed endometriosis and then reprogrammed to iPSCs. Using these iPSCs, protocols were followed to generate monocytes and macrophages. iPSCs were treated with mTeSR1 media, including monocyte differentiation kit A (BMP4) and kit B (VEGF, SCF and bFGF), for 3 days and 5 days respectively to differentiate into hematopoietics. Then, HSCs were cultured in StemPro 34 media with factors, including Flt-ligand, GM-CSF, and M-CSF, to differentiate to monocytes, which are float cells and CD14 marker positive, and then collected for further differentiation. In the continuous culture, monocytes are reseeded and cultured at RPMI media with M-CSF to generate M0 macrophage. Flow cytometry was performed to identify M0 macrophages, which are CD68 positive, and then M0 macrophages were cultured in the polarization media for one more day to differentiate into M1 macrophages or M2 macrophages. We performed immunostaining to identify the cells using macrophage-specific markers. What we observed was that after one day of treatment, the cells treated with IFN- γ are CD80 and 86 positive, M1 macrophage markers, and the cells treated with IL-4 are CD163 and 206, M2 macrophage markers. All types of macrophages are attached to the bottom of well. In future studies, we will perform transcriptomic analysis of macrophages from iPSCs derived from endometriosis and non-diseased patients to identify differences. Furthermore, coculture endometrial stromal cells and macrophages derived from the same iPSC line, to investigate the interaction between the two tissues. Our new iPSC endometriosis model will allow new discoveries towards better diagnosis and treatment of endometriosis.

Luteal Response to PGF2 α is Altered after Maternal Recognition of Pregnancy in Cattle

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Abstract Text

In cattle, embryonic interferon-tau promotes luteal maintenance during the classical period of maternal recognition of pregnancy (~d15-22) by downregulating uterine secretion of prostaglandin F2 α (PGF2 α). After cessation of embryonic secretion of interferon-tau, the CL is maintained despite upregulation of uterine PGF2 α secretion. Conversely, about 50% of pregnancy losses are associated with inappropriate luteal regression. The mechanisms of luteal maintenance beyond secretion of interferon-tau remains poorly understood. We aimed to investigate the luteal responsiveness to endogenous and exogenous PGF2 α on gestational d50 to gain insight into the mechanisms of luteal maintenance. In experiment 1, pregnant cows on gestational d50 were randomly allocated to receive saline (control; n=4) or oxytocin treatment (n=6). Treatment consisted of administration of 50 IU of oxytocin intramuscularly at Hours 0, 12, and 24. Blood was collected for 120 hours. In experiment 2, pregnant cows on d50 were randomly allocated to receive different doses of exogenous PGF2 α (dinoprost tromethamine): 0 mg (control), 4 mg, 8 mg, 16 mg, or 25 mg (clinical dose to promote luteolysis) (n=6/group). Blood was collected for 72 hours post-treatment. Data were analyzed with mixed linear models on SAS. In experiment 1, plasma PGF2 α metabolite, PGFM, was not different between groups prior to treatment (48.4 ± 3.9 vs. 58.6 ± 12.9 pg/mL; $P=0.4$). After oxytocin treatment, plasma PGFM increased ($P<0.0001$) within 30 minutes, peaked in about 2 hours, and returned to pre-treatment concentrations within 4 hours. In the oxytocin group, maximal post-treatment PGFM was 378 ± 58 pg/mL; i.e., well above natural-occurring luteolytic pulses of PGFM during luteolysis at the end of estrous cycle (~100-200 pg/mL). Nevertheless, none of the cows in the oxytocin treatment group underwent luteolysis (progesterone < 1 ng/mL). Overall, there was no effect of treatment on circulating progesterone ($P>0.2$). In experiment 2, circulating progesterone was not different among groups prior to treatment (6.0 ± 0.4 ng/mL; $P=0.3$). Plasma progesterone was normalized to hour 0; there were significant effect of treatment and treatment by time interaction ($P<0.03$). Specifically, circulating progesterone was unaltered in cows receiving 4 mg of PGF2 α ($P=0.7$) whereas cows receiving 8 mg had a transient decrease in progesterone at one hour post-treatment (26.4% decrease) but returned to pre-treatment concentrations; in both groups, at 72 hours post-treatment progesterone was similar to pre-treatment values (4 mg: 6.8 ± 0.8 vs. 8.0 ± 0.7 and 8 mg: 6.3 ± 1.2 vs. 5.8 ± 1.6 ng/mL; $P>0.3$) and none of the cows had luteolysis. For cows receiving 16 mg of PGF2 α , progesterone decreased 13% within 1 hour of treatment and continued to decrease to 30% by hour 72 ($P=0.04$); 50% of the cows had luteolysis within 48 hours of treatment. For cows receiving 25 mg of PGF2 α , progesterone decreased 16% within 1 hour of treatment and continued to decrease 76% by hour 72 ($P=0.001$); 50% of the cows had luteolysis within 48 hours of treatment. Fetal mortality as indicated by cessation of heartbeat occurred by 72 hours post-treatment in all cows with luteolysis. Taken together, luteal responsiveness to PGF2 α is altered on gestational d50. It appears that an unknown mechanism modulates the luteal response to PGF2 α after the classical period of maternal recognition of pregnancy so that the CL is maintained despite increase in frequency and amplitude of endogenous, natural-occurring pulses of PGF2 α .

Characterization of Embryo-Maternal Communication in PAG1 and PAG7 Knockout Pregnancies in Cattle

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Abstract Text

Bovine maternal recognition of pregnancy is mediated by conceptus-derived products, most notably interferon tau (IFNT), which, in part, prevents luteolysis and stimulates expression of interferon-stimulated genes (ISGs) in maternal tissues. Pregnancy associated glycoproteins (PAGs), which are also secreted by the trophoblast, and have been implicated in conceptus-maternal communication. However, the relationship between PAGs and IFNT signaling or downstream maternal responses during the peri-implantation period remains unclear. We hypothesized that disruption of PAG1 or PAG7 would alter embryo-maternal communication and impact the establishment of pregnancy. Embryos were generated using abattoir derived oocytes, and CRISPR/Cas9-mediated knockout of PAG1 or PAG7. Grade 1, developmental stage 7-8 embryos were fresh transferred into synchronized recipient cows at 8 days post-estrus. Treatment embryos were transferred as follows: control (CTRCas9; $n = 4$), PAG1 knockout (PAG1KO; $n = 7$), and PAG7 knockout (PAG7KO; $n = 8$), along with negative control cows that did not receive an embryo (NoEmbryo; $n = 3$). Cervical cytobrush samples were collected on 14, 17, and 20 days post-estrus. Pregnancy outcome was determined at 28 days post-estrus via transrectal ultrasonography and visualization of embryonic heartbeat. Total RNA was extracted from cytobrushes, and RT-qPCR was performed to quantify expression of *ISG15*, *MX2*, and *OAS1*. Expression of *ISG15* was affected by day ($P = 0.0001$) and pregnancy outcome ($P = 0.0014$), but not embryo treatment ($P = 0.81$). In cows confirmed pregnant by ultrasonography, *ISG15* expression increased from 14 to 17 days post-estrus and remained elevated or further increased until 20 days post-estrus. Pregnant cows exhibited a 7-fold increase ($P = 0.0014$) in *ISG15* expression compared to cows that returned to estrus, consistent with sustained IFNT signaling during conceptus elongation. Similar temporal patterns were observed for *MX2* and *OAS1*; expression of *MX2* was approximately 7-fold higher and *OAS1* 4-fold higher in pregnant cows relative to those that returned to estrus (*MX2*: day, $P = 0.026$; pregnancy, $P < 0.001$; *OAS1*: day, $P = 0.006$; pregnancy, $P < 0.001$). No differences were detected among embryo treatments (CTRCas9, PAG1KO, PAG7KO) for *ISG15*, *MX2*, or *OAS1* ($P > 0.10$), indicating that disruption of PAG1 or PAG7 did not impair induction of ISGs during the maternal recognition window. Pregnancy maintenance by 28 days post-estrus was limited ($n = 3$ total; PAG7KO = 2, PAG1KO = 1), with no confirmed pregnancies from control embryo transfers. Therefore, analyses focused on ISG expression from 14 to 20 days post-estrus, when IFNT signaling is expected to be maximal. Findings indicate that PAG1 and PAG7 are not required for initiation of IFNT-mediated maternal signaling during early pregnancy. Collectively, these data demonstrate that embryos lacking PAG1 or PAG7 retain the capacity to elicit a maternal interferon response, indicating that IFNT signaling is established independently of individual PAGs. Future studies will determine whether the observed knockout phenotype is also reflected by altered PAG secretion in the maternal circulation, providing additional insight into the role of PAGs in embryo-maternal communication.

Dietary Omega-6 Supplementation Alters Blastocyst Lipid Accumulation and Cell Number in Beef Cattle

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Abstract Text

Previous research has demonstrated that dietary supplementation with omega-6 fatty acids increases the concentrations of omega-6 within plasma, the endometrium, and conceptus. Furthermore, beef cows supplemented with omega-6 fatty acids have improved pregnancy rates to artificial insemination compared to controls. However, it is presently unknown whether dietary supplementation with omega-6 fatty acids can influence the developmental competence for the pool of ovarian follicles. The objective of this experiment was to assess the influence of dietary fats on follicular fluid composition and *in vitro* embryo development. We hypothesized that dietary supplementation of omega-6 fatty acids would modulate follicular fluid composition and improve cumulus oocyte complex (COC) development to blastocyst stage following *in vitro* fertilization. Angus-influenced beef cows ($n = 33$) were provided a 40-day adaptation period. Dietary treatments were initiated at fixed-time artificial insemination (day 0) and continued until slaughter (day 22). Dietary treatments included either omega-6 (CSSO) or control (CON) at two doses (100 or 250 g/day). Omega-6 was supplemented via soybean oil in the form of calcium salts (100CSSO, $n = 8$; 250CSSO, $n = 8$). Control was supplemented as an iso-lipidic, isocaloric mix of saturated and monounsaturated fats (100CON, $n = 9$; 250CON, $n = 8$). Ovaries were obtained and transported to the lab in warmed (38°C) phosphate-buffered saline within one hour after slaughter. Visible antral follicles were counted and classified as small (< 5 mm), medium (5–8 mm), or large (> 8 mm). Follicular fluid was aspirated and ovaries were slashed with a sterile scalpel blade. All viable COCs were collected ($n = 1,317$) and subjected to standard *in vitro*-maturation (IVM; day -1), fertilization (day 0), and culture (days 1–7) protocols. Recovered follicular fluid and spent IVM (sIVM) medium were analyzed for glucose concentrations and subjected to liquid chromatography-mass spectrometry for untargeted metabolite analysis. Day-7 blastocysts were fixed in 4% paraformaldehyde and stained with 1 µg/mL Nile Red and 1 µg/mL Hoechst 33342. ImageJ software was used to quantify mean fluorescence intensity of lipid accumulation and total cell number. There was no effect ($P > 0.10$) of dietary treatment on the number of recovered COCs, cleavage rate, blastocyst development rate, concentration of glucose in follicular fluid or sIVM, or antral follicle count. Lipid accumulation and total cell number were greatest in the 100CSSO compared to all other treatments ($P < 0.05$). There was no difference in lipid accumulation or total cell number between 100CON or 250CON ($P > 0.10$). Total metabolite abundance was greater in follicular fluid than sIVM ($P = 0.0001$). Both 100CSSO and 250CSSO had reduced metabolite abundance in sIVM compared to CON treatments ($P < 0.05$). Collectively, these data indicate that short-term (22-day) omega-6 dietary supplementation with 100 g/day may increase cell number and lipid accumulation of *in vitro* produced blastocysts. While a greater cell number indicates improved embryo quality, the implications of increased lipid accumulation remains inconsistent in literature. Further studies are warranted to better understand how dietary lipid supplementation influences oocyte, and subsequent embryo quality and metabolism prior to *in vitro* embryo production.

PKA Signaling Upregulates Acetyl-CoA Producing Enzymes to Enhance Protein Acetylation and Steroidogenesis in Differentiating Bovine Granulosa Cells

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Abstract Text

Progesterone (P4) is a steroid hormone synthesized by the corpus luteum (CL), an endocrine gland formed after ovulation from remnants of the ruptured ovarian follicle (OF). Luteinizing Hormone (LH)/Protein Kinase A (PKA) signaling pathway triggers the transcriptional and metabolic reprogramming required for CL formation. Acetyl-CoA (AcCoA) is a lipogenic precursor that serves as an acetyl group donor for histone acetylation and is considered an essential epigenetic regulator. AcCoA can be generated from citrate produced in the tricarboxylic acid (TCA) cycle by citrate synthase (CS), followed by citrate hydrolysis via cytoplasmic ATP-citrate lyase (ACLY). In addition, AcCoA synthetase short-chain family member 2 (ACSS2) can supply cytosolic AcCoA from acetate. Herein, we hypothesize that PKA signaling facilitates granulosa cell (GC) differentiation into a luteal phenotype by metabolic reprogramming that upregulates AcCoA-producing pathways, thereby enhancing nuclear protein acetylation required for chromatin remodeling and further gene expression. Bovine GC and luteal cells (n=3-6 per group) were isolated from ovarian follicles (OFs) measuring 2-5 mm and 6-9 mm in diameter, and from mid-luteal phase CL, respectively, and lysed for subsequent western blot analysis. In addition, bovine GC isolated from OFs measuring 2-5 mm in diameter (n=4) were treated with PKA activator (Forskolin; FSK; 10 μ M) in the presence or absence of BMS-303141 (25 μ M, ACLY inhibitor) for 24 h. Next, GC were subjected to further immunofluorescence and western blotting analysis. Media samples were collected to assess progesterone synthesis under experimental conditions. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test or Student's t-test. The content of CS, ACLY, and ACSS2 progressively increased ($P < 0.05$) from GC to luteal cells, suggesting enhanced AcCoA availability during GC differentiation toward the luteal phenotype. Alongside this, elevated levels of steroidogenic enzymes such as CYP11A1 (Cytochrome P450 Family 11 Subfamily A Member 1), StAR (Steroidogenic Acute Regulatory Protein), and 3 β HSD (3 β -Hydroxysteroid Dehydrogenase), as well as decreased expression of CYP19A1 (Cytochrome P450 Family 19 Subfamily A Member 1), were observed ($P < 0.05$), indicating a transition from estradiol to P4 synthesis in luteal cells. Treatment with PKA activator enhanced ($P < 0.05$) P4 secretion, content of ACLY, acetylated histone H3 (lysine 9 and 27; H3K9 and H3K27), and steroidogenic proteins in GC. Immunofluorescence analysis further demonstrated elevated cytoplasmic and nuclear localization of ACLY in FSK-treated GC. ACLY inhibition using BMS significantly decreased ($P < 0.05$) basal and FSK-induced content of acetylated proteins, including acetylated H3K27, as well as content of steroidogenic protein StAR and CYP11A1. A drop in protein acetylation was less prominent in the bovine GC cultured in the presence of FSK and BMS in comparison to BMS treatment alone, suggesting that PKA signaling may drive alternative metabolic pathways to supply acetyl groups for protein acetylation, independent of ACLY activity. Inhibition of ACLY effectively ($P < 0.05$) attenuated basal and FSK-stimulated progesterone synthesis by the bovine GC. These results collectively demonstrate that PKA signaling upregulates ACLY-mediated AcCoA supply to coordinate both nuclear and cytoplasmic proteins acetylation, which is essential for the steroidogenic function of differentiating GC. The partial rescue by FSK under ACLY inhibition further highlights the metabolic flexibility of differentiating GC, warranting further investigation into alternative AcCoA-producing pathways.

Primary Ovarian Granulosa Cell Response to *Arid1a* Loss and *Kras*^{G12D} Gain

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Abstract Text

Cancer-driving mutations in AT-rich interaction domain 1A (ARID1A) and Kirsten rat sarcoma viral oncogene homolog (KRAS) are frequent in endometriosis. Ovarian endometriosis develops in the *Arid1a*^{flox/flox}; *Kras*^{lox-stop-lox-G12D}; *Amhr2*^{Cre/+} (AKA) mouse model. Cre-mediated deletion primarily occurs in ovarian granulosa cells, which are crucial for ovarian folliculogenesis. While ARID1A loss has been extensively studied in epithelial tumor cells, its role in granulosa cells remains poorly defined, particularly within the context of oncogenic *Kras*^{G12D}. The objective is to investigate the expression of ovarian folliculogenesis and cell cycle genes in primary mouse granulosa cells upon deletion of *Arid1a* and gain of *Kras*^{G12D} in vitro. Mice underwent ovarian superstimulation, dissection, and granulosa cell isolation. Primary granulosa cells were treated with 15 U of TAT-Cre recombinase or vehicle for 1 h. After 48h, recombination was confirmed by PCR. Cells were examined for ARID1A, KRAS^{G12D}, and cyclin-dependent kinase inhibitor-2A (CDKN2A) by immunofluorescence. Ovarian folliculogenesis and cell cycle genes were evaluated by qPCR. Successful recombination of *Arid1a* and *Kras*^{G12D} was confirmed. Granulosa cells with deletion of ARID1A and gain of KRAS^{G12D} showed significant dysregulation of genes important in ovarian folliculogenesis, including growth differentiation factor-9 (*Gdf9*), 1.8-fold decrease ($p < 0.05$; unpaired, one-sided *t* test; $n \geq 3$); follicle-stimulating hormone receptor (*Fshr*), 6.1-fold decrease ($p < 0.05$); luteinizing hormone/choriogonadotropin receptor (*Lhcgr*), 11.6-fold decrease ($p < 0.0001$); and inhibin subunit beta-A (*Inhba*), 2.7-fold decrease ($p < 0.05$). Additionally, the cell cycle regulator gene *Ccna2* (Cyclin A2) was downregulated 2.3-fold ($p < 0.01$; unpaired, one-sided *t* test; $n = 6$). Notably, the senescence marker *Cdkn2a* (cyclin-dependent kinase inhibitor-2A or p16) showed a 15-fold increased expression following TAT-Cre treatment (nonparametric, one-sided Mann-Whitney test, $p = 0.057$, $n \geq 3$). This was confirmed by CDKN2A immunofluorescence. TAT-Cre-mediated recombination in primary ovarian granulosa cells likely promotes oncogene-induced senescence, a well-described characteristic of oncogenic KRAS^{G12D}. Increased expression of *Cdkn2a* likely leads to cell cycle arrest, leading to loss of proliferation. The downregulation of *Fshr* is the suspected primary event affecting downstream expression of *Lhcgr* and *Inhba*. Future studies will focus on the mechanism of independent loss of ARID1A or gain of KRAS^{G12D} in granulosa cells. This project is funded by R01HD109707.

Integration of Splicing Architecture, mRNA Modifications, and Poly(A) Dynamics in the Aging Ovary

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Abstract Text

Female reproductive aging is characterized by a progressive decline in ovarian function, but the isoform-level regulatory mechanisms underlying this decline remain poorly defined. Post-transcriptional processes, including alternative splicing, mRNA modifications, and poly(A) tail regulation, can alter mRNA isoform diversity, stability, and translation efficiency, and may be especially important in the hormonally dynamic ovary, but how they are coordinated remains unknown. We previously identified widespread dysregulation of alternative splicing in reproductively aged (14-month) versus young (6-week) C57BL/6 mouse ovaries using nanopore direct RNA-sequencing and proteomics. Here, we extend that work by integrating nanopore-derived RNA modification calls (m6A, m1A, ac4C) and poly(A) tail measurements with age-associated splicing changes in ovaries following gonadotropin stimulation. RNA modification patterns were reproducible and correlated with splicing event type.

We asked three biological questions. First, does aging shift global RNA modification composition? In the aged ovary, m1A increased and ac4C decreased in both contexts. Following PMSG, m1A increased by +16.4% (0.627 vs 0.539, $q=5.54e-72$), ac4C decreased by -30.7% (0.212 vs 0.306, $q=4.25e-103$), and m6A changed by +3.7% (0.161 vs 0.155, $q=0.120$; not significant). Following PMSG+hCG, m1A increased by +1.9% (0.490 vs 0.481, $q=6.35e-04$), ac4C decreased by -5.1% (0.323 vs 0.341, $q=7.02e-12$), and m6A increased by +4.7% (0.187 vs 0.179, $q=5.33e-05$). Together, these results show consistent age-associated shifts in m1A and ac4C, while m6A changes were modest and more apparent after PMSG+hCG, with larger overall effect sizes in the PMSG condition.

Second, does aging change how RNA modifications are distributed across different alternative splicing event classes? At alternative transcript-end events, m1A was enriched relative to non-alternative-end events (+29.9% following PMSG, $q=1.17e-21$; +12.8% following PMSG+hCG, $q=9.63e-17$), while ac4C and m6A were lower (ac4C: -9.8% and -9.7%, $q=1.20e-05$ and $q=8.10e-06$; m6A: -44.4% and -29.3%, $q=1.50e-17$ and $q=8.84e-12$). At alternative 3' splice-site events, m1A was consistently lower than non-alternative-3' events (-28.2% following PMSG, $q=9.05e-12$; -22.6% following PMSG+hCG, $q=2.26e-22$), while ac4C and m6A were higher (ac4C: +17.0% and +28.6%, $q=1.29e-05$ and $q=3.58e-13$; m6A: +45.5% and +41.6%, $q=2.64e-04$ and $q=8.10e-06$). This shows a shared aging-linked redistribution of RNA modifications across alternative splicing event classes rather than random mixing.

Third, do poly(A) tail dynamics change with age in the same way across stimulation conditions? Poly(A) tail dynamics were context-specific: with aging, median tail length increased by +12.38 nt following PMSG ($p<1e-300$), but decreased by -0.93 nt following PMSG+hCG ($p=3.36e-02$, $q=7.85e-02$). These results support a multi-layer model in which RNA modification state and splice-event localization show strong shared aging signals, while poly(A) tail dynamics are more context-dependent.

Together, these results identify hormone-context-specific coordination between splicing state, RNA modification architecture, and poly(A) regulation in the aging ovary, highlighting post-transcriptional regulation as a major axis of female reproductive aging. By integrating multiple types of post-transcriptional regulatory features, this model will provide a more comprehensive understanding of the aging ovarian transcriptome.

Aging Alters FSH-Responsive Chromatin Accessibility and Transcriptional Control in Granulosa Cells

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Abstract Text

Age-related decline in female fertility signifies a pressing clinical challenge, driven by progressive disturbance of ovarian regulatory frameworks. Despite the central role of follicle-stimulating hormone (FSH) in orchestrating granulosa cell (GC) proliferation, differentiation, and steroidogenesis, the molecular foundation for age-dependent shifts in FSH-responsiveness remains unclear. FSH signals fundamentally through the cAMP-PKA-CREB axis, where phosphorylated CREB stimulates transcriptional events crucial for ovulation and oocyte competence. Hence, understanding how ovarian responsiveness to FSH changes with aging is crucial for detecting mechanisms that trigger reduced fertility and enhance reproductive health.

To explore this, we implemented integrative epigenomic and transcriptomic characterization of acute FSH responses in granulosa cells from young (2-month) and aged (10-month) mice. Diestrus-stage mice received a single intraperitoneal dosage of FSH (1 µg) for 2 hours, beside age-matched controls. For young mice, GCs were examined with parallel ATAC-seq and RNA-seq to identify FSH-responsive regulatory dynamics and gene expression profiles. ATAC-seq was implemented in GCs of aged mice to elucidate age-dependent distinctions in chromatin accessibility and regulatory competence.

In young mice, FSH prompted a robust transcriptional response manifested by prompt induction of immediate-early genes, involving *Ereg*, *Areg*, *Creb3l1*, *Ptgs2*, *Nr4a1* ($P_{adj} < 0.05$, $\log_2FC \geq 1.5$), aligned with follicular maturation and ovulatory competence. Upstream regulator analysis recognized CREB and cAMP as key mediators of FSH-responsive cascades. Chromatin accessibility exploration uncovered strong enrichment of AP-1 (FOS/JUN) and CREB motifs ($p < 0.001$), reinforcing canonical cAMP-facilitated chromatin remodeling. Pathway analyses emphasized synchronized involvement of gonadotropin-releasing hormone, VEGF, and dopamine-DARPP32 cAMP feedback loops, indicating endocrine and vascular integration during follicle stimulation. Combined ATAC-seq/RNA-seq interpretations uncovered enhanced accessibility at CREB-motif-containing regulatory domains accompanied by consistent upregulation of *Pde4d* and *Tnfrsf6*, supporting heightened FSH-responsiveness and ovulatory competence.

Differential footprinting analysis of GCs from older mice reflected significantly higher binding activity of EGR1/3, KLF10/12, SP1 ($p < 0.001$) in FSH-treated versus control, contemplating age-dependent reprogramming of chromatin accessibility. *In support of this data*, FSH-responsive loci were enriched for pathways governing signaling pathways, cytoskeletal remodeling, and chromatin/histone modification rather than proliferative outputs, highlighting an age-dependent transition toward adaptive and maintenance programs.

Prompted by our interest in delineating the integrated effects of FSH and aging, we performed paired comparisons of age-matched controls (2 versus 10 months) and FSH-treated groups (2 versus 10 months). GCs from the ovaries of young control mice were enriched for steroid receptor motifs (AR, NR3C1/2) ($p < 0.001$), whereas GCs from the ovaries of older control mice exhibited CTCF and ETS (ELK, FEV) ($p < 0.001$) accessibility, signifying a transition from hormone-mediated transcription to genome-architectural and signaling-adaptive regulation. In response to FSH, GCs from young ovaries triggered AP-1 and steroidogenic regulators (FOS/JUN, NR5A2, GATA2, AR) ($p < 0.001$), whereas chromatin accessibility in GCs from older ovaries oriented toward NRF1, EGR1/2/3, CTCF ($p < 0.001$) networks, prioritizing early-response and chromatin architectural programs over steroidogenic stimulation.

Collectively, these findings exhibit that ovarian FSH-responsiveness is considerably remodeled with aging. GCs from young mice elicit canonical CREB-mediated proliferative and ovulatory processes, whereas GCs from aged mice transition toward adaptive and structural regulatory frameworks. This comprehensive atlas of acute FSH signaling across the reproductive lifespan determines the molecular logic underlying reduced ovarian competence and discovers potential targets to enhance ovarian function and assisted reproductive outcomes.

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Discovery of a novel *Cdc14b* transcript in mouse oocytes

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Abstract Text

Research into CDC14B's function in female fertility is limited, despite knockout models having female specific subfertility. Notably, previously published studies have observed that mouse oocytes and pre-implantation embryos have a unique CDC14B localization, suggesting an oocyte specific function. Canonically, CDC14B is sequestered in the nucleolus during interphase. CDC14B is never observed in the nucleolus or even enriched in the nucleus of prophase I oocytes up to morula embryos. Mice have two confirmed *Cdc14b* transcript variants, canonical *Cdc14b1* and non-canonical *Cdc14b2*. Both transcript isoforms contain the catalytic site but differ significantly in regions that regulate protein localization, specifically *Cdc14b1* contains a nucleolus localization signal (NLS) and *Cdc14b2* does not. We, therefore, hypothesized that mouse oocytes did not express canonical *Cdc14b1* which contains the NLS. Initial studies explored transcriptomic data to answer this question. Preliminary studies aligned *Cdc14b1* and *Cdc14b2* transcripts via EMBOSS Needle Pairwise alignment and found perfect sequence alignment from the exon 2 coding region to the end of the transcript, including the 3' UTR. Conversely, the 5' UTR and exon 1 regions showed no alignment. This suggests alternative transcription start sites as the cause for this transcript difference rather than alternate splicing. In confirmation of this the *Cdc14b* gene had two ENCODE Registry of candidate cis-Regulatory Elements annotated promoter-like signatures immediately upstream of *Cdc14b1*'s exon 1 and *Cdc14b2*'s exon 1 (hereafter called Alt-exon 1). We next sought to investigate which *Cdc14b* transcript was expressed in mouse oocytes. Four publicly available mouse oocytes transcriptomes were aligned to the mouse genome. No read counts were observed for *Cdc14b1* exon 1. Conversely a modest number of reads >13 aligned to Alt-exon 1 of *Cdc14b2*. Notably, a previously annotated intronic region between exon 1 and Alt-exon 1 was observed with read counts of >14, suggesting the presence of an unknown exon. Sashimi plot visualization revealed that Alt-exon 1 and this novel exon both spliced to exon 2. Importantly, the unannotated exon region was in the same reading frame as exon 2 and observed in all transcriptome data sets. Furthermore, an NCBI blast search for this putative exon found no validated transcripts. Taken together this evidence points towards a novel unannotated *Cdc14b* exon only utilized in oocytes, hereafter called n-exon 1. A series of RT-PCR experiments confirmed tissue expression (liver, ovary, uterus, lungs and kidney) of *Cdc14b1* and *Cdc14b2*. Conversely, RT-PCR found *Cdc14b2* transcripts, but not *Cdc14b1* transcripts in mouse oocytes. Continuing studies are investigating the expression pattern of our novel *Cdc14b* transcript in oocytes and other tissues. Ultimately, our investigation discovered an oocyte specific transcript pattern, including a novel *Cdc14b* transcript, that clearly point to a specialized role for CDC14B in mammalian oocyte meiosis which requires follow-up studies.

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Extracellular Matrix Stiffness and Nuclear Confinement Drive Granulosa Cell Morphological Shifts and Enhance Estradiol Production

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Abstract Text

Cancer therapies such as chemotherapy and radiation have transformed childhood cancer into a survivable disease, but these life-saving treatments are profoundly gonadotoxic. Nearly 60% of female survivors develop premature ovarian insufficiency (POI), resulting in infertility and early loss of ovarian hormones vital for cardiovascular, skeletal, and neurological health. These deficiencies shorten both quality of life and life expectancy. Current hormone replacement options are static and fail to replicate the dynamic, multi-hormonal output of the ovary, highlighting the urgent need for innovative approaches to restore fertility and endocrine function.

Granulosa cells (GCs), the key hormone-producing cells of the ovary, are central to this challenge. GCs convert androgens to estradiol and secrete additional endocrine factors that support systemic health. Importantly, GCs are morphologically and functionally dynamic: squamous and quiescent in primordial follicles, cuboidal and proliferative in growing follicles. These shape transitions across folliculogenesis coincide with dramatic changes in gene expression and steroidogenic output. Emerging discoveries in physical genomics establish nuclear shape as a regulator of chromatin organization and gene expression, raising the possibility that GC morphological transitions actively drive, rather than simply reflect, follicle activation and steroidogenic output. Here, we investigated whether these physical transitions regulate follicle activation and GC steroidogenic function.

In the ovary, GC shape is governed in part by the surrounding extracellular matrix (ECM). In bovine ovaries, the collagen-rich cortex, which contains quiescent primordial follicles, is approximately 8-fold stiffer than the medulla, where growing follicles reside. Our laboratory has demonstrated that tissue regions surrounding non-growing follicles are significantly stiffer than those surrounding growing follicles in human ovaries, implicating ECM-mediated mechanical constraint as a potential regulator of follicle dormancy, though a causal relationship remains to be established. To directly test whether mechanical constraint regulates follicle activation, we performed partial enzymatic digestion of bovine cortical ECM, which significantly reduced collagen content as validated by picrosirius red staining. BrdU incorporation analysis revealed that ECM disruption increased the proportion of BrdU+ GCs across primordial and transitional follicle stages compared to PBS controls at 48 hours, suggesting that disruption of mechanical constraint is sufficient to drive GC entry into S-phase as an early marker of follicle activation.

Our preliminary studies demonstrate that GC nuclear morphology shifts systematically across stages of human follicle development, with nuclear eccentricity decreasing from 0.90 ± 0.00008 in primordial follicles to 0.72 ± 0.0098 in primary follicles ($p < 0.0001$), consistent with the progressive morphological changes accompanying follicle activation. We therefore asked whether imposing nuclear deformation alone is sufficient to enhance GC steroidogenic output, independent of the signals that normally accompany follicle activation. Primary bovine GCs cultured on engineered micropillar substrates exhibited measurable nuclear deformation and secreted approximately 3.5-fold more estradiol than those grown on flat substrates under identical conditions, suggesting that nuclear-level physical constraint is sufficient to enhance steroidogenic activity.

Ongoing studies employing FISH, ATAC-seq, scRNA-seq, and ChromSTEM imaging will define how physical confinement reshapes chromatin architecture and transcriptional programs at steroidogenic loci. By integrating mechanical, biochemical, and genomic analyses, this work will establish a novel framework for understanding and manipulating GC fate and function, generating foundational knowledge to inform next-generation fertility and hormone replacement technologies for pediatric cancer survivors and others at risk for POI.

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Acute Activation of the FSH Receptor is Detectable at All Stages of Follicle Growth in Mice

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Abstract Text

When mammalian ovarian follicles reach the late preantral/early antral stage, they become dependent on follicle-stimulating hormone receptor (FSHR) signaling for further growth to the preovulatory stage. Interestingly, expression of FSHR mRNA has been reported in follicles well before the preantral stage, and isolated follicles as small as the secondary stage (~100 μm diameter) were reported to exhibit FSH-induced growth over 24 hours, suggesting early expression of FSHR protein. However, acute activation of functional FSH receptors in small follicles has not been conclusively demonstrated or quantified. To address this, we isolated individual follicles ranging from the small primary stage (~40-60 μm diameter) to the late antral stage (~300-330 μm diameter) from mice that express the cAMPFIRE-M fluorescent sensor for cAMP, the dominant second messenger involved in FSHR signaling. Follicles were plated on organotypic membranes over a channel of medium and imaged on a confocal microscope within a stage-top incubator that maintained consistent levels of CO₂, temperature, and humidity. After a 10-minute baseline recording period, follicles were exposed to a physiological dose of 0.3 nM ovine FSH for 1 hour, followed by 1 nM FSH for 1 hour. We found that even the smallest isolated follicles (~40-60 μm diameter) responded dose-dependently to FSH with an increase in granulosa cell cAMP levels, peaking between 10-15 minutes after FSH ($n = 11$). In secondary and small preantral follicles up to ~125 μm in diameter ($n = 67$), responses to both concentrations of FSH were similar to those of primary follicles, suggesting that FSHR expression is stable during this growth period. FSH responsiveness increased dramatically in larger follicles, suggesting an upregulation of FSHR expression that may reflect the transition to FSH dependence. Follicles ranging from ~180-330 μm in diameter (large preantral to large antral) exhibited similar response profiles to both 0.3 nM and 1 nM FSH, with a tendency for larger responses in larger follicles ($n = 27$). The roles and functions of FSH signaling in follicles before the preantral stage have not yet been determined. Although FSH is not required for growth in these follicles, there is evidence that FSH supplementation can augment growth in isolated follicles from the secondary stage. Furthermore, our data demonstrate that FSH signaling occurs in the granulosa cells of follicles as early as the primary stage. Supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (R03 HD104879 to J.R.E.).

PP1 β exhibits dynamic and changing localization throughout mouse oocyte meiosis

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Abstract Text

Protein phosphatase 1 (PP1) is a serine/threonine phosphatase that is responsible for approximately half of the dephosphorylation events in mammalian cells. In mouse oocyte meiosis, PP1 is essential for accurate M-Phase re-entry, progression, and exit. PP1 has three catalytic subunits in mammals: PP1 α , PP1 β , and PP1 γ . PP1 β is the only isoform that is essential for survival in mice, whereas PP1 α and PP1 γ knockout mice are viable, suggesting that PP1 β has unique roles that cannot be compensated by the other isoforms. The subcellular localization of PP1 β during oocyte meiosis is largely unknown. Therefore, the aim of this study is to determine PP1 β localization at distinct meiotic stages (prophase I arrest (pro I), nuclear envelope breakdown (NEBD; 3 h post-meiotic arrest release), and metaphase I (met I; 8 h post-meiotic arrest release). PP1 β localization was assessed in mouse oocytes using indirect immunofluorescence followed by confocal imaging and quantitative image analysis. Specific regions of interest (ROIs) were selected based on the meiotic stage, and the fluorescence intensity of these regions were measured. For pro I oocytes, the ROIs were the nucleus and cytoplasm, whereas for NEBD and met I, the chromosomes and cytoplasmic regions were measured. For each oocyte, a nucleus/cytoplasmic ratio (pro I) or a chromosome/cytoplasmic ratio (NEBD and met I) was calculated by dividing the normalized fluorescence of the nucleus or chromosomes by the normalized fluorescence of the cytoplasm. A ratio of >1.2 was defined as nuclear or chromosomal enriched; a ratio of 0.8 to 1.2 was defined as evenly distributed, and a ratio of <0.8 was defined as cytoplasmic enriched. For statistical analysis, the Fisher's exact test was performed with a p-value of $p \leq 0.05$ considered statistically significant. At pro I, PP1 β displayed three distinct localization patterns: nuclear enriched (52.7%), nuclear excluded (4.3%), and an even distribution between the nucleus and cytoplasm (43.0%). Pro I oocytes were then divided into two categories based on DNA morphology: non-surrounded nucleolus (NSN; meiotically incompetent) and surrounded nucleolus (SN; meiotically competent). Chromatin configuration at pro I is an indication of meiotic competence, as SN oocytes are more likely to resume meiosis and support embryo development than NSN oocytes. PP1 β exhibited significant nuclear enrichment in NSN oocytes compared to SN oocytes (NSN: 52.9% vs. SN: 13.9%; $p=0.0032$). In contrast, PP1 β even distribution was observed more often in SN oocytes (SN: 19.4% vs NSN: 8.3% $p=0.0262$). Following NEBD, 28.6% of oocytes exhibited PP1 β enrichment on the chromosomes and 71.4% of oocytes exhibited even distribution between chromosomes and the cytoplasm. Finally, at met I, PP1 β exhibited an even localization between the cytoplasm and the chromosomes (100% of oocytes). Taken together, these results point to PP1 β localization at pro I as a potential indicator of meiotic competence. Additionally, PP1 β appears to be transiently associated with the chromosomes in a subset of NEBD oocytes but largely has no clear enrichment at NEBD and met I. Future research aims to elucidate PP1 β localization at later stages of oocyte meiosis including anaphase/telophase I and metaphase II.

A Novel Function of Bucky Ball in Regulating Oocyte-Specific Alternative Splicing in Zebrafish

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Abstract Text

In most metazoans, transcription is largely silenced during the oocyte-to-embryo transition (OET) and early embryogenesis. Consequently, early embryonic development initially relies on maternal RNAs and proteins synthesized and stored during oogenesis. Although proper transcriptional regulation during oogenesis is known to be essential for the initiation of embryonic development, relatively few studies have investigated the mechanisms that govern oocyte-specific transcriptional regulation. The Bucky Ball (Buc) protein plays a key role in the formation of biomolecular condensates such as Balbiani body and germ plasm in zebrafish oocytes. In *Buc*^{-/-} oocytes, the animal-vegetal polarity is disrupted. Embryos derived from the *Buc*^{-/-} oocytes fail to form the blastodisc and die shortly after fertilization. However, the mechanisms by which *Buc* deficiency leads to these defects remain unclear. To investigate the dynamic interactions between maternal RNAs and protein condensates during the OET, we developed a fractionation protocol to separate relatively free RNAs from RNAs associated with protein condensates. Fully grown oocytes and unactivated eggs from wild-type and *Buc*-null (*Buc*^{-/-}) female zebrafish were fractionated into soluble fractions (enriched for free RNAs) and insoluble fractions (enriched for condensate-associated maternal RNAs), followed by RNA sequencing. We analyzed the sequencing data by calculating the “insolubility” of maternal RNAs, defined as the percentage of each transcript present in the insoluble fraction. We found that in *Buc*^{-/-} oocytes, the insolubility of snRNAs was significantly reduced, indicative of defective snRNP assembly. Consistently, paired-end RNA-seq of stage II and fully grown oocytes revealed an increase in aberrant alternative splicing events in *Buc*^{-/-} mutants. To determine whether these defects result from the loss of Balbiani body in *Buc*^{-/-} oocytes, we performed fractionation RNA-seq in *macf1*-null (*macf1*^{-/-}) mutants, in which the Balbiani body fails to properly disassemble. In contrast to *Buc*^{-/-} mutants, snRNA insolubility and alternative splicing remained normal in *macf1*^{-/-} oocytes, suggesting that *Buc* regulates snRNP assembly independently of the Balbiani body. Consistent with this interpretation, we found that snRNAs do not colocalize with the Balbiani body. Instead, Cajal bodies, nuclear condensates responsible for snRNA maturation and snRNP assembly, exhibit structural defects in *Buc*^{-/-} oocytes. Together, these findings suggest that *Buc* plays a novel role in regulating snRNA maturation and snRNP assembly. By identifying a *Buc*-dependent regulatory mechanism crucial for alternative splicing during oogenesis, our work provides new insight into oocyte-specific transcriptional regulation.

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.

High-Resolution Multiscale Mapping of The Mouse Ovary Reveals Mechanical, Structural, and Functional Differences Across Age and Between Contralateral Ovaries.

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Abstract Text

All tissues contain multiple cell types that are organized in compartments and have unique biochemical and mechanical properties. Within the ovary, primordial follicles reside in a quiescent state in the cortex and develop largely in the interior part of the ovary, termed the medulla. The prevailing model suggests that the stiffer ovarian cortex helps maintaining primordial follicle dormancy, whereas the softer medulla supports follicle growth and expansion. Moreover, our work and that of others has shown that ovarian stiffness increases with age in both mice and humans, implicating mechanical properties in age-related fertility decline. However, it remains unclear whether the ovary exhibits spatial mechanical heterogeneity that influences function, and whether these mechanical differences are preserved with aging. To address these questions, we developed a multiscale, high-resolution analysis method based on three steps: 1) Piuma Nanoindenter (probe 0.44N/m, 27mm diameter; stiffness-*Young's Modulus*) to make measurements 200mm apart across the live isolated ovary. 2) Optical Coherence Tomography (OCT) to live-imaging ovarian 3D structure. 3) Histologic analysis on fixed tissues to identify cell types and quantify extracellular matrix components (ECM). By maintaining ovarian orientation throughout these steps, we can superimpose the mechanical, structural, cell type and ECM data. Using our system, we investigated how ovarian mechanical properties change along an aging *continuum* (6-12 weeks, 7-9 months, >15 months; N=10/age group). As previously shown, ovaries become stiffer with age (6-12w=1.30±0.4kPa, >15mo=4.57±1.8kPa; p< 0.0001). This increase was progressive, as mid-age ovaries showed *Young's Modulus* of 2.70±1.5kPa. Surprisingly, when comparing contralateral ovaries, we found that the left ovary (OvL) was 2.5 times stiffer than the right ovary (OvR) in reproductively old mice (OvR 3.44±1.23kPa, OvL 5.69±1.7kPa, p=0.007), but this was not observed in young (OvR 1.33±0.5kPa, OvL 1.26±0.4kPa), and a tendency in differences starts in mid-age mice (OvR 2.02±0.9kPa, OvL 3.39±1.7kPa). OCT analysis revealed that aging increased 3D structural heterogeneity, and this phenotype is more evident in OvR. We further investigated these differences and found that the OvR was significantly larger in size than the OvL in young (N=20: 4.92±1.4mg vs. 3.79±1.4mg; p=0.014) and mid-age mice (N=13: 5.70±0.6mg vs. 4.58±0.7mg; p=0.002), but not in the old mice (N=15: 3.27±0.7mg vs. 2.76±0.8mg), suggesting persistent structural and functional differences between contralateral ovaries. We then analyzed the spatial mechanical properties between the ovarian cortex and medulla. Ovaries were encapsulated in 2% agarose, bisected, and each half was used to probe either cortex or medulla. Agarose did not alter tissue's mechanics (Agarose stiffness: 7.51±0.5kPa). Surprisingly, in young (N=3) and mid-age (N=5) mice, we detected no significant differences in stiffness between compartments (6-12w: cortex 1.28±0.9kPa vs. medulla 1.22±1.17kPa; 7-9mo: cortex 2.50±3.36kPa vs. medulla 2.04±3.18kPa). However, the cortex was consistently slightly stiffer, and in mid-age mice, the medulla became highly mechanical heterogenous. We are currently analyzing old ovaries. Overall, using our novel multiscale approach, we mapped the mechanical and structural properties of the murine ovary with sub-compartment resolution as a function of age. The age-associated increase in stiffness appears gradual, potentially initiating locally in the medulla and then expanding within this compartment. Surprisingly, no differences in stiffness were observed between the cortex and medulla, and we are currently analyzing other mechanical properties. Ongoing studies examine the intrinsic difference between contralateral ovaries, and the cellular basis of these changes with histology, single-cell RNA sequencing, and protein expression. This work was supported by R00-HD108424 to F.A.R.

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. PCOMECS 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 PCOMECS proliferation. These findings indicate that androgens increase zinc influx and proliferation via SLC39A9 in HOMECS but not in PCOMECS. HOMECS and PCOMECS responses via AR were determined by allowing cells to migrate through a semi-permeable membrane in the direction of an androgen stimulus gradient. HOMECS migration was increased by 62% in the presence of T ($p < 0.05$), but not BSA-T, whereas PCOMECS 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. PCOMECS 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.

Decoding the Biphasic Hypoxia Inducible Factor (HIF)1 α Response and Mitochondrial Adaptation in Granulosa Cells

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Abstract Text

Granulosa cells within developing ovarian follicles reside in a physiologically low oxygen microenvironment, yet how sustained reductions in oxygen tension regulate steroidogenesis remains poorly understood. Hypoxia-inducible factor-1 α (HIF1 α) is a central mediator of oxygen-dependent signaling and a key transcriptional regulator in steroid production, exerting both direct and indirect effects on cAMP-mediated STAR-dependent pathways. Acute hypoxia studies have shown that moderate oxygen reduction (10% O₂) enhances STAR protein, whereas severe hypoxia (1% O₂) suppresses it, indicating that the degree of oxygen deprivation influences granulosa cell function. However, those studies relied on short exposures, which may have introduced oxygen fluctuations during stimulation and sample processing, thereby limiting their physiological relevance. Here, we present, to our knowledge, the first integrated transcriptomic and proteomic analysis of granulosa cells exposed to a continuous (chronic) hypoxia system that prevents oxygen bursts during cAMP stimulation and sample collection, thereby enabling a precise evaluation of oxygen-dependent steroidogenic regulation. Our objective was to define molecular adaptations governing steroidogenesis during prolonged oxygen deprivation and following suppression of HIF1 α activity. Immortalized murine granulosa cells (KK-1) were cultured for 24h under normoxia, moderate hypoxia (10% O₂), or severe hypoxia (1% O₂). Steroidogenesis was induced with cAMP, and HIF1 α involvement was examined using its activity inhibitor echinomycin. RNA-seq and LC-MS/MS identified differentially expressed genes (DEGs; $P < 0.05$, FDR < 0.01) and proteins (DEPs; ≥ 2 peptides, FDR < 0.1) associated with altered oxygen availability and HIF1 α signaling. Compared with normoxia, cAMP stimulation under moderate and severe hypoxia induced 2,488 and 7,461 DEGs, respectively, and 286 DEPs under severe hypoxia. Chronic moderate hypoxia promoted expression of genes supporting mitochondrial adaptation, lipid mobilization, and fine-tuning of cAMP/PKA signaling, consistent with preservation of steroidogenic capacity. Chronic severe hypoxia, on the other hand, impaired mitochondrial function and protein localization to the organelle, and shifted granulosa cells away from cAMP/PKA-dependent steroidogenic signaling toward growth factor-driven PI3K/MAPK survival pathways and metabolic suppression programs. Transcriptomic profiling showed suppression of *Star* and cholesterol homeostasis pathways in both moderate and severe hypoxia, whereas proteomic analysis showed that loss of STAR, CYP11A1, LDLR, LIPE, ABCA1, and SQLE protein expression was restricted to severe hypoxia. Interestingly, *Hif1a* transcripts decreased in both moderate and severe hypoxia, alongside induction of its negative regulators *Egln1*, *Egln3*, and *Vhl*, suggesting compensatory mechanisms that limit chronic HIF1 α accumulation. Indeed, time-course analysis under 1% O₂ revealed an early, transient increase in HIF1A protein in the first 6h followed by marked suppression 24h later, further supporting the dynamic adaptation of HIF1 α during prolonged hypoxia. Inhibition of HIF1 α in cAMP-stimulated cells under normoxia and moderate hypoxia yielded 8,249 and 9,030 DEGs and 11 and 67 DEPs, respectively, affecting DNA transcription, chromatin remodeling, protein phosphorylation, intracellular protein transport, cholesterol biosynthetic process, and insulin receptor pathway, predominantly in the moderate hypoxic environment. Novel candidate factors and pathways potentially regulated by HIF1 α include cholesterol trafficking factors LDLR, ABCA1 and ASTERB, the PI3K–Akt–mTOR and AMPK signaling pathways, and factors involved in mitochondrial protein import and function. Together, these findings define a biphasic role for HIF1 α in steroidogenesis and demonstrate that regulated HIF1 α activity is required for cholesterol homeostasis and mitochondrial function in granulosa cells. This work provides a mechanistic framework for oxygen-dependent regulation of ovarian steroidogenesis, with relevance to female fertility and disorders characterized by dysregulated hormone production. (Supported by SNSF grant nr: 310030_207895)

The Role of Kir6.1 in Ovarian Smooth Muscle Contractility and its Relationship to Female Fertility

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Abstract Text

Cantú syndrome is an autosomal dominant disorder characterized by hypertrichosis, distinctive coarse facial features, and cardiovascular abnormalities. It is caused by gain-of-function mutations in ATP-sensitive potassium channels composed of pore-forming Kir6.1 and regulatory SUR2 subunits. Overactivity of these ATP-sensitive potassium channels inhibits smooth and striated muscle contraction, disrupting muscle functions in various tissues.

Notably, it has been observed that some females with Cantú syndrome have menstrual abnormalities, secondary amenorrhea and infertility. Ovarian agenesis has been reported in one case. However, the presence or function of ATP-sensitive potassium channels in the mammalian ovary remains unexplored. Appropriate contractile activity of smooth muscle is essential to ovarian physiology, including ovulation and steroidogenesis. Follicle rupture during ovulation is driven by contractions of smooth muscle cells in the theca layer of the follicle. Vascular tone, arising from vascular smooth muscle and pericyte contractions, ensures sufficient blood supply to deliver oxygen, nutrients, and hormones for follicular development, luteinization and steroidogenesis.

Here we show that Kir6.1 is specifically expressed in smooth muscle cells, including pericytes, in the murine ovary. We also identify a unique mouse model exhibiting subfertility associated with smooth muscle dysfunction due to a gain-of-function mutation in Kir6.1 that has been observed in Cantú syndrome patients. This mouse showed a prolonged estrus cycle and ovarian histopathology, recapitulating female subfertility associated with human Cantú syndrome, providing not only a model in which to test new therapeutic strategies for Cantú syndrome patients, but also broadening our understandings of the role of smooth muscle in ovarian physiology.

Effectiveness of an Individualized Lifestyle Intervention in Women with PCOS Desiring Conception: Findings from the IPOS Multicenter Randomized Controlled Trial

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Abstract Text

Polycystic Ovary Syndrome (PCOS) is a leading cause of infertility and metabolic disturbances in reproductive-aged women. While lifestyle modification is recommended as the first-line therapy for PCOS, high-quality, large-scale data from South Asia evaluating its impact on fertility outcomes is lacking. The IPOS (Individualized lifestyle intervention in PCOS) trial is a multicenter, open-label, randomized controlled trial designed to assess whether individualized lifestyle interventions improve live birth rates among Indian women with PCOS desiring conception.

The primary objective is to evaluate the effect of a structured individualized lifestyle intervention on live birth rates. Secondary objectives include assessment of metabolic, anthropometric, menstrual, and quality of life outcomes.

This ongoing trial aims to recruit 1200 women with PCOS (aged 19–37 years) across five tertiary care centers in India. Participants are randomized 1:1 to receive either individualized lifestyle intervention (diet + physical activity counseling by trained personnel) or standard care. Outcomes are assessed at 3 and 6 months, with further follow-up for conception and pregnancy outcomes.

As of 31st December 2024, a total of 449 participants have been randomized (intervention: 225; control: 224). Baseline characteristics were comparable across arms. At 6 months, the mean weight loss was 2.61 ± 2.58 kg overall, with no significant between-group difference. Clinical pregnancy rates were 20.49% in the intervention arm and 17.4% in the control arm. Positive pregnancy rates were slightly higher in the intervention group (12.88% vs 10.71%). Dietary adherence at 6 months was 83.9% in the intervention group. The intervention arm showed trends towards improved waist circumference, mood, and quality of life, though differences were not statistically significant.

Preliminary findings suggest that individualized lifestyle interventions are feasible and show encouraging trends in improving fertility-related outcomes in Indian women with PCOS. Final trial results will inform national guidelines and address a critical evidence gap in preconception care for PCOS in South Asia.

NS309 Restores Membrane Potential and Autophagy to Delay Post-Ovulatory Aging in Porcine Oocytes

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Abstract Text

Post-ovulatory aging (POA) of oocytes causes progressive deterioration of oocyte quality, leading to reduced fertilization, impaired embryonic development, and an increased risk of abnormal offspring. Disruption of calcium (Ca^{2+}) homeostasis, mitochondrial dysfunction, membrane depolarization, and defective autophagy are key drivers of this decline. NS309, a selective activator of small- and intermediate-conductance calcium-activated potassium (SK/IK) channels, can regulate membrane potential and restore cellular function, but its role in oocyte aging is unknown.

Here we tested whether NS309 attenuates POA-induced defects in porcine oocytes by stabilizing membrane potential, improving Ca^{2+} handling in the endoplasmic reticulum (ER) and mitochondria, and alleviating oxidative stress and autophagy impairment. In vitro matured porcine oocytes were cultured under POA conditions with or without NS309, and mitochondrial activity, ER distribution, Ca^{2+} dynamics, and autophagy-related markers were analyzed using immunofluorescence, Western blotting, and functional assays. We also evaluated whether NS309 protects against glibenclamide-induced KATP channel inhibition, which aggravates membrane depolarization and aging phenotypes.

We anticipate that NS309 treatment will reduce oxidative stress, restore mitochondrial and ER function, promote protective autophagy, and maintain a more stable membrane potential in aged oocytes, ultimately preserving developmental competence. These findings will provide new mechanistic insight into how potassium channel modulation regulates oocyte quality and may support a novel strategy to prevent age-related infertility.

Investigating the Connection Between Nexilin, a Protein Involved in Actin Binding and Membrane Contact Sites, and Vesicle Trafficking in Mouse Oocytes.

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Abstract Text

A hallmark of female meiosis are two asymmetric divisions that give rise to a large egg and two small polar bodies. Asymmetric division ensures that the egg retains half of the maternal DNA, as well as cytoplasmic resources that support early embryonic development. To achieve asymmetric division, the egg relies on accurate organization and positioning of the meiotic spindle. The actin cytoskeleton and various actin-associated proteins are necessary for driving these meiotic events. However, there are many aspects of actin function and dynamics during spindle positioning that remain poorly defined. We have identified the actin-binding protein nexilin (NEXN), best characterized for its function in cardiac muscle, as a regulator of meiotic spindle positioning in mouse oocytes. Acute, Trim-Away-mediated depletion of nexilin revealed that nexilin-deficient oocytes progress through meiosis I abnormally. Notably, a subset underwent near symmetric division, while another subset displayed a normal first polar body but abnormal metaphase II spindle positioning. Interestingly, these phenotypes resemble those observed following vesicle trafficking perturbation with Brefeldin A (BFA). We hypothesize that the link between nexilin and processes mediated by membrane-bound vesicles contributes to nexilin's function during oocyte maturation. In mammalian oocytes, Rab11a-positive vesicles drive meiotic spindle positioning by acting as a scaffold for actin nucleators and motor proteins. Disruption of Rab11a function results in a centrally positioned meiotic spindle and failed asymmetric division, mirroring some phenotypes observed with acute nexilin depletion and with BFA treatment. We first examined if perturbation of vesicle trafficking with BFA would cause abnormalities in nexilin localization. Prophase I oocytes were treated with a range of concentrations of BFA (0.5, 2.5, 5 μ M) and matured in vitro for 16 hours. Eggs were fixed and stained with anti-nexilin antibody to assess nexilin localization. We observed similar meiosis I outcomes in BFA-treated oocytes as previously reported, with almost all oocytes exiting from prophase I arrest but then progressing through meiosis I abnormally. Notably a subset displayed germinal vesicle loss and no polar body emission, but with the meiotic spindle stuck in the center of the oocyte, while another subset displayed near symmetric division producing a large first polar body. We found that 2.5 and 5 μ M of BFA produced comparable phenotypes, with substantially more modest disruptions to cytokinesis with 0.5 μ M (the original study presented findings with 5 μ M BFA only). In BFA-treated oocytes, nexilin was found to be enriched in the cortex with some cytoplasmic signal, similar to what was observed in untreated oocytes, although with the caveat that untreated oocytes underwent normal meiosis I division. These findings suggest that nexilin localization in oocytes is not directly dependent on BFA-sensitive vesicle trafficking. With our overall hypothesis that there is a connection between vesicle trafficking in oocytes and nexilin, we will next examine if nexilin acts upstream of vesicle trafficking, such that nexilin deficiency would perturb vesicles. Studies will test if nexilin depletion affects Rab11a localization, as well as the localization of other membrane-bound structures, such as the cis- and trans-Golgi, and the endoplasmic reticulum. These studies will provide broader insight into nexilin's role in membrane organization in oocytes, particularly since it has been observed that nexilin depletion disrupts endoplasmic reticulum organization in developing cardiomyocytes. This work is supported by NIH grant R01HD091117 and the EMBRIO Institute under contract #2120200, funded by the National Science Foundation (NSF) Biology Integration Institute.

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.

Mapping Ovarian Stiffness and Proteomic Profile across Reproductive Transitions in the Murine Model

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Abstract Text

Background

Ovarian aging is associated with increased extracellular matrix deposition, resulting in fibrosis, tissue stiffening, and impaired function. Notably, the ovary is the first organ to exhibit age-related functional decline, with reproductive aging occurring more rapidly than somatic aging in other tissues; however, the mechanisms underlying this accelerated decline remain poorly understood. This is clinically exemplified in conditions such as premature ovarian insufficiency, highlighting the need to identify processes governing ovarian aging that may represent therapeutic targets to extend ovarian lifespan as a potential alternative to hormone/menopause replacement therapy. Recent studies have begun to characterize the biomechanical changes associated with ovarian aging using nano-indentation and atomic force microscopy, demonstrating increased stiffness in aged compared to young ovaries. Despite these findings, it remains unknown how transitions through puberty, reproductive attainment, and aging, influenced by dynamic microenvironmental and hormonal cues, affect stiffening.

Aim

1. To determine how ovarian stiffness changes across reproductive transitions and understand the contribution of different ovarian structures to these biomechanical properties.

2. To temporally map the ovarian proteome across reproductive transitions.

Methods

Mouse ovaries were collected from 6 timepoints, based on key reproductive transitions: 14, 21 days (pre-puberty); 3, 6 months (reproductive prime); 12, 18 months (aged). Ovarian stiffness was assessed using atomic force microscopy and quantified via Young's Modulus. Analyses included (1) intra-organ spatial heterogeneity, (2) biological variance within the replicates of any given age group, and (3) longitudinal shifts across reproductive stages. n=4 biological replicates. A novel extracellular matrix enrichment protocol was devised to isolate the insoluble proteome for mass spectrometry. n=5 technical replicates.

Results and Discussion

Analysis of ovarian stiffness across reproductive transitions revealed a parabolic trend across the lifespan. Young's Modulus was highest in pre-pubertal (day 14 and 21) and aged ovaries (12 and 18 months), with median values of 271.1 Pa and 148.0 Pa, respectively. While stiffness increases before gonadotrophin exposure and as oestrus cyclicity is perturbed/arrested, it is contrastingly lowest at reproductive prime (ages 3 and 6 months), median of 90.4 Pa, indicating a softer and uniquely composed microenvironment at this stage. Spatial stiffness and proteomics heterogeneity was evident in individual ovaries within all ageing timepoints assessed. Interestingly, this was not attributable to simple cortex-medulla differences, suggesting that heterogeneity originates from follicle subtype and stromal microenvironment. Extracellular matrix-enriched proteomic analysis revealed that while several matrix components remained conserved across age, others exhibited age-associated variation, supporting a compositional basis for the observed mechanical changes. These findings demonstrate changes in ovarian stiffness across reproductive transitions, with important next steps towards mapping the structural and mechanical machinery mediating these processes.

Conclusion

These findings demonstrate that ovarian stiffness is highest at the pre-pubertal stage, decreases at reproductive maturity, and increases again during reproductive senescence. Mapping these mechanical and associated proteomic shifts provides a foundational framework for understanding how changes in the physical microenvironment may regulate functional decline in the aging ovary.

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Boar Responses to Reduced Expression of Steroid Reductase: A Pilot Study

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Abstract Text

Steroid five alpha reductase is one of many enzymes involved in steroid metabolism in the boar. Products of the enzyme are dihydrotestosterone and androstenone. The type 2 enzyme appears essential for human male reproductive tract development and fertility but its presence has no effect in male mice. Expression of this type 2 enzyme but not type 1 enzyme was previously demonstrated in the juvenile boar testis. A guide RNA was designed to reduce expression in animals produced using CRISPR/Cas9 technology. The founder male had a 7 bp deletion on one chromosome and a 2 bp deletion on the homologous chromosome. After breeding the founder male to the founder female, F1 male offspring were produced with a combined 3 bp alteration, a combined 6 bp deletion, a combined 8 bp alteration, or a combined 11 bp deletion. Fourteen males from two litters were further evaluated with tissues collected at 6 months of age. Preliminary comparisons with unrelated control boars suggested the editing was effective with means for specific edits consistent with a graded response. Samples from boars with a 3 bp change were compared with samples from full siblings with a combined 11bp deletion on the pair of homologous chromosomes. Testis size was similar between these two groups (621 vs 566 g, SEM $\pm$ 79). Androstenone and skatole were measured in scapular fat using liquid chromatography tandem mass spectrometry (LC-MS/MS). Numerically, boars with the larger edit had a little less than one fourth the concentration of androstenone compared with the boars with the smallest edit (n=3 in each group, P = 0.10). Skatole was reduced (P < 0.05) approximately six-fold in the boars with the largest edit compared with those with the smallest edit. Androstenone and skatole concentrations in scapular fat were correlated (R = 0.75) although this did not quite reach statistical significance (P < 0.10) with the small sample size; correlation is consistent with the accepted hypothesis that androstenone inhibits skatole metabolism. A panel of 108 untrained participants evaluated cooked pork loin from these edited boars in a sensory panel. The loins from the 11 bp combined deletion were numerically better than those from barrows but equivalent for the overall evaluation and were statistically different from loins from control boars (Kruskal Wallis test), consistent with observed skatole data. The sensory panel also detected an unpleasant barnyard fragrance in samples from the sibling boars with the minimal 3 bp change. Reduced steroid reductase activity is consistent with normal health, normal fertility, reduced androstenone, and reduced skatole. Supported in part by UC Davis Academic Senate New Initiatives Award, USDA NIFA Exploratory Grant 2019-67030-29097, UC Davis Clear Center Award, and California Pork Producers Association.

A Putative Metabolic Bypass in the Tricarboxylic Acid Cycle of Mammalian Sperm

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Abstract Text

Mature sperm are a unique biological system. An overlooked, yet important facet of their physiology is their metabolism. Three unique features make sperm metabolism interesting:

- < ! 1. Mature sperm do not grow or divide, therefore sperm metabolism is focused on generating energy and reducing equivalents.
- < 2. Sperm undergo a rapid increase in motility during maturation in the female genital tract, which demands a significant increase in energy production.
- < ! 3. Sperm display a unique cellular anatomy, mitochondria are located in the midpiece while glycolytic enzymes are colocalised in the flagellum.

Despite these features, the intricacies of sperm metabolism remain unclear. Progress in the field has been impeded by a lack of suitable methodologies. A key metabolomic technique, which our lab has applied to sperm metabolism is stable isotope labelling.

Sperm are able to metabolise various substrates, but little is known about which energy sources are used *in vivo*. Our lab has applied stable isotope labelling to mouse sperm using ^{13}C labelled glucose, fructose or pyruvate. Unexpectedly, we have found a significant difference in the distribution of ^{13}C in tricarboxylic acid (TCA) cycle metabolites when sperm are given pyruvate as their energy source compared to glucose or fructose.

Our findings are exiting due to the centrality of the TCA cycle (also known as the Krebs cycle or citric acid cycle) in respiration. This cyclical metabolic pathway oxidizes nutrient derived acetyl-Coenzyme A to carbon dioxide and is the main producer of energy (ATP), reductant (NADH) and amino acid precursors. Dysfunction in the TCA cycle is associated with metabolic diseases, cancers, and neurodegeneration.

To explain these puzzling results, we hypothesise that that sperm, under certain conditions, use an alternative version of the TCA cycle that bypasses most enzymatic steps. We suggest that the key enzyme in this bypass is ATP citrate lyase, which could be used as a target for non-hormonal contraceptive or as part of an explanation of idiopathic infertility.

The presence of this alternative pathway introduces a completely new facet to our understanding of sperm metabolism. Our current work explores this unexpected twist in a classical metabolic pathway which has been studied for nearly a century.

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.

A Novel Inducible MEIOSIN Mouse Model for Studying the Regulation of Meiotic Entry During Spermatogenesis

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Abstract Text

The prevalence of azoospermia, a condition that causes male infertility, increased 39% between 2006 to 2023, and some studies predict that it will continue to rise for at least the next two decades. Though aberrant meiosis is a common cause of nonobstructive azoospermia, the mechanisms required for germ cells to initiate and complete meiosis remain poorly defined. STRA8 and MEIOSIN are transcription factors that function in a complex to induce meiotic gene expression, which is required for meiotic initiation. Loss of either *Meiosin* or *Stra8* prevents germ cell entry into meiosis, with both knockout models displaying germ cells that stall in the preleptotene stage, causing infertility. Mitotically dividing differentiating spermatogonia transiently express *Stra8* but not *Meiosin*, whose absence is thought to be the reason that precocious initiation of meiosis does not occur in these cells. To test this, we developed a novel Cre-lox inducible Meiosin-IRES-eGFP transgenic mouse model. Using *Ngn3-Cre* to induce constitutive MEIOSIN expression beginning in undifferentiated spermatogonia, we have shown that nearly 100% of seminiferous tubules exhibit induced MEIOSIN expression. Interestingly, histological analysis of this mouse model revealed that STRA8-positive differentiating spermatogonia with induced MEIOSIN expression only rarely exhibit precocious entry into meiosis and fail to show robust MEIOSIN-dependent gene expression changes. This finding indicates that merely expressing STRA8 and MEIOSIN simultaneously is insufficient to significantly activate the meiotic cell cycle program or to promote initiation of meiosis in all differentiating spermatogonia. This suggests that there are additional uncharacterized mechanisms that regulate the specific timing of meiotic initiation to prevent premature meiotic entry. Future studies will use our inducible MEIOSIN model to examine meiotic initiation defects in *Meioc* knockout spermatocytes as these cells display disrupted meiotic initiation, reduced endogenous *Meiosin* expression, and resultant reduced expression of critical meiotic cell cycle genes. We hypothesize that induced MEIOSIN expression at stage appropriate times is sufficient to rescue meiotic gene expression and cellular development in the *Meioc* knockout.

From Bedside to bench: Establishing a workflow to study the causative factors of hypospadias in humans

Submission Type: Late Breaking Abstract Submission

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Abstract Text

Hypospadias is a common malformation in fetal development of the penis in which the urethra does not open at the head of the penis. Even though hypospadias affects about 1 in 200 boys, its etiology is still largely unknown. Human male genitalia foreskin (FSK, prepuce) is involved in proper urethra closure. The foreskin of the penis is a rich, easily accessible source of highly proliferative mesenchymal cells. Genetic mutations that negatively impact mesenchymal cell function consistently result in hypospadias. Here we establish a workflow to isolate mesenchymal cells from the FSK and test their ability to form spheroids. We also examine the functionality and physiological relevance of spheroids by treating them with 5 α -dihydrotestosterone (DHT), a potent biologically active androgen metabolite, important in external male genitalia development.

We collected human foreskin tissues of circumcised and hypospadias patients from The University of Missouri Hospital in Columbia, Missouri. Isolated mixed cell populations from the FSK of circumcised and hypospadias patients (N=3) were cultured for 16 days after which the cells were subjected to negative fluorescence-activated cell sorting (FACS) with antibodies directed against cell surface markers EpCAM (Epithelial Cell Adhesion Molecule) and PECAM1 (Platelet Endothelial Cell Adhesion Molecule-1). We found very low epithelial and endothelial cells (up to 1%) in the preparations from circumcised and hypospadias patients. The purity of the negatively sorted cells was confirmed by flow cytometry against FAP (Fibroblast Activated Protein) antibody marker (99-100% cell purity). In support of the flow cytometry data, the mesenchymal cells from circumcised and hypospadias patients did not immunoreact to EpCAM and PECAM-1 antibodies but were 100% immunoreactive to FAP antibody, when tested on mesenchyme cell cultured for 24 hrs. These data indicated that both cells from circumcised and hypospadias patients did not differentiate into or possess the characteristics of epithelial or endothelial cells and were mesenchyme cells.

We also investigated the potential of the mesenchyme cells to form spheroids and react to 10 nM DHT. The ability of our mesenchymal cells to form spheroids is a key indicator of their ability for self-organization, enhanced cell-cell interactions, and elevated therapeutic potential.

Overall, our preliminary data support the creation of a novel model system that can be used for both the identification of crucial regulators of human hypospadias and for therapeutics.

NEK5 Regulates the Mitochondrial Function during Mouse Preimplantation Embryonic Development

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Abstract Text

NEK5, a member of NIMA (Never in Mitosis A)-associated kinase, has multiple physiological functions including the regulation of cell cycle, centrosome integrity maintenance, and mitochondrial function modulation. Our previous study demonstrated that *Nek5* depletion severely impaired preimplantation embryonic development. In this study, the mechanism of the regulation of NEK5 during mouse preimplantation embryonic development was further investigated. *Nek5* knockdown was implemented via siRNA-mediated depletion at the 1-cell stage of mouse embryos and the effects were examined by Western blot, immunofluorescence, quantitative real-time PCR, and other assays, with at least three replicates per experiment. NEK5 is continuously expressed throughout all stages of mouse preimplantation embryonic development, with higher expression level at the 2-cell stage compared to the 1-cell stage. It is mainly localized in the cytoplasm during interphase and colocalizes with the spindle during mitosis. We found that most *Nek5* depletion embryos arrested at the 2-cell stage and blastocyst formation rate was extremely low. Transcriptome sequencing analysis revealed that downregulated expression of genes associated with mitochondrial function and energy metabolism pathways. qPCR validation confirmed the expression of mitochondrial-related genes was significantly decreased. Further functional assessments demonstrated that *Nek5* knockdown resulted in decreased mitochondrial quantity, reduced mtDNA copy number, mitochondrial membrane depolarization, and significantly elevated levels of reactive oxygen species. Immunofluorescence analysis demonstrated that NEK5 colocalizes with mitochondria proteins, and the fluorescence intensity of these proteins was decreased after *Nek5* knockdown. In conclusion, this study demonstrates that NEK5 plays indispensable roles during mouse preimplantation embryonic development by regulating mitochondrial function. This study was supported by the National Key Research and Development Program of China (2022YFC2702200) to Z.H.

Tfap2a and *Tfap2b* Are Required for Urethral Tube Formation and Ventral Fusion During Penile Development

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Abstract Text

Birth defects of the penis are some of the most common congenital malformations in the world. Some of the most common penile birth defects are caused by incomplete urethral tube formation and failure of ventral fusion during genital tubercle development. In the United States, hypospadias affects approximately 1 in 125 male births. Although surgical repair can correct the urethral defect, complications such as urethrocutaneous fistula, meatal stenosis, and the need for repeat interventions are frequent. Despite its clinical prevalence, the cellular and gene regulatory mechanisms governing urethral morphogenesis during penis development remain incompletely understood. Normal penile development requires two key fusion events: 1) fusion of the genital swellings during early patterning of the penis and 2) subsequent fusion of the urethral folds to generate a closed urethral tube. Disruption of either process results in congenital penile defects, including bifid penis and hypospadias. Members of the AP-2 transcription factor family, including TFAP2A and TFAP2B, are established regulators of epithelial fusion in other developmental contexts, including cleft palate and ventral body wall closure. Our central goal was to identify whether *Tfap2a* and *Tfap2b* are essential for the fusion events during early penile patterning and urethra closure. First, to determine whether TFAP2A and TFAP2B are expressed during penile development, we performed immunofluorescence across key developmental stages. TFAP2A expression was detected as early as embryonic day (E) 11.5 in mesenchyme surrounding the urethral epithelium along the penis and in the skin. TFAP2B expression was first observed at E12.5 and persisted through E18.5, localizing primarily to the skin and distal mesenchyme adjacent to the urethra. These expression patterns support the role for AP-2 factors during urethral morphogenesis. Next, we generated an *Isl1*-Cre-driven conditional knockout model to compare control embryos with *Tfap2a/b* single- and double-knockout embryos across stages spanning urethral fusion (E12.5–P0). *Isl1*-Cre targets all the progenitor cells of the penis, being active prior to penis formation (E10.5). Whole-mount imaging of external genitalia was performed to evaluate gross morphology. Single-gene knockout analysis revealed distinct contributions. *Tfap2a* single-knockout embryos exhibited ventral urethra fusion defects indicating that *Tfap2a* is required for normal urethral morphogenesis. In contrast, *Tfap2b* single-knockout embryos displayed genital morphology comparable to controls, suggesting *Tfap2b* alone is insufficient to disrupt gross development and may play a supportive or partially redundant role to *Tfap2a*. *Tfap2a/b* double-knockout embryos exhibited variable but consistently penetrant abnormalities in genital morphology. The most severe defect observed was a fully bifid penis, indicating there was a disruption in the fusion of the genital swellings during early patterning of the penis. The mild phenotypes were dramatic cases of hypospadias where there is a complete loss of urethral fold formation and urethral fusion. Although severity varied, all double knockouts demonstrated impaired ventral fusion and abnormal urethral morphogenesis. Histological analysis (H&E) at E15.5, E18.5, and P0 revealed underlying tissue-level defects. *Tfap2a* single-knockout and *Tfap2a/b* double-knockout genital tubercles showed disrupted urethral tube architecture and irregular epithelial organization. Together, these findings demonstrate that TFAP2A and TFAP2B function cooperatively to regulate both genital swelling fusion and urethral tube formation during penis development. These results open new avenues to study the transcriptional programs essential for genital tubercle morphogenesis and to gain mechanistic insights into the developmental origins of hypospadias.

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.

Sperm mRNAs: Comparison Between Stallion and Donkey Extraction Assays in Cooled Semen

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Abstract Text

Spermatozoa are highly specialized and transcriptionally inactive cells, hosting a very low amount of mRNA. This together with the high chromatin condensation complicates RNA extraction. Due to differences in the membrane composition of sperm among species, specific protocols for mRNA extraction are necessary. There is an increasing interest if sperm RNA can be targeted to predict fertility. Information in this regard is sparse in equid species. The aim of this study was to find the best assay for the extraction of mRNA from cooled semen originating from horses and donkeys, and to compare mRNA from these related but still very different species.

Ejaculates from 4 horses and 4 donkeys were diluted with BotuSemen extender to a final concentration of 50×10^6 sperm/ml. Horse semen was collected in Vienna and stored refrigerated (4°C). Donkey semen was collected and processed in Barcelona, then shipped refrigerated (4°C) to Vienna. All samples were analyzed 24 hours after collection. In each sample, sperm motility was assessed by computer-assisted sperm analysis. The remaining sample was processed by density gradient centrifugation (Equipure) followed by two centrifugation steps in PBS. The RNA was extracted using a total of eight different assays, four preceded by somatic cell lysis through a specific buffer (SCLB) and four excluding this step (NSCLB). Extraction assays, with and without somatic cell lysis, included: A) incubation with Trizol (room temperature) (T); B) bead-based homogenization (3x) in Trizol (BH); C) heated trizol (65°C 30 min) (HT); or D) bead-based homogenization (3x) + heated Trizol (65°C 30 min) (HTBH). In all samples, mRNA was isolated using Direct-zol columns. Following extraction, mRNA contamination by solvents (260/230 ratio) and by proteins (260/280 ratio), mRNA integrity (DV200 Index) and yield (TapeStation) were assessed for each species and extraction assay. Differences in the abundance of genes expression previously proven to be correlated with fertility (PRM1, PRM2, PRM3, CRISP3, PLCz1, ZBPB) regarding treatment and species were determined by RT-qPCR (reference genes: ACTB and RPL32; somatic cell markers: CDH1 and PTPRC). Statistical analysis was performed with two-way ANOVA (IBM-SPSS statistics 29.0.1.0) with $p < 0.05$ considered significant.

SCLB-HT provided better results for 260/230 in stallion compared to the other assays ($p < 0.001$), but overall results for this parameter were better in donkey sperm ($p < 0.05$). RNA integrity and yield were higher increase ($p < 0.001$) in donkey sperm, and in samples treated with HT and HTBH ($p < 0.001$). Regarding gene expression, PRM1 and PRM3 were higher ($p < 0.05$) in donkey sperm, while PLCz1 ($p < 0.001$), ZBPB ($p < 0.05$), and CRISP3 ($p < 0.001$) were higher in horse sperm. The extraction procedure did not influence mRNA abundance.

Our study demonstrates that horse and donkey sperm require different assays for optimal mRNA extraction. This is most likely caused by differences in sperm membrane composition but this needs to be addressed in further studies. Species-specific assays for mRNA extraction are thus required.

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 Cterminus 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 headkinking amplitude, the ability to exhibit a circular trajectory, and are essential for sperm competition in double matings

Seasonal Modulation of Reproductive Traits, Semen Quality Parameters, and Testicular Hemodynamics in Subtropical Male Goats

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Abstract Text

Pakistan has the third-largest goat population with 80.3 million heads. Although goats are classified as short-day breeders, their reproductive dynamics under subtropical conditions with moderate photoperiod variation remain insufficiently characterized. This study evaluated monthly and seasonal variations in reproductive traits, semen quality, and testicular doppler hemodynamics in adult Beetal bucks. Five mature bucks (2–3 years; BCS 3.5 ± 0.1) were monitored over 12 months across all seasons (Spring, Summer, Autumn, and Winter) during 2023-24. Scrotal circumference (SC), testicular volume (TV), body weight, semen characteristics (volume, concentration, total motility TM, progressive motility (PM), rapid motility (RM), slow motility (SM), CASA-derived kinematics, testicular echogenicity (TE), and Doppler indices (RI, PI, CFM) were recorded fortnightly. Results revealed significant monthly and seasonal variations in SC and TV ($P < 0.05$), with peak SC in summer (June-August) and maximum TV in October, followed by winter decline. BW significantly influenced SC and TV and showed interaction effects with month ($P < 0.05$). Semen volume and concentration varied monthly ($P < 0.001$) but not seasonally. TM and PM were significantly affected by month and BW, with optimal values in July, whereas seasonal differences were non-significant. TE and Doppler indices (RI, PI) demonstrated marked monthly variation ($P < 0.05$), with lower RI and PI during June and July, indicating enhanced testicular perfusion. However, after BW adjustment, seasonal differences in RI and PI disappeared. Correlation analysis revealed strong positive associations between SC and motility parameters during summer, and a strong negative relationship between RI/PI and RM ($r = -0.927$ and -0.827 , respectively), confirming that reduced vascular resistance is linked to improved sperm functional competence. CASA kinematic parameters (VSL, BCF, STR) exhibited selective monthly sensitivity, while most remained seasonally stable after BW adjustment. In conclusion, Beetal bucks under subtropical conditions exhibit pronounced monthly but attenuated seasonal reproductive variations. Body weight exerts a stronger influence on semen quality and testicular hemodynamics than broad seasonal categorization. These findings suggest partial seasonal resilience in short-day breeders raised in subtropical environments and emphasize the importance of accounting for BW in breeding soundness evaluation and semen production programs.

Counteractive effects of VitaminC in PFOS exposed mouse Spermatogonial cells

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Abstract Text

Perfluorooctane sulfonate (PFOS), a widely distributed and well-known environmental endocrine disruptors (EEDs), is recognized as a “forever chemical” and has been associated with reproductive toxicity and subfertility. Growing evidence indicates that PFOS exposure during both early development and adulthood can lead to significant reproductive impairments, including reduced sperm motility and count, disrupted or abnormal gametogenesis, increased sperm aneuploidy, and elevated sperm DNA fragmentation, alters the circulating testosterone level. However, the mechanisms by which PFOS-induced oxidative stress contributes to epigenetic alterations in male germ cells and the mitigation strategy remain poorly understood.

In the present study, GC-1 cells (a mouse type B spermatogonia cell line) were used to investigate whether PFOS-induced oxidative stress operates through epigenetic modifications *in vitro*. GC-1 cells were exposed to 1 μ M, 10 μ M, and 50 μ M PFOS, while a separate group was co-treated with 75 μ M vitamin C for 24 hours. All experimental endpoints were assessed under the same conditions. These doses were selected based on preliminary cell-viability results that demonstrated low, moderate, and substantial reductions in viability across the concentration range. Cell-viability assays and gene-expression analyses revealed a dose-dependent decline in cell viability as well as alterations in the expression of epigenetic-modifying enzymes such as *ezh2*, *hdc2*, *nanos2* genes in PFOS-treated cells, compared with the vitamin-C co-treated groups. Staining with anti- γ -H2AX antibody showed PFOS exposure leading to DNA damage, which was significantly reduced by vitamin C co-treatment. Flow cytometry and DCFDA-based immunofluorescence staining confirmed that vitamin C effectively attenuates PFOS-induced reactive oxygen species (ROS) production and restores gene expression impaired by PFOS exposure. RNA-seq data demonstrated that PFOS (50 μ M) exposure significantly downregulated genes such as *VegFa*, *Slc2a1* and other metabolic regulators. Conversely, Vitamin C treatment enhanced the expression of steroidogenic and junctional genes including *Star*, *Cyp11a1*, and members of the *Gja* family (gap junction cornerstone in testis) highlighting their involvement in pathways essential for germ-cell maturation. To further substantiate present *in vitro* results, we are conducting *in vivo* studies in both medaka and mouse models.

The present findings suggest that PFOS exposure induces oxidative stress and DNA damage in type B spermatogonia and that these harmful effects can be mitigated/blocked by vitamin C co-treatment.

Histone Demethylase KDM2A in Sertoli Cells Regulates Retinoic Acid Signalling by Targeting Zeb1 to Facilitate Spermatogenesis

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Abstract Text

Sertoli cells are pivotal in supporting germ cell development and are the primary source of retinoic acid (RA), thereby facilitating the testicular microenvironment and spermatogenesis within the seminiferous tubules. Nevertheless, the molecular mechanisms that regulate the Sertoli cell-dependent RA pathway remain to be fully elucidated. Herein, we demonstrate that histone demethylase KDM2A in Sertoli cells plays an essential role in spermatogenesis by modulating the RA signaling pathway. Conditional knockout of KDM2A in mouse Sertoli cells results in impaired spermatogonial differentiation and defective meiotic initiation. This is attributable to compromised cell adhesion and disrupted blood testis barrier (BTB) function within seminiferous tubules, which ultimately leads to male infertility due to spermiogenesis arrest. Multi-omics analysis of purified Sertoli cells revealed that KDM2A interacts with various splicing factors and regulates the alternative splicing of RA-related molecules, such as *Rail4* and *Cyp2d22*. We also identified many differentially expressed genes involved in cell-cell adhesion and RA signaling upon KDM2A depletion in Sertoli cells. Furthermore, we demonstrate that KDM2A can directly target *Zeb1* and finetune H3K36me2 at *Zeb1* promoter to regulate the expression of RA synthases, including *ALDH1A2* and *ALDH1A3*, in Sertoli cells. Interestingly, exogenous RA supplementation could partially rescue defective spermatogenesis in Sertoli cell-Kdm2a knockout mice. Collectively, our findings uncover an essential role for KDM2A in Sertoli cells and provide a potential therapeutic approach for treating male infertility caused by dysfunctional Sertoli cell-derived testicular microenvironments.

Poster Session B

Griffin Hall

Tuesday, July 21,

8:00 AM – 9:45 AM

Educating Future Scientists: The Importance of Metadata in Scientific Research

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Abstract Text

In addition to storing information, data repositories are designed to disseminate information widely for reuse in new studies or educational applications. The FAIR (Findable, Accessible, Interoperable, and Reusable) guiding principles for data sharing underscore the need for metadata, which are attributes that describe the data being shared. Metadata are critical for the reuse of the shared data and include, for example, usage licenses, contributors to the collection of the data and metadata, as well as methods used to obtain the data. In addition, there are often specific metadata requirements based on the domain of the data. However, while metadata are collected in different ways (lab notebooks, spreadsheets, etc.) over the course of a study, they often are not organized with the intent for broad dissemination via a data repository. This presentation covers metadata broadly, and provides recommendations for collecting interoperable metadata with ideas for training students to think about metadata in their research studies.

To ensure that data are findable and reusable, data repositories require basic information about the study, e.g., its title, an abstract, and the individual(s) who collected the data. Metadata are more specialized for the specimens/samples. For example, the Gene Expression Omnibus stores high-throughput sequence data and thus requires information about the protocols used and data processing steps that resulted in the sequence being submitted. The Ecological Metadata Language (EML) requires study and specimen information along with information describing an environmental dataset such as geographic and temporal coverage. The Multispecies Ovary Tissue Histology Electronic Repository (MOTHER) project collects metadata based on EML with MOTHER-specific metadata for ovarian histology slide images. This includes information about the donor animal, slide preparation, and the microscope and camera used to obtain the digital image. A critical aspect of metadata in biological research is the need to use standardized nomenclatures for biological entities including species, organs, cells, proteins and genes. Standardized nomenclature greatly enhances the findability of data since it reduces ambiguity, minimizes the use of inaccurate or imprecise names for biological entities, and ensures that researchers can consistently identify and retrieve data across studies, databases, and disciplines. Examination of metadata requirements prior to conducting a study can be helpful in ensuring the metadata are collected and recorded concurrent with the experiment, when the metadata is most readily available.

Educating future scientists about the need to record metadata will make data dissemination through online repositories more effective. For sharing ovary histology slides in MOTHER, one of the most time-consuming steps is compiling the required metadata. Incorporating this training into a science curriculum could be initiated by the requirement of a metadata section in lab reports. More ambitious approaches include creating courses focused on data literacy, research methods, or a STEM success class focused on data sharing and reproducibility. Embedding metadata literacy into the science curriculum can transform a time-consuming process into a foundational skill that will promote the longevity, reproducibility and impact of reproductive science and biological research in general.

Effects of Sperm Energy Restriction and Recovery Prior to ICSI on Fertilization and Early Embryo Development Using a Novel Automated Imaging Pipeline

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Abstract Text

Assisted Reproductive Technologies (ART) are widely used to address infertility in clinical settings and to support selective breeding in livestock production. Among them, Intracytoplasmic Sperm Injection (ICSI) is particularly valuable for cases of low sperm motility or low sperm count.

It has been shown that sperm energy restriction and recovery (SER) prior to *in vitro* fertilization (IVF) improves fertilization rates, early embryonic development, and implantation rates following non-surgical embryo transfer in mice. The aim of this work was to evaluate the effects of SER combined with ICSI on fertilization and embryo development in mice, in order to assess the broader applicability of SER across ART platforms and its potential as a therapeutic approach for male infertility.

ICSI was performed using C57BL/6J sperm and CD-1 oocytes, with sperm subjected to either SER or control treatment prior to injection. Briefly, for the SER treatment, sperm were first incubated in the absence of energy substrates (glucose and pyruvate) until they became motionless (approximately 20–45 minutes), after which substrates were added back. Control sperm were incubated in the presence of substrates throughout. Fertilization was evaluated by cleavage to the 2-cell stage of the surviving injected oocytes, and *in vitro* embryo development was assessed by progression to the blastocyst stage. In addition, cell number and lineage specification were evaluated by immunostaining for the trophectoderm marker CDX2 together with DAPI, followed by confocal imaging. To support quantitative analysis of blastocyst quality, we developed an automated image-analysis pipeline using Fiji and MATLAB that integrates 3D nuclear information to quantify total cell number and nuclear staining in whole blastocysts. Compared with controls, SER significantly increased fertilization rates (CON: $70.3 \pm 5.9\%$ vs SER: $83.7 \pm 5.1\%$, $n = 7$, $p = 0.0289$). Preliminary analyses also showed a higher proportion of SER embryos reaching advanced developmental stages at 96 hours post injection (hpi) (CON: $56.1 \pm 10.6\%$ vs SER: $78.5 \pm 8.8\%$, $n = 2$), although no difference was observed at 120 hpi (CON: $81.5 \pm 6.3\%$ vs SER: $78.5 \pm 7.5\%$, $n = 6$, $p = 0.476$); additional studies are needed to confirm these early observations. SER did not alter total cell number or cell lineage specification at the blastocyst stage at 120 hpi.

Overall, our results suggest that combining SER with ICSI has potential value for improving fertilization in sperm with reduced fertilizing capacity, supporting the broader applicability of SER across multiple ART techniques. The automated quantification pipeline developed here also provides a reproducible tool for assessing blastocyst quality in future studies. Together, these findings indicate that a sperm-only treatment can influence fertilization and early embryo development, highlighting the role of sperm in shaping early developmental outcomes.

Assessing Contraceptive Efficacy of ADCY10 Inhibitors in Cervical Mucus

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Abstract Text

Male fertility depends on sperm motility, which enables sperm to traverse the female reproductive tract to the site of fertilization. Soluble adenylyl cyclase (sAC) is a key regulator of sperm motility defining it as a promising non-hormonal contraceptive target for women and men. To evaluate the contraceptive efficacy of sAC inhibitors under conditions mimicking the female reproductive tract, we employed a capillary assay using cervical mucus isolated from humans or cows as well as methylcellulose, a viscoelastic liquid that simulates cervical mucus. We have shown that the addition of a sAC inhibitor either directly to semen, modeling an on-demand male contraceptive, or to the cervical mucus, approximating a female contraceptive, effectively blocked sperm migration. These studies provide proof-of-concept for the efficacy of sAC inhibitors under physiologically relevant conditions. We now explore the metabolic requirements for sperm movement through viscoelastic media.

Investigating Differences in Germline-Specific Antibody Affinities Across Avian Species

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Abstract Text

The quantification and enrichment of avian Spermatogonial Stem Cells (SSCs) in adult testicular tissue via Fluorescent Activated Cell sorting (FACS) is a pinpoint method towards the preservation of critical genetic lines through bio banking and cell culture. As such, Stage-Specific Embryonic Antigens (SSEAs) are commonly used cell surface markers in avian research to identify and isolate these germline stem cells. In non-commercial avian species, the bioavailability of germline cells through adult testicular tissue is significantly greater than those which are derived from embryonic and adolescent specimens, especially in critical or endangered species. Spermatogonial Stem Cells make up a small percentile of avian testicular tissue; thus, in any given sample, SSC populations typically tend to be low. The majority of testicular cell populations are comprised of somatic support cells such as Leydig, Sertoli, and Peritubular Myoid cells which may be excluded using surface markers such as Follicle Stimulating Hormone Receptor (FSHR). In previous studies, SSEA-1, SSEA-3, and SSEA-4 have been used to identify germline stem cells in chickens, with SSEA-1 having the strongest binding in early germ cells of embryonic chicks (Jung et al 1). However, along with other known surface markers, SSEA-1 lacks specificity leading to the importance of SSEA-3 and SSEA-4 for targeting SSCs (Gough et al 2). While these markers are fairly conserved across avian species, binding patterns change as differentiation of germ cells occurs. Therefore, studying how these markers behave in different species is critical to knowing how to identify critical germline cells.

Cryopreservation of Sunflower Sea Star (*Pycnopodia helianthoides*) Sperm and Larvae to Support Conservation

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Abstract Text

Sunflower sea star (*Pycnopodia helianthoides*; *P. hel*) populations along the western coast of North America have declined by more than 90% since 2013, primarily due to sea star wasting disease. In response, collaborative captive propagation and restoration programs have been initiated. Assisted reproductive technologies may support these efforts by preserving genetic diversity and enabling managed breeding. Sperm was collected from spawning males ($n = 6$) by gently lifting individuals partially out of seawater and aspirating concentrated sperm from the gonopores. Motility, progression (0–5 scale), and viability were assessed (mean $\pm$ SEM: $70.0 \pm 6.3\%$, 3.8 ± 0.2 , and $74.9 \pm 3.9\%$, respectively). Sperm (100 μ L) was diluted 1:1 in sterile seawater (8°C) and extended 1:1 with 10, 20, 30, or 40% dimethyl sulfoxide (DMSO) in sterile seawater, resulting in final concentrations of 5, 10, 15, and 20% DMSO. Duplicate aliquots (200 μ L) were equilibrated for 15 min at 4°C, placed in a dry shipper for 10 min (approximate cooling rate $-50^\circ\text{C}/\text{min}$), and plunged into liquid nitrogen. Samples were thawed in a 25°C water bath for 1 min and diluted 1:4 with sterile seawater. A one-way repeated-measures ANOVA detected no statistically significant effect of DMSO concentration on post-thaw motility ($F(3,12)=3.18$, $p=0.063$, $\eta^2=0.38$), although 10 and 15% DMSO produced the highest values ($26.0 \pm 2.2\%$ and $22.0 \pm 1.8\%$, respectively) compared with 5% and 20% DMSO ($16.0 \pm 3.4\%$ and $13.0 \pm 2.7\%$, respectively). Fertilization capacity was evaluated for the 10% and 15% DMSO treatments using sperm from three males. Approximately 1000 eggs were combined with 1×10^6 thawed sperm and incubated overnight at 8°C. Fertilization success, defined as development to the blastula stage, averaged $42.0 \pm 17.6\%$ for 10% DMSO and $13.7 \pm 13.7\%$ for 15% DMSO, with progression to the gastrula stage averaging $31.3 \pm 18.9\%$ and $14.0 \pm 14.0\%$, respectively. In additional trials, sperm cryopreserved in 10% DMSO yielded larvae that developed into adults capable of producing viable gametes. Preliminary protocols were also developed to cryopreserve early developmental stages. Gastrula-stage larvae (4 gastrula/ μ L sterile seawater) were diluted 1:1 with Amphibian Ringer's solution containing 20% DMSO and 2.0M trehalose, resulting in final concentrations of 2 gastrula/ μ L, 10% DMSO, and 1.0M trehalose. Samples (500 μ L) were equilibrated for 5 min at 20°C, frozen at $-1^\circ\text{C}/\text{min}$ to -40°C using a programmable freezer, held for 10 min, and plunged into liquid nitrogen. Samples were thawed at 22°C, diluted with 30 mL sterile seawater (8°C), and held overnight. Of four crosses evaluated, two exhibited initial post-thaw survival of 9% and 28%, with surviving larvae ingesting algae but not surviving beyond two weeks in culture. Post-thaw survival demonstrates that gastrula-stage larvae can withstand cryopreservation, although longer-term outcomes likely reflect interactions between cryopreservation stress and culture conditions. Together, these findings demonstrate that *P. hel* sperm can be cryopreserved while retaining fertilization capacity and that early developmental stages tolerate cryopreservation though further work is needed to improve survival. These tools provide a foundation for biobanking and assisted breeding strategies supporting species recovery.

Beyond Anatomy: Circulating and EV-Associated Prenatal Plasma miRNAs as Noninvasive Biomarkers in Congenital Diaphragmatic Hernia

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Abstract Text

Risk stratification in fetal anomaly remains largely dependent on anatomic imaging and invasive procedures that offer limited physiologic insight. Circulating maternal plasma miRNAs represent a promising noninvasive alternative, yet translating sequencing-based discovery into clinical application requires validated, reproducible quantification workflows. Building on our prior work identifying distinct prenatal miRNA signatures associated with disease presence and severity in congenital diaphragmatic hernia (CDH), this study evaluates PCR-based validation strategies for a CDH-specific miRNA panel. We systematically compare whole plasma versus extracellular vesicle (EV)-derived fractions, PCR platforms, and normalization approaches to establish reproducible workflows and clarify how methodological choices affect biological interpretation.

Maternal peripheral blood plasma samples were collected from patients at our fetal center during the second and third trimesters of pregnancy and snap-frozen for storage at -80°C. Extracellular vesicles (EVs) were isolated using the exoEasy kit (Qiagen) and characterized by nanoparticle tracking analysis (NanoSight). miRNAs were extracted from plasma and EVs using the miRNeasy Serum/Plasma kit (Qiagen). Quantification was performed using real-time PCR (RT-PCR) and droplet digital PCR (ddPCR) with Advanced TaqMan assays (Thermo Fisher Scientific); miRNA concentration was measured by fluorometric Qubit assay. Statistical analyses were conducted in R.

CDH-associated miRNA expression patterns identified by sequencing were largely conserved across PCR platforms, supporting the biological validity of the original discovery findings. However, RT-PCR results were highly sensitive to normalization strategy, with global mean, endogenous, and exogenous approaches yielding divergent interpretations. Notably, ddPCR's capacity for absolute quantification offered a normalization-independent alternative, highlighting its utility in settings where normalization strategy meaningfully affects interpretation and where endogenous controls have not been established. EV-isolated miRNA profiles were distinct from total plasma fractions, suggesting biologically meaningful compartmentalization of circulating miRNA signal. Conservation of CDH miRNA signatures across platforms supports their potential as noninvasive prenatal biomarkers.

Compartment-specific differences between EV-associated and whole plasma fractions likely underlie reported inconsistencies in the miRNA biomarker literature, suggesting EV-enriched profiling may be better suited for mechanistic studies. Together, these findings provide a methodological foundation for translating circulating miRNA discovery into clinical risk stratification in CDH, with ongoing work aimed at resolving tissue-specific signals and pathways relevant to CDH pathogenesis.

Manual Tissue Slicer Designed for Ovarian Tissue Cryopreservation

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Abstract Text

Fertility preservation strategies, such as ovarian tissue cryopreservation and subsequent autotransplantation, offer critical options for patients at risk of or experiencing primary ovarian insufficiency. A fundamental requirement for these approaches is the preparation of uniform cortical slices from fresh ovarian tissue, typically 0.5–1.5 mm in thickness, that preserve follicular architecture and stromal integrity before cryopreservation. However, the discontinued production of the Stadie-Riggs slicer, the primary tool previously used for fresh tissue slicing, has limited the progress of ovarian tissue cryopreservation in translational research and clinical application. Therefore, a compact, manual ovarian tissue slicer was designed and specifically optimized to address this unmet need. The slicer was constructed using biocompatible 316L stainless steel and borosilicate glass, with overall dimensions of 8 × 8 × 2.5 cm and a mass of 0.6 kg. The device is compatible with No. 36 disposable surgical scalpel blades and is sterilizable via either ethylene oxide gas or autoclaving (up to 132 °C), ensuring suitability for research and clinical environments. To evaluate slicer performance, bovine ovaries were collected from a local slaughterhouse. The corpus luteum was removed prior to tissue processing. A portion of the ovarian cortex was immediately fixed in 4% paraformaldehyde as an unsliced control. The remaining cortex was cut into 10 × 10 × 1 mm slices using the slicer. Each tissue slice was cut into two pieces with one serving as the fresh control and the other undergoing cryopreservation. The fresh control and cryopreserved-warmed tissue pieces were fixed in 4% paraformaldehyde. All tissue samples were paraffin-embedded, sectioned, and stained with hematoxylin and eosin for evaluation using light microscopy. The tissue slicer design is user-friendly with intuitive assembly, disassembly, and cleaning instructions. Slices with ~1 mm thickness were consistently obtained from fresh bovine ovarian cortex using the slicer. Fresh control slices retained typical ovarian tissue morphology similar to that of the unsliced tissue, including condensed fibrous tunica albuginea and intact primordial, primary, and secondary follicles with minimal mechanical disruption. The stromal tissue and follicle integrity, as well as cryodamage observed, in cryopreserved-warmed tissues were comparable to results from previously published ovarian tissue cryopreservation studies using large animal models. These findings demonstrate that the manual slicer built in this study can consistently generate high-quality ovarian cortical slices, a reliable and reproducible performance suitable for ovarian tissue cryopreservation. This newly developed tool holds significant potential to advance fertility preservation for female patients by applying a standardized procedure in a timely manner for ovarian tissue slicing. Additionally, the slicer provides a valuable platform for ovarian biology and physiology research involving ovarian tissue culture.

Bisphenol A Exposure Alters Testicular Cell Proportions in Medaka: Single-Cell Annotation Reveals 13 Distinct Cell Types

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Abstract Text

Bisphenol A (BPA) is a widespread endocrine-disrupting chemical associated with adverse effects on male reproductive health. Although BPA has been linked to defects in spermatogenesis and testicular function, the extent to which specific testicular cell populations respond to exposure is not yet fully resolved. To address this gap, we used medaka (*Oryzias latipes*) as a vertebrate model and performed single-cell RNA sequencing (scRNA-seq) on control and BPA-treated testis samples using the Parse Biosciences single-cell RNA sequencing workflow. Raw sequencing data were processed with the Parse Biosciences Trailmaker pipeline aligned to the medaka reference genome. Parse quality control metrics indicated strong data quality and robust single-cell recovery. The control sample yielded 5,229 high-quality cells from 56,315,523 reads (median 1,107 transcripts, 782 genes per cell), while the BPA-treated sample yielded 3,442 cells from 34,286,936 reads (median 887 transcripts, 654 genes per cell). Sequencing saturation was 50.8% in both samples, and barcode/cDNA Q30 scores exceeded 0.90. The datasets were CCA-integrated in Seurat using graph-based clustering (resolution 0.5) and UMAP dimensionality reduction. Integrated clustering resolved 12 testicular cell populations and revealed significant differences in cellular composition between conditions. BPA-treated testis showed increased proportions of preleptotene spermatogonia (36.3% vs 30.9% in control), undifferentiated type A spermatogonia (13.0% vs 11.5%) and macrophages (3.3% vs 1.7%), but decreased proportions of diplotene/diakinesis spermatocytes (4.2% vs 6.8%) and Leydig cells (4.2% vs 9.9%). Cell identities were assigned by comparing cluster-specific marker expression with published markers from orthologous species and medaka orthology mapping, enabling annotation of major germ-cell stages (undifferentiated spermatogonia, preleptotene spermatogonia, primary/diplotene spermatocytes, round/elongating spermatids, mature spermatozoa) and somatic populations (Leydig cells, peritubular myoid/stromal cells, macrophages, erythroid progenitors, testicular neurons). These findings reveal BPA-associated shifts in testicular cell proportions and provide a high-resolution single-cell reference of medaka testis cell types for future studies of endocrine disruption effects. *AI-assisted tools were used to support code development and manuscript editing. All content was reviewed and verified by the authors, who take full responsibility for all data analysis, interpretation, and conclusions.* The study was funded by the National Institutes of Health (R01ES032452 & R21ES027123) to RKB.

Spatiotemporal Patterns of Vascular Remodeling Across the Estrous Cycle

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Abstract Text

Despite the absence of menstruation, the murine uterus undergoes significant remodeling and regeneration across the estrous cycle. Deficiencies in regenerative capacity of the endometrium have been correlated with poor outcomes for fertility and pregnancy. While the regenerative capacity of the epithelium is better understood, the regenerative capacity of other networks, like the uterine vasculature, remain almost completely unknown. Understanding how blood vessels participate in and are affected by hormone-mediated cyclic remodeling is instrumental to the field of reproductive science. We recently performed a first-of-its-kind high-resolution spatial transcriptomics array on uterine sections at all four stages of the rodent estrous cycle (proestrus, estrus, metestrus, and diestrus). From this, a novel endothelial cell population was identified, distinct from artery and vein endothelium. Unique transcriptional signatures for proliferation were upregulated in this population, along with normal expression of pan-endothelial markers. Interestingly, the spatial arrangement of these cells was dynamic: positioned near the myometrium at proestrus and estrus and toward the endometrium at metestrus and diestrus. This shift in where angiogenesis is happening through the estrous cycle implies different oxygenation, metabolic, or immune cell needs in different areas of the uterus depending on cycle stage. Preliminary immunofluorescence data suggest that protein expression closely matches the gene expression data obtained from the spatial array. We observed myometrial endothelial cells exhibited relatively stable levels of proliferation, but endometrial proliferation increased in metestrus and diestrus. Further, more Vcam1 protein was expressed on vessels in diestrus compared to proestrus, indicating possible immune cell recruitment and endothelial activation at this stage. We now hope to demystify the molecular mechanisms and physiological effects exhibited by this cell population. In the context of uterine remodeling, several biological processes come to mind. We hypothesize that proliferative endothelium could be facilitating remodeling through monocyte and neutrophil recruitment and extravasation into degenerating endometrial tissue, or it could be preparing the uterus for future embryo implantation and pregnancy demands by preemptive distribution of blood vessels to the luminal epithelium and uterine muscles. While angiogenesis during tissue remodeling may be a given, patterns of spatiotemporal organization of proliferating blood vessels have yet to be described. This work marks an exciting new way to consider the mechanisms of uterine remodeling.

RNAs Undergo the Soluble-to-Insoluble Phase Transition during Vertebrate Oocyte-to-Embryo Transition.

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Abstract Text

The oocyte-to-embryo transition (OET) is one of the most important developmental transitions. During the OET, transcription is largely inactive, and development is regulated primarily by maternally deposited RNAs and proteins through post-transcriptional mechanisms. How maternal products are precisely remodeled to dictate the OET remains largely unclear.

To investigate this process, we developed a centrifugation-based fractionation approach to characterize RNA solubility states during the OET. In this assay, RNAs associated with a limited number of RNA-binding proteins remain in the supernatant and are defined as “soluble,” whereas RNAs sequestered in membrane-less protein condensates resist detergent extraction and accumulate in the pellet, representing an “insoluble” state. Using fractionation RNA-seq, we previously identified transcripts undergoing soluble-to-insoluble (S-I) and insoluble-to-soluble (I-S) phase transitions during *Xenopus* OET.

Here, we extend this framework to zebrafish and perform fractionation RNA-seq across the OET. By integrating multi-omics datasets, including poly(A)-tail sequencing, ribosome profiling, and N6-methyladenosine (m⁶A) RIP-seq, we systematically investigate the cis-regulatory features underlying RNA phase transitions. Our results reveal that RNA solubility dynamics are evolutionarily conserved during vertebrate OET. While ribosome association during translation promotes the S-I transition of many mRNAs, a subset of transcripts undergoes phase transitions through ribosome-independent mechanisms. Further analyses suggest that specific sequence motifs and m6A modifications contribute to the regulation of RNA phase behavior.

Together, these findings support a model in which RNA solubility is governed by a coordinated cis-trans regulatory code and represents a conserved layer of post-transcriptional control during early embryogenesis. Ongoing work aims to identify the trans-acting factors that mediate these transitions and to define their functional roles in developmental regulation.

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Comparative analysis of pluripotency in bovine embryonic stem cells of *Bos taurus* and its hybrid with *Bos indicus*

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Abstract Text

Bos indicus (BI) and *Bos taurus* (BT) have distinct evolutionary and domestication histories, leading to pronounced differences in stress tolerance and environmental adaptation. Crossbred *Bos indicus* × *Bos taurus* (BI × BT), hereafter referred to as CIT cattle (e.g., Girolando and Charbray), are economically important for heat tolerance and valuable for genomic imprinting studies. However, research on bovine pluripotent stem cells has been predominantly confined to the BT lineage. To address this gap and investigate whether divergent parental genomes integrate to achieve proper pluripotency, we established embryonic stem cell (ESC) lines from CIT embryos.

Specifically, we generated cross-bred blastocysts by fertilizing BT oocytes with BI sperm. The embryos were cultured on mitomycin C-treated mouse embryonic fibroblasts (MEFs), yielding 12 independent bESC lines from 12 blastocysts, including one line that has been maintained for over 30 passages and indicating long-term self-renewal capacity. To systematically assess the impact of genetic background and culture conditions on pluripotency, we selected two previously established and well-characterized BT lines and two newly derived CIT lines for comparative analysis. These cell lines were cultured under two distinct medium conditions: a published formulation (TIF2) and a newly developed N2B27-based medium (TIF3), each combined with two different feeder layers: MEFs and bovine fetal fibroblasts (BFFs), with BT-ESC cultured on MEFs and in TIF2 regarded as the control.

Both BT- and CIT-bESCs formed compact, dome-shaped colonies characteristic of naïve pluripotency, with no detectable morphological differences between media or feeder types. All bESC lines exhibited strong alkaline phosphatase (AP) activity and robust nuclear expression of core pluripotency transcription factors (OCT4, SOX2, and NANOG characterized by immunofluorescence). Upon embryoid body (EB) induction, both BT- and CIT-bESCs differentiated into derivatives of the three germ layers, as confirmed by the expression of TUJ1 (ectoderm), SMA (mesoderm), and GATA6 (endoderm). These results demonstrated that CIT-bESCs were pluripotent and preserved multilineage differentiation capacity. Proliferation analysis revealed that the control condition exhibited significantly slower growth than any of the treatment groups in pairwise comparisons. However, when only breed was considered, there was no significant difference between BT- and CIT-derived bESCs ($p>0.05$). Quantitative RT-PCR analysis showed comparable expression levels of key pluripotency genes, including *OCT4*, *SOX2*, *NANOG*, *LIN28*, *KLF4*, and *CDH1*. Although individual genes displayed variable expression levels depending on medium and feeder type, no systematic differences were detected between BT- and CIT-bESCs (2-way ANOVA, $p>0.05$).

Collectively, these data demonstrate that CIT-bESCs exhibit comparable pluripotency characteristics to those of BT-bESCs, and that TIF3 medium and BFF feeders support bovine pluripotency as effectively as the TIF2 and MEF culture system. This study establishes an in vitro model for understanding how crossbred genetic influence the cell pluripotency and developmental capability.

Interindividual Variability of Sperm DNA Methylation is Associated with Reduced Fecundability in Normospermic Men Attempting Natural Conception

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Abstract Text

Sperm Epigenetic Variability Outliers and Stochasticity (EVOS) captures the accumulation of deviations from normal site-specific DNA methylation patterns throughout an individual's epigenome. The aim of this study was to investigate the association between the sperm EVOS and couples' fecundability in the general population.

Sperm EVOS was calculated as the sum of sperm DNA methylation CpG outliers on the Illumina EPIC (v1) array in the general population cohort: the Longitudinal Investigation of Fertility and the Environment (LIFE). Downstream analysis was restricted to couples with normospermic men based on 5th percentile values from WHO 2010 and no other male-factor infertility diagnosed ($n = 277$). The association of sperm EVOS (continuous log-transformed or categorized by quartiles) with time to pregnancy was examined using the Cox proportional hazards model (CoxPH) and Kaplan-Meier survival analysis (KM). Pregnancy odds ratios for EVOS quartiles at the 3rd, 6th, and 12th months were assessed using logistic regression. Models were performed with and without adjustments for potential confounders, including male and female age, BMI, smoking status, and average methylation at the DLK1 region as an indicator for potential somatic cell contamination.

Of the 277 couples, 233 continued in the study for one year, with 8% failing to conceive at the 12th month of attempting. Sperm EVOS displayed a right-skewed distribution, with median numbers of EVOS CpGs of 640. In both crude and adjusted CoxPH models, sperm EVOS was associated with reduced fecundability of couples attempting natural conception (Fecundability Odds Ratio (FOR) = 0.82, 95% CI: 0.68, 0.99; $p = 0.04$). Furthermore, for couples with EVOS in the highest quartile (Q4), we observed 46% reduction in per-cycle pregnancy probability (FOR = 0.54, 95% CI: 0.36, 0.81; $p = 0.003$), compared to Q1. KM analysis revealed that couples in the Q4 of EVOS had a longer time to pregnancy compared to those in the middle (Q2+Q3) and lowest (Q1) quartiles ($p = 0.026$). Pregnancy odds ratios for couples in the highest sperm EVOS quartile indicated 65%, 70%, and 73% reduction in conception probabilities at 3, 6, and 12 months, respectively, compared to men in Q1.

In conclusion, our findings indicate that sperm EVOS represents a novel measure of DNA methylation variability and is associated with a reduced likelihood of conception and a longer time to pregnancy among couples from the general population, including couples in which men are classified as having normal semen parameters. These findings highlight the importance of maintaining stable sperm epigenetic profiles in achieving pregnancy.

Biomarker-Informed Flow Cytometric Sorting for Selection of Morphologically Normal Sperm

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Abstract Text

Semen processing frequently encounters ejaculates with elevated sperm morphological abnormalities, particularly in beef sires, resulting in discarded collections and reduced availability of usable semen. Current quality control relies on threshold-based acceptance of whole ejaculates, preventing recovery of functional sperm from otherwise unusable samples. Methods that enrich morphologically normal, fertilization-competent sperm rather than rejecting entire ejaculates could improve semen utilization and genetic progress. Flow cytometry was developed in the 1970s and later adapted for bovine sperm sex selection; however, morphology-directed applications remain largely unexplored. Recent advances in high-resolution and image-based cytometry provide an opportunity for next-generation morphology sorting. The objective of this study was to determine whether fluorescence biomarkers and flow cytometric sorting can isolate morphologically abnormal sperm to enable enrichment of normal populations. Peanut agglutinin conjugated to Alexa Fluor 647 (PNA-AF647) was used to identify sperm with disrupted or ruffled acrosomes. Labeled sperm populations were gated and sorted using BD FACSDiscover S8 instruments and verified by 40× differential interference contrast microscopy (n=3 bulls). Sperm with ruffled acrosomes were identified with 89.5% accuracy (range 81.3-96.1%). Next, CellMask Orange was used to differentiate sperm head shape. Using the eccentricity image feature, pyriform and normal sperm heads were segregated into two separate populations with >95% purity rates. Biomarkers targeting cytoplasmic droplet retention and mitochondrial midpiece integrity as well as purity of sorted populations will be evaluated in follow-up studies. These results demonstrate the feasibility of biomarker-informed morphology sorting to enrich fertilization-competent sperm. Long-term investigations could include proteomics to understand fertilization difference between normal and abnormal populations. Integration of morphology sorting into existing sex-sorted semen production pipelines could further improve the purity of fertilization-competent sperm, reduce discarded ejaculates, and increase pregnancy outcomes and producer profitability. This project was supported by the National Association of Animal Breeders (NAAB) through the Doak Fellowship Program (M.R.) and 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.

Tisseel-Induced Sterile Lesions in the Non-Human Primate Maternal-Fetal Interface Disrupt Decidual NK Cell Networks

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Abstract Text

Placental inflammation, encompassing villitis of unknown etiology (VUE), thrombotic malperfusion, and related pathologies, complicates 5-25% of pregnancies and has no targeted treatment. Within the Developmental Origins of Health and Disease framework, adverse *in utero* immune exposures are linked to childhood asthma and inflammatory bowel disease. While systemic maternal immune activation is mechanistically coupled to fetal immune programming through inflammatory cytokine cascades, the consequences of local, sterile placental inflammation on fetal immune development remain poorly understood. To fill this gap, we have developed a model of placental inflammation in the rhesus macaque (*Macaca mulatta*).

Tisseel fibrin sealant at 250 μ L (n = 3) or 725 μ L (Tisseelx3, n = 3) was injected into the intervillous space, under ultrasound guidance, of pregnant (~GD90) rhesus macaques. Saline-injected animals served as controls (n = 4). C-sections were performed at ~GD155 (term = GD165). Decidual mononuclear cells (MCs) were isolated by mechanical and enzymatic dissociation. Single-cell RNAseq was performed using the Parse Bioscience Evercode Platform. Resulting data were aligned to the rhesus macaque reference genome and processed using Seurat and CellChat pipelines. Differential cluster abundance was assessed using negative binomial generalized linear models with BH-FDR correction. All reported p-values are BH-FDR adjusted.

Tisseel treatment did not alter overall immune cell distribution but produced significant changes in decidual NK cell subsets. Immature NK cells were markedly expanded in Tisseel vs Saline (log2FC = 2.54, p = 0.032), while Cytotoxic NK cells were suppressed (log2FC = -3.27, p = 0.005). Tisseelx3 showed divergent effects - suppression of mature tissue-resident dNK1 cells (log2FC = -1.49, p < 0.0001) without immature NK induction - consistent with fibrin resorption kinetics at the higher dose. CellChat analysis revealed that Tisseel uniquely induced stromal-to-dNK1 communication via the SEMA3A-NRP1-PLXNA2 axis, absent in both Saline and Tisseelx3, alongside upregulation of extracellular matrix remodeling pathways including periostin and fibronectin signaling.

These data demonstrate that Tisseel-induced sterile lesions produce persistent, dose-dependent remodeling of the decidual NK cell compartment and stromal-immune crosstalk. We propose this NHP model as a reproducible platform for studying sterile placental inflammation, analogous to VUE and thrombotic malperfusion, to define how local immune activation shapes fetal immune development.

Transient Mild Scrotal Hyperthermia Modifies Epididymal Extracellular Vesicles, Suggesting a Role in Maintaining Sperm Homeostasis During Epididymal Maturation

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Abstract Text

Elevated environmental temperatures reduce reproductive performance in mammals. In swine, the scrotum needs to be maintained 2-8°C below abdominal temperature for males to produce high-quality semen. The mechanisms of how scrotal hyperthermia affects sperm production are not clear. Preliminary experiments in which scrotal insulation was used to elevate the scrotal temperature showed that the abundance of 4 proteins (SPATA18, TOMM34, VDAC1, and GPX4) was increased 2-5 fold as early as 6 days after insulation removal. The collected sperm would have been in the epididymis during scrotal insulation, suggesting that epididymal maturation was affected by scrotal hyperthermia, but there are few studies of the heat sensitivity of the epididymis.. Epididymal extracellular vesicles (EVs) fuse with sperm and add proteins as they move through the epididymis, allowing sperm to gain the ability to be motile and fertile. All 4 proteins that are elevated following hyperthermia are membrane proteins and may be secreted by the epididymis via EVs and added to sperm. Therefore, we hypothesized that the change in sperm protein profile following hyperthermia may be due to heat stress on the epididymis, affecting the secretion of EVs, causing alterations in EV protein content, or affecting the interactions of EVs with sperm. We collected caput and cauda epididymal fluid and isolated EVs by ultracentrifugation. The average diameter of the caput and cauda EVs was 133.1±47.3 nm and 105.3±40.5 nm and they contained the EV marker proteins CD81 and CD9. After characterizing the EVs, we performed western blots for EV and epididymal sperm protein lysates and found that all 4 proteins that were elevated following hyperthermia are present in EVs and sperm collected from both that caput and cauda epididymis. This suggests that those 4 proteins are added to the sperm via epididymal EVs. To further investigate the role of EVs under heat stress, caput and cauda epididymal EVs, fluid, and sperm were collected at 0, 3, and 6 days following scrotal insulation. Comprehensive analyses revealed that sperm metabolomic profiles were unchanged and sperm proteomic alterations were minimal in response to heat stress. In contrast, EV proteomic profiles showed significant alterations. Gene Ontology (GO) analysis of differentially expressed EV proteins indicated enrichment of pathways associated with oxidative stress response, inflammatory response, regulation of gene expression, mRNA stability, and protein synthesis. Analysis of EV-sperm interactions using imaging flow cytometry showed that EVs mostly interact with sperm head compared to the tail. Further, heat stressed EVs had a modest increase in interactions with caput sperm compared to control EVs. Collectively, these findings suggest epididymal EVs as the major epididymal component responding to mild heat stress. The differences between EV and sperm responses to mild heat stress combined with increased EV–sperm interactions and enrichment of stress related pathways in EVs, suggest a potential role for epididymal EVs in the recovery of sperm and the epididymis from heat stress and in maintaining sperm homeostasis during epididymal maturation. This work was supported by USDA.

Perfluorobutane Sulfonic Acid Exposure Increases Ovarian Follicular Atresia and Disrupts Steroidogenic, Inflammatory, and Metabolic Protein Signaling in the Ovary and Uterus

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Abstract Text

Perfluorobutane sulfonic acid (PFBS) is increasingly used as a purportedly safer alternative to perfluorooctane sulfonate (PFOS), yet its reproductive toxicity remains poorly defined. The hypothesis that PFBS disrupts ovarian and uterine function by altering key steroidogenic, metabolic, and inflammatory signaling pathways was thus tested. Seven-week-old female C57BL/6J mice were exposed to saline or PFBS (2.5 mg/Kg BW) per os for 15 d. The ovarian and uterine protein abundance of cytochrome P450 (CYP) family 1, subfamily A, polypeptide 1 (CYP1A1), CYP family 1, subfamily B polypeptide 1 (CYP1B1), CYP family 11, subfamily A, polypeptide 1 (CYP11A1), CYP family 19, subfamily A, polypeptide 1 (CYP19A1), peroxisome proliferator-activated receptor (PPAR) α (PPAR α), δ (PPAR δ), γ (PPAR γ), and Toll-like receptor 4 (TLR4) were quantified by western blotting and unadjusted unpaired t-test. In the ovary, PFBS exposure tended to increase ($P \leq 0.1$) ovary weight and the abundance of CYP1A1 and CYP19A1 but tended to decrease ($P \leq 0.1$) the abundance of TLR4 protein. PFBS also increased ($P \leq 0.05$) the number of atretic follicles, indicating impaired follicular health. In the uterus, PFBS decreased ($P \leq 0.05$) PPAR γ and CYP11A protein abundance, without altering uterine weight ($P \geq 0.1$). Collectively, these findings demonstrate that PFBS is not biologically inert and may disrupt reproductive function by modulating steroidogenic enzymes and signaling pathways in both the ovary and uterus. This study raises important concerns about the reproductive safety of PFBS and highlights the need to re-evaluate short-chain PFAS as “safer” alternatives.

Natural Genetic Variation in Damage Tolerance—Rather Than Repair Capacity—Is a Primary Determinant of Chemotherapy-Induced Oocyte Loss

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Abstract Text

Chemotherapy-induced premature ovarian insufficiency (POI) affects 8–40% of young female cancer survivors. The alkylating agent cyclophosphamide (CTX), widely used to treat malignancies and immune disorders, is particularly ovotoxic and can deplete the immature primordial follicle (PF) pool that constitutes the finite ovarian reserve. Despite increasing understanding of CTX-induced ovarian damage, we cannot predict which patients are at higher risk of POI. Genetic variation influences drug response in other contexts, yet the genes and pathways driving inter-individual differences in ovarian toxicity remain unknown. Previous studies focused primarily on drug metabolism—particularly hepatic CYP450 enzymes—but the complete picture likely involves DNA damage recognition and repair, and cellular quality control mechanisms. Here, we leverage the natural genetic diversity of laboratory mouse strains to dissect the molecular and genetic basis of differential ovarian response to CTX. C57BL/6J (B6) and CAST/EiJ (CAST) exhibit strikingly different primordial follicle outcomes: all PF oocytes are depleted in B6 ovaries following *in vivo* CTX treatment, whereas they survive in CAST. Importantly, CAST primordial follicles are also resistant to the CTX analog mafosfamide *ex vivo*, ruling out differences in hepatic drug metabolism. DNA damage markers confirm that CTX induces damage in both strains, indicating that PF survival in CAST reflects genetically determined differences in the cellular response rather than differential drug exposure. By comparing ovarian bulk transcriptomes between the CTX-sensitive (B6) and CTX-resistant (CAST) strain at baseline, 6h, and 18h after exposure, we identified co-regulated gene modules underlying divergent follicle fates. Both strains activate p53 transcriptional targets, but B6 mounts stronger apoptotic response over time (*Pmaip1* logFC: +1.1 at 6h to +3.2 at 18h; *Cdkn1a*: +1.7 to +3.2), than CAST (*Pmaip1*: +0.6 to +1.2; *Cdkn1a*: +0.4 to +1.0). B6 also uniquely upregulates *Fas* (+0.99) and *Casp3* (+0.24), engaging both intrinsic and extrinsic death pathways, while CAST downregulates pro-apoptotic BH3 members *Bad* (-0.47) and *Bak1* (-0.39), effectively raising the apoptotic threshold. Unexpectedly, CAST expresses lower baseline levels of most DNA repair genes: 34 of 56 assessed are lower (22 significantly; e.g., *Fancc* -0.73, *Xrcc3* -1.06, *Exo1* -1.09, *Lig4* -1.25), and CTX-induced repair upregulation is also weaker. Strikingly, *Chek2*—the kinase that activates the oocyte-specific Tap63 apoptotic response—is expressed at roughly half the B6 level at baseline (logFC = -1.02, FDR = 1.6×10^{-8}) and remains low after treatment. In parallel, CAST uniquely activates the Kit/PI3K-Akt/Foxo3 dormancy-preservation axis (*Kit* +0.63, *Foxo3* +0.35, *Tsc1/Tsc2* +0.50/+0.34, *Rictor* +0.60, *Akt3* +0.39), maintaining primordial follicle quiescence. Together, these findings argue that DNA repair capacity is not the limiting factor for PF survival after CTX. Instead, oocyte fate is determined by the apoptotic threshold set by the CHEK2 checkpoint and other death/survival related pathways. B6 oocytes, with high CHEK2 expression, rapidly activate Tap63/p53-dependent death before repair can complete, while CAST oocytes tolerate comparable damage through restrained apoptosis and active dormancy signaling, buying time for repair. CAST/EiJ carries 173 private SNPs in *Chek2* gene including a missense variant, a splice-site variant, a dense 3' UTR cluster, and promoter variants—any of which could reduce CHEK2 expression or activity and will require experimental validation. Our results establish that natural genetic variation in damage tolerance—rather than repair capacity—is a primary determinant of chemotherapy-induced oocyte loss and identify the CHEK2–Tap63 axis as a candidate pathway for genetic prediction of ovarian toxicity in cancer patients.

Psilocybin Exposure Adversely Impacts Male Reproductive Health in a Rhesus Macaque Model

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Abstract Text

Psilocybin use is increasing in both clinical and non-clinical settings, especially among individuals of reproductive age. However, the effects of psilocybin use on male reproductive health remains largely unknown. To address this, we leveraged our first-in-kind rhesus macaque model of chronic psilocybin use recapitulating human microdosing (n = 6) and a single-case experimental design where male rhesus macaques served as their own controls. Males were exposed to increasing oral doses of psilocybin (0.1 and 0.2 mg/kg), and testicular volume was assessed longitudinally using scrotal ultrasound. Semen samples were collected in triplicate samples every few days prior to each dose increase and analyzed using computer-assisted sperm analysis (CASA), with additional staining to assess sperm viability, mitochondrial activity, and acrosome integrity.

Psilocybin exposure was associated with a dose-dependent reduction in testicular volume, with a 12% decrease at 0.1 mg/kg (p = 0.18) and a significant 40% decrease at 0.2 mg/kg (p = 0.0166). Semen quality was similarly impaired at 0.2 mg/kg, with a ~27% reduction in sperm viability (p < 0.0001), a 23% reduction in mitochondrial activity (p < 0.0001), and a 52% increase in liquid fraction volume (p = 0.0102). Acrosome integrity showed a 3% decline that did not reach statistical significance (p = 0.088). Conventional CASA parameters including sperm concentration, total motility, and progressive motility were not significantly altered.

These findings provide the first evidence that psilocybin exposure adversely affects male reproductive health, demonstrating both structural and functional changes within a translational microdosing paradigm. Future studies are needed to determine whether psilocybin use may affect key male reproductive hormones and time to conception.

Carbendazim Induces Cytotoxicity and Mitochondrial Dysfunction in Bovine Mammary Epithelial Cells

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Abstract Text

Carbendazim is a benzimidazole-type fungicide targeting fungal diseases in various crops, fruits, and vegetables. Due to its extensive use in agriculture, its metabolites and residues are frequently detected in environmental media, including soil and water. Recent studies have highlighted risks associated with its embryotoxicity and reproductive toxicity, along with its potential carcinogenicity in humans. However, its specific effects on bovine mammary epithelial cells (MAC-T) have not yet been elucidated. This study demonstrated that carbendazim treatment reduces the viability of MAC-T cells in a concentration-dependent manner and significantly increases early apoptotic cell death. Furthermore, relative levels of cytosolic and mitochondrial calcium ions were markedly elevated by carbendazim exposure in MAC-T cells. Additionally, carbendazim exposure caused a collapse of the mitochondrial membrane potential, a marker of mitochondrial function and induced cell cycle arrest at the G2-M phase. These findings suggest that carbendazim exerts cytotoxic effects on bovine mammary cells by disrupting mitochondrial homeostasis and regulating the cell cycle.

Phthalate Mixture, but Not Individual Phthalates, Disrupts Uterine Epitranscriptomic Homeostasis and Causes Severe Dysfunction

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Abstract Text

Phthalates are ubiquitous endocrine-disrupting chemicals (EDCs) commonly used in plastics, cosmetics, and personal care products. Human exposure occurs primarily through diet and typically involves complex mixtures of phthalates rather than individual compounds. Importantly, phthalate metabolites are detected in nearly all urinary samples from women, underscoring the widespread nature of exposure. Although previous studies have examined the effects of phthalates on ovarian, renal, and pulmonary physiology, comparatively little is known about their impact on uterine function. In this study, we investigated the effects of chronic exposure to an environmentally relevant phthalate mixture, as well as the individual effects of di(2-ethylhexyl) phthalate (DEHP) and di-isononyl phthalate (DiNP), on uterine homeostasis. Female CD-1 mice were exposed through dietary incorporation for 6 months and 1 year at doses of 0.15 ppm and 1.5 ppm, representing environmentally relevant and occupational exposure levels, respectively, while control mice received corn oil as a vehicle. Uterine tissues were analyzed using histological, immunohistochemical, and molecular approaches to assess fibrosis, hyperplasia, and expression of estrogen-responsive and extracellular matrix-associated genes. Chronic exposure to the phthalate mixture induced severe uterine pathology characterized by increased myometrial thickness, excessive collagen deposition, and a marked increase in endometrial gland number, consistent with fibrotic remodeling and uterine hyperplasia. In contrast, exposure to DEHP or DiNP individually did not produce comparable pathological changes, suggesting that phthalate mixtures exert unique and potentially synergistic effects on uterine physiology. Mechanistically, exposure to the phthalate mixture caused a significant disruption of the uterine epitranscriptomic landscape. Specifically, we observed upregulation of the m6A “writer” proteins METTL3 and RBM15, accompanied by downregulation of the m6A “eraser” ALKBH5, alterations that were absent following exposure to DEHP or DiNP alone. These epitranscriptomic changes were associated with activation of Wnt/ β -catenin signaling, a pathway linked to abnormal differentiation, inflammation, and fibrosis. Collectively, our findings demonstrate that chronic exposure to an environmentally relevant phthalate mixture, but not individual phthalates, disrupts uterine m6A regulatory pathways and activates β -catenin signaling, leading to severe uterine dysfunction and emphasizing the importance of evaluating mixture-based EDC exposures in reproductive health studies.

Exposure to Concentrated Ambient Fine Particulate matter (PM_{2.5}) in Wild Type and Human Amyloid-beta-knock-in Mice: Impacts on Reproductive and Neurological Endpoints

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Abstract Text

Our prior work showed that exposure to ambient PM_{2.5} decreased the ovarian follicle reserve in females; others reported PM_{2.5} exposure decreased epididymal sperm counts in male mice. Epididymal sperm counts are expected to recover due to the presence of testicular germline stem cells, while the post-natal ovary lacks germline stem cells. We hypothesized that 1) PM_{2.5} exposure permanently depletes the ovarian follicle reserve and has reversible effects on testicular sperm production; 2) PM_{2.5} exposure induces inflammatory changes in the brains of both males and females that contribute to neurodegeneration and that ovarian follicle depletion caused by PM_{2.5} enhances Alzheimer's pathology progression.

We exposed 2-months old, late adolescent, male and female wild type (WT) C57BL/6J mice and mice that express the human amyloid- β protein sequence (hA β -KI mice) to concentrated ambient PM_{2.5} (average concentration $169 \pm 113 \mu\text{g}/\text{m}^3$) or filtered air ($1.9 \pm 1.2 \mu\text{g}/\text{m}^3$) for 5h/day, 4d/wk for 12 weeks. Mice were euthanized 24h after the last exposure at age 5 mos or 9 mos after the last exposure at age 14 mos. Females of both strains exposed to PM_{2.5} and euthanized at age 5 mos had statistically significantly decreased total number of ovarian follicles/ovary ($P < 0.001$, effect of exposure), as well as primordial ($P = 0.002$) and antral follicles/ovary ($P = 0.004$), while the decrease in primary follicles approached significance ($P = 0.089$). Automated follicle counts were performed on cleared ovaries using wholemount imaging with antibody against oocyte cytoplasmic-specific protein MSY2. Cauda epididymal sperm counts were significantly decreased 24h after the last exposure to PM_{2.5} at age 5 mos ($P = 0.009$). By contrast testicular sperm counts and seminiferous tubule histology were not significantly altered by exposure to PM_{2.5} in either strain at age 5 mos, nor were cauda epididymal sperm counts at 9 mos after exposure, age 14 mos. Behavioral testing for object recognition memory (ORM) at 14 mos revealed no main effects of PM_{2.5} exposure, sex, or genotype. X-maze behavioral testing at age 5 mos revealed no statistically significant effects of PM_{2.5}, sex, or strain in color mode or contrast mode. However, PM_{2.5} exposure significantly increased time spent in contrast mode path 1 and decreased time spent in path 3, a pattern reported in double transgenic Alzheimer's mice. Immunofluorescence of cortical and hippocampal expression of amyloid precursor protein C-terminal, amyloid plaques, microglia related proteins Iba1, CLEC7A, Tmem119, or GFAP, astrocyte secreted C3, presynaptic cytomatrix protein Bassoon, or postsynaptic scaffold protein Homer1 revealed no statistically significant effects of exposure, strain or sex. By contrast, colocalization of Bassoon and Homer1 in the hippocampus, but not the cortex, was found to increase significantly with PM_{2.5} exposure ($P = 0.011$). Colocalization of these proteins has been reported to increase after treatment with tetrodotoxin or NMDA receptor antagonists, suggesting that increased colocalization may be an indicator of decreased synaptic function. Conclusions: 1. Exposure to PM_{2.5} during adolescence significantly depleted the ovarian follicle reserve in WT and hA β -KI mice, and significantly decreased cauda epididymal sperm counts at 24 h after the end of exposure, while having no significant effects on epididymal counts 9 mos post-exposure or on testicular sperm counts. 2. PM_{2.5} exposure increased co-localization of Bassoon and Homer1 in the hippocampus, which may be associated with synaptic dysfunction. 3. There was a statistically significant effect of PM_{2.5} exposure on behavior in the x-maze contrast mode test at 9 mos after the exposure ended.

Exposure to Diethyl Phthalate Does Not Alter Key Regulators of Ovarian Function in Primary Mouse Granulosa Cells

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Abstract Text

Diethyl phthalate (DEP) is a phthalate used as a plasticizer in a wide range of products including cosmetics, medical tubing, and food packaging. Our lab has previously shown that a human relevant mixture of phthalates negatively affects the female reproductive system. Although DEP is the main component of the mixture, limited data exist regarding its individual effects on ovarian health. Thus, study tested the hypothesis that exposure to DEP alters granulosa cell gene expression and sex steroid hormone levels in vitro. Ovaries were collected from CD-1 mice between 21 and 24 days of age. Granulosa cells were collected and incubated for 96 hours with vehicle control (DMSO) or varying doses of DEP (0.01, 0.1, 1, 10, 100 µg/L). Cells were collected for gene expression analysis via qPCR and media were collected for hormone assays via enzyme-linked immunosorbent assay (ELISA). The results indicated that DEP exposure did not significantly alter expression of cell cycle regulators including cyclin D2 (*Ccnd2*), cyclin-dependent kinase inhibitor 1A (*Cdkn1a*), cyclin E1 (*Ccne1*) or cyclin-dependent kinase 4 (*Cdk4*). DEP also did not affect the expression of markers of steroidogenesis such as hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*Hsd3b1*), and cytochrome P450 family 11 subfamily A member 1 (*Cyp11a1*). However, DEP at the 0.01 µg/L dose borderline increased steroidogenic acute regulatory protein (*Star*) compared to control. DEP also did not significantly affect markers of oxidative stress in the granulosa cells. Although DEP at the 100 µg/L dose, borderline significantly increased the expression of superoxide dismutase 1 (*Sod1*) compared to control, it did not affect other markers of oxidative stress including superoxide dismutase 2 (*Sod2*), kelch-like ECH-associated protein 1 (*Keap1*), nuclear factor erythroid 2-like 2 (*Nfe2l2*), catalase (*Cat*), BCL2-associated agonist of cell death (*Bad*), glutathione peroxidase (*Gpx*), glutathione-disulfide reductase (*Gsr*). DEP also did not affect estradiol levels compared to control. These data indicate that DEP, at human relevant doses, does not independently alter the expression of key functional markers in mouse granulosa cells. Supported by NIH R35ES034988.

PFOS Induces Aberrant Activation of Progenitor Signaling Pathways in TM3 Leydig Cells via Oxidative Stress

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Abstract Text

Environmental endocrine disruptors (EEDs) are well-established environmental pollutants that interfere with hormonal signaling, leading to impaired male reproductive function, reduced testosterone levels, and decreased fertility. Exposure to perfluorooctanesulfonic acid (PFOS), a representative per- and polyfluoroalkyl substance (PFAS), has been associated with declining male fertility and reduced circulating testosterone levels. Leydig cells, the steroidogenic cells responsible for testicular androgen production, represent a primary target of PFOS toxicity. It is not clearly understood whether PFOS exposure-induced oxidative stress impairs Leydig cell function through alterations in their transcriptomic landscape. Moreover, questions remain if EED-induced fertility defects can be prevented or mitigated.

Using TM3 cell line, we examined if PFOS induces oxidative stress-driven DNA damage and cell-cycle dysregulation in Leydig cell *in vitro*, and whether vitamin C co-treatment mitigates these effects. TM3 cells were exposed to increasing concentrations of PFOS (5, 25, and 50 μ M) with and without vitamin C co-treatment. Reactive oxygen species (ROS) generation was assessed using DCFDA fluorescence, DNA damage was evaluated by γ H2AX immunostaining, and cell-cycle progression was analyzed by flow cytometry. Transcriptomic alterations were characterized using bulk RNA sequencing followed by pathway analysis. Pathway enrichment analysis in TM3 Leydig progenitor cells demonstrated dose-dependent effects of PFOS (5–50 μ M) characterized by aberrant activation of PI3K–Akt and MAPK signaling pathways alongside enrichment of oxidative stress-associated pathways. PFOS-induced signaling changes were accompanied by modulation of pathways involved in cytoskeletal organization, cell–matrix interactions, and steroid biosynthesis, suggesting stress-driven reprogramming of Leydig progenitor signaling networks. Vitamin C co-treatment attenuated oxidative stress signaling and normalized PFOS-induced PI3K–Akt and MAPK pathway activation. Collectively, these findings indicate that PFOS promotes oxidative stress-mediated dysregulation of Leydig progenitor signaling rather than physiological activation, with vitamin C being capable of restoring signaling balance relevant to testicular steroidogenic function.

The present study suggests that PFOS exposure resulted in a dose-dependent increase in intracellular ROS levels, accompanied by elevated γ H2AX signaling, indicating increased DNA damage. RNA-sequencing analysis revealed PFOS-induced transcriptional reprogramming of genes involved in oxidative stress responses, DNA damage signaling, and cell-cycle regulation. Notably, vitamin C co-treatment attenuated PFOS-induced ROS production, reduced DNA damage markers, partially restored PFOS-responsive gene expression patterns, suggesting that vitamin C may serve as an widely acceptable micronutrient to mitigate EED-induced testicular injury and male fertility defects.

Di(2-ethylhexyl) Phthalate and Diisononyl Phthalate Alter the Nrf2 Signaling Pathway in the Mouse Ovary

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Abstract Text

Di(2-ethylhexyl) phthalate (DEHP) and diisononyl phthalate (DiNP) are plasticizers used in a wide range of consumer products including building materials, food and beverage containers, and children's toys. This is concerning because DEHP and DiNP can leach out of products and enter the human body. Furthermore, both short-term and long-term exposures DEHP and DiNP have been shown to exhibit endocrine disrupting abilities and lead to early reproductive aging in animal models. However, little is known about the mechanisms underlying phthalate-induced reproductive aging. Previous studies indicate that increased oxidative stress can accelerate the loss of ovarian follicles and lead to reproductive aging. Thus, this study tested the hypothesis that DEHP and DiNP increase oxidative stress in the mouse ovary. Specifically, this study focused on the effects of DEHP and DiNP on the Nrf2 signaling pathway because Nrf2 is a transcription factor that is responsible for regulating the expression of antioxidant proteins such as glutathione peroxidase, glutathione S-transferases, NAD(P)H quinone dehydrogenase 1, and peroxiredoxin-1. To test this hypothesis, female CD-1 mice aged 39-40 days were dosed through the chow with either vehicle control (corn oil), DEHP (0.15, 1.5, 1500 parts per million (ppm)), or DiNP (0.15, 1.5, 1500 ppm) for 1, 6, 9, 12, and 15 months. The ovaries from these mice were then collected immediately post-dosing and subjected to quantitative polymerase chain reaction analysis of the expression of factors in the Nrf2 signaling pathway. At 1 month exposure, DEHP and DiNP at 1500 ppm significantly decreased the expression of NFE2 like bZIP transcription factor 2 (*Nfe2l2*), the gene that encodes the protein NRF2, when compared to control. At 6 months exposure, DEHP and DiNP at 1500 ppm significantly increased the expression of *Nfe2l2* when compared to control. At 9 months exposure, DEHP at 0.15 ppm and 1.5 ppm and DiNP at 1.5 ppm and 1500 ppm significantly increased the expression of *Nfe2l2* when compared to control. At 15 months exposure, DEHP at 0.15 ppm and 1.5 ppm and DiNP at 1.5 ppm and 1500 ppm significantly increased the expression of *Nfe2l2* when compared to control. Collectively, our data indicate that DEHP and DiNP exposure significantly affect the Nrf2 signaling pathway in the ovary. Given that the Nrf2 signaling pathway is crucial for regulating oxidative stress in the ovary, these data suggest that long term exposure to DEHP and DiNP can significantly affect oxidative stress pathways in the ovary. Supported by NIH T32 ES007326 and NIH R01 ES034112.

Wildfire emitted particulate matter induces ovarian hyperandrogenism through aryl hydrocarbon receptor activation

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Abstract Text

Wildfires have become more frequent and intense over the past decades, a trend expected to continue. Recent wildfires in California and Canada have significantly affected the air quality across vast areas in the US. Wildfire-generated particulate matter 2.5 (WFPM_{2.5}), particularly its nano-sized fraction (PM_{0.1}) can be more toxic than urban background PM_{2.5} due to a higher proportion of polar organic compounds, including polycyclic aromatic hydrocarbons (PAHs). WFPM has been linked to higher risks of respiratory and cardiovascular diseases, yet the effects on female reproductive health remain largely unknown. Here, we used a mouse model and a 3D in vitro ovarian follicle culture model to investigate the effects of nano-sized WFPM (WFPM_{0.1}) on ovarian functions and potential mechanisms involved. We first exposed 8-week-old CD-1 female mice with 443 µg lab-synthesized WFPM_{0.1} (LS-WFPM_{0.1}) in 50 µl water once through intratracheal lung instillation. LS-WFPM_{0.1} was generated by complete combustion of pine wood, the major wood in California and Canadian wildfires, using a wildfire smoke simulator (WiSS). Blood sera were collected on days 3, and 7 post-instillation for measuring sex hormones using ELSIA. Late-stage antral follicles were isolated from ovaries, and RT-qPCR was performed to analyze the expression of genes involved in follicle development, steroidogenesis, and aryl hydrocarbon receptor (AhR) signaling, given that PAHs are known AhR activators with endocrine-disrupting potential.

ELISA results revealed elevated circulating estradiol and testosterone levels between LS-WFPM_{0.1}-treated and control mice, although the differences were not statistically significant. RT-qPCR data revealed that antral follicles from LS-WFPM_{0.1}-treated mice had altered expression levels of androgen synthesis related genes and highly induced AhR target genes. These results suggest that in vivo exposure to LS-WFPM_{0.1} through intratracheal lung instillation interferes with ovarian steroidogenesis and activates AhR in antral follicles.

We next used a 3D hydrogel encapsulated in vitro follicle growth (eIVFG) system to examine the direct ovarian impacts of LS-WFPM_{0.1}. Immature mouse follicles were cultured in eIVFG for 6 days and treated with LS-WFPM_{0.1} at 0, 1, 10 and 100 µg/mL from days 4-6 during follicle maturation. Follicles were treated with human chorionic gonadotropin (hCG) on day 6 to induce ovulation. While LS-WFPM_{0.1} treatment did not affect follicle growth, ovulation, or oocyte meiotic resumption, it concentration-dependently increased testosterone secretion, altered androgen synthesis and activated AhR target genes, consistent with our in-vivo findings. Single-follicle RNA sequencing (RNA-seq) analysis confirmed the RT-qPCR results and discovered new differentially expressed genes (DEGs) related to inflammation. Single oocyte RNA-seq analysis revealed changes in oocytes transcriptomics, with DEGs associated with mitochondrial respiratory chain.

The same concentration range of real world WFPM_{0.1} collected from central New Jersey during 2023 Canadian wildfire (C-WFPM_{0.1}) were used for in-vitro exposure in eIVFG. Both C-WFPM_{0.1} and benzo[a]pyrene (BaP), a high molecule weight PAH, resulted in similar ovarian hyperandrogenism and AhR activation. Mechanistic studies using eIVFG revealed that co-treatment of 10 µM resveratrol, a selective AhR inhibitor, reversed LS-WFPM_{0.1} induced ovarian hyperandrogenism and transcriptional induction of androgen synthesis and AhR target genes.

In summary, our study indicates that exposure to WFPM_{0.1} interferes with ovarian functions by promoting testosterone secretion, inducing ovarian inflammation, and altering oocyte transcriptomics via AhR activation.

The Effect of Simulated Natural Light Cycles on Hormonal Regulation and Fertility in Aye-Ayes's (*Daubentonia madagascariensis*) Under Managed Care

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Abstract Text

The aye-aye (*Daubentonia madagascariensis*) is an endangered, nocturnal primate endemic to Madagascar. While *in situ* populations face threats such as poaching and habitat degradation, *ex situ* populations often exhibit low reproductive success. In zoological settings, aye-ayes are often kept on a reverse light cycle to align nocturnal activity with visitor hours. These environments may introduce light pollution during the dark phase and insufficient light intensity during the light phase, which poses the risk of circadian rhythm disruption. Circadian dysregulation may negatively affect the synthesis and secretion of ovarian steroid hormones, ultimately impairing estrous cyclicity and reproductive success. The objective of this study is to assess whether increasing light intensity during the light phase of a reverse light cycle can improve estrous cycle dynamics and ovarian steroid hormone production in a reproductively unsuccessful aye-aye breeding pair. To characterize baseline endocrine profiles, voluntary fecal and saliva samples were collected from both individuals and analyzed for concentrations of salivary melatonin and fecal estrogen, progesterone and testosterone metabolites. In addition, voluntary vaginal cytology was conducted to monitor and characterize estrous cycle dynamics based on epithelial cell morphology and relative proportions of cell types across cycle phases. To provide a comparative framework and serve as a positive control, samples from four reproductively proven male and female aye-aye housed at a separate institution were collected and analyzed concurrently. Fecal hormone monitoring assays were validated by testing for parallelism between serial fecal extract dilutions and the respective standard curve for multiple enzyme-linked immunosorbent assays (ELISAs). These included estradiol (E2, R0008), estrone conjugate (EC, R522), progesterone (P4, K025-H5), pregnanediol-3-glucuronide (PdG, R13904), and testosterone (T, K032-H5). Female fecal pools consisted of ten samples, with five collected during the follicular phase as determined by vaginal swelling and five collected during the presumed luteal phase. Male fecal pools included six samples, three corresponding to the female's follicular phase and three associated with her presumed luteal phase. Serial dilutions ranging from neat to 1:64 demonstrated parallel displacement with the testosterone standard curve for both male ($F(6,6) = 1.23$, $P = 0.403$) and female ($F(6,6) = 0.94$, $P = 0.472$) extracts. Female extracts also showed parallelism with the P4 ($F(6,4) = 1.80$, $P = 0.296$), PdG ($F(8,6) = 2.05$, $P = 0.18$) and E2 ($F(6,6) = 1.272$, $P = 0.389$) ELISA standard curves. In contrast, no parallel response was observed for the EC ($F(7,4) = 6.83$, $P = 0.041$) assay, indicating limited suitability of this assay for fecal estrogen metabolite detection in this species. Biological validation of baseline data from the unproven pair during the October 2025 estrous cycle revealed clear endocrine patterns. Peaks in female estradiol and testosterone concentrations coincided with maximal vaginal swelling during estrus, while male testosterone concentrations increased at the onset of the female's estrus and vaginal swelling. Conversely, the lowest male testosterone levels corresponded with the female's lowest estradiol concentrations during the same cycle. Experimental data collection is currently underway, during which light intensity and spectral composition are modified to more closely approximate natural lighting conditions for the unproven breeding pair. Findings from this study are expected to clarify the influence of lighting environments on circadian regulation and reproductive physiology in nocturnal primates, contributing to evidence-based husbandry strategies aimed at enhancing reproductive success in *ex situ* aye-aye populations.

Adropin in Porcine Pituitary Cells: Expression and in Vitro Effects on Viability and Hormone Secretion During Estrous Cycle

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Abstract Text

Adropin is a metabolism-related hormone primarily expressed in the liver, but also in adipose tissue, heart and brain. Its expression is strongly associated with the body's metabolic status. Proper regulation of energy metabolism is crucial for female reproduction. Many metabolic hormones, such as leptin, are part of the regulatory system in the hypothalamus-pituitary-ovary axis. A proper understanding of the molecular pathways underlying the action of metabolic hormones in the anterior pituitary is key to a full understanding of reproductive modulation. The main aim of this study was to address an important knowledge gap regarding adropin expression and role in anterior pituitary cells, depending on the stage of the estrous cycle in pig.

Firstly, we examined gene and protein expression of adropin and its receptor (GPR19) in female porcine anterior pituitary at days 2-3, 10-12, 14-16, and 17-19 of the estrous cycle by RT-qPCR and Western blot ($n=5$, $p<0.05$) as well as its cellular localisation (immunocytochemistry). Additionally, we assessed adropin levels in serum throughout the estrous cycle by ELISA ($n=6$, $p<0.05$). Then, we examined the *in vitro* effect of adropin (0.1, 1, 10, 100 nM) on anterior pituitary cell viability after 24 h of incubation (alamarBlue assay) ($n=5$, $p<0.05$). Lastly, we assessed adropin (1, 10 nM) effect on luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion after 24 h of incubation (ELISA) ($n=5$, $p<0.05$). Statistical analyses were performed in GraphPad Prism using one-way ANOVA followed by Tukey's post hoc test.

Our results demonstrated the expression and cellular localisation of adropin and GPR19, as well as changes in blood adropin levels in pigs throughout the estrous cycle. Interestingly, the highest adropin levels in blood were observed in the luteal phase of the estrous cycle (10-12 and 14-16), also GPR19 exhibits elevated expression in pituitaries from the luteal phase. Furthermore, we showed that adropin has dose- and phase-dependent effects on anterior pituitary cell viability; at days 2-3 of the estrous cycle, we observed no impact of adropin regardless of the dose, whereas at days 10-12 of the estrous cycle, we described a downregulating effect of adropin (1-100 nM). Finally, we observed that there was no impact of adropin on FSH secretion throughout the estrous cycle, whereas it increased LH secretion in the mid-luteal phase of the cycle.

From this study, we conclude that adropin has estrous stage-dependent effects on anterior pituitary cells, and its role, especially in the luteal part of estrous cycle, should be studied thoroughly. These findings provide a foundation for a better understanding of how metabolic hormones influence reproductive processes.

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Phthalate Exposure Alters Genes Involved in Reproduction and Neuroinflammation in the Mouse Arcuate Nucleus

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Abstract Text

Phthalates are chemical plasticizers humans encounter in daily life, occupational or medical settings. Classified as endocrine disrupting chemicals (EDCs), phthalates interfere with hormone activity and these compounds are known to decrease human fertility and contribute to inflammation and oxidative stress. Ovarian health and function, in terms of follicle formation, steroidogenesis, and reproductive cyclicity, have been shown to be directly affected by phthalate exposure. However, the ovary is part of the larger hypothalamic-pituitary-gonadal (HPG) axis and less is known about the effects of phthalates on the neural control of reproduction. The hypothalamic arcuate nucleus (ARC) contains kisspeptin-producing neurons which control pulsatile gonadotropin releasing hormone (GnRH) secretion and receive hormone feedback from the ovaries. To examine the effects of phthalates on gene expression in the ARC, adult mice were exposed to the commonly used phthalate di(2-ethylhexyl) phthalate (DEHP) as well as its replacement diisononyl phthalate (DiNP). Phthalate dosing was given acutely for 10 days via oral administration during a period when the mice were reproductively mature or in chronic exposure through diet for 9 months or 15 months. Acute exposure to DEHP and DiNP showed no effects on reproductive gene expression in the ARC. DiNP exposure slightly increased mRNA levels of translocator protein (*Tspo*), a gene shown to be involved in neuroinflammation compared to controls. After 9 months of continuous phthalate exposure, mRNA levels of *Kiss1* were increased with DiNP relative to control. Interestingly, Kisspeptin immunostaining showed that DiNP reduced peptide levels in ARC tissue from treated mice compared to control. Also, both DEHP and DiNP increased ARC *Tspo* gene expression at multiple concentrations. By 15 months of exposure, neither *Kiss1* nor *Tspo* mRNA expression was changed by phthalates. Overall, these data show that 9 month chronic exposure to phthalates can alter ARC reproductive gene and protein expression that could influence GnRH secretion. *Tspo* protein expression appears localized to ARC tanycytes and vascular endothelia suggesting that phthalates may contribute to neuroinflammation of specialized ARC cells. Finally, since gene expression changes seen at 9 months of phthalate exposure are no longer present by 15 months, we propose that during the transition to reproductive senescence, the HPG axis may be susceptible to endocrine disruption by phthalates.

Dynamic Transposable Element Expression and Isoform-Resolved Transcriptome in Bovine Preimplantation Embryos

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Abstract Text

Mammalian preimplantation development requires extensive epigenetic and transcriptomic reprogramming to transform two terminally differentiated gametes into a totipotent embryo. In cattle, in vitro embryo production (IVP) is essential for reproductive technologies and sustainable livestock production. However, only 40% IVP embryos reach the blastocyst stage, limiting the efficiency of assisted reproductive technologies. A key developmental milestone is zygotic genome activation (ZGA), when transcriptional control shifts from maternally inherited RNAs to the embryonic genome. Disrupted ZGA can lead to gene dysregulation, developmental arrest, and reduced blastocyst formation. My laboratory integrates embryology, functional genomics, and bioinformatics to define the molecular mechanisms regulating bovine embryonic reprogramming, with a focus on epigenetic regulation, transposable element activity, and isoform-level transcriptome dynamics during early development. To determine how embryo production systems affect early development, we integrated RNA-seq datasets from 163 bovine samples across 14 studies, including in vivo-derived (IVV), in vitro-produced (IVP), and somatic cell nuclear transfer (SCNT) embryos. Comparative transcriptome analysis revealed delayed genome activation in IVP and cloned embryos compared to IVV embryos, with major transcriptional activation occurring at the 8-cell stage rather than the 4-cell stage. Cloned embryos showed pronounced gene downregulation at the 4-cell stage and disrupted developmental trajectories, including abnormal activation of pluripotency-associated genes. We further characterized transposable elements, which are increasingly recognized as regulators of gene expression and chromatin state during early development. Thousands of self-expressed transposable element loci were identified across embryo types. ERVK elements were activated at the 16-cell stage in in vivo embryos but delayed until the blastocyst stage in in vitro-produced and cloned embryos. ZGA-specific MLT1D elements were enriched with transcription factor (TF) DUX4 motif and H3K27ac, suggesting potential TF-TE interactions and enhancer-like functions during bovine ZGA. In a second study, we established the first isoform-resolved transcriptome atlas of bovine preimplantation embryos using ultra-low-input PacBio long-read RNA sequencing from oocyte to blastocyst stages. Because current bovine transcriptome annotations are primarily based on short-read sequencing, they do not fully resolve transcript complexity, novel isoforms, or alternative splicing events during embryogenesis. Each long-read sample generated more than 20 million reads, with an average read length of approximately 1.6 kb. Across preimplantation stages, we identified 286,711 unique isoforms mapping to 14,353 annotated genes and 14,084 novel genes. More than 140,000 isoforms were classified as novel, substantially expanding the known transcript diversity of bovine embryos. Novel isoforms were supported by transcription start site signals and active histone modifications data, indicating that they represent biologically relevant transcriptional products. Differential isoform expression revealed a major surge between the 8-cell and 16-cell stages, corresponding to bovine major ZGA, and identified approximately 500 candidate ZGA genes. Together, these studies provide a comprehensive view of gene, transposable element, and isoform-level regulation during bovine preimplantation development. By identifying dysregulated genes, developmentally dynamic transposable elements, and novel isoforms associated with embryo developmental competence, this work provides new molecular targets and foundational resources for improving bovine embryo quality, reproductive efficiency, and the success of assisted reproductive technologies in cattle.

Functional Role of Maternal Factors MOF and H4k16ac in Bovine Embryonic Genome Activation

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Abstract Text

Zygotic genome activation (ZGA) is a critical developmental transition that prepares embryos for cell differentiation and ensures normal development. During this period, fertilized embryos transition from dependence on maternal RNA/proteins to active transcription of the zygotic genome, producing new zygotic RNAs and proteins. ZGA is conserved across animals, although its timing differs by species: nuclear cycle 14 in *Drosophila*, 2-cell in mice, 4-cell in humans and pigs, and 8-16 cell stage in bovine. Many maternal factors regulate ZGA, such as pioneer transcription factors (TFs) and histone modifiers. Males-Absent-On-the-First (MOF) is a known histone acetyltransferase of histone 4 Lysine 16 acetylation (H4K16ac), a modification associated with chromatin activation and transcriptional competence. In *Drosophila*, MOF has been found essential for male X chromosome dosage compensation with both MSL complex-dependent and -independent mechanisms, as well as its critical role in ZGA. Besides, the roles of maternally deposited MOF in embryo development were also investigated in mouse, pig, and sheep. However, how MOF and H4K16ac contribute to euchromatin during bovine ZGA remains poorly understood. In addition, it is unclear whether maternal H4K16ac can remain stably on embryonic genomes during reprogramming and if there is any biological significance underlying it. In this study, we investigate the role of MOF during bovine ZGA and early embryo development. We first profiled the *MOF* mRNA expression via qPCR and quantified the protein levels of MOF and H4K16ac across developmental stages. Our results showed that MOF is a maternally deposited factor in bovine oocytes and exhibits dynamic expression across embryonic developmental stages. To assess its functional role, we knocked down *MOF* expression in bovine zygotes using *MOF* RNA interference (RNAi) via micro-injection. qPCR results confirmed the knockdown efficiency, and the reduction of *MOF* level persisted through the blastocyst stage. Functionally, *MOF*-knockdown embryos arrested and degenerated at the morula stage and exhibited a significantly low blastocyst rate compared with controls. Consistent with the reduction of *MOF* mRNA, nuclear MOF protein levels were markedly reduced, accompanied by a global decrease in H4K16ac across developmental stages. These results suggest that MOF is required for maintaining H4K16ac levels and supporting normal bovine embryo development. To define the role of MOF and H4K16ac during bovine ZGA regulation, we will perform RNA-seq, CUT&Tag, and ATAC-seq on control and *MOF*-knockdown embryos. These analyses will enable us to profile MOF/H4K16ac-dependent transcriptional changes, map MOF and H4K16ac genomic occupancy, and assess changes in chromatin accessibility. Together, these approaches will provide a comprehensive view of how MOF-mediated chromatin acetylation regulates the establishment of transcriptionally active chromatin and orchestrates ZGA in bovine embryos.

Supplementation of Methionine and 5mM Guanidinoacetic Acid During Oocyte Maturation Influences *In-Vitro* Embryo Production

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Abstract Text

Nutrient composition of media for *in-vitro* embryo production can influence maturing oocyte quality and subsequently early embryonic development. A specific group of nutrients, one-carbon metabolites (OCMs), influences methylation status and can alter early embryonic development. Two specific OCMs, methionine (Met) and guanidinoacetic acid (GAA) can alter global methylation by providing or sequestering methyl groups, respectively. A recent study in our lab demonstrated negative implications of supplementing GAA during oocyte maturation on early embryonic development. Our hypothesis is that supplementation of 5mM GAA during oocyte maturation *in-vitro* will lead to methyl deficiency while incorporation of Met will rescue methylation status and alter early embryonic development. Follicles (2-7mm) from slaughterhouse ovaries were aspirated to obtain cumulus oocyte complexes (COC; n = 800/treatment) and matured for approximately 23 hr in maturation medium containing: Control, Met (21 μ M), GAA (5 mM), or Met + GAA. After maturation, a subset of oocytes were randomly selected for transcript abundance analysis of markers associated with methylation and oocyte quality, 5-methylcytosine analysis, and chromatin and microtubule analysis. The remaining oocytes underwent *in-vitro* fertilization with a single sire, followed by embryo culture *in-vitro*. A subset of the presumptive zygotes were cultured in a timelapse incubator to monitor timing of developmental stages. Blastocysts were collected for transcript abundance of markers associated with methylation and quality or stained for 5-methylcytosine and total cell counts (inner cell mass, ICM and trophectoderm, TE). Percent cleavage and blastocyst rate, ICM: TE, oocyte microtubule distribution and chromatin configuration were collected and analyzed using the GLIMMIX procedure, while 5-methylcytosine and transcript abundance were analyzed using the MIXED procedure with the main effect of supplementation as fixed effects. Timing of developmental stages was analyzed using the GLM procedure with the main effect of supplementation as fixed effects and time as a repeated measure. Five-methylcytosine was reduced ($P < 0.05$) in matured oocytes supplemented with Met or GAA alone, while the combination of Met and GAA increased 5-methylcytosine in matured oocytes compared to controls, and no differences were observed at the blastocyst stage. No differences were observed in matured oocyte transcript abundance; however, an increase ($P < 0.05$) was observed for *CKB*, *CKTM1*, and *HNF4A* in blastocysts developed from GAA-supplemented oocytes compared to controls. A reduction ($P < 0.05$) in *DMNT3A* transcript abundance was observed in blastocysts from oocytes supplemented with Met or Met and GAA compared to controls. Timing to the 2-cell stage was longer ($P < 0.05$) in the Met only and GAA only matured oocyte groups compared to the control, while the combination of both Met and GAA took longer to reach the 4-cell stage. No differences in developmental timing were observed in later embryonic stages. Total number of cells, ICM and TE numbers were reduced in Met only and GAA only oocyte supplementation compared to controls. Taken together, supplementation of Met only or GAA only during oocyte maturation had a negative influence on the quality of the developing embryo *in-vitro*, while the combination of Met and GAA supplementation during oocyte maturation did not impact early embryonic development. Using this model, we can begin to understand the implications of supplementing OCM during oocyte maturation *in-vitro* and long-term influences on early embryonic development. USDA is an equal opportunity employer and provider.

Telomere Elongation Kinetics During Rhesus Macaque Preimplantation Embryo Development

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Abstract Text

Telomere length (TL), the repetitive caps at chromosome ends, is crucial for protecting genome integrity. As cells divide, telomeres shorten, and when too short, the cell can no longer divide, a process linked to senescence and multiple age-related diseases. TL, established in the inner cell mass (ICM) of the blastocyst, influences telomere length throughout life; however, the kinetics of telomere elongation during preimplantation development have been documented only in the mouse. We sought to characterize the pattern of preimplantation TL elongation in a nonhuman primate (NHP) model that more closely replicates human reproductive physiology, including ovarian cellular dynamics and oocyte function. To this end, adult rhesus macaques (aged 9-15 years) underwent controlled ovarian stimulation (COS; n=4) and oocyte collection, in accordance with protocols used in human infertility treatment. A subset of mature oocytes (MII) underwent in vitro fertilization with sperm from male donors of proven fertility. Mature oocytes (MII), cleavage-stage embryos, morulae, and blastocysts were collected for TL determination. Blastocysts underwent laser biopsy, yielding trophectoderm-only (TE) and inner cell mass (ICM) plus TE (ICM+TE) samples. TL was assessed using a qPCR assay amplifying telomeric sequences, calibrated to the single-copy housekeeping ribosomal gene S10. TL was lowest in MII oocytes, then increased to its highest level at the morula stage ($p < 0.05$). TL remained elevated in both TE+ICM and TE samples relative to MII oocytes and cleavage-stage embryos. TL was significantly higher in oocytes that failed to reinitiate meiosis (GV) relative to mature MII oocytes ($p < 0.05$). MII TL was negatively correlated with maternal age ($R^2 = 0.94$), such that older animals yielded mature oocytes with shorter telomeres, as has also been observed in humans. Notably, a maternal age-related decline in TL is also observed in TE cells ($R^2 = 0.97$). Collectively, these findings demonstrate that 1) telomere elongation in primates occurs after fertilization, peaking more rapidly than in mice, and 2) that maternal age is associated with shorter telomeres in the egg that are maintained in the TE of the blastocyst.

Refining Views of the Oocyte Cortex Through the Oocyte-to-Egg and Egg-to-Embryo Developmental Transitions

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Abstract Text

Recent research has shown that the actomyosin cytoskeleton in the oocyte cortex plays a critical role in the oocyte-to-egg and egg-to-embryo developmental transitions. Disruptions to cortical actin, and proteins that bind actin and link actin to the membrane, lead to abnormalities in the oocyte's progression through meiosis and into early embryogenesis. The goal of our work is to quantify changes in actin organization and mechanical properties in the cortex. We also aim to understand cortical actin organization in the context of the picket-fence model, that describes cortical actin as a meshwork beneath the plasma membrane, in which anchoring proteins functioning as "pickets" link the actin filaments to the membrane. The arrangement of picket proteins along the actin filaments creates compartments within the actin meshwork referred to as "actin corrals" that allow for specialized diffusion of membrane proteins. We complement our studies of cortical actin organization with analysis of the mechanical properties of the oocyte cortex throughout these transitions. Our studies utilize total internal reflection fluorescence (TIRF) microscopy to capture images of cortical actin in fixed eggs labeled with phalloidin, at near super-resolution. We use a post-image processing computational algorithm, enhanced super resolution radial fluctuations (eSRRF), to achieve ~100 nm resolution images from which the area of the actin corrals can be measured. This imaging and subsequent analyses revealed an approximately 24% decrease in actin corral size in fertilized and parthenogenetically activated eggs compared to unfertilized metaphase II eggs. We will build upon these findings through additional analyses of cortical actin organization throughout the oocyte-to-egg transition. Additionally, we aim to investigate if there is a difference in cortical actin organization between eggs activated by fertilization by sperm and eggs that are parthenogenetically activated via increased intracellular calcium. In context of the picket-fence model, we also aim to characterize changes in the organization of candidate picket proteins along the actin filaments. Among the candidate picket proteins are CD9, a tetraspanin that is required for fertilization, and tetraspanin-associated proteins, IgSF8, ezrin, and radixin, proteins that are involved in linking cortical actin to the plasma membrane. For analysis of the mechanical properties of the oocyte cortex, we use atomic force microscopy (AFM) and contact mechanics modeling to extract the Young's modulus, a metric that describes stiffness, from indentation experiments. Preliminary force-indentation data on live zona pellucida-free oocytes shows that the Young's modulus decreases in the oocyte-to-egg transition. Future studies will examine cortical stiffness in sperm-fertilized and parthenogenetically activated eggs. Analyses of cortical actin organization and cortical stiffness will provide valuable insight into how the oocyte cortex reorganizes to promote successful developmental transitions. Studies have shown that cortical actin thickening is associated with a necessary decrease in cortical tension during meiosis I. We aim to get greater understanding of this apparent paradox, that the actin cortex becomes thicker, yet the cortex becomes softer. Additionally, abnormal mechanical properties of oocytes are associated with developmental abnormalities. By quantifying the cortical actin organization, in context of the picket-fence model, we aim to define how patterns in the meshwork influence oocyte mechanics, providing new views of the dynamic oocyte cortex and insights into parameters that define oocyte quality. Copilot plugin for MATLAB was used to debug code. Supported by the EMBRIO Institute under contract #2120200, funded by the National Science Foundation (NSF) Biology Integration Institute.

The Mammalian Egg-To-Embryo Transition: Consideration of Effectors Beyond CaMKII γ and Events Occurring at the Egg Cortex and Membrane

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Abstract Text

The egg-to-embryo transition (egg activation) is the process by which a metaphase II-arrested egg initiates embryonic development following fertilization. Calcium/Calmodulin-dependent protein kinase II gamma (CaMKII γ) is one key integrator of the sperm-induced calcium signal: (a) in the absence of CaMKII γ , the egg is unable to activate to exit from metaphase II arrest, and (b) a constitutively active (CA) form of CaMKII γ induces parthenogenetic exit from metaphase II arrest. However, other events of egg activation, particularly events occurring at the cell cortex (the cytoplasmic region underlying the plasma membrane), appear not to be entirely dependent on CaMKII γ . These include cortical granule (CG) exocytosis and establishment of the membrane block to polyspermy. This suggests that some events of egg activation are regulated by factors beyond CaMKII γ . The goal of this project is to investigate egg activation events occurring at the egg cortex and additional potential effectors of egg activation. First, we evaluated the dependence on calcium signaling of another cortical/membrane event of the egg-to-embryo transition: sperm-induced cortical remodeling, which involves chromatin-associated signals and formation of an actin-rich fertilization cone over the sperm DNA. We compared control fertilized eggs to fertilized eggs with suppressed calcium signals (treated with the intracellular calcium chelator BAPTA-AM) or with inhibition of calmodulin (treated with KN-93). We observed that substantially reduced circularity (as a measure of cell shape change) associated with sperm-induced cortical remodeling occurs in BAPTA-AM-treated and in KN-93-treated eggs. Second, we investigated factors that facilitate changes in the egg cortex upon sperm incorporation, focusing first on the Rho/Rac/Cdc42 family of small GTPases. These proteins are of interest because of their well-characterized effects on actin filaments, and evidence of functions related to sperm-induced cortical remodeling and potentially calcium signaling. Our studies utilized a Cdc42/Rho/Rac activator, CN04, to observe the effects of increased small GTPases activity. We started studies with metaphase II-arrested eggs, which have chromatin-induced cortical remodeling with resting levels of calcium. The goal is to determine if increased small GTPase activity results in changes around the maternal DNA first in metaphase II eggs. Live-cell imaging experiments reveal that CN04 treatment increased F-actin levels surrounding the maternal DNA in comparison to controls. In addition, CN04-treated cells developed a transient protrusion, measured as decreased circularity and roundness. This transient response to CN04 occurs as early as 30 mins or as late as 4 hours; the cells then recover a normal metaphase II morphology. These data show that increased small GTPase activity promotes increased chromatin-induced cortical remodeling around the metaphase II DNA. Third, with an interest in identifying egg activation effectors beyond CaMKII γ , we examined if other isoforms of CaMKII are expressed in eggs, focusing on CaMKIId, as CaMKIId mRNA is detected in mouse eggs. Our immunoblot analyses confirmed that an anti-CaMKIId antibody does not cross-react with the α , β , or γ isoforms of CaMKII, and detects CaMKIId protein in prophase I oocytes, metaphase II eggs, and fertilized eggs. Immunofluorescence experiments with this antibody also detect CaMKIId in female gametes at these different stages. Taken together, these studies expand our view of egg activation and provide insights into additional effectors downstream of sperm incorporation and calcium signaling and of egg activation events occurring at the egg cortex.

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Expression, Regulation and Function of Tumor Necrosis Factor (TNF) and TNF Receptors at the Maternal-Conceptus Interface in Pigs

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Abstract Text

The tumor necrosis factor superfamily is an important mediator for inducing apoptosis through its death receptors. During pregnancy, apoptosis has been observed in endometrial tissues and plays an important role in the establishment and maintenance of pregnancy in many species. However, the expression and function of tumor necrosis factor- α (TNF) and its receptors, TNF receptor superfamily 1A (TNFRSF1A) and TNFRSF1B, in the endometrium during pregnancy have not been well studied in pigs. Thus, we determined the expression of TNF, TNFRSF1A, and TNFRSF1B in the endometrium during the estrous cycle and pregnancy in pigs. Real-time RT-PCR analysis showed that *TNF*, *TNFRSF1A*, and *TNFRSF1B* were expressed in the endometrium during the estrous cycle and pregnancy in a pregnancy status- and stage-specific manner. Levels of *TNF* and *TNFRSF1B* mRNAs on D15 of pregnancy were significantly greater than those on D15 of the estrous cycle. Immunohistochemistry analysis showed that TNF, TNFRSF1A, and TNFRSF1B proteins were localized to endometrial epithelial and chorionic epithelial cells. Immunostaining signals of TNFRSF1A and TNFRSF1B increased in chorioallantoic tissues during mid- to term pregnancy. In endometrial explant cultures, the expression of *TNF*, *TNFRSF1A*, and *TNFRSF1B* was up-regulated by interferon- γ . To understand the function of TNF, we treated porcine endometrial epithelial (pUE) and conceptus trophectoderm (pTr) cells with increasing doses of TNF *in vitro* and measured cell proliferation, migration, and the expression of select cytokines and chemokines. We found that TNF did not affect proliferation of pUE and pTr cells, but increased migration of pTr cells and the expression of cytokines and chemokines in pUE and pTr cells. These results in pigs showed that TNF and its receptors are expressed at the maternal-conceptus interface and TNF regulates the expression of cytokines and chemokines in pUE and pTr cells. These suggest that TNF may play an important role in the establishment of pregnancy by regulating cell migration of trophectoderm cells and cytokine production in endometrial epithelial and trophectoderm cells at the maternal-conceptus interface during pregnancy in pigs. [This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (Ministry of Science & ICT; #RS-2025-00518549), Republic of Korea]

Connecting Preeclamptic Placental Pathology and Transcriptomics at the Maternal-Fetal Interface

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Abstract Text

The maternal-fetal interface (MFI) of the placenta is a critical determinant of pregnancy health; where genetically distinct tissues must work in tandem to sustain fetal development. Failure of MFI integration can lead to placental malperfusion pathologies, immune-associated injury, and insufficient exchange, further resulting in adverse pregnancy outcomes such as Preeclampsia (PE). The only known treatment for PE is delivery of the placenta; making this organ, and the interface of which it constitutes, critical to disease pathogenesis. We sought to better categorize the preeclamptic MFI of the placenta, through integration of advanced, spatially resolved histopathological and RNA expression analyses. We hypothesized that, compared to healthy controls, PE placentae would demonstrate structural changes, pathological increases, and expression profiles indicative of placental malperfusion and immune incompatibility.

Thus, we recruited control (n=5) and preeclamptic (n=4) pregnancies for participation in our study. MFI biopsies from control (n=6) and PE (n=6) placentae were obtained shortly after birth, FFPE-processed, H&E-stained, scanned at 20x magnification, and submitted to the 10x Genomics Visium platform. Resulting images were assessed for placental architecture (placental villi, intervillous space, intervillous blood, extravillous blood, decidual basalis), placental pathologies (fibrin deposition, villous agglutination, inflammatory villous agglutination, and stromal mineralization), and RNA expression. Placental ST data was processed via 10x Genomics Space Ranger software, deconvoluted, clustered, and assessed through differential expression (DE) and pathway analyses. Cluster spots were superimposed onto histological images using scripts generated with Claude by Anthropic. Placental histopathological features and cluster-histopathological overlap were annotated and quantified via pixel count in GIMP (version 3.2.4) and reported as a ratio of total tissue/cluster area to account for sample size/MFI location variability. All condition-level analyses utilized Wilcoxon rank-sum tests for non-parametric data.

Spatial RNA expression analysis revealed six unique placental clusters, half confined to the villous region (Cluster 1: terminal villous syncytia, Cluster 2: villous cores, Cluster 3: stem/anchoring villi) and half within the decidual region (Cluster 4: decidual stroma, Cluster 5: extravillous trophoblasts, Cluster 6: immune-enriched fibrin). Villous clusters 1 and 2 demonstrated the highest number of DE genes, sharing a broad immune-inflammatory hallmark program that included allograft rejection, interferon gamma response, and inflammatory response. Histopathological analysis revealed an increase of stromal mineralization within PE villi (p=0.0360), with Cluster 2 specifically further demonstrating an increase in mineralization within PE placentae. Cluster 5 demonstrated the clearest matrix-remodeling pathway profile and showed increased transcription within PE placentae. In the decidual region, stromal and extravillous trophoblasts clusters (4 and 5) of PE placentae displayed general transcriptional suppression, alongside decreased agglutination at the MFI. In addition, physiological and pathological fibrin deposition within the decidua significantly overlapped with cluster 6, demonstrating a general lack of transcription, with moderate immune enriched activity of NK CD16 populations.

The increased fetal vascular malperfusion pathology and expression of genes associated with oxidative stress and immune activation within the villous region, demonstrated here in PE placentae all evidence placental insufficiency. In addition, the increase of MFI remodeling activity within anchoring villi of PE tissue may suggest a degree of compensatory action from fetal tissue. At the same time, the maternal compartment largely demonstrated transcriptional suppression in PE, alongside decreased injury at the MFI. These patterns demonstrate that preeclampsia-associated transcriptional changes are spatially compartmentalized: broad suppression in decidual regions coexists alongside active upregulation of structural and invasive villous cell populations at the MFI.

Expression of the Interferon-Stimulated Genes ISG15 and OAS1 Differs between Pigs and Ruminants, with OAS1 Additionally Upregulated in Endometrial Glands by Estrogen

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Abstract Text

Interferon (IFN)-stimulated genes (ISGs) increase in the endometrium of ruminants and pigs in response to IFN tau (IFNT) and IFN gamma (IFNG), secreted by the trophectoderm of elongating conceptuses (embryo and extraembryonic membranes), respectively. ISG15 and 2',5'-oligoadenylate synthetase 1 (OAS1) were two of the first ISGs to be characterized in ruminants and their endometrial expression is used to assess secretion of IFNT by elongating bovine conceptus. In addition, their expression by the peripheral blood leukocytes (PBLs) of ruminants can confirm pregnancy, and they are also expressed by the corpus luteum (CL) of these species. Expression of ISG15 and OAS1 has not been fully examined in pigs. This study characterized expression of ISG15 and OAS1 in the endometrium, PBLs, and CL of pigs. In Study 1, pigs were ovariectomized on various days of the estrous cycle and early pregnancy and endometrium, CL, and whole blood were collected. Endometrial ISG15 mRNA was high on Day 15, remained high through Day 18 and then declined by Day 20 of pregnancy. ISG15 protein was localized to the stratum compactum stroma from Days 15 through 20, with expression limited to implantation sites. In contrast, endometrial OAS1 mRNA was high on Days 10 through 20, then decreased on Day 24 compared to days 10 and 18. OAS1 protein was present in luminal epithelium on Day 12, but not thereafter, while OAS1 was present in glandular epithelium (GE) from Days 12 through 20. OAS1 was also present in the fibroblasts and endothelium of the stratum compactum stroma from Days 15 through 20. Neither ISG15 nor OAS1 mRNA increased in the PBLs or CL from pregnant pigs. In Study 2, endometrial explants were derived from the uteri of pigs on Day 11 of pregnancy and were treated with (a) extracellular vesicles (EVs) isolated from uterine flushings of Day 15 pregnant pigs, (b) EVs from uterine flushings of Day 15 cyclic pigs, or (c) recombinant IFNG. Both EVs from pregnant pigs and recombinant IFNG increased ISG15 and OAS1 mRNA in endometrial explants while EVs from cyclic pigs did not. Since the biphasic peaks of endometrial OAS1 mRNA on Day 10 and Day 18, and expression of OAS1 protein in the GE prior to secretion of IFNG, observed in study 1, were unique to the pig, the role of steroid hormones in the regulation of OAS1 expression was of further interest. Therefore, in Study 3, pigs were ovariectomized on Day 4 post-estrus and given IM injections on Days 4 through 12 of either progesterone, estrogen, or corn oil (CO). On Day 12, pigs were hysterectomized, and endometrial tissues analyzed using immunofluorescence microscopy. OAS1 protein was detected in the GE of ovariectomized pigs treated with estrogen, but not in pigs treated with progesterone or CO. ISG15 protein was not regulated by estrogen or progesterone. Results suggest that ISG15 and OAS1 increase in the endometrial stratum compactum stroma at implantation sites in response to conceptus-derived IFNG. This is similar to IFNT upregulation of ISG15 and OAS1 in ruminants. However, unlike ruminants, ISG15 and OAS1 do not increase in PBLs and CL. Further, regulation of expression of endometrial OAS1 is not limited to conceptus IFNG but also appears to be induced in GE by estrogen. This is a novel finding differentiating epitheliochorial pigs from synepitheliochorial ruminants.

Luminal Epithelium Remodeling Synchronized with Stromal Disruption Drives Endometrial Regeneration in Menstruation and Pregnancy

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Abstract Text

The endometrium undergoes extensive tissue loss, remodeling, and repair as a normal part of uterine physiology. The endometrium consists of a thick layer of stromal tissue topped with a monolayer of luminal epithelium that sprouts epithelial glands into the surrounding stroma during neonatal development. These components face drastic disruption during pregnancy and menstruation and their subsequent return to a pre-injury state is essential to sustain the capacity for pregnancy. Despite this importance, the scale and nature of epithelial disruption during menstruation and pregnancy, and the mechanisms of its subsequent repair remain incompletely understood. Current models postulate that menstruation proceeds through shedding of the superficial portion of the endometrium, exposing truncated glands harboring epithelial progenitors that carry out post-menstrual re-epithelialization of the exposed luminal surface. Here, we use a $Cxcl15^{Cre} R26^{nTnG}$ mouse line to assess the contributions of glandular cells to maintaining and restoring the luminal epithelium. We find that glandular lineages do not contribute to luminal populations in young or aged mice, although we could induce glands to produce functional epithelium using extensive chemical ablation of the mucosa, suggesting that these cells can serve as facultative progenitors upon extreme damage. Luminal and glandular compartments remained separate during physiologically relevant endometrial disruptions, including menstrual-like tissue shedding, gestation and parturition. Through systematic analysis of tissue damage and regeneration in menstruating samples, we determined that the luminal epithelium undergoes extensive proliferation and envelops the shedding tissue while simultaneously covering retained endometrium, effectively sheltering the underlying stroma before it becomes exposed. During pregnancy, similar luminal epithelium structures envelop the differentiated tissue surrounding developing embryos, suggesting that the mechanisms of perimenstrual repair and gestational epithelial remodeling share previously unappreciated mechanisms. Together, these studies propose a new paradigm in which the uterus deploys a unifying morphogenetic event across menstruation and pregnancy to achieve scarless restoration.

Effects of diet-induced obesity on uterine epithelial glucose metabolism

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Abstract Text

Obesity is an increasingly common issue that affects health, often causing metabolic problems and lowered fertility. Studies have shown that embryo transfer in obese women results in lower implantation and birth rates than in lean women, indicating that obesity impairs uterine function. Whereas fertility outcomes in diet-induced obese (DIO) mouse models are inconsistent. The uterine epithelium stores glucose as glycogen, which peaks during proestrus, and is mobilized during preimplantation for embryo development. The glucose liberated from glycogen could be secreted into the uterine lumen or used to synthesize other nutrients that support embryo development. It could also be used to support the needs of the epithelium. However, the effect of obesity on epithelial glucose metabolism before implantation is not well studied. Therefore, we examined how obesity influences epithelial glucose metabolism at proestrus in two DIO models. We randomly divided eight-week-old mice into three groups: low-fat diet (LFD, 10% kcal from fat), high-fat diet (HFD, 45% kcal from fat), and very high-fat diet (VHFD, 60% kcal from fat) for 18 weeks. Weekly weight monitoring showed significant increases in the high-fat diet groups by week 7. Glucose tolerance was assessed at weeks 5, 10, and 15 via intraperitoneal glucose tolerance test (IPGTT). Glucose tolerance gradually worsened in VHFD mice from weeks 5 to 15, with HFD mice showing intolerance at weeks 10 and 15. After fasting for 5-6 hours, mice were sacrificed in the proestrus stage at week 18. Both DIO groups exhibited increased body fat and serum leptin levels. Hepatic glycogen stores were reduced, but liver mass and triglycerides increased in the obese groups. Although the glucose tolerance was reduced and liver glycogen stores were affected, serum insulin levels remained unaffected in DIO mice. The estrous cycle, uterine mass, and endometrial thickness were unaffected by obesity. Metabolomics identified 109 metabolites in uterine tissue; of these, only one metabolite, i.e., 3-hydroxy butanoic acid, was significantly different ($FDR < 0.05$) in the VHFD group. Pathway enrichment analysis with a P-value cutoff of 0.05 showed increased amino acid metabolism in both high-fat diet groups. Furthermore, pathways such as nicotinate and nicotinamide metabolism, fructose and mannose metabolism, pantothenate and CoA biosynthesis were also enriched. Whereas the VHFD group showed enrichment in pathways including purine metabolism, glyoxylate and dicarboxylate metabolism, pyruvate metabolism, and the citrate cycle, along with lipid and fatty acid metabolism pathways. Immunohistochemistry of two key glycolytic enzymes (phosphofructokinase, PFKM; pyruvate kinase, PKM2) showed no difference in the staining in the uterine epithelium of the DIO mice compared to LFD mice. PAS staining showed no differences in glycogen levels in the uterine epithelium across groups. Similarly, there was no difference in immunohistochemical staining of glycogen-metabolizing enzymes (hexokinase 1, HK1; glycogen synthase, GYS; glycogen phosphorylase, PYGM; glucose-6-phosphatase, G6PC3) in the uterine epithelium in all three groups. Insulin signaling markers (phospho-IRS and phospho-AKT) also showed no differences in the epithelium of the three groups. In summary, our DIO models exhibit obesity traits such as metabolic dysfunction, but glucose metabolism in the uterine epithelium remains largely unaffected, suggesting that the endometrium did not become insulin resistant. Future work will explore how obesity impacts uterine glucose metabolism during decidualization.

Keywords: Diet-induced obesity, glucose, endometrium, insulin signaling.

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Impact of Reprogramming History on the Identity of Induced Human Trophoblast Stem Cells

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Abstract Text

Human trophoblast stem cells (TSCs) provide a powerful platform for investigating early placental development and trophoblast lineage specification. Advances in cellular reprogramming have enabled the derivation of induced TSCs (iTSCs) from either somatic cells or induced pluripotent stem cells (iPSCs), thereby expanding opportunities to model patient-specific placental disorders. However, whether distinct reprogramming routes converge on equivalent TSC states remains unclear. Here, we generated human iTSC lines using two alternative strategies: direct reprogramming of umbilical cord-derived fibroblasts (uc-iTSCs) and stepwise conversion following iPSC reprogramming (iPSC-TSCs). The iTSCs generated by both approaches closely resembled primary human TSCs in canonical marker gene expression and global transcriptomic profiles, confirming successful iTSC establishment. Notably, donor-matched uc-iTSC/iPSC-TSC pairs derived from the same infant did not exhibit enhanced transcriptional correlation in the TSC state. They differed markedly in oxygen responsiveness. Under physiologically relevant (5% O₂) vs elevated (20% O₂) oxygen conditions, 2,019 genes showed route-specific oxygen responses in iPSC-TSCs compared to only 21 genes in uc-iTSCs. The iPSC-TSCs exhibited pronounced transcriptional remodeling under 5% O₂ vs 20% O₂ in pathways encompassing hypoxia-responsive and epigenetic regulatory programs. In contrast, uc-iTSCs maintained comparatively stable expression profiles while preserving donor-specific features across oxygen conditions, with oxygen-dependent changes largely confined to a small subset of genes shared with iPSC-TSCs. Upon differentiation into syncytiotrophoblast (STB) or extravillous trophoblast (EVT), transcriptional organization was predominantly governed by oxygen tension rather than by two contrasting reprogramming routes. A subset of donor-matched uc-iTSC/iPSC-TSC pairs displayed strong transcriptional concordance (Pearson $r > 0.85$ across highly variable genes) when cultured under oxygen conditions optimal for each differentiation state, i.e., 20% O₂ for STB and 5% O₂ for EVT. Differential expression analysis between uc-iTSC and iPSC-TSCs identified *PEG3*, an imprinted gene involved in placental hormone regulation, metabolic control, and programmed cell death, as strongly repressed in iPSC-TSCs across oxygen conditions, whereas uc-iTSCs retained substantially higher expression comparable to primary TSCs (adjusted $P < 10^{-22}$ for iPSC-TSC vs uc-iTSC comparisons in the TSC state). Together, these findings demonstrate that reprogramming history exerts durable, state-dependent effects on trophoblast identity, shaping environmental responsiveness and epigenetic configuration beyond canonical lineage marker expression. This study provides a framework for selecting appropriate reprogrammed trophoblast models for studies of human placental development and pregnancy-associated disorders. This research is supported by grant R01HD094937 from the National Institutes of Health R01HD094937.

Comparative Analysis of Primary Stromal Fibroblast Isolation Across Endometriosis-Associated Tissues

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Abstract Text

Endometriosis is a chronic inflammatory disease, defined by the growth of endometrial-like tissue outside the uterus. Endometriosis typologies include superficial peritoneal, deep-invasive, abdominal wall, adenomyosis, and ovarian endometriomas. A hallmark of endometriosis is adhesive disease with significant fibrosis, likely driven by stromal fibroblasts within the microenvironment.

However, primary stromal fibroblasts from endometriosis remain less well characterized than primary stromal fibroblasts from eutopic endometrium. This may be due to limited surgical samples or challenges in isolating cells from endometriosis tissue.

This study correlated the endometriosis typology and clinical features with the successful isolation of primary stromal fibroblasts and characterized the molecular features of primary stromal fibroblasts isolated from endometriotic tissues.

With IRB approval, primary stromal fibroblasts were isolated using mechanical and enzymatic digestion, size filtration, and selective growth. Immunofluorescence was used to confirm positive expression of stromal fibroblast markers (vimentin, VIM) and the absence of expression of epithelial (cytokeratin-7, KRT7) and endothelial cell markers (CD31, PECAM1). Samples were categorized as endometriosis, adenomyosis, or non-endometriosis, non-adenomyosis (control) based on final surgical pathology. Anatomical location was used to delineate endometriosis typology. Descriptive statistics were used to summarize demographics. Primary stromal fibroblasts underwent *in vitro* decidualization, and QPCR was used to evaluate the expression of prolactin (*PRL*), insulin-like growth factor-binding protein-1 (*IGFBP1*), and bone morphogenetic protein-2 (*BMP2*).

A total of 117 fresh tissue samples were procured: benign ovarian cyst wall (non-endometriosis, n=42), ovarian endometrioma (EOMA, n=41), eutopic endometrium (control, n=12), eutopic endometrium (with adenomyosis, n=10), superficial peritoneal endometriosis (n=5), eutopic endometrium (with endometriosis, n=4), and abdominal wall endometriosis (n=3). There was no difference in age (endometriosis: median 38years (range 21-49); control: median 35years (range 15-52), Mann-Whitney-U, p=0.08). Primary stromal fibroblast cultures were successfully established from 21/41 (51.2%) EOMA, while benign ovarian cyst walls demonstrated the lowest success rate of 10/42 (23.8%). Benign ovarian cyst wall pathology was mostly serous cystadenoma. Primary stromal fibroblast cultures were successfully established from control (8/12, 66.7%), adenomyosis (6/10, 60.0%), and endometriosis (2/4, 50.0%) endometria. Lower success was noted in peritoneal endometriosis (1/5, 20.0%) and abdominal wall endometriosis (1/3, 33.3%) samples. Across all tissue groups, primary stromal fibroblast cultures showed strong diffuse cytoplasmic VIM staining, consistent with fibroblast identity. KRT7, an epithelial cytoplasmic marker, was detected only sporadically, appearing in two EOMA samples and one adnexal mass sample, suggesting limited, possibly incidental, epithelial contamination. PECAM1, an endothelial membrane marker, was similarly observed in only one EOMA sample, consistent with minimal, likely isolated endothelial contamination. Stromal fibroblasts from EOMA treated with decidualization cocktail exhibited a statistically significant blunted induction of decidualization markers compared to control induction [EOMA: *PRL*: 93.5-fold increase; *IGFBP1*: 2.98-fold increase; *BMP2*: 1.85-fold increase (n=3); Control: *PRL*: 319.4-fold increase; *IGFBP1*: 16.3-fold increase; *BMP2*: 2.84-fold increase (n=3), unpaired t-test (two-sided), p<0.05].

These data demonstrated that successful culture of primary stromal fibroblasts was possible across multiple endometriosis tissue types. *In Vitro* decidualization revealed robust responses in control fibroblasts, whereas EOMA-derived stromal fibroblasts exhibited a blunted induction, consistent with progesterone-resistant disease. Progesterone is considered anti-inflammatory and may exhibit anti-fibrotic properties. Future studies will characterize the pro-fibrotic properties of EOMA stromal fibroblasts.

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Investigating The Role of Uterine Perfusion in Corpus Luteum Maintenance During Pregnancy in Cattle: Insights From a Pregnancy Loss Model

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Abstract Text

Induced conceptus demise at the onset of the second month of gestation consistently results in luteolysis within a predictable timeframe, providing a valuable model to study the mechanisms responsible for corpus luteum (CL) maintenance beyond classical maternal recognition of pregnancy. Luteolysis, after conceptus demise, is mediated by PGF2 α and is preceded by a 24% decrease in uterine perfusion, suggesting a potential role of perfusion in CL maintenance. This study aimed to test the hypotheses that 1) sildenafil citrate, a vasodilator, will increase uterine artery perfusion and 2) increased uterine perfusion will reduce transport of PGF2 α and delay luteolysis following pregnancy loss. In Experiment 1, non-pregnant cows were randomly assigned to receive intrauterine administration of sildenafil citrate (100mg; n=9) or vehicle (n=7) on day 8 $\pm$ 1 of the estrous cycle. Uterine artery (UA) perfusion index and diameter, ipsilateral to the CL, were assessed using ultrasonography at 0, 1, 3, and 6 h. Sildenafil citrate increased UA perfusion index (+31% vs. -6.6%, $P < 0.01$) and diameter (+22.2% vs. -0.4%, $P = 0.05$) relative to controls, with the greatest effect observed at 1 h post-administration. In Experiment 2, conceptus demise was induced in pregnant cows (n=11) at day 35 of gestation (study day 0) through intrauterine administration of 7.2% hypertonic saline. In addition, all cows received intrauterine administrations of a cyclooxygenase inhibitor (240mg flunixin meglumine) every 8h from day 5 to 7, and four intrauterine pulses of PGF2 α (0.5mg) every 8h on day 6. Cows were randomly assigned to receive intrauterine administration of sildenafil citrate (100mg; n=5) or vehicle (n=6) 40 min before each PGF2 α pulse. Progesterone was assessed twice daily to determine luteolysis, while UA characteristics and prostaglandin F2 α metabolite (PGFM) were assessed before and after sildenafil and PGF2 α administration, respectively. The change in UA perfusion index, relative to pretreatment baseline, was greater in sildenafil than control cows (+27% vs. -10.4%, $P = 0.07$); however, UA diameter did not differ ($P = 0.25$). Basal circulating PGFM did not differ between control and sildenafil cows (24.8 $\pm$ 3.3 vs. 22.1 $\pm$ 3.9 pg/mL; $P = 0.55$); however, PGFM 10 min post-PGF2 α administration was greater for sildenafil than control cows (484.8 $\pm$ 87.8 vs. 306.7 $\pm$ 59.5 pg/mL; $P = 0.05$). The day of luteolysis did not differ ($P = 0.46$) between control (7.5 $\pm$ 0.3) and sildenafil (7.2 $\pm$ 0.2) cows. In conclusion, sildenafil citrate increases uterine perfusion but does not alter CL lifespan after conceptus demise, indicating that enhanced uterine perfusion is not the primary mechanism supporting CL maintenance beyond maternal recognition of pregnancy.

Expression and Regulation of Histamine Metabolic Enzymes and Receptors and the Function of Histamine at the Maternal–Conceptus Interface in Pigs

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Abstract Text

Histamine, a biogenic monoamine, plays a critical role in regulating interactions between the embryo and the endometrium during the implantation period in mammals. Histamine is synthesized by histidine decarboxylase (HDC) and degraded by histamine N-methyltransferase (HNMT) and diamine oxidase (AOC1) and its actions are mediated through four histamine receptors, histamine receptor (HR) H1 (HRH1), HRH2, HRH3, and HRH4. In humans, maternal blood histamine levels increase during early pregnancy, and HRH1 and HRH2 are expressed in both the decidua and trophoblast. In mice, histamine is synthesized in the luminal epithelium of the endometrium during the preimplantation period. However, the biogenesis and metabolism and the roles of histamine during the implantation period in pigs remains poorly understood. To initiate the study on the roles of histamine during pregnancy, we examined the expression of histamine metabolic enzymes and receptors in the endometrium during the estrous cycle and pregnancy in pigs. We found that *HDC*, *HNMT*, *AOC1*, *HRH1*, *HRH2*, *HRH3*, and *HRH4* were expressed in the endometrium during the estrous cycle and pregnancy. The expression of *HDC* in the endometrium was greater on Day 12 of the estrous cycle than Day 12 of pregnancy, while the expression of *HNMT* and *HRH2* in the endometrium were greater on Day 15 of pregnancy than on Day 15 of the estrous cycle. Histamine metabolic enzymes and receptors were also expressed in conceptuses during early pregnancy and in chorioallantoic tissues during mid- to late pregnancy. In addition, *HDC*, *HNMT*, *AOC1*, *HRH1*, and *HRH2* proteins were localized to luminal and glandular epithelial cells and blood vessels during the estrous cycle and pregnancy. Culture of porcine uterine epithelial cells with increasing doses of histamine *in vitro* affected the expression of pro-inflammatory cytokines, cyclooxygenases, and matrix metalloproteinases. These results suggest that histamine may play an important role in the establishment and maintenance of pregnancy in pigs by regulating endometrial epithelial cell function during pregnancy. [This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (Ministry of Science & ICT; #RS-2025-00518549), Republic of Korea]

Noonan Syndrome-Associated SHP2-Q79R Mutation in Trophoblast Disrupts Placental Development, Resulting in Intrauterine Growth Restriction

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Abstract Text

Noonan syndrome (NS) is an autosomal dominant genetic disorder, with approximately 50% of cases caused by activating mutations of SHP2—a ubiquitously expressed non-receptor tyrosine phosphatase that regulates multiple signaling pathways essential for development and homeostasis. While activating SHP2 mutations have been extensively linked to embryonic developmental defects, their impact on the placenta—a temporary but vital organ for intrauterine fetal development and postnatal health—remains poorly understood. In the present study, trophoblast-specific activation of the NS-associated SHP2-Q79R mutation in mice led to intrauterine growth restriction (IUGR) and disrupted placental architecture, characterized by aberrant expansion of glycogen trophoblast (GlyTs) cells in the labyrinth. Mechanistically, SHP2-Q79R upregulates the RNA-binding protein PTBP3, which stabilizes *Nr2f2* mRNA and enhances the expression of COUP-TFII (encoded by *Nr2f2*). This signaling cascade triggers up regulation of GlyT-associated genes and the expansion of GlyTs in the placental labyrinth. Notably, trophoblast-specific activation of SHP2-Q79R mutation also induced abnormal fetal cardiac development, partially recapitulating the clinical phenotype of NS. These findings not only provide unappreciated insights into the etiology of placental pathology, but also reveal cardiac developmental abnormalities arising from placentas carrying NS-causing mutations, identifying the placenta as a potential therapeutic target for intrauterine fetal developmental abnormalities.

Pregnancy-Associated Glycoproteins (PAGs) Induce Endometrial Changes Associated with Early Pregnancy: Maintenance of the CL of Pregnancy by PAGs may Involve Novel Immunomodulatory Mechanisms

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Abstract Text

We recently found that PAGs maintain bovine corpus luteum (CL) during pregnancy; however, the mechanisms underlying this action remain undefined. Open Holstein heifers (n=25) were synchronized and ovulated spontaneously (defined as D0) by ultrasonography. Blood flow to CL was assessed daily by color Doppler and P4 measured daily by ¹²⁵I radioimmunoassay and PAGs by BioPRYN Flex ELISA. PAGs were extracted from cotyledons collected at parturition from Holstein cattle and purified via multiple protein-exclusion steps. Heifers received daily intrauterine infusions from D14–D24 post-ovulation: 2 mg bovine serum albumin in horn ipsilateral to CL (Control; n=8) or 2 mg PAGs in horn contralateral (ContraLat; n=9) or ipsilateral (IPSI; n=8) to CL to evaluate local versus systemic effects of PAGs on CL maintenance. Blood was sampled every 12h (D14–24) and daily thereafter (D25–30). Uterine cytobrush was collected on D16 for RNA-seq analysis. In RStudio, linear models included treatment, day, block, and their interactions; residuals were assessed, Box–Cox transformations applied when needed, and Tukey-adjusted pairwise contrasts generated. Transcriptomic counts were analyzed using a negative binomial model followed by Wald test. Circulating PAGs from D14.5 (12h post-infusion) to D19 (day before mean luteolysis day in ContraLat group) did not differ between IPSI (3.8±1.6 ng/mL) and ContraLat (3.3±1.7ng/mL) but were undetectable for Control (P< 0.0001). IPSI heifers exhibited prolonged CL lifespan (D25) compared to ContraLat (D20) and Control (D16; P< 0.0001) indicating that there was an ipsilateral effect of PAGs to maintain the CL of pregnancy. From RNA-seq analysis, 127 genes were upregulated and 35 downregulated by PAG treatment including upregulation of CXCL8, CXCL5, CCR1, CCR2, MMP9, MMP19, MMP25 CD36, RAB20, ILRAP. This transcriptomic analysis indicated four major effects of PAGs on the endometrium: 1) In the absence of an embryo, PAG treatment was sufficient to induce coordinated expression of CXCL8 and CXCL5 together with their cognate receptors CXCR1 and CXCR2, indicating that PAGs establish a GPCR-driven chemokine landscape that primes the endometrium for controlled myeloid responsiveness while preserving immune balance; 2) Concurrently, the upregulation of CCR1 and CCL2 indicates activation of a complementary CC chemokine axis networks that regulate controlled recruitment of myeloid cells independently of embryo attachment. Downstream, this chemokine landscape is accompanied by transcriptional features of enhanced innate immune competence and homeostatic clearance, including CD36 and RAB20, consistent with macrophage-associated phagocytosis and endosomal trafficking that limit secondary inflammatory signaling and preserve epithelial integrity. 3) Enhancement of innate immune functional readiness coupled with active suppression of inflammatory amplification through IL-1 buffering and regulated NF-κB signaling. A constrained inflammatory architecture is further supported by induction of IL1RAP, indicating selective tuning of IL-1 receptor signaling at the maternal interface, compatible with localized inflammatory cues that remain tightly regulated; 4) Activation of metabolic, phagocytic, and extracellular matrix remodeling programs that support a tolerogenic, implantation-permissive endometrial environment. For example, PAG exposure activates extracellular matrix remodeling programs through induction of MMP9, MMP19, and leukocyte-associated MMP25, enabling pericellular proteolysis, immune cell migration, and structural plasticity without pathological inflammation. Our results indicate that PAGs are critical for the maintenance of the CL of pregnancy. The mechanisms appear to involve a chemokine network that regulates neutrophils and monocyte/macrophages to maintain endometrial immune balance during the peri-implantation period and are likely to be critical for maintaining the optimal uterine environment and the CL during the remainder of pregnancy.

Siglecs in the Porcine Oviduct and Sialylated Ligands on Sperm: Roles in the Formation of an Immune-Quiescent Sperm Reservoir

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Abstract Text

Following semen deposition, seminal fluid and sperm elicit uterine immune cell infiltration, facilitating sperm clearance. Unlike the uterus, sperm do not trigger phagocytic responses in the oviduct in the absence of disease. Thus, the oviduct possesses a distinct immunological microenvironment that tolerates sperm while maintaining the capacity to respond to pathogens.

The mechanisms underlying this sperm-selective immune response remain poorly understood. The sperm glycocalyx has been proposed as a key contributor to innate oviductal tolerance, yet the cell and molecular mechanisms involved remain unclear. The current investigation focused on the role of sialic acid-containing glycoconjugates on sperm and their potential to elicit innate tolerance via cognate sialic acid-binding immunoglobulin-type lectins (Siglecs) expressed in the oviduct. Consistent with this, we identified eight Siglecs expressed in the porcine lower oviduct, five of which are immune inhibitory (Siglecs-2, -3, -5, -10, and -11). Mass spectrometry analysis of porcine sperm revealed the presence of a mixture of α 2,3 and α 2,6-linked sialic acids, with α 2,3-linked sialic acids being the predominant linkage type. Of the detected glycans, several sialic acid-containing glycoconjugates were identified as potential ligands for Siglecs, including O-linked glycans: NeuAc1GalNAc1, NeuGc1GalNAc1, NeuAc2Gal1GalNAc1, and glycolipids: NeuAc2Gal1GalNAc1Gal1Glc1, Fuc1Gal1GalNAc1NeuAc1Gal1Glc1. Lectin staining confirmed the location of sialoglycans in the apical region of the sperm head. Spheroids were formed with primary oviduct epithelial cells to model oviduct physiology, enabling the study of the immune response to sperm sialoglycans via qPCR. Results demonstrated that innate sialic acids contribute to sperm immune tolerance, while the addition of α 2,6-linked sialic acids triggered an immune response, upregulating IL1B expression. Our findings highlight the expression of five oviductal inhibitory Siglecs and their potential interactions with sperm sialoglycans to regulate the oviduct's unique immune response to sperm. This research was supported by NIH R24GM137782 to PA, 1R21HD111954-01A1 to DM and AA, and NIH 1F31HD108959 to LM.

Deconstructing the Role of a Physical Stimulus in Peri-implantation Uterine Luminal Remodeling

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Abstract Text

Differentiation of uterine stromal cells, decidualization, is essential for a successful pregnancy, yet the initiating factors differ between species. In humans, decidualization begins spontaneously every menstrual cycle, independent of the embryo. In contrast, mice require a specific trigger, such as an embryo or an artificial stimulus to initiate decidualization. During early pregnancy in mice, embryomediated uterine architectural changes could act as a signal to initiate decidualization. However, how these structural transitions aid in natural and artificial decidualization remains unclear. To address this question, we used multiple estrogen-dependent implantation and decidualization models. These included natural pregnancy and several artificial stimuli like sesame-oil, uncoated agarose beads, and Concanavalin A (ConA)-coated agarose beads that were introduced into pseudo-pregnant female mice to evaluate early uterine remodeling and its relationship to implantation chamber formation and decidualization. During pre-implantation gestational day (GD) 3.5, luminal epithelial folding occurred across all conditions irrespective of whether an object, embryo or egg was present in the lumen. The number of folds correlated strongly with uterine horn length rather than embryo number, indicating a hormone-driven response rather than dependence on a specific physical or molecular stimulus. During implantation at GD4.5, embryos, oil droplets, and ConA-coated beads induced a V-shaped implantation chamber together with stromal PTGS2 expression under the implantation chamber. In contrast, uncoated agarose beads failed to initiate a chamber or PTGS2 expression, although they were present in the lumen. Thus, the mere presence of a physical object is insufficient to trigger implantation chamber formation. Additionally, lectins with carbohydrate-binding properties similar to ConA also initiated an implantation chamber, suggesting that specific sugar-based interactions between the stimulus and uterine epithelium can drive the structural changes associated with implantation. By post-implantation stage GD5.5, PTGS2 expression extended laterally along the elongated chamber in the presence of an embryo, oil droplet or ConA-beads. Second harmonic generation imaging further showed that collagen fibers were arranged radially around the elongated implantation chamber during decidualization, a pattern that was consistent regardless of the stimulus. Taken together, these results demonstrate that the uterus does not respond uniformly to all physical objects; rather, decidualization is triggered only when the uterus encounters a focal stimulus capable of initiating communication with the luminal epithelium that results in a structural transformation of the lumen into the V-shaped chamber. These findings reveal key similarities and differences between embryo-dependent and embryo-independent models of decidualization. These embryo-independent models of decidualization in the mouse can help uncover the physical and molecular pathways that underlie decidualization initiation in other mammals, including humans.

Protective Effects of Defatted *Tenebrio molitor* Extract Against LPS-Induced Inflammation and Oxidative Stress in Porcine Luminal Epithelial Cells

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Abstract Text

Edible insects have emerged as potential therapeutic agents due to their bioactive properties. This study aimed to investigate whether *Tenebrio molitor* (TM) extract attenuates lipopolysaccharide (LPS)-induced inflammation and oxidative stress in porcine luminal epithelial (pLE) cells. The TM extract was prepared using an aqueous extraction method under optimized pH and temperature conditions, followed by a defatting process (TM-DF). To determine the optimal experimental concentrations, cell viability assays were performed; the concentration of LPS that inhibited viability by approximately 50% was identified, and the non-cytotoxic concentration of TM-DF was established. Subsequently, pLE cells were pre-treated with 5 µg/mL of TM-DF for 2 hours prior to treatment with LPS (50 µg/mL) for 48 hours. Pre-treatment with TM-DF significantly restored cell viability that had been inhibited by LPS. Furthermore, TM-DF effectively suppressed the LPS-induced reactive oxygen species (ROS) elevation, indicating a potent antioxidant effect. These findings suggest that defatted *Tenebrio molitor* extract (TM-DF) possesses protective properties against LPS-induced cellular damage and oxidative stress in pLE cells, highlighting its potential as a functional feed additive or therapeutic agent.

Transcriptomic Analysis of Neonatal Cortical Bone and Bone Marrow in a Porcine Model of Biological Extremes in Intrauterine Crowding

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Abstract Text

Intrauterine growth restriction (IUGR) affects ~10% of human pregnancies and is associated with short- and long-term complications. The objective of the present study was to assess the impact of IUGR on cortical bone (CB) and bone marrow (BM) gene expression in a surgically-induced porcine model of biological extremes in intrauterine crowding. Commercial crossbred gilts (N=12) were allocated to one of two surgical groups to produce biological extremes in uterine crowding and IUGR incidence. A total of n=6 gilts underwent a unilateral hysterectomy-ovariectomy (UHO), wherein one uterine horn and the ipsilateral ovary were removed, effectively decreasing uterine space by ~50% while maintaining ovulation rates by compensatory activity within the contralateral ovary. Conversely, n=6 gilts underwent a unilateral oviductal ligation (UOL) in which a single oviduct was ligated to reduce litter size by ~50% while maintaining uterine space. Following a minimum of 21 days postoperative recovery, gilts were artificially inseminated using pooled Duroc semen and allowed to gestate to term. At approximately 18 hours after farrowing, the resultant piglets were euthanized, and in-depth phenotypes including weights of the liver (LVR), brain (BRN), and body were collected. Additionally, the midshaft of one femur was dissected out, and CB and BM separated by centrifugation. From the resultant population of n=98 piglets, a subset of four biologically-extreme, male UOL piglets with a low BRN/LVR weight ratio ($\bar{x}_{BRN/LVR}=0.56\pm0.04$) and four biologically-extreme, male UHO piglets with a high BRN/LVR ($\bar{x}_{BRN/LVR}=1.55\pm0.21$) were selected for transcriptomic analysis via bulk RNA-Seq. In the CB, 11,499 genes were expressed at a CPM $\geq$ 5 in at least four samples, while in the BM, 10,752 genes were expressed at or above this threshold. At a threshold of log2FC >|1| and $p_{adj}<0.05$, 106 and 63 differentially-expressed genes (DEGs) were identified in CB and BM, respectively. Of these genes, 84 were downregulated, and 22 upregulated in the CB of UHO relative to UOL males, while in the BM of UHO males, 34 genes were downregulated and 29 genes were upregulated. A subset of DEGs with a log2FC >|1.5| and $p_{adj}<0.01$ were selected for further assessment by qPCR, with 14 genes differentially-expressed in CB and 14 genes differentially-expressed in BM at this cutoff. For qPCR, a larger subset of male ($n_{UOL}=12$; $n_{UHO}=12$) and female ($n_{UOL}=12$; $n_{UHO}=12$) piglets with the most-extreme BRN/LVR were selected. Within this subset, the BRN/LVR was more than doubled in UHO ($\bar{x}_{BRN/LVR}=1.34\pm0.33$) relative to UOL ($\bar{x}_{BRN/LVR}=0.64\pm0.09$) piglets, while body weight was markedly reduced in UHO ($\bar{x}=922.92\pm307.94$ g) relative to UOL ($\bar{x}=1876.04\pm227.59$ g). A total of five DEGs identified by RNA-Seq were assessed in the CB of this larger subset, of which three, GRIA1, LOC100511639, and NGFR were differentially-expressed by qPCR, with median fold-changes of 6.38, 2.97, and -1.88, respectively, in UHO relative to UOL piglets ($P<0.001$). Ten additional hypothesis-driven genes with roles in regulating bone remodeling were also assessed in CB. Expression of CTSK, RUNX2, and SPP1 was significantly upregulated in the CB of UHO relative to UOL piglets (median fold-changes of 1.53, 1.17, and 1.29, respectively; $P<0.05$), while expression of ADAMTS14, BMP1, COL1A1, COL1A2, SOST, TNFSF11, and MEPE did not differ significantly between groups. Collectively, these results provide valuable information on the transcriptomic profiles of neonatal CB and BM and give novel insights into the consequences of intrauterine crowding and IUGR on CB and BM gene expression.

Exploring How Defects in Uterine Hedgehog Signaling Result in Thin Murine Endometrium

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Abstract Text

In the mouse uterus, the expression of members of canonical Hedgehog signaling rapidly and significantly change across the estrous cycle. Despite compelling prior studies, the individual roles of the numerous pathway members have not yet been fully elucidated. Using a Progesterone Receptor (PGR)-mediated conditional knockout (cKO) of *Gli2*, the primary activating transcription factor propagating downstream Hedgehog signaling, we have uncovered a striking phenotype in the uterus. Across the estrous cycle, mice lacking uterine *Gli2* possess a thin endometrium. In humans, a thin pre-gestation endometrium is associated with increased risk of implantation failure, pregnancy loss, and complications such as pre-eclampsia and fetal growth restriction. The use of medications to thicken the endometrium prior to pregnancy yield inconsistent results. And so, in many cases, thin endometrium appears to be an indication of other functional defects in this tissue which cannot be resolved by simply improving the measured thickness. The mechanisms by which thin endometrium later negatively affect gestation are unknown. Excitingly, however, the thin endometrium of *Gli2* cKO mice may recapitulate the patterns observed in humans. Fertility trials in these animals have revealed decreased fertility, by the number of pups born in each litter. Our investigations are establishing what distinguishes a thin endometrium from a normal endometrium. Our results show that the cyclic thickening of the endometrium between diestrus and proestrus is dependent on *Gli2* for proper stromal expansion. RNA sequencing shows decreases ($p < 0.05$) in epithelial and keratinocyte differentiation, as well as various cellular immune responses in the *Gli2* cKO uteri. These results offer exciting new downstream impacts of Hedgehog signaling in the uterus and a model to investigate the negative impacts of thin endometrium on fertility.

Examining Menstrual Fluid Proteins for Non-Invasive Detection of Heavy Menstrual Bleeding

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Abstract Text

Heavy menstrual bleeding (HMB) affects 1 in 3 women and is a leading cause of iron deficiency anaemia. Women with HMB experience symptoms for an average of 3 years before seeking help. A non-invasive method to identify women with HMB is required to reduce risk of anaemia. Menstrual fluid (MF) can be collected non-invasively. We hypothesized that MF protein content differs between women with objectively quantified normal menstrual blood loss (NMB) and HMB. MF might provide markers to allow identification of women with HMB.

With informed consent and ethical approval (REC23/WS/0125), MF was collected on day 1 or 2 of menstruation. Participants were classified as NMB (< 80mls, n=35) or HMB (≥80mls, n=18) after objective quantification by the alkaline haematin method. All had regular cycles (24-38 days), were not using exogenous hormones and had a pelvic ultrasound scan. Digested peptides from MF supernatants (n=10 NMB, n=10 HMB) were analysed by nano LC–MS/MS using an Ultimate RSLCnano LC system coupled to a timsTOF Flex mass spectrometer. Data acquisition via diaPASEF was analysed using a neural network-based DIA workflow. Protein identifications were defined by the presence of 2 or more unique peptides and data was analysed using Qiagen IPA. Proteins identified as differentially expressed in HMB vs NMB ($p < 0.05$, fold change > 1.2) from mass spectrometry data were examined in MF supernatant (n=35 NMB, n=18 HMB) by ELISA.

Mass spectrometry analysis identified 2,591 proteins in MF. Of these, 178 proteins showed statistically significant differences in abundance between HMB and NMB ($p < 0.05$). Applying an additional fold-change threshold, 71 proteins met both criteria ($p < 0.05$ and absolute fold change ≥ 1.2 ; HMB vs NMB) and were defined as differentially expressed. Pathway analysis predicted activation of immune and vascular interaction pathways in the HMB group, including complement cascade, scavenger receptor-mediated uptake, and immune cell communication. Upstream regulator analysis using Qiagen IPA inferred inflammatory cytokines and immune regulatory factors, including TNF, IL17A, and IL1B, as potential drivers of these pathway changes. Of the 71 differentially expressed proteins, 23 were increased and 48 were decreased in HMB relative to NMB. Proteins were ranked based on statistical significance in expression between NMB and HMB, and selected proteins were further examined by ELISA (n = 35 NMB, n = 18 HMB). Levels of serum amyloid A-1 protein were significantly higher in individuals with HMB compared with NMB ($p = 0.023$).

To our knowledge, this study is the first to examine the proteome of MF from women with and without objective HMB. Our findings of increased levels of serum amyloid A-1 and activation of immune and vascular interaction pathways in HMB suggest an increased inflammatory response at menstruation in those with HMB. This study supports the feasibility of using MF to distinguish between NMB and HMB. Validation of protein markers in larger, diverse populations is required to make progress towards using MF to identify women with HMB and reduce risk of anaemia.

Functional roles of extracellular vesicles in the oviduct on mouse embryo development *in vivo*

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Abstract Text

Preimplantation embryos interact with oviduct epithelial cells during the first few days after fertilization *in vivo*. Oviductal extracellular vesicles (called oEVs) are crucial for preimplantation embryo development *in vitro*. EVs shuttle molecular cargo such as lipids, mRNAs, miRNAs, and proteins to other cells. Several studies show that the addition of oEVs to culture media increases blastocyst developmental rates and reduces reactive oxygen species and apoptotic cells in preimplantation embryos. Our previous work shows that epithelial cell-specific oEVs labeled with green fluorescent protein, using the *Wnt7a*^{Cre/+};*Cd9*-TIGER^{f/+} mouse model, from the oviduct are taken up by 4- to 8-cell-stage embryos *in vivo* and detected in the perivitelline space. Proteomic analysis has shown that these EVs contain proteins enriched for embryo protection, including protease inhibitors, heat-shock proteins, and DNA repair. Because embryos can develop *in vitro*, the requirement and functional roles of oEVs have yet to be investigated *in vivo*. Based on prior studies and our current work, we hypothesize that oEVs enhance preimplantation embryo developmental trajectory *in vivo*. To test the function of EVs, an EV inhibitor (GW4869) was used. Adult *Wnt7a*^{Cre/+};*Cd9*-TIGER^{f/+} female mice treated with GW4869 (75 µg/mouse), beginning at the estrus stage and continuing for a total of 4 days, showed a significant reduction of EV-GFP signals in the epithelial cells of the oviduct and uterus. Transmission electron microscope images illustrated that multivesicular body formation was severely reduced in the GW4869-treated oviduct, indicating a successful suppression of EV biosynthesis and release. However, when these mice were allowed to mate, the number of blastocysts (assessed by morphology) was similar between the vehicle- and GW4869-treated group. Nevertheless, the transcriptional signature and chromatin accessibility of these blastocysts are being investigated to assess whether the inhibition of EVs could lead to changes in embryo developmental potentials. In summary, we have shown that the production of oEVs can be inhibited, which allows us to further investigate their role in the development of preimplantation embryos *in vivo*. The findings from our study may provide insights into whether the use of oEVs would be beneficial for embryos developed *in vitro* in a clinical setting. Funding R01 HD087097.

Glycogen Mobilization and Redox Capacity in the Intercaruncular Endometrium Contribute to Form of Selenium-Induced Increase in Conceptus Length at Maternal Recognition of Pregnancy in Beef Heifers

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Abstract Text

Selenium (Se) supplementation to grazing cattle is necessary in regions where the soil, and therefore forages, are deficient in this trace mineral. Supplementation typically occurs using an inorganic form of Se (ISe) even though organic, chelated forms (OSe) are available when cattle consume forage. We have studied the effect of supplementing beef heifers with ISe alone versus a mixture of the two forms (MIX, 1:1 ISe:OSe) on ovarian and uterine responses during the early luteal phase and at maternal recognition of pregnancy (MRP), and identified significant effects on the corpus luteum (CL), intercaruncular (ICAR) and caruncular (CAR) endometrium, and serum metabolites. Critically, these findings culminated in a MIX-induced increase in conceptus length at MRP. Relevantly, we observed significant form of Se-induced changes in circulating glucose, cholesterol, and low- and very-low density lipoproteins during early pregnancy. In ICAR, MIX-supplemented heifers had an increased abundance of mRNA encoding myostatin (MSTN) which may increase glucose in histotroph, with glucose the primary energy source to nourish the preimplantation conceptus. These findings led us to consider whether metabolomic changes in the ICAR would contribute to the mechanism supporting the observed larger conceptus length. The objective of this study was to determine the effect of supplementation with ISe versus MIX on the metabolome of ICAR samples collected from these heifers at MRP. Angus-cross heifers received no Se for 45 days (depletion) followed by ISe alone for 45 days (repletion), then at least 90 days of supplementation with either ISe or MIX (treatment) prior to synchronization of estrus and artificial insemination. On d17, heifers were killed and their reproductive tracts collected. ICAR samples from pregnant heifers (n = 6 per treatment) were collected and processed for untargeted metabolomics. Samples were analyzed using an Ultra-High Performance Liquid Chromatography (UHPLC) system coupled to a Thermo Fisher Scientific Q Exactive HF (QEHF) mass spectrometer. Separations were performed on C18 and HILIC columns under positive (POS) and negative (NEG) ion modes. Compound Discoverer 3.3 SP3 was used for metabolite identification, and multivariate analysis was performed using MetaboAnalyst6.0. On the C18 column, we identified a total of 101 annotated metabolites in POS ion mode and 60 annotated metabolites in NEG ion mode. On the HILIC column, we identified a total of 127 annotated metabolites in POS ion mode and 151 annotated metabolites in NEG ion mode. In MIX-supplemented heifers there was a greater abundance ($P < 0.05$) of tetraglucoside and spermidine indicating mobilization of glycogen by ICAR, whereas in ISe-treated heifers, increased abundance ($P < 0.05$) of L-(+)-Lactate was observed which indicates a shift towards anaerobic glycolysis. We observed a MIX-induced increase ($P < 0.05$) in acylcarnitines indicating a shift to fatty acid oxidation within the ICAR of that treatment group. This then contrasted by a greater abundance ($P < 0.05$) of free fatty acids in the ICAR of ISe-treated heifers, indicating the absence of carnitine-mediated oxidation. Finally, there was a greater abundance ($P < 0.05$) of glutathione and ent-Prostaglandin $F_{2\alpha}$ in the ICAR of MIX-treated heifers, suggesting a MIX-induced environment supporting the mitigation of oxidative stress, and an increase ($P < 0.05$) of the luteotropic prostaglandin E_2 indicating a shift towards a pro-gestational state. Collectively, the MIX-form specific metabolome contributes to a nutrient-rich environment that supports MRP and rapid elongation of the pre-implantation conceptus.

Loss of a Key RNA Splicing Mechanism Disrupts Lysosomal Homeostasis and Inflammatory Control in the Uterus, Leading to Progressive Fibrosis and Infertility

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Abstract Text

RNA splicing is a fundamental mechanism regulating tissue homeostasis, yet its role in maintaining uterine integrity remains poorly understood. Polypyrimidine tract-binding protein 1 (PTBP1) is a key RNA-binding protein that regulates alternative splicing of transcripts involved in metabolism, vesicular trafficking, and cellular stress responses. To define PTBP1's role in the uterus, we generated a mutant mouse model in which the *Ptbp1* gene is conditionally deleted in the uterus using progesterone receptor (PR)-driven Cre and examined reproductive function and uterine homeostasis. Although *Ptbp1* mutant females were initially subfertile when bred with wild-type males, they became sterile after the third delivery, failing to deliver pups despite normal mating behavior, whereas control females continued to produce normal litters. Histological analysis of the uteri of non-pregnant pubertal females revealed extensive uterine fibrosis in *Ptbp1* mutant uteri by three months of age, which became markedly more pronounced by six months, indicating progressive disruption of uterine architecture. To elucidate the molecular mechanisms underlying this phenotype, we performed RNA sequencing of mutant and control uteri, revealing dysregulation of genes linked to lysosomal function, cellular senescence, and inflammation. Importantly, loss of *Ptbp1* led to alternative splicing of pyruvate kinase (PKM), reducing expression of the PTBP1-dependent PKM2 isoenzyme. PKM2 is a critical regulator of lysosomal biogenesis, coupling cellular metabolic status to AMPK-mTOR-TFEB signaling to promote transcription of lysosomal and autophagy-related genes and sustain lysosomal formation. In control and *Ptbp1* mutant uteri, immunostaining for lysosome-associated membrane glycoprotein 1 (LAMP1) revealed abundant lysosomes in both epithelial and stromal compartments of control uteri, whereas *Ptbp1* mutant uteri showed a drastic reduction in lysosomes in both compartments. Further analysis of RNA sequencing results revealed that defective lysosomal homeostasis was accompanied by activation of inflammatory and senescence-associated programs, including chemokine upregulation, NLRP6 inflammasome activation, macrophage accumulation, and induction of extracellular matrix remodeling genes such as collagens, consistent with progressive fibrosis. Together, these findings identified PTBP1 as a previously unrecognized regulator of uterine homeostasis, acting through alternative RNA splicing to preserve critical metabolic activity via PKM2 expression, promote lysosomal biogenesis, and suppress chronic inflammatory signaling. Loss of PTBP1 leads to lysosomal insufficiency, persistent inflammation, and progressive uterine fibrosis, ultimately compromising reproductive health, fertility, and pregnancy outcomes.

Endometriosis Alters Pain-Associated Gene Signatures in the Dorsal Root Ganglion and Driving Peripheral Sensitization and Pelvic Pain

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Abstract Text

Endometriosis is a chronic inflammatory gynecological disease of reproductive-age women. Chronic pelvic pain in endometriosis is a combination of visceral, inflammatory, and neuropathic components. In a subpopulation of endometriosis patients, pain persists after removing endometriotic lesions, and the underlying mechanisms are unknown. We used a xenograft model of human endometriosis, measured pelvic pain, and analyzed gene signatures in the dorsal root ganglion by RNA-Seq, and determined the expression of proteins involved in nociception and neurogenic inflammation. Results indicated that endometriosis altered gene signatures associated with nociception, neuroinflammation, glutamate/GABA biosynthetic and signaling components in the dorsal root ganglion. The results support the concept that endometriotic lesions alter gene expression profiles in the dorsal root ganglion, the first component in the pain pathways from the periphery to central pain circuits. Our ongoing studies will determine the gene expression profiles in the spinal cord and brain pain-processing regions in endometriosis mouse models.

The Pathogenic Properties of Human Endometriotic Epithelial Cells Requires Steroid Receptor Coactivator-2

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Abstract Text

Introduction: Endometriosis is a chronic debilitating gynecological disorder defined by the presence of heterogeneous endometriotic lesions outside the uterine cavity. Endometriotic lesions primarily develop on the pelvic peritoneum, uterosacral ligaments, ovaries, and fallopian tubes, leading to pelvic pain, dyspareunia, subfertility, and infertility. Despite the high prevalence of endometriosis reported for reproductive age women worldwide, the mechanisms that underpin endometriotic lesion pathology are not clearly understood at the molecular level. The steroid receptor coactivator (SRC) family, comprising SRC-1, SRC-2, and SRC-3, controls distinct transcriptional programs across a wide range of physiological and pathological processes. While studies demonstrate that endometrial SRC-2 is essential for both human and murine endometrial cell function that leads to pregnancy establishment, the involvement of this coactivator in endometrial pathologies is less well defined. To address this insufficiency, we investigated whether SRC-2 has a functional role in the pathogenic properties—migration, invasion, and proliferation—of human endometriotic epithelial cells. This line of investigation is particularly relevant since therapeutic approaches to inhibit the action of SRC family members have recently been reported for a number of pathologies, including cancer.

Methods: Human and non-human primate matched eutopic endometrium and ectopic endometriotic lesion was used for immunohistochemical studies. Immortalized human endometriotic epithelial cells in culture were used for expression and functional analyses of SRC-2 and its target genes. Quantitative real time PCR and western techniques were used for expression studies while cell viability/proliferation, clonogenic survival, and transwell cell migration and invasion assays were used to measure cell proliferation, migration and invasion respectively. Lastly, RNA-seq revealed the genome-wide transcriptome in human endometriotic epithelial cells that are dependent upon SRC-2 expression and required for endometriotic epithelial cell pathology. **Results:** We demonstrate that SRC-2 expression levels are elevated in human and non-human primate endometriotic lesions when compared with matched eutopic endometrial tissue. Using a series of *in vitro* functional assays, we demonstrate that SRC-2 is critical for the pathogenic properties of human endometriotic epithelial cells, which include cellular proliferation, migration and invasion. Global transcriptome analysis highlights distinct gene expression programs that mediate SRC-2's contribution to the pathogenic properties of human endometriotic cells, which encompass cytokine signaling, cellular inflammatory responses, adaptation to hypoxia, epithelial-mesenchymal transition, and cell-cell communication. To underscore the utility of this SRC-2 transcriptome dataset as an information resource to identify new molecular drivers of endometriosis progression, we show that S100 calcium-binding protein A10 (S100A10) is a crucial SRC-2 responsive molecular target for maintaining the pathogenic properties of the human endometriotic epithelial cell. As a multifunctional protein with a wide range of physiological activities, elevated S100A10 mRNA levels have been reported in human endometriotic lesions when compared with matched eutopic endometrial tissue (GSE5108 and GSE7305). Considering SRC-2 and S100A10 represent promising targets for cancer therapeutic intervention, our data support SRC-2 and S100A10 as new molecular vulnerabilities in the pathology of endometriosis that should be considered when formulating more effective treatment regimens to clinically manage this endometrial pathology in the future.

Evaluation of the Stability of Expression of Reference Genes in 12Z and KGN Cell Lines with and Without TNF- α Treatment

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Abstract Text

Background: Both 12Z and KGN cells are used as *in vitro* models for studying reproductive diseases. 12Z is an immortalized human epithelial-like endometriotic cell line, frequently used to study endometriosis, particularly inflammatory effects. KGN is a human granulosa cell tumor cell line, frequently used to study granulosa cell biology. Accurate quantification of differential gene expression by qPCR is central to many studies; however, reliability depends on the selection of stable housekeeping genes, a factor that is frequently overlooked.

Objective: To evaluate the stability of seven candidate housekeeping genes: actin beta (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), ribosomal protein L19 (*RPL19*), ribosomal protein S18 (*RPS18*), beta-2-microglobulin (*B2M*), and hypoxanthine phosphoribosyl-transferase 1 (*HPRT1*) across KGN and 12Z cells under tumor necrosis factor alpha (TNF- α) and control (vehicle) conditions.

Methods: Candidate genes were selected through an extensive literature search for common housekeeping genes reported in reproductive tissues or cell lines. TaqMan probes were selected based on inventoried and intron-spanning assays. 12Z and KGN cells were seeded, allowed to attach and proliferate overnight, then treated with either 10 ng/mL TNF- α or vehicle (distilled water) in serum-free media for 24 hours, with three biological replicates per treatment per cell line. RNA was isolated and reverse transcribed into cDNA. RNA and cDNA quantity and quality were consistent across samples. Using TaqMan probes, with three technical replicates per biological replicate, qPCR was performed. Across replicates for both treatment groups, gene stability was assessed using (1) the coefficient of variation (CV). (2) Stability values were computed by the relative quantification (RQ Δ Ct) method implemented in the R package NormFinder. (3) Equivalence was assessed using nonparametric two one-sided tests (TOST), and expression shift was estimated using the Hodges-Lehmann (HL) estimator, with equivalence bounds of -0.5 to 0.5 (SAS9.4). The most appropriate housekeeping gene would have a low CV, a low stability score, and a minimal expression shift, with a 95% confidence interval (CI) within the (-0.5, 0.5) equivalence bounds.

Results: For 12Z, *ACTB* had the lowest CV (0.1176), followed by *YWHAZ* (CV=0.3212), *RPS18* (CV=0.3539), *HPRT1* (CV=0.3654), *B2M* (CV=0.5079), *RPL19* (CV=0.6237), and *GAPDH* (CV=0.7588). *RPS18* had the best stability score, followed by *ACTB*, *RPL19*, *YWHAZ*, *GAPDH*, *HPRT1*, and *B2M*. *ACTB* showed the least median shift between treated and control groups [median difference=-0.113 Ct; 95% confidence interval (-0.429, 0.159)]. For KGN, *ACTB* had the lowest CV (0.2512), followed by *HPRT1* (CV=0.3263), *B2M* (CV=0.362), *RPS18* (CV=0.4224), *YWHAZ* (CV=0.4603), *RPL19* (CV=0.469), and *GAPDH* (CV=0.47). *B2M* had the best stability score, followed by *RPS18*, *YWHAZ*, *GAPDH*, *RPL19*, *HPRT1*, and *ACTB*. None of the candidates passed the nonparametric TOST equivalence testing in KGN cells.

Conclusion: *ACTB* showed the lowest variation (CV) in 12Z and KGN cells receiving either TNF- α or vehicle treatment. However, NormFinder analysis, which accounts for intra- and inter-group variation relative to the other candidates, showed *RPS18* and *ACTB* as the most stable genes in 12Z cells, and *B2M* and *RPS18* in KGN cells, making *RPS18* a potential housekeeping gene candidate for both cell types. These data provide a foundation for more robust and reproducible quantification of differential gene expression by RT-qPCR in both cell lines, particularly in response to TNF- α treatment. Future work will include testing additional perturbation conditions, including oxidative stress, other cytokines, or hormonal manipulations.

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Developmental Transition of Estrogen Receptor Lineages in the Mouse Uterine Epithelium

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Abstract Text

Estradiol (E2) is essential for uterine development and function, acting primarily through the nuclear estrogen receptors ESR1 and ESR2. While ESR1 is considered the dominant mediator of estrogen signaling in the uterus, the developmental contribution of ESR2 and its relationship to ESR1 remain incompletely understood. To investigate potential transitions between ESR2- and ESR1-expressing cell populations, we generated a conditional knockout mouse model in which *Esr1* is deleted in *Esr2*-expressing lineages (*Esr1^{fllox/flox}; Esr2-iCre; Esr2-Esr1KO*). Unexpectedly, these mice exhibited robust uterine inflammation and epithelial hyperplasia—phenotypes not observed in global ESR1 or ESR2 knockout models. Despite widespread ESR1 expression in the WT uterine epithelium, a distinct epithelial subpopulation lacked ESR1, suggesting that the pathological phenotype originates from disruption of a specific epithelial lineage rather than from global epithelial dysfunction. Based on these findings, we hypothesized that ESR2 marks a discrete subpopulation of uterine epithelial cells, including stem or precursor cells, that normally undergoes a developmental transition from ESR2 to ESR1 expression, and that loss of ESR1 within this lineage disrupts uterine homeostasis. To test this hypothesis, we mapped the spatial and temporal expression of ESR1 and ESR2 lineages in the developing and adult mouse uterus. We employed a heterozygous *Esr2-iCre* knock-in mouse model to label cells that currently expressed *Esr2*, using Cre immunoreactivity as a lineage tracer, in combination with ESR1 immunohistochemistry. Uteri were collected at postnatal days (PND) 2, 6, 16, 40, and ≥80, capturing early epithelial organization, pubertal remodeling, and adult homeostasis. To complement tissue-level analyses, we analyzed publicly available single-cell RNA sequencing datasets (Spencer *et al.*, 2023) from PND 1, 5, 12, and 15 to assess *Esr1* and *Esr2* expression dynamics in isolated uterine epithelial cells using cell markers to distinguish luminal and glandular epithelial cells. At PND 1, 5, 12, and 15, *Esr1* was expressed in 11.7%, 39.9%, 64.0%, and 73.3% and *Esr2* was expressed in 9.6%, 8.7%, 3.4%, and 1.6% of luminal epithelium cells, respectively. At PND 12 and 15 *Esr1* was expressed in 75.4% and 83.3% and *Esr2* was expressed in 3.6% and 1.7% of glandular epithelium cells, respectively. Immunohistochemical analyses of ESR1 corroborated these findings. At PND 2, 6, 16, 40, and ≥80, ESR1 was expressed in 1.0%, 9.0%, 82.0%, 71.0%, and 98.1% of luminal epithelium cells, respectively. At PND 16, 40, and ≥80, ESR1 was expressed in 66.0%, 93.8%, and 98.3% of glandular epithelium. Interestingly, we did not detect Cre (hence ESR2) protein expression by immunohistochemistry. The inability to detect ESR2 expression potentially indicates a very low level of ESR2 expression or an extremely transient ESR2 expression. In conclusion, these data support a model in which ESR2 defines a transient epithelial lineage that normally transitions to ESR1-dependent states during uterine development. Disruption of ESR1 within this lineage provides a plausible mechanistic basis for the uterine inflammation and epithelial pathology observed in *Esr2-Esr1KO* mice. This study identifies estrogen receptor switching as a previously unrecognized regulatory mechanism in uterine epithelial biology and offers new insight into estrogen-dependent uterine pathologies.

Type 1 Diabetes Impairs Ovarian Function By Altering The Ovarian Mechanical Environment In Mice

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Abstract Text

Only in the United States, 1.8 million people have Type 1 Diabetes (T1D). Diabetes mellitus affects all tissues, and the ovary is no exception. Women with T1D experience irregular menses, lower antimüllerian hormone levels, and early menopause onset. Mouse models of T1D also demonstrate that ovarian reserve and oocyte quality are severely reduced. However, the mechanism by which T1D impacts ovarian function and oocyte quality remains poorly understood. In other tissues, a common pathophysiology of T1D is increased collagen deposition and crosslinking. Therefore, we hypothesized that T1D similarly promotes an ovarian pro-fibrotic environment and alters stromal cell composition, resulting in a decline in follicle numbers and oocyte quality. To test this hypothesis, we utilized the Akita mouse model, a well-established genetic model of T1D (N = 5 Akita, N = 5 wild-type [WT]). We first confirmed the diabetic phenotype of these mice. Fasting serum glucose levels were significantly elevated in Akita mice compared to WT controls (WT: 192.7 ± 7.8 mg/dL; Akita: 314.2 ± 43.5 mg/dL; $p=0.01$), consistent with early-onset hyperglycemia. Body weights were comparable between groups (WT: 18.2 ± 0.9 g; Akita: 17.5 ± 0.6 g; ns), as were ovarian weights (WT: 3.3 ± 0.2 mg; Akita: 3.0 ± 0.1 mg; ns), indicating that T1D does not affect overall growth. We then analyzed ovarian function. First, we monitored estrous cyclicity daily over 15 days. Akita mice required approximately four additional days to complete an estrous cycle compared to WT mice. Stage-specific analysis revealed significantly reduced time spent in proestrus (WT: $32 \pm 2.4\%$; Akita: $20 \pm 4.6\%$; $p=0.05$) and a trend toward increased diestrus (WT: $38.6 \pm 3.4\%$; Akita: $56 \pm 8.5\%$; $p=0.09$). Second, at 11 weeks of age, ovaries were collected for histological evaluation and follicle quantification. Surprisingly, the total number of follicles did not differ between genotypes (WT: 230.2 ± 41.4 follicles/ovary; Akita: 279.0 ± 45.8 follicles/ovary; ns). However, Akita ovaries exhibited reduced corpora lutea (CL) formation (WT: 6.2 ± 1.4 ; Akita: 2.4 ± 1.2 CL/ovary; $p=0.08$), indicating reduced ovulation capacity, consistent with the observed estrous disruption. Given the ovulatory defect, we investigated whether T1D increases collagen-mediated ovarian stiffness, which may impair oocyte release. Using the Piuma nanoindenter (30 indentations/ovary), we found that Akita ovaries were four times stiffer than WT counterparts (Akita: 5.93 ± 2.00 kPa; WT: 1.4 ± 0.31 kPa; $p<0.01$). Akita ovaries had highly heterogeneous mechanical properties, with stiffness ranging from ~ 1 kPa to ~ 14.8 kPa, similar to our previous published data from reproductively old mice. Collagen levels were increased in Akita mice (Akita: $6.38 \pm 1.09\%$; WT: $3.32 \pm 0.67\%$; $p = 0.06$), suggesting that collagen deposition may drive the T1D-associated increase in ovarian stiffness. To understand how the fibrotic environment in the ovary is generated, we are currently exploring stromal cellular composition. Preliminary analysis revealed reduced 3β -HSD protein expression (theca, theca-lutein, and steroidogenic stromal cells; Akita: 116.9 ± 2.41 a.u.; WT: 128.1 ± 2.59 a.u. $p = 0.01$) suggesting that T1D impairs steroidogenic capacity and reprograms the ovarian stromal compartment. Together, these findings demonstrate that T1D induces early ovarian dysfunction and biomechanical remodelling in reproductively young mice, consistent with premature ovarian aging. We are currently analyzing additional stromal cell populations and performing RNA-seq analysis. This project is supported by the DRC at Washington University (NIH P30DK020579).

Caprine Preantral Follicle Isolation: Comparison of Different Methodologies to Improve Efficiency

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Abstract Text

As the global population continues to grow, methods to improve the reproduction of important milk, meat, and hide animals, such as goats, become increasingly imperative. In this way, research that explores the use of oocytes from preantral follicles, which make up a majority of the follicular population in the mammalian ovary, has become an essential aspect for the development of assisted reproductive technologies in livestock. Currently, the isolation of high numbers of preantral follicles is a bottleneck to advancements in this research area, where the standard in goats is the use of a tissue chopper machine and Pasteur pipets. Therefore, the present study was developed to test the hypothesis that the use of a homogenizer machine in combination with a tissue chopper machine and centrifugation can improve the isolation of primordial, primary, and secondary caprine follicles. To evaluate this hypothesis, hemi-ovaries from a total of 5 slaughtered meat goats aged 1–4 years were processed using one of the four following methods: (i) tissue chopping at 500 μm , followed by homogenization and centrifugation (SIU500), (ii) tissue chopping at 75 μm , followed by homogenization and centrifugation (SIU75), (iii) tissue chopping at 500 μm , followed by Pasteur pipetting (TC500), and (iv) tissue chopping at 75 μm , followed by followed by Pasteur pipetting (TC75). In total, 3,122 preantral follicles (2,291 primordial, 699 primary, and 132 secondary) were isolated across all four groups. Overall, when data were combined regardless of tissue chopping interval, the use of tissue chopping in tandem with homogenization and centrifugation (SIU) facilitated the isolation of more ($P < 0.002$) preantral follicles than tissue chopping followed by Pasteur pipetting (TC). Furthermore, both homogenization and centrifugation groups (SIU500 and SIU75) were able to isolate more ($P < 0.02$) preantral follicles than the TC500 group; however, all aforementioned groups were similar to the TC75 group. This is further reflected in the number of preantral follicles (both overall and viable only) expected to be isolated per mg or per cm^3 of ovarian tissue, with the SIU500 and SIU75 groups isolating more follicles per mg ($P < 0.04$, $P < 0.05$ for overall and viable, respectively) and cm^3 ($P < 0.03$ for both overall and viable) than the TC500 group, but with similar ($P > 0.05$) yields to the TC75 group. However, while not statistically significant due to high inter-animal and inter-ovarian variance, it should be noted that both SIU groups isolated 2–3 fold higher numbers of preantral follicles than the TC75 group. Moreover, because the percentages of viable follicles isolated between all groups were similar ($P > 0.05$), it is clear that homogenization and centrifugation do not appear to negatively impact preantral follicle viability. Overall, the results from this study show, for the first time, that the use of homogenization and centrifugation post-tissue chopping is an enticing method to collect higher numbers of overall and viable caprine preantral follicles in a manner that is competitive with the current industry standard. Furthermore, this study provides tangible, applicable results by providing expected yields of follicles per mg and per cm^3 of caprine ovarian tissue for each of the explored isolation methods. In summary, the present study highlights the importance of the combined use of tissue chopping, homogenization, and centrifugation to isolate high numbers of preantral follicles in the caprine species.

Ovarian Aging Phenotypes Differ in Genetically Diverse Mouse Strains

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Abstract Text

The ovary is the first organ in the human body to show age-related declines in function. Human genome-wide association studies have revealed that genetics is the main determinant of menopause onset and ovarian reserve, contributing to ovarian aging. In mice, differences in the number of primordial follicles and in vitro fertilization success rates have been reported in different strains of mice. We previously showed that, with age, increased collagen deposition leads to ovarian fibrosis and stiffness, impaired follicle development, and reduced oocyte quality. However, CB6F1 mice showed signs of ovarian fibrosis earlier than C57BL/6J mice, suggesting that ovarian functional capacity is influenced by both age and genetic background. Here, we aimed to explore natural genetic variation among commonly used strains of mice to dissect the cellular and molecular basis of ovarian aging phenotypes. We first focused on two common mouse strains (CD1 and C57BL/6J) at two ages (2 months: young; 9 months: mid-aged) and evaluated the following parameters: 1) estrous cyclicity, 2) body and ovarian weights, 3) number of follicles, and 4) ovarian stiffness. CD1 mice spent fewer days in estrus than C57BL/6J mice at both ages (Age: $p < 0.01$, Strain: $p < 0.0001$; young: $n=10/\text{strain}$, mid-age: $n=15/\text{strain}$), but the number of days in proestrus increased with age in both strains. Body weight increased with age more in CD1 mice than in C57BL/6J mice (Age: $p < 0.0001$, Strain: $p < 0.001$; $n=10/\text{age and strain}$), and ovarian weight was more variable in CD1 mice than in C57BL/6J mice (young: $F=12.21$, $p=0.001$; mid-age: $F=91.56$, $p < 0.0001$; $n=10$). Ovary-to-body weight ratio was lower in C57BL/6J mice than in CD1 mice at both ages. The ratio decreased with age in C57BL/6J mice but not in CD1 mice (Age: $p=0.001$, Strain: $p < 0.0001$; $n=10/\text{age and strain}$). The two strains had similar total numbers of follicles per ovary at mid-aged (CD1: 1013 ± 901.0 , C57BL/6J: 670 ± 449.5 ; $n=5$), but C57BL/6J mice had a higher percentage of late-stage follicles than mid-aged CD1 mice ($33.22 \pm 9.235\%$ vs. $14.48 \pm 9.430\%$; $n=5$). Furthermore, mid-aged CD1 mice had 5.11-fold more corpora lutea per section than mid-aged C57BL/6J mice ($p < 0.0001$; $n=5$). Finally, we used a Piuma Nanoindenter to measure ovarian stiffness. Ovaries from young C57BL/6J mice tended to be softer than those from CD1 mice, though the findings were not statistically significant (1.632 ± 0.7401 vs. 2.730 ± 1.339 , $p=0.2085$; $n=5$). Ovaries from mid-aged C57BL/6J were 1.96-fold stiffer compared to the young group (young: 1.632 ± 0.7401 vs. mid-age: 3.192 ± 2.277 ; $n=5$) but not in the CD1 mice (1.06-fold change; young: 2.730 ± 1.339 vs. mid-age: 2.905 ± 1.167 , $n=5$). Together, these data reveal strain-dependent differences in ovarian aging phenotypes. In ongoing work, we are analyzing hormone concentrations, assessing in vitro fertilization success, and performing ovary RNA sequencing for CD1 and C57BL/6J mice as well as CB6F1 and C57BL/6NJ mice. We are also including an older cohort (>15 months) for the study. Our findings will provide insights into age- and genetic-related factors underlying ovarian mechanical and functional properties.

Effect of Form of Selenium on Cholesterol Trafficking and Luteal Function in Beef Cattle

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Abstract Text

Soils deficient in the trace mineral selenium (Se) are widespread across the southeast US, necessitating supplementation of Se to the diet of cattle consuming forages grown on these soils. Selenium is typically supplemented in a free-choice vitamin mineral mix in an inorganic form (ISe); however, organic forms of Se (OSe) are found in forages. Our lab has previously reported that supplementation with an isomolar 1:1 mix (MIX) of ISe and OSe, versus ISe alone as the industry standard, increases early luteal phase concentrations of progesterone (P4) and decreases systemic concentrations of circulating low, and very-low density lipoproteins (LDL/VLDL). Additionally, caruncular and intercaruncular endometrial transcriptomics are altered, and an increase in conceptus length is observed at maternal recognition of pregnancy.

The objective of this research was to identify the mechanism responsible for the increase in luteal uptake and trafficking of cholesterol by the corpus luteum (CL), by determination of the effect of form of Se on transcripts within the Liver-X-Receptor – Retinoid-X-Receptor (LXR/RXR) activation pathway. The LXR/RXR activation pathway plays a central role in cholesterol homeostasis as a key transcriptional regulator of genes associated with cholesterol biosynthesis and transportation. Corpora lutea were collected from Angus-cross cows on Day 7 post-estrus. All cows had been maintained on ISe or MIX for at least 120 days prior to the collection of luteal samples, and were in a stable, Se-adequate status (n=5 per treatment) at the time of sample collection. Reverse transcription and real-time PCR were performed on RNA extracted from luteal samples, with qPCR performed in triplicate. The relative expression of each transcript was assessed against the geometric mean of 3 housekeeping genes (*Actb*, *Hprt1*, and *Sdha*).

In CL retrieved from ISe versus MIX treated cows, the abundance of mRNA encoding *Hmgcs2*, the rate limiting enzyme involved in de novo synthesis of cholesterol was decreased. In contrast, the abundance of mRNA for *Abca* family members (strongly correlated to the LXR/RXR activation pathway and intracellular transportation), *March6* (degrades *Hmgcr*), *Mylip* (degrades LDLR), *Nr1h2* (regulator of cholesterol homeostasis), *Scap* (cholesterol sensor), *Srebf2* (inducer of HMG-CoA reductase) and *Usp2* and *11* (regulators of protein stability and turnover) were all increased ($P < 0.05$).

Results indicate that in the CL of ISe versus MIX treated cows, an active mechanism regulating cholesterol biosynthesis and shuttling is apparent. The altered transcripts within this pathway appear to be responsible for the decrease in systemic levels of LDL/VLDL and concurrent increase in P4 that is observed in MIX vs. ISe treated cows.

Extracellular Vesicles Derived from Adipose Tissue Stem Cells Are Capable of Promoting Ex Vivo Murine Follicular Activation

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Abstract Text

Infertility may be associated with the depletion of the ovarian follicular reserve, whose maintenance depends on signaling pathways such as PI3K/Akt/mTOR. Extracellular vesicles derived from adipose tissue-derived stem cells (ADSC-EVs) have been described as modulators of cellular homeostasis through the transfer of bioactive molecules. Thus, the aim of this study was to isolate and characterize ADSCs cultured under starvation conditions or in EV-depleted medium and to investigate the regulatory effects of their EVs on follicular activation. Inguinal adipose tissue from female C57BL/6J mice aged 12 weeks (CEUA #9348070622; n=44) was collected for ADSC isolation and characterization regarding viability, potency, and immunophenotype. ADSC-EVs were isolated by ultracentrifugation and characterized by Nanoparticle Tracking Analysis, flow cytometry, and transmission electron microscopy. Ovaries from 3-day-old neonates (n = 72) were cultured ex vivo and supplemented with different concentrations of ADSC-EVs, followed by follicular quantification and gene expression analysis. ADSCs exhibited differentiation capacity and positive immunostaining for CD44 and CD29 ($p < 0.0001$), and cell viability was higher in the EV-depleted group compared with the starvation group after 24 h ($p = 0.0045$), with further enhancement after 48 h ($p < 0.0001$). ADSC-EVs showed a modal diameter of 164 nm and a concentration of 2.9×10^9 particles/mL, with predominance of Alix⁺ and CD63⁺ markers and low expression of Calnexin⁺. Supplementation of ovarian explants with ADSC-EVs promoted an increase in transitional follicles at the concentration of 8.8×10^7 particles/mL compared with the control group ($p=0.02$), as well as a concentration-dependent modulation in the proportion of secondary follicles ($p < 0.0001$). ADSCs cultured in EV-depleted medium produced a higher number of EVs (2.49×10^{10} particles/mL), with a modal diameter of 190 nm and increased expression of Alix⁺, CD63⁺, and CD9⁺. Ovarian supplementation with these ADSC-EVs resulted in increased transitional and primary follicles, particularly at the concentration of 17.6×10^7 particles/mL, compared with the control group, higher concentrations tested, and non-cultured ovaries from 3- and 6-day-old mice ($p < 0.05$), reflecting a significant increase in the follicular activation rate in this group ($p < 0.05$). Relative expression of *Amh* and *Fshr* was altered after 72 h of treatment ($p=0.001$), whereas *Foxo3* was upregulated at 24 h ($p=0.011$) and 72 h ($p=0.001$), with no changes in *Pten* expression ($p > 0.05$). The *Cpeb1* gene was upregulated after 72 h ($p=0.001$), and *Bcl2* showed significant alterations at both 24 and 72 h ($p=0.001$). Taken together, these results demonstrate that ADSC-EVs modulate follicular activation ex vivo in a concentration- and culture-condition-dependent manner, highlighting their potential as a regulatory and therapeutic strategy for modulating the ovarian follicular reserve.

Investigating the Role of Thrombospondin-1 During the Establishment of the Mammalian Ovarian Reserve

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Abstract Text

During fetal development, the ovary undergoes major morphological changes and cellular remodeling events that lead by birth to the formation of two functionally distinct regions where follicles reside. Follicles in the center (medullary) and surface (cortical) regions have different activation outcomes at birth, where center follicles are activated, while surface follicles remain quiescent to form the ovarian reserve. These regional differences suggest the local niche plays a role in determining follicle fate. The ovarian reserve is a finite pool of primordial follicles, which are the functional reproductive unit of the ovary, and is responsible for the female's reproductive lifespan. As the ovary develops, key structural events occur, such as the formation of germ cell cysts and ovigerous cords (OCs) that ultimately break down into follicles. Disruption of structural events during fetal ovarian development could disrupt the regionalization or structural integrity of follicles, ultimately affecting the size of the ovarian reserve. Thus, disrupted structural events during fetal ovarian development could be an underappreciated source of infertility. However, the molecular and spatial cues regulating structural patterning in development are poorly understood. Additionally, how distinct cortical and medullary niches are established is unknown.

Using 10x Genomics Visium HD spatial transcriptomics we identified thrombospondin-1 (Thbs1, TSP-1) as the first regionally enriched gene in the center of fetal ovary as early as Embryonic day (E)14.5. TSP-1 expression appears specifically in the cells that anchor the OCs, suggesting a potential function in OC formation. TSP-1 is important in many contexts for remodeling of vasculature and extracellular matrix (ECM), but it has not been characterized in the developing ovary. We know that adult Thbs1^{-/-} mice are subfertile (Lawler et al., 1998), but the initial establishment of the ovarian reserve during development was not investigated as a possible explanation of the subfertility phenotype.

To fill this gap, we used our automated follicle counting pipeline OoCount (Folts et al., 2025) on ovaries collected from P2 Thbs1-null versus control females. Our preliminary data suggest that Thbs1^{-/-} ovaries had a 38-58% decrease in the raw count of oocytes compared to the average counts for Thbs1^{+/+} ovaries. We hypothesize that TSP-1 is critical for establishment of a robust reserve of ovarian follicles. Investigating how TSP-1 may influence regionalization of follicles, vascular and ECM remodeling, and follicle formation in the developing fetal ovary will provide additional insight into the mechanisms governing establishment of the ovarian reserve and the etiology of subfertility.

Investigating Ribosome Biogenesis and Translation in the Aging Mammalian Oocyte

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Abstract Text

Oocyte quality is the greatest barrier to fertility at advanced maternal age. Though the impact of oocyte quality is appreciated, our understanding of its molecular culprits is limited. One aspect of oocyte quality that remains understudied is translation, the production of proteins by macromolecular machines called ribosomes. Oocytes are unique in that they rely entirely upon translation to modulate gene-regulation during meiotic maturation, fertilization, paternal genome unpacking, and the oocyte-to-embryo transition. Furthermore, the ribosomes made by the oocyte provide for the zygote until its own ribosome biogenesis pathway becomes active. Ribosomes are therefore an important - but poorly characterized - factor of oocyte quality. If ribosome production or activity is perturbed with age, oocyte quality would be significantly compromised.

To address this gap in knowledge, we conducted single-cell RNA sequencing from oocytes isolated from reproductively young (2-month-old) and old female (14-month-old) mice. The pathways most enriched with age were those associated with ribosome biogenesis and translation. We found that ribosomal RNA also significantly increases in oocytes with age. This observation compliments both published and new findings that cytoplasmic ribosome populations and large and small ribosome subunit interactions significantly increase with age in oocytes.

To determine the functional output of these ribosomes, global translation assays were conducted in germinal vesicle oocytes from reproductively young and old female mice. Surprisingly, the results revealed significantly reduced translation levels in old oocytes, despite greater cytoplasmic ribosome density. This finding led us to hypothesize that old oocytes lose translation efficiency with age and become increasingly prone to ribosome stalling, a molecular phenomenon in which translating ribosomes become stuck on transcripts, halting peptide synthesis, leading to an accumulation of assembled ribosomes. To test this hypothesis, we have designed a series of injectable translation fidelity reporters to measure the incidence of stalling and translation error, which are currently being evaluated.

Taken together, we surmise that ribosome numbers increase while translation activity decreases with age. Ribosome stalling may be one source of age-dependent translation decline, which could impact many cellular processes, especially those that rely on timely and coordinated protein production, such as meiosis. Continued investigation of age-associated changes in ribosome production and translation activity in oocytes will provide new insight into oocyte quality and how it changes with age.

Interplay Between Oncostatin-M and Interleukin-4 in Regulating Human Periovulatory Granulosa Cell Function

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Abstract Text

Ovulation is a complex physiological process that resembles an acute inflammatory response, involving a dynamic interplay between various cytokines within the periovulatory follicle. While Oncostatin-M (OSM) has been recently identified as a critical mediator of ovulatory changes in the human ovary; specifically, OSM dramatically upregulates the expression of Interleukin-4 receptor (IL4R) in granulosa cells. Given that IL4 is known to be present in human ovulatory follicular fluid, these findings suggest that the OSM-induced sensitization of granulosa cells to IL4 may play a fundamental role in the ovulatory process. In this study, we investigated the synergistic effects of OSM and IL4 on human granulosa cells. In vivo analysis using qPCR confirmed levels of IL4R were increased in human periovulatory granulosa cells after ovulatory human chorionic gonadotropin (hCG) administration. Furthermore, RNA-seq analysis of primary human granulosa-lutein cells (hGLC) cultures identified the hCG-upregulated IL4R mRNA levels and the expression of IL2RG (encoding the common gamma chain, γ C, a partner for IL4R), indicating that the periovulatory granulosa cells possess the essential components required to transduce IL4-mediated signals. Functional assays demonstrated using Western blot analyses that while OSM and IL4 individually activate STAT3 and STAT6, respectively, their co-treatment results in a synergistic enhancement of STAT3, STAT6, and ERK1/2 signaling. Global transcriptomic profiling via RNA-seq revealed a highly dynamic landscape of gene expression. Heatmap, Venn diagram, and Upset plot analyses of RNA-seq datasets collectively demonstrated that while individual treatments with hCG, OSM, or IL4 each induce distinct transcriptomic changes, their combination, particularly the di- and tri-treatments, triggers the formation of massive unique gene clusters. These clusters include both robustly up-regulated and significantly down-regulated transcripts, representing a profound reprogramming of the granulosa cell transcriptome. Functional enrichment indicated that various genes in these synergistic clusters are primarily associated with JAK-STAT signaling (JAK3, STAT3, STAT5B, MYC, SOCS3, PTPN2), protein glycosylation (NPC1, GFPT2, GALNT1, GALNT4, GALNT9, GALNT12), and glucose homeostasis (CISH, LEPR, WFS1). In conclusion, our findings demonstrated for the first time that OSM and IL4 cooperatively reprogram the transcriptomic landscape of human granulosa cells, characterized by the emergence of extensive gene networks uniquely regulated by their synergistic interaction. This study suggests that the IL4-IL4R axis, significantly amplified by OSM, is a key regulatory pathway that coordinates essential biological functions during the human periovulatory period. Supported by NIH grants: R01HD115554 and R03HD109497.

Bile Acids Alter Steroidogenic Gene Expression in Equine Granulosa Cells *In Vitro*

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Abstract Text

Bile acids are increasingly recognized as signaling molecules that extend beyond their classical role in lipid metabolism. Emerging evidence suggests that bile acids influence reproductive physiology, however, their direct effects within the ovarian follicle remain poorly defined. Granulosa cells play a central role in follicular metabolism and steroid hormone synthesis and are highly sensitive to metabolic stress, cholesterol availability, and intracellular signaling cues. Disruption of these processes can impair follicular development and fertility, particularly in metabolically dysregulated states. Our preliminary findings indicate that equine granulosa cells express key bile acid receptors and transporters, supporting the possibility of active bile acid signaling within the follicular microenvironment. Individual bile acid species exert distinct signaling effects, with chenodeoxycholic acid functioning as a potent farnesoid X receptor agonist, whereas ursodeoxycholic acid and its taurine-conjugated form are associated with cytoprotective signaling pathways. Given these mechanistic differences, we examined whether chenodeoxycholic acid and ursodeoxycholic acid differentially regulate metabolic and steroidogenic gene expression in equine granulosa cells. We hypothesized that exposure to chenodeoxycholic acid and taurochenodeoxycholic acid would negatively impact granulosa cell function by downregulating steroidogenic and metabolic pathways, whereas ursodeoxycholic acid and tauroursodeoxycholic acid would exert protective effects. Cholesterol supplementation and pharmacologic modulation of the farnesoid X receptor were included to further define pathway-specific regulation. Together, these data aim to clarify how distinct bile acid species and related signaling mechanisms modulate ovarian granulosa cell function. To test this hypothesis, we investigated the effects of chenodeoxycholic acid, taurochenodeoxycholic acid, ursodeoxycholic acid, tauroursodeoxycholic acid, cholesterol supplementation, and guggulsterone, a farnesoid X receptor antagonist, on gene expression in primary equine granulosa cells (n = 6). Cells were cultured under controlled conditions and exposed to physiologically relevant concentrations of each treatment, followed by targeted analysis of genes involved in steroidogenesis, cholesterol metabolism, bile acid signaling, and cellular stress responses. Preliminary analysis (n = 2 biological replicates) indicates that increasing cholesterol concentration reduced nuclear receptor subfamily 1 group H member 4 (0.45-fold, P = 0.15) and solute carrier family 51 alpha expression (0.28-fold, P = 0.52). Ursodeoxycholic acid increased solute carrier family 51 alpha expression relative to chenodeoxycholic acid (approximately 5-fold, P = 0.19). In steroidogenic pathways, ursodeoxycholic acid also increased forkhead box L2 (approximately 2.6-fold, P = 0.18) and steroidogenic acute regulatory protein (approximately 1.9-fold, P = 0.16) relative to chenodeoxycholic acid, while tauroursodeoxycholic acid increased 3-beta-hydroxysteroid dehydrogenase (approximately 1.8-fold, P = 0.11) compared to taurochenodeoxycholic acid. Higher cholesterol increased forkhead box L2 (approximately 2.8-fold, P = 0.19) but reduced 3-beta-hydroxysteroid dehydrogenase (approximately 0.5-fold, P = 0.27). Although not statistically significant, the magnitude and direction of change support sterol-dependent modulation of bile acid signaling and steroidogenic gene regulation in equine granulosa cells. This work advances understanding of bile acid signaling in the ovary and identifies potential mechanisms linking metabolic dysregulation to impaired follicular function. Defining bile acid-specific effects on granulosa cell biology may reveal novel targets for improving reproductive outcomes in metabolically compromised individuals.

Excess Ovarian Androstenedione is Associated with Early-Life Systemic Hematologic Signatures of Low-Grade Inflammation in Developing Beef Heifers

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Abstract Text

Excess androgen exposure disrupts ovarian function by impairing follicular progression and ovulation, ultimately leading to subfertility or infertility, as observed in conditions such as polycystic ovary syndrome (PCOS). Within our physiology research herd, we have identified a subpopulation of cows exhibiting elevated androstenedione concentrations (High A4) in the follicular fluid of dominant ovarian follicles as well as cultured cortex pieces. These High A4 cows experience a 17% reduction in calving rate and display increased ovarian inflammation and fibrosis, accompanied by elevated concentrations of inflammatory cytokines within the dominant follicle. Despite this well-characterized ovarian phenotype, the systemic hematologic and immune profile associated with excess ovarian androgen production has not been fully defined. Based on our previous findings of inflammation within the ovary, we hypothesized that High A4 cows would exhibit systemic hematologic signatures consistent with enhanced inflammatory activity. Blood samples were collected monthly from pre-weaning through breeding in 2019 and 2020. Complete blood counts were analyzed using a HemaTrue® Veterinary Hematology Analyzer to quantify white blood cells, red blood cells, platelets, granulocytes, lymphocytes, and monocytes. Heifers were classified based on A4 production from cultured ovarian cortex tissue with Control being A4 secretion < 1 ng/mL/day (n=9) and High A4 being > 1 ng/mL/day. (n = 6). Data were analyzed using GraphPad Prism, utilizing a two-way ANOVA and a Tukey post hoc multiple comparisons test. Mean corpuscular volume (MCV), an indicator of red blood cell (RBC) size, was elevated in High A4 heifers one week prior to weaning (p = 0.01) compared to controls and increased again toward the end of the trial (collections 7-9). In addition, RBC size variability was greater in High A4 heifers, as indicated by elevated red blood cell distribution width (RDW). This RDW was increased in High A4 females during pre-weaning and the following collection period (1-week post-weaning) compared to controls (p < 0.05). Although platelet numbers did not differ between groups (p=0.17), High A4 heifers exhibited reduced mean platelet volume during pre-weaning compared with controls (p< 0.05). Notably, alterations in these hematologic parameters have also been altered in women with PCOS, an anovulatory disorder, characterized by chronic inflammation and endocrine dysfunction. Our laboratory has previously demonstrated that High A4 females share multiple phenotypic traits with PCOS. The presence of such hematologic changes early in life suggests that systemic inflammatory dysregulation is present prior to puberty and reproductive maturity. Collectively, these findings indicate that heifers with excess ovarian androstenedione production exhibit early and persistent hematologic changes consistent with low-grade systemic inflammation, and whole blood indices, particularly red blood cell-associated parameters, may serve as early biomarkers for identifying High A4 females prior to breeding. Thus, early identification of this subpopulation could improve heifer retention strategies, thereby enabling producers to cull potentially sub-fertile animals before substantial reproductive investments are incurred.

Ovarian Tissue-Resident Macrophages as Potential Coordinators of Sex-Specific Neuroinflammatory Aging

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Abstract Text

Reproductive aging is among the earliest systemic aging processes in females, preceding many classical hallmarks of organismal decline. Beginning in the mid-30s and accelerating through perimenopause, women experience reduced fertility and diminished endocrine function, which contribute to systemic aging and sex differences in chronic disease burden. Notably, neurodegenerative disorders such as Alzheimer's disease occur more frequently and often progress more severely in women; however, the biological basis for this disparity remains unclear. Emerging evidence suggests that immune signaling across organs may play a critical role in shaping these outcomes. Here, we investigate whether ovarian tissue-resident macrophages (TRMs) contribute to sex-specific neuroinflammatory trajectories during aging. We employed a tamoxifen-inducible Cx3cr1Cre/ERT2-NuTRAP mouse model to selectively label and profile ovarian TRMs and brain microglia using Translating Ribosome Affinity Purification (TRAP). Transcriptomic analyses were performed in ovary and brain across three aging stages: young (5-6 months), reproductively aged (12-14 months), and reproductively ceased (18 months). We observe a progressive shift in ovarian TRMs toward pro-inflammatory and senescence-associated transcriptional states with age. Female microglia exhibit a delayed but parallel acquisition of these signatures, a pattern not observed in males. In parallel, aging ovarian TRMs show increased expression of exosome-associated transcripts, with similar enrichment emerging later in female - but not male - microglia. These findings suggest a female-specific axis of immune activation linking ovarian aging to the brain. Together, our results support a model in which ovarian immune remodeling precedes and may shape neuroinflammatory changes in females, potentially contributing to sex-biased vulnerability to neurodegenerative disease. Ongoing studies are examining circulating proteomic and extracellular vesicle profiles, as well as the functional impact of TRM depletion, to further define mechanisms of ovary-to-brain communication during aging.

Omentin Modulates Key Cellular Processes and Signaling Pathways in Porcine Granulosa Cells: An RNA-seq Analysis

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Abstract Text

Omentin-1 (ITLN1) is an anti-inflammatory adipokine involved in insulin sensitivity, glucose metabolism, and energy homeostasis, with emerging evidence suggesting its role in the regulation of reproductive processes. Current data indicate that ITLN1 may affect follicular growth, granulosa cells (GCs) proliferation and survival, steroidogenesis, apoptosis, and follicular maturation, potentially linking metabolic status with ovarian function. However, the molecular mechanisms underlying its action in GCs remain unclear. Therefore, this study aimed to investigate the effects of ITLN1 on porcine GCs using transcriptomic profiling.

GCs were collected from ovarian follicles of gilts during the mid-luteal phase of the estrous cycle (days 10-12). Cells were cultured in control medium or medium supplemented with ITLN1 (10-100 ng/mL). Following RNA isolation and quality assessment, libraries were sequenced on the Illumina NovaSeq X platform. Reads were quality-filtered, aligned to the reference genome, and analyzed for differential gene expression. Genes with adjusted p-value < 0.05 and fold-change ≤ 0.83 or ≥ 1.2 were considered significant. Functional analyses included Gene Ontology, pathway enrichment, and targeted evaluation of GCs-related genes. Selected findings were validated using RT-qPCR, co-culture experiments, ELISA and metabolic assays, and pharmacological inhibition.

Transcriptomic analysis indicated that ITLN1 promotes a more differentiated, metabolically active, and steroidogenically competent phenotype in porcine GCs, as evidenced by increased expression of BMP2 $\uparrow$, LHCGR $\uparrow$, IGF1 $\uparrow$, and HMGCR $\uparrow$, together with reduced expression of FSHR $\downarrow$, AMH $\downarrow$, AMHR2 $\downarrow$, and INHBA $\downarrow$, consistent with acquisition of a more advanced follicular phenotype and potentially enhanced responsiveness to gonadotropic and metabolic cues. ITLN1 also increased the expression of genes involved in cell cycle progression and proliferation, including CDK1 $\uparrow$, CCNA2 $\uparrow$, CCNB1/2 $\uparrow$, CDC20 $\uparrow$, FOXM1 $\uparrow$, MKI67 $\uparrow$, ANLN $\uparrow$, and AURKA $\uparrow$, while reducing expression of the cell cycle inhibitors CDKN1A $\downarrow$ and CDKN1C $\downarrow$, indicating activation of proliferative and mitotic programs in GCs. Furthermore, ITLN1 upregulated anti-apoptotic factors including BCL2L1 $\uparrow$, BIRC2 $\uparrow$, BIRC3 $\uparrow$, BIRC6 $\uparrow$, and BAG3 $\uparrow$, together with selected pro-apoptotic genes such as FAS $\uparrow$ and BOK $\uparrow$, while reducing BBC3/PUMA $\downarrow$, suggesting fine regulation of apoptotic balance and mechanisms supporting GCs survival. ITLN1 additionally modulated steroidogenesis- and lipid metabolism-related genes, including SRD5A1 $\uparrow$, HMGCS1 $\uparrow$, IDI1 $\uparrow$, SC5D $\uparrow$, SQLE $\uparrow$, LDLR $\uparrow$, SCARB1 $\uparrow$, and FITM2 $\uparrow$, while HSD3B1 $\downarrow$ and ABCG1 $\downarrow$ were reduced, indicating enhanced cholesterol synthesis, uptake, intracellular lipid accumulation, and remodeling of steroidogenic activity. Co-culture of GCs and theca cells confirmed reduced progesterone production together with increased estradiol secretion following ITLN1 treatment. Moreover, ITLN1-induced alterations in cholesterol metabolism were validated using a metabolic assay, demonstrating increased cholesterol and cholesteryl ester accumulation. Transcriptomic data also demonstrated modulation of the insulin/IGF, MAPK, cAMP-PKA, and, to a lesser degree, PI3K-AKT signaling pathways, characterized by increased expression of IRS1 $\uparrow$, APPL1 $\uparrow$, AKAP1 $\uparrow$, AKAP11 $\uparrow$, AKAP13 $\uparrow$, ATF2 $\uparrow$, ATF3 $\uparrow$, and INSIG1 $\uparrow$, together with reduced expression of IGFBP2 $\downarrow$, IGFBP3 $\downarrow$, IGFBP4 $\downarrow$, IGFBP6 $\downarrow$, IGFALS $\downarrow$, ADCY3 $\downarrow$, BAD $\downarrow$, BBC3 $\downarrow$, and BAMBI $\downarrow$, suggesting altered IGF signaling, insulin responsiveness, and cell differentiation-associated pathways in porcine GCs. Pharmacological inhibition experiments using S961 (insulin receptor antagonist), PD98059 (MAPK/ERK inhibitor), and KT5720 (PKA inhibitor) further confirmed the involvement of insulin, MAPK, and cAMP-PKA signaling pathways in the regulation of steroid hormone production by ITLN1.

Collectively, these findings indicate that ITLN1 regulates pathways associated with GCs proliferation, cell cycle progression, apoptosis, differentiation, steroidogenesis, cholesterol and lipid metabolism, and insulin/IGF-, MAPK-, PI3K-AKT-, and cAMP-PKA-related signaling in porcine GCs. Hence, ITLN1 may function as a local regulator of ovarian follicular activity.

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Mitigation of Reproductive Aging by Vitamin C in Japanese Medaka

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Abstract Text

Aging is a universal biological process characterized by the progressive loss of cellular function, including DNA damage, epigenetic alterations, and oxidative stress. These molecular changes contribute to functional decline across tissues, including reproductive tissues, where aging is associated with reduced fertility and impaired gamete quality. As reproductive aging continues, the gonadal cells undergo increased oxidative stress and experience epigenetic alterations. Vitamin C is a known antioxidant and epigenetic regulator that may help mitigate molecular damage associated with aging, since preliminary studies in medaka suggest that vitamin C can rejuvenate mitochondrial function and blocks epigenomic alterations in the liver. It is not clearly understood if vitamin C can decelerate the aging of reproductive organs and improve reproductive outcomes. The objective of the present study was to determine aging related abnormalities in testicular tissues and whether vitamin C supplementation can mitigate reproductive decline. We hypothesized that vitamin C exposure would slow reproductive aging both at the structural and molecular levels. We used Japanese medaka (*Oryzias latipes*) as a model organism. Like humans, Japanese medaka cannot synthesize vitamin C endogenously. Male and female medaka (n=15, each sex) were treated with vitamin C (1 mg/L) or control (no vitamin C) for 8 months starting from 30 days post fertilization (dpf), which corresponds to sexual differentiation of gonads. Medaka become sexually mature in 90 dpf and gonad samples were collected from fish at 9 months old age. To assess the effects vitamin C on reproductive decline, testes and ovaries were removed from the fish and analyzed histologically using hematoxylin and eosin staining to evaluate germ cell differentiation, sperm density, and gonadal tissue structure. To measure the reproductive fitness, eggs were collected from fish that were under exposure and fertilization rates were compared. Early developmental outcomes, mainly embryo development and survival were recorded. Vitamin C exposure increased egg production and improved fertilization rates compared to controls. Histological analysis revealed more synchronized germ cell differentiation, increased sperm density in the seminiferous tubules, and robustness of seminiferous tubule structure in vitamin C-exposed fish compared to controls. These findings suggest that vitamin C supplementation can mitigate age-associated reproductive decline through enhanced maintenance of testicular tissue integrity. Ongoing transcriptomic and epigenetic analyses of testes and ovaries will further elucidate the molecular mechanisms underlying improved reproductive longevity offered by continuous supplementation of Vitamin C. *The study was supported by funds from NIH National Institute of Environmental Health Sciences (R01ES032452, R21ES027123) to RKB and Mizzou Forward Undergraduate Research Grant to JK.*

Aurora-C Safeguards Egg Euploidy by Regulating Cytokinesis Timing and Abscission

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Abstract Text

After chromosome segregation in meiosis I, oocytes will undergo cytokinesis, culminating in abscission to produce a mature metaphase II egg and the first polar body (PB). Proper cytokinesis timing is essential for maintaining chromosome integrity, with abscission serving as the final opportunity to correct segregation errors. However, the temporal control of oocyte cytokinesis remains poorly defined. Our data identify Aurora kinase C (AURKC) as a key regulator of cytokinesis timing and abscission that protects against egg aneuploidy. Inhibition of AURKC at anaphase I onset markedly advanced the initiation of PB extrusion and increased the incidence of aneuploidy. Live-cell imaging revealed that AURKC inhibition significantly shortened cytokinesis timing from anaphase I onset to abscission completion (96.2 ± 4.0 min vs. 134.9 ± 4.6 min in controls; $P < 0.0001$), thereby reducing the time available to detect and prevent lagging chromosomes. Additionally, 15.6% of AURKC-inhibited oocytes ($P < 0.05$) exhibited abscission anomalies, such as the spindle rejoining despite maintaining a morphologically normal PBs, or complete cytokinesis failure. AURKC inhibition also impaired chromosome alignment in metaphase II eggs, with 75.3% of eggs displaying spindles containing stray chromosomes. Our preliminary data further suggest that AURKC might regulate cytokinesis timing and abscission via phosphorylation of KIF2C/MCAK and KIF23/MKLP1, respectively. Together, these findings demonstrate that precise control of cytokinesis timing and abscission is essential for maintaining euploidy and reveal a unique role for AURKC in coordinating these processes to ensure accurate chromosome inheritance.

Metabolic Changes in the Bovine Corpus Luteum During Early Luteolysis

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Abstract Text

The corpus luteum (CL) is a transient structure that forms on the ovary from the remnant of the ovulatory follicle and produces progesterone, the hormone necessary to maintain pregnancy. In cattle, in the absence of a pregnancy, uterine prostaglandin $F_{2\alpha}$ (PGF2A) results in luteal regression. Inadequate luteal function or mistimed luteolysis is a cause of early pregnancy loss in cattle, leading to losses in productivity. An improved understanding of luteolysis could lead to technology to prevent early pregnancy loss. Processes mediating the early response of the CL to PGF2A are poorly understood, and little is known about global changes in the metabolome and lipidome of the CL during this time. The goal of this study was to identify early metabolomic and lipidomic changes in the CL following induction of luteolysis with PGF2A. Corpora lutea were collected from cows on days 10–12 of the estrous cycle, prior to PGF2A injection (0 hr, n=4), 0.5 hours post-injection (0.5 hr, n=4), and 1 hour post-injection (1 hr, n=4). Untargeted metabolomics was performed by LC-MS, using a Thermo Vanquish Horizon liquid chromatography system. Untargeted lipidomics was performed on the same samples by LC-MS/MS using a Thermo Orbitrap Fusion Lumos Tribrid mass spectrometer coupled with a Thermo Vanquish Horizon UHPLC system and a Waters CSH C18 column. Using MetaboAnalyst, data were normalized and analyzed via ANOVA with a Benjamini-Hochberg multiple comparisons correction to identify which metabolites and lipids changed during early luteolysis. Among the 272 metabolites identified in the luteal tissue samples, 96 changed over time during early luteolysis (padj < 0.05). In contrast, although 423 lipids were identified, none were altered (padj < 0.05). Next, cluster analysis was performed to evaluate temporal patterns of change in the differentially abundant metabolites. This analysis revealed two distinct patterns of metabolite changes across time points. The 18 metabolites in the first cluster rapidly declined between 0 hr and 0.5 hr, but did not change between 0.5 hr and 1 hr. These metabolites were associated with glycine, serine, and threonine metabolism. The 77 metabolites in the second cluster declined between 0 hr and 0.5 hr, and declined further between 0.5 hr and 1 hr. These metabolites were associated with functions such as arginine biosynthesis and metabolism, histidine metabolism, beta alanine metabolism, and pyrimidine metabolism. Among the many amino acids that declined (padj < 0.05) during luteolysis were six of the essential amino acids for cattle: arginine, histidine, threonine, methionine, phenylalanine, and tryptophan. This suggests that PGF2A rapidly induces a global depletion of luteal amino acids. Only one metabolite increased temporally during luteolysis, dicyclohexyl phthalate. Overall, these results demonstrate that luteolysis leads to rapid metabolic changes in the CL within one hour, while lipid profiles remain unchanged, at least during this early part of luteolysis. Perhaps most interesting is the rapid depletion of amino acids induced by PGF2A. We hypothesize that as luteolysis progresses, cells may become amino acid deficient, and this may be one mechanism leading to their death. This work improves our understanding of the early events involved in luteal regression and may contribute to identifying mechanisms associated with early pregnancy loss in dairy cattle. This work was supported by Penn State start-up funds to CHKH. The co-authors would like to acknowledge the Huck Institutes' Metabolomics Core Facility (RRID:SCR_023864).

Polyamine alleviates postovulatory oocyte aging by restoring mitochondrial function and NAD⁺ biosynthesis via hypusinated eIF5A in pigs

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Abstract Text

Although polyamines play important roles in oocyte maturation, their mechanisms in postovulatory oocyte aging (POA) in pigs, characterized by impaired NAD⁺ biosynthesis and reduced developmental competence, remain unclear. Here, we identify spermidine (Spd) as an essential polyamine that enhances oocyte maturation capacity and quality in porcine POA through eIF5A hypusination (eIF5A^{hyp}) and NAD⁺ biosynthesis. Immature porcine oocytes were matured *in vitro* for 44 h and subsequently cultured for an additional 24 h to induce POA model. The Spd-treated group was cultured under identical conditions with the addition of 50 μ M Spd for the same duration. First, transcriptomic analysis using RNA sequencing (RNA-seq) identified a total of 304 differentially expressed genes (fold change > 1.5) between the IVM and POA groups, including 115 upregulated and 189 downregulated genes. Next, mitochondrial function, NAD⁺ biosynthesis, and eIF5A^{hyp} related genes expressions were evaluated using immunofluorescence (IF) staining, Western blotting, and qPCR. Compared with untreated aged oocytes, Spd-treated POA oocytes exhibited significantly reduced expressions of aging-associated genes, including *CLU* ($p < 0.01$) and *FAM111A* ($p < 0.001$), along with restored ATP production ($p < 0.05$), mitochondrial membrane potential (MMP, $\Delta\Psi$ m; $p < 0.05$), and NAD⁺ biosynthesis ($p < 0.01$). Importantly, Spd supplementation improved structural hallmarks of oocyte quality by stabilizing cortical F-actin, promoting proper spindle assembly, and reducing chromosome disorganization. Consistently, protein analysis demonstrated that eIF5A and hypusine levels were decreased in POA oocytes, accompanied by reduced LC3B expression, a marker of mitophagy activation, these alterations were effectively reversed by 50 μ M Spd treatment. In conclusion, Spd enhances eIF5A^{hyp} activation, alleviates mitochondrial dysfunction and NAD⁺ depletion during POA, and preserves both mitochondrial integrity and cytoskeletal architecture, thereby maintaining oocyte quality and highlighting its potential as a therapeutic anti-aging modulator in postovulatory oocytes.

Keywords: Post-ovulatory aging, Spermidine, NAD⁺ synthesis, hypusinated eIF5A, Porcine oocytes

Weighted Gene Co-Expression Network Analysis Reveals Distinct Ovarian Signaling Differences Between Early and Non-Cycling Pubertal Classifications and FSH stimulation

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Abstract Text

Five pubertal classifications of bovine heifers were identified in our herd: Typical (T), Early (E), Start-Stop-Start (SSS), Start-Stop-Discontinuous (SSD), and Non-Cycling (NC). These classifications are moderately heritable and predictive of reproductive efficiency. NC heifers exhibit follicular arrest and reduced reproductive performance, yet when stimulated with FSH, they have increased numbers of ovarian follicles that develop to ≥ 7 mm and a tendency to develop to ≥ 10 mm compared to Typical (controls). We hypothesized that granulosa cells from NC females differ in their responsiveness to FSH compared to those with Typical or Early classifications. To test this hypothesis, females were assigned to either a Non-Stimulated or FSH-Stimulated treatment. In the Non-Stimulated group, reproductive cycles were synchronized using two PGF2 α injections 14 days apart, and ovaries were collected 36 h after the second injection. In the FSH-Stimulated group, cycles were similarly synchronized, followed by dominant follicle aspiration on day 10, and seven FSH injections administered every 12 h (250 IU total), with PGF2 α given with the final two injections. Granulosa cell RNA was collected from estrogen active follicles > 10 mm, poly-A+ libraries were sequenced (151 bp), and reads were mapped to ARS-UCD2.0. Weighted Gene Co-Expression Network Analysis (WGCNA) is a bioinformatics tool used to create clusters of genes, known as modules, with similar expression patterns. WGCNA was conducted using R and used to identify gene network modules and their correlation with pubertal classification, FSH treatment, and age class (heifer vs. cow). Modules were analyzed using DAVID or Ingenuity Pathway Analysis. In the WGCNA analysis, two modules differed significantly ($p < 0.05$) between Early and Non-Cycling females. These modules were linked to biological pathways for actin cytoskeleton regulation and actin-dependent signaling pathways involving genes such as Actin Gamma 1 (*ACTG1*), Cofilin 1 (*CFL1*), and Integrin-linked kinase (*ILK*), suggesting altered cytoskeletal dynamics in NC granulosa cells. An additional module related to autophagy, containing CASP8 and FADD-like apoptosis regulator (*CFLAR*), TNF receptor associated factor 6 (*TRAF6*), and autophagy related 101 (*ATG101*), tended to show stronger association with the Early group, consistent with increased follicle dynamics observed in this classification. When Non-Stimulated and FSH-Stimulated groups were compared seven modules were identified with genes associated with follicle growth and ovulation—such as Jun Proto-oncogene, Ap-1 (*JUN*), Fos Proto-oncogene, AP-1 (*FOS*), and Prostaglandin E Synthase (*PGES*) increased in the FSH-stimulated group. Conversely, pathways related to FOXO signaling, including F-box protein 25 (*FBXO25*), Insulin Receptor (*INSR*), Phosphatase and Tensin Homolog (*PTEN*), and Sirtuin 1 (*SIRT1*) along with autophagy networks discussed above were increased in the Non-Stimulated females. Only one module differed between heifers and cows; this module was independent of pubertal classification and enriched for metabolic processes including peroxisomal protein import and fatty acid β -oxidation. Together, these findings demonstrate distinct differences in granulosa cell signaling networks between Early and Non-Cycling classifications. The consistent patterns observed in both heifers and cows support the idea that reproductive phenotypes established before puberty persist into adulthood. Moreover, coordinated changes in cytoskeletal and autophagy-related modules provide new insight into potential mechanisms underlying follicular arrest in Non-Cycling females and their altered response to FSH stimulation.

Modeling Ovarian Remodeling and Functional Plasticity Following Peripubertal Exposure to Masculinizing Endocrine Environments Using a Mouse Kidney Capsule Transplantation Platform

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Abstract Text

Masculinizing hormone therapy using testosterone is increasingly initiated during peripubertal development for gender affirmation in transmasculine individuals. However, the direct effects of sustained androgen-dominant systemic environments on juvenile ovarian tissue - and the potential for subsequent recovery of ovarian function - remain poorly understood. As many individuals who receive early gender-affirming treatment may later desire fertility restoration, there is a critical need to define how developing ovaries respond to masculinizing endocrine exposure and whether these effects are reversible. Here, we hypothesized that exposure of juvenile ovarian tissue to a masculinizing endocrine milieu suppresses follicular growth and induces stromal remodeling, and that these changes may be reversible upon restoration of a female hormonal environment. To isolate systemic endocrine effects from ovarian-intrinsic programs, ovaries from prepubertal female donors (C57BL/6, 1-month old) were surgically transplanted beneath the renal capsule of adult 2-month-old female or male recipients (C57BL/6, n=3/sex), allowing revascularization and exposure of genetically identical ovarian tissue to sex-specific systemic endocrine environments. Two weeks post-transplantation, ovarian grafts were analyzed by H&E staining to assess tissue architecture and by immunohistochemistry for Ki67 and α -smooth muscle actin (α -SMA) to evaluate granulosa cell (GC) proliferation and thecal organization, respectively.

While corpora lutea were absent in grafts from both recipient groups, ovaries transplanted into male hosts exhibited pronounced structural remodeling, including visibly expanded theca layers surrounding developing follicles. Quantitative analysis demonstrated significantly reduced GC proliferation in primary and secondary follicles of male recipients (Ki67+ cells: 40.25%) compared to female recipients (62.69%). In parallel, α -SMA staining revealed significantly thicker theca layers in ovaries exposed to the male endocrine environment (14.33 μ m) relative to those in female hosts (8.00 μ m). Together, these findings demonstrate that exposure of juvenile ovarian tissue to a masculinizing systemic endocrine milieu suppresses GC proliferation and promotes androgen-associated thecal remodeling, indicating early follicular growth impairment and stromal reorganization. This transplantation platform provides a novel experimental framework to directly assess ovarian tissue responses to androgen-dominant environments during developmental windows relevant to peripubertal gender-affirming care, with translational implications for fertility preservation strategies. Ongoing studies will incorporate circulating hormone measurements and extended transplantation intervals. Importantly, we will test whether androgen-exposed ovarian grafts regain follicular activity following relocation into ovariectomized female hosts, enabling direct assessment of ovarian functional plasticity and reversibility. Collectively, this model allows mechanistic dissection of systemic endocrine regulation of juvenile ovarian structure and function and offers a powerful approach for studying reproductive tissue adaptability following early masculinizing hormone exposure.

ESR2 acts as a tumor suppressor in granulosa cells

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Abstract Text

Adult granulosa cell tumors (AGCTs) are rare, typically unilateral ovarian neoplasms arising from granulosa cells, characterized by distinctive histology, including Call–Exner bodies and coffee-bean nuclear grooves, and strong FOXL2 expression. Over 95% of AGCTs harbor the FOXL2 C134W mutation and frequently retain estrogen-producing steroidogenic machinery, often associated with elevated estradiol (E2), anti-Müllerian hormone (AMH), and inhibin A/B. Despite this, the pathogenesis of AGCT remains poorly understood, and the contribution of estrogen receptor signaling to tumorigenesis has not been fully defined. During our studies on estrogen receptor conversion in granulosa cells, we serendipitously observed that aged (≥ 13 months) Esr1-Esr2KO mice, lacking estrogen receptor β (ER β , ESR2) in the granulosa cell lineage, developed unilateral ovarian masses resembling human AGCT. This unexpected finding prompted us to investigate the role of ESR2 in granulosa cell tumor development and whether it shares downstream molecular features with human AGCT. To test this hypothesis, we generated a granulosa cell-specific Esr2 knockout mouse model (Esr1-Esr2KO) and characterized age-dependent tumor formation, molecular phenotypes, and their microenvironment. Aged Esr1-Esr2KO females with masses (14–15 months, $n = 5$) exhibited reduced body weight compared to wild-type (WT) controls ($n = 14$, $p = 0.01$) or Esr1-Esr2KO mice without masses ($n = 11$, $p = 0.03$), but no significant differences between WT and Esr1-Esr2KO without masses ($p = 0.27$). Histological analysis revealed ovarian masses with disrupted follicular architecture and Call–Exner bodies, a hallmark of AGCT. MRI at 14 months identified ovarian masses in 10% of Esr1-Esr2KO mice, with significantly larger ovarian volumes compared to WT ($11.3 \pm 4.4 \text{ mm}^3$ vs. $4.7 \pm 0.7 \text{ mm}^3$), and tumor incidence rose from 0% at 10 months to 75% by 15 months, resulting in an overall significant incidence of 23.8% (5/21), while WT mice remained tumor-free (0/17, $p = 0.0405$). Sanger sequencing revealed no canonical Foxl2 390C >G (p.C134W) mutation in the tumors, indicating a FOXL2 mutation-independent mechanism of tumorigenesis. Hormonal profiling revealed persistently elevated serum estradiol across ages 1–11 months ($34.7 \pm 2.1 \text{ pg/ml}$ vs. $15.0 \pm 0.9 \text{ pg/ml}$ in WT, $n=10-11/\text{genotype}$, $p < 0.0001$), indicating early endocrine dysfunction. Gene expression analysis showed that key granulosa cell markers, such as *Foxl2*, *Inha*, *Amh*, and *Cyp19a1*, were maintained in Esr1-Esr2KO ovaries without masses, but *Gata4* expression was significantly increased in early-stage follicles ($n=3-5/\text{genotype}$, $p < 0.05$). Additionally, *Sox9* expression, a Sertoli cell marker, was localized in Esr1-Esr2KO ovarian masses, suggesting partial lineage reprogramming. Immune analysis revealed elevated white blood cell and lymphocyte counts in Esr1-Esr2KO mice with ovarian masses ($14.67 \times 10^3/\mu\text{L}$ vs. $7.94 \times 10^3/\mu\text{L}$ in WT, $n=2-3/\text{genotype}$, $p = 0.002$), indicating systemic immune activation. Hypoxia-related gene expression, particularly *Hif1a*, was also localized in the ovarian masses, suggesting activated hypoxic pathways ($n=3/\text{genotype}$). These findings demonstrate that ESR2 loss in granulosa cells leads to age-dependent ovarian mass formation with tumor-like features, independent of the FOXL2 mutation, and reveal ESR2 as a critical regulator of granulosa cell identity and ovarian tumor suppression. This study provides novel insights into non-FOXL2-dependent mechanisms of granulosa cell tumorigenesis and underscores the role of estrogen receptor signaling in ovarian tumor development.

Synchronized Spermatogenesis for Stepwise Analysis of the Developmental Program of Spermatid Morphogenesis

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Abstract Text

To form fertilization-competent sperm, haploid round spermatids must complete the developmental program of spermiogenesis. It is during this morphogenetic program that round spermatids transform into highly polarized sperm by condensing their nuclei, reshaping their heads, shedding cytoplasm, developing acrosomes, and growing flagella. These events are accompanied by considerable gene expression changes and formation of transient Sertoli- and spermatid-specific structures including the acroplaxome, manchette, tubulobulbar complex, and apical ectoplasmic specialization. While these unique gene expression changes and spermiogenesis-specific structures have been well characterized, the detailed mechanisms regulating their formation and their specific role(s) in spermatid morphogenesis remain unclear. To address this considerable gap in knowledge, we synchronized spermatogenesis in mice and collected testes enriched with spermatids at each step of their development. From these mice, we isolated for the first time millions of spermatids at steps 1 (Golgi phase), 5 (cap phase), 8 (acrosome phase, round), and 9 (acrosome phase, elongating) for 'omics analyses to define specific transcriptome and proteome changes at discrete steps of spermatid morphogenesis. In addition, we defined sperm transit time from the testis to the cauda epididymis to be 2-3 days, which is considerably shorter than estimates in the literature (5-14 days). Importantly, we also determined the first sperm generated from synchronized spermatogenesis were fertilization-competent, generating large litters of healthy offspring. In conclusion, this *in vivo* manipulable system provides a novel means that our lab and others can utilize to study the stepwise molecular changes in gene expression, epigenetics, and signaling pathway activation that underlie the unique cellular events during the program of spermiogenesis in both wild-type and genetically modified mice.

Transcriptional Regulation of *PRSS* Genes by Bivalent Chromatin and Silencer Activity in the Human Testis

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Abstract Text

Spermatogenesis is a complex process that involves not only mitotic and meiotic divisions but also extensive morphological remodeling during spermiogenesis, requiring precise temporal and cell-type-specific transcriptional regulation. The *PRSS* gene locus, encompassing six protease genes, provides a valuable model for elucidating the mechanisms of transcriptional regulation in the testis, and studies in the mouse have revealed its important roles and strict regulation in spermatogenesis. However, despite the evolutionary conservation among eutherians, little is known about the *PRSS* locus in species other than mouse; even their testicular expression profiles remain unknown. In this study, we focused on the primate *PRSS* locus, especially human, and examined their expression patterns and a part of their transcriptional regulatory mechanisms. We analyzed public RNA-seq data sets of the testis of six primate species and found that one or two different *PRSS* genes were predominantly expressed in the testis of each species. In the human testis, the *PRSS50* gene was predominantly expressed, although qRT-PCR using human testis RNAs confirmed low levels of expression of the other five *PRSS* genes. Furthermore, public single-nucleus RNA-seq data revealed that the human *PRSS50* gene was predominantly expressed from spermatogonium to spermatocyte stages and in round spermatids. Interestingly, public ChIP-seq data sets with human and mouse germ cells revealed that promoter regions of the four human *PRSS* genes with low expression uniquely possessed both H3K4me3, associated with gene activation, and H3K27me3, associated with gene inactivation, whereas the corresponding mouse genes with high expression were marked only with H3K4me3. This suggests that bivalent chromatin domains contribute to low expression of the four human *PRSS* genes. Next, we focused on the transcriptional regulatory mechanism of *PRSS45* gene, which showed low expression in human testis but high expression in chimpanzee. As a result, while a human-specific sequence identified by genome comparison showed no significant transcriptional regulatory activity by our reporter assays, an ATAC-seq peak identified downstream of the bivalent chromatin region significantly decreased *PRSS45* promoter activity. Therefore, we suggest that the human *PRSS45* gene was expressed at a low level due to the bivalent chromatin structure and silencer activity. Our current findings reveal unique regulatory mechanisms underlying *PRSS* gene expression in human testis and provide a foundation for understanding transcriptional regulation in human spermatogenesis.

Potential Post

Translational Modifications Underlying Aggresome Formation in Bovine Sperm

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Abstract Text

Recent work from our group has shown a sire-specific phenotype, an accumulation of protein aggregates in the sperm head, that leads to impaired embryo development, increased autophagy, and cellular stress. The purpose of this study is to identify proteomic differences that may contribute to the formation of aggresomes in sperm. Samples from sires characterized as either high (n=3) or low (n=3) aggresome content were purified using density gradient centrifugation and then subject to proteomic analysis, including the following post-translational modifications (PTMs): phosphorylation, tri-oxidation, acetylation, and palmitoylation. Spermatozoa were lysed by sonication, and proteins were extracted via methanol:chloroform:water precipitation. Proteins were then digested using a dual-stage ArgC/LysC protocol, desalted by C18 SPE, and labeled with tandem mass tags. Labeled peptides were analyzed by nanoLC-MS/MS on an Orbitrap Fusion™ Lumos™ with a 203-minute gradient. Data were processed in Proteome Discoverer 3.1.0.638 using Sequest HT against the *Bos taurus* reference proteome. Reporter ion abundances were normalized to the total peptide amount intensity. Differential abundance between groups was assessed by ANOVA on individual proteins without imputation. There were no differences in protein abundance (FDR) between groups. We further evaluated two PTMs with nominal P values < 0.05. The first one was palmitoylation of Pleckstrin homology and RUN domain containing M1 (PLECKHM1), which was found to be 1.423 times more prevalent in HA sires compared to LA (P=0.0326). Palmitoylation is the addition of a 16-carbon fatty acid to a thiol group on a cysteine residue to form a thioester; it is commonly involved in the shuttling of proteins to different cellular compartments. PLECKHM1 is an adapter protein that regulates the fusion of the autophagosome with the lysosome in conjunction with the GABARAP family of proteins and TRIM22. The addition of a large palmitoyl group to PLECKHM1 may interfere with its interaction with TRIM22. This could prevent autophagosome fusion with the lysosome, which has been shown to cause aggregation of proteins in other cells, and could be underlying the formation of the aggresome in HA sires. The second PTM of interest is the phosphorylation of the non-selective voltage-gated ion channel (VDAC2). VDAC2 phosphorylation was found to be 1.396 times more prevalent in HA sires compared to LA (P=0.0007). Phosphorylation is a PTM that commonly regulates protein activity. In neurological pathologies, hyperphosphorylation has been shown to cause aggregation of proteins. During periods of cellular stress, VDAC2 interacts with TMX2 to promote autophagy. This is consistent with previous work from our group, showing that embryos produced from HA sires exhibit increased cellular stress and autophagy. Autophagy is an essential cellular process, however, its dysregulation can impact cell viability and lead to aggresome formation. There were no differences in acetylation (P< 0.5), and all differences in tri-oxidation had FDR values exceeding 0.70. Further analysis with a greater sample size is being undertaken to validate the results of this pilot study. However, identification of differentially modified PTMs provides important preliminary insights into potential mechanisms underlying aggresome formation.

Sperm Motility in the Mosquito *Culex pipiens* is Regulated by Bicarbonate and cAMP

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Abstract Text

Understanding sperm activation and motility are essential for identifying potential targets for mosquito population control. In many taxa, bicarbonate (HCO_3^-) stimulates intracellular cAMP signaling that promotes phosphorylation and motility-associated changes in flagellar beating. However, how extracellular bicarbonate concentration affects mosquito sperm activation and progressive motility is not well understood.

In this study, we examined the effects of bicarbonate and cAMP on sperm motility patterns in the mosquito *Culex pipiens*. Previous studies from our laboratory established that sperm motility was initiated through the action of a serine protease (trypsin) stored in the male accessory glands. To determine the effect of bicarbonate sperm were isolated from mature seminal vesicles from individual adult males and incubated in Insect Ringer containing trypsin and 0.0, 1.2, 5, 10, or 20 mM NaHCO_3 . Sperm motility was recorded under darkfield optics at 100 frames per second at two and ten minutes and analyzed using SpermQ, a software package that analyzes sperm flagellar motility patterns. Using the SpermQ preparator allowed us to identify three motility patterns that we named Waves, Ribbons, and Rockets, representing increasing progressive motility patterns following activation. It was previously reported that *C. pipiens* sperm in Insect Ringer containing 1.2 mM NaHCO_3 achieve >90% forward progressive motility. When we removed the bicarbonate from the Insect Ringer, we found that the addition of 2 $\mu\text{g/mL}$ trypsin could still activate sperm motility at 2 minutes, but that progressive motility was not sustained at 10 minutes. Further, progressive sperm motility was NaHCO_3 concentration dependent with maximal progressive sperm motility achieved at 5 mM NaHCO_3 .

To determine if bicarbonate might act on a soluble adenylate cyclase (sAC), we activated sperm in the presence of a cell permeant and non-hydrolyzable form of cAMP, 8-Br-cAMP, in the absence of trypsin. Sperm treated with 8-Br-cAMP in insect Ringer rapidly activated sperm motility at levels comparable to those achieved through the action of trypsin. Progressive motility consisting of mostly Ribbon and Rocket waveforms persisted at 10 minutes suggesting that cAMP is sufficient to achieve progressive sperm motility in *C. pipiens*. These findings were supported through the addition of 1 mM IBMX, a compound that acts by inhibiting phosphodiesterases, thereby elevating intracellular cAMP concentrations. In the absence of bicarbonate, the predominate observed waveforms were Ribbons but these shifted to Rockets in the presence of 1.2 mM NaHCO_3 .

These findings support a working model in which cAMP/PKA signaling can rapidly activate vigorous progressive motility in *C. pipiens* sperm. We are currently refining a signaling model that integrates cAMP/PKA activation with our previous studies on Ca^{2+} -dependent and MAPK-associated regulation of waveform switching and persistence.

Mitochondrial Production of Reactive Oxygen Species during Sperm Capacitation and its Association with Human Fertility.

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Abstract Text

Sperm capacitation is a critical process that enables spermatozoa to acquire the ability to fertilize an ovocyte. The precise mechanisms underlying sperm capacitation remain incompletely understood. One aspect that has garnered attention in recent years is the role of mitochondria in this process. In this regard, we found that in normozoospermic donors, after four hours of incubation in capacitating medium, mitochondrial activity increased, accompanied by an increase in mitochondrial superoxide production as well as extracellular H_2O_2 release. These changes were not associated with oxidative stress damage, suggesting that these molecules may act as modulators of capacitation in fertile men. We hypothesize that this may be one of the mechanisms altered in patients with fertility problems.

Herein, we simultaneously assessed, in real time during the four hours of capacitation, changes in mitochondrial function (oxygen consumption as well as mitochondrial membrane potential [MMP]) and H_2O_2 production. These measurements were performed using the Fluo-Oroboros instrument for the first time in human sperm cells. We observed that the three parameters did not show identifiable breakpoints over the four-hour recording period, indicating that the increase was continuous throughout capacitation. To determine whether these mechanisms are dysregulated in abnormal semen samples, we analyzed H_2O_2 production and MMP in samples with parameters below World Health Organization (WHO 2021) reference ranges. As expected, MMP was lower in pathological samples compared with normozoospermic samples and did not increase during capacitation. Moreover, H_2O_2 production did not change over the four hours; however, H_2O_2 levels in non-capacitated (NC) sperm were higher than in capacitated normal samples.

Taken together, these findings indicate that sperm mitochondrial function is modified during capacitation, and that an altered balance in mitochondrial ROS production may contribute to impaired capacitation in infertile men.

Temperament Scoring and Substance P Outcomes In Peripubertal Bulls Undergoing Weekly Electroejaculation

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Abstract Text

Electroejaculation (EEJ) is a common method for semen collection but is subject to welfare concerns due to occasional vocalization. Therefore, we assessed hormonal and behavioral metrics associated with aversive stimuli over a multiple EEJ schedule in a between and within subject design. The first objective was to compare plasma Substance P concentrations, Pen Score (PS), Chute Score (CS), and Exit Velocity (EV) between control (n=4) and collected (weekly EEJ; n=7) peripubertal bulls for ~5 months. The second objective was to compare PS, CS, and EV of collected bulls on EEJ dates to themselves on alternating non-EEJ dates for the last two weeks of the study. We hypothesized that there would not be significant differences found for either objective. At all processing dates, PS and EV were determined. Blood sampling and CS occurred every 2 weeks during the EEJ period and all processing dates during the last two weeks. Based on 30 second videos taken within ~2 minutes of EEJ, CS was determined by 3 masked observers and averaged. Approximately 15 minutes after EEJ (collected) or a comparable time (control), blood was collected in K3EDTA tubes with aprotinin for plasma Substance P quantification via ELISA. Data were analyzed using generalized linear mixed-effects models for treatment and time via RStudio. Results of Objective 1 demonstrated that there was no significant interaction between time and treatment for PS, CS or EV ($p > 0.05$), but they decreased over time ($p > 0.05$). Neither interaction nor main effect of treatment was significant for Substance P concentration ($p > 0.05$), but time was significant ($p < 0.05$). Results of Objective 2 demonstrated that neither the interaction nor main effects were significant ($p > 0.05$). Based on the preliminary results, there is no indication of long-term welfare concerns. Extrapolation of these results of this study must be interpreted carefully due to the relatively small sample size on peri-pubertal bulls and differences of values assigned using a subjective scoring system.

Comparative Proteomic Analysis of Bovine Seminal Plasma from Holstein Bulls with Varying Field Fertility

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Abstract Text

Bovine seminal plasma (SP) is the fluid component of semen and contains proteins, minerals, electrolytes, hormones, and enzymes that support sperm function and influence interactions with the female reproductive tract. During commercial semen processing for cryopreservation, SP is commonly removed or diluted, which may limit the presence of SP-derived factors in the final insemination dose. Recent studies suggest that variation in SP composition may contribute to fertility differences among bulls with similar semen quality. Thus, characterizing SP protein components may provide insight into the role of SP in bovine fertility. To answer this question, 6 Holstein bulls were assigned to high-fertility (HF; n = 3) or low-fertility (LF; n = 3) groups based on field sexed semen fertility data. HF bulls had greater pregnancy per artificial insemination (P/AI; 56.7%) and sire conception rate (SCR; 0.04493) compared with LF bulls (P/AI: 47.5%; SCR: -0.01799), with both fertility measures differing significantly between groups ($P < 0.01$). For each bull, two consecutive ejaculates were collected on the same day, and the procedure was repeated one week later. Ejaculates were pooled within bull, supplemented with protease inhibitor, digested with trypsin, and analyzed using bottom-up liquid chromatography–tandem mass spectrometry (LC-MS/MS). Proteomic profiles of bovine seminal plasma were analyzed using a robust bioinformatics pipeline implemented in Python (v3.12). Raw protein abundance values were log-transformed to normalize the distribution. To account for missing values inherent in mass spectrometry data, a left-shifted distribution imputation strategy was used. Differential expression between HF and LF groups was evaluated using Welch's t-test. P-values were adjusted using the Benjamini-Hochberg False Discovery Rate (FDR) procedure. Proteins were classified as significantly differentially expressed proteins (DEPs) based on an FDRadjusted $P < 0.05$ and an absolute $\log_2$ fold-change $\log_2FC > 1.0$. Multivariate data integration was performed using Principal Component Analysis (PCA) for unsupervised dimensionality reduction and Linear Discriminant Analysis (LDA) to assess group supervised classification accuracy. Overall, 1,118 proteins were identified. Group-specific proteins were first defined as those detected in at least one animal from one fertility group and absent from all animals in the other group, resulting in 177 HF-specific and 157 LF-specific proteins. Because this approach may include proteins detected in only one individual, a stricter criterion requiring detection in at least two animals per group was applied. Using the more conservative approach, 29 HF-specific and 42 LF-specific proteins were identified and ranked by mean abundance among quantified samples. The top HF-specific proteins included CDH1, PIGR, MMP2, PDCL2, and START3, while the top LF-specific proteins included CEP131, SERPINB6, UBE2V1, KIF11, and EBD. Several of these proteins have been identified in reproductive processes. These findings suggest that differences in fertility-associated seminal plasma may involve extracellular remodeling, sperm development, cell adhesion, immune regulation, and germ-cell-derived proteins. Further analyses will evaluate the effects of HF/LF seminal plasma on semen quality parameters and in vitro fertilization outcomes to better determine their reproductive relevance.

Acute Regulation of Steroidogenesis: RhoA-centered Signaling Integrates Endocytosis, Organelle Remodeling, and Transcription Downstream of LH

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Abstract Text

Luteinizing hormone (LH) stimulates steroidogenesis within minutes, but the signaling mechanisms that connect receptor activation to mitochondrial cholesterol delivery remain poorly defined. Although canonical models emphasize Gs-cAMP-PKA signaling, the broader signaling architecture of the acute steroidogenic response has not been systematically examined. To define early signaling events downstream of LH receptor activation, we performed global phosphoproteomic profiling in MA-10 Leydig cells at 15, 45, and 90 minutes following LH stimulation. Strikingly, canonical PKA substrate phosphorylation was not detected, despite robust steroidogenic activation. Instead, pathway enrichment consistently identified the Rho GTPase cycle as the most prominent signaling module across all timepoints, accompanied by phosphorylation of proteins involved in endocytosis, cytoskeletal remodeling, organelle dynamics, and transcriptional regulation. These phosphorylation events were temporally coordinated with changes in proteins linked to ER remodeling, autophagy, and mitochondrial fission, indicating rapid cellular reorganization to support steroid biosynthesis. Functional validation supported this signaling framework: induction of a constitutively active RhoA recapitulated all features of LH-stimulated steroidogenesis, whereas disruption of clathrin-mediated endocytosis using dominant-negative clathrin, dominant-negative adaptor AP180, and dominant-negative dynamin, markedly suppressed steroid hormone production. These findings demonstrate that Rho-dependent signaling serves as a central organizing principle, and that clathrin-mediated endocytosis is an essential process for the acute steroidogenic response. Together, these findings support a model in which LH receptor activation rapidly engages a RhoA-centered signaling network that coordinates cellular processes to drive mitochondrial cholesterol delivery and acute steroid hormone biosynthesis.

Ca²⁺ oscillations at Fertilization Ensure Genomic Stability by Maintaining Proper Embryonic Chromosome Segregation in Mice

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Abstract Text

The sperm-specific factor phospholipase C zeta (PLC ζ) triggers repetitive increases in intracellular calcium (Ca²⁺) concentrations, known as Ca²⁺ oscillations, in the oocyte cytoplasm during mammalian fertilization. In humans, infertility associated with mutations in *Plcz1* (the gene encoding PLC ζ) has been reported, indicating that PLC ζ -induced Ca²⁺ oscillations are essential for successful fertilization and early embryonic development. In our previous study, we demonstrated that B6:*Plcz1*^{-/-} (KO) male mice fail to induce Ca²⁺ oscillations during in vitro fertilization (IVF). Despite the absence of Ca²⁺ oscillations, KO-derived embryos were capable of developing to the blastocyst stage and implanting in the uterus, suggesting that PLC ζ may have roles beyond oocyte activation. To further investigate these roles in vivo, we mated wild-type (WT) and KO males with mature females and comprehensively assessed fertility and embryonic development. Embryos and fetuses were collected after mating to evaluate preimplantation development, implantation efficiency, chromosome dynamics, and genomic copy number variations in fetal chromosomes. To visualize chromosome behavior in real time, TagGFP2-H2B mRNA was microinjected into embryos, followed by live-cell imaging. Karyotype analysis was performed to determine the origin and nature of chromosomal abnormalities. In a subset of KO embryos, strontium chloride (SrCl₂) treatment was applied after IVF to assess its potential rescue effect. KO males exhibited severely reduced fertility, and blastocyst formation rates were significantly lower than those of WT controls. Although implantation of KO-derived embryos was confirmed, both the number and weight of implantation sites were significantly reduced at embryonic day 19.5 (E19.5), indicating compromised developmental potential. Live imaging revealed a markedly increased frequency of abnormal chromosome segregation in KO embryos. Karyotype analysis further demonstrated that most chromosomal abnormalities originated during the first mitotic division. Notably, SrCl₂ treatment partially rescued these mitotic defects, supporting a causal relationship between Ca²⁺ oscillation failure and chromosome segregation errors. In addition, genomic copy number variations were detected in KO-derived fetuses, indicating persistent genomic instability. Collectively, these findings demonstrate that PLC ζ -induced Ca²⁺ oscillations are not only required for fertilization but also play a critical role in safeguarding genomic stability by ensuring accurate chromosome segregation during early embryogenesis in mice.

Follicle-Stimulating Hormone Knockout Impairs Reproduction in Male Sheep

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Abstract Text

Follicle-stimulating hormone (FSH) plays essential roles in Sertoli cell function, spermatogenesis, and male fertility. To investigate the physiological consequences of FSH deficiency in a large mammal model, sheep embryos were produced *in vitro* and edited via electroporation 6h post-fertilization using CRISPR-Cas9 and a dual-single guide RNA (sgRNA) approach, targeting the *FSHB* gene. Embryos reaching the blastocyst stage were transferred into synchronized recipient ewes and gestated to term. Three male lambs with confirmed biallelic loss-of-function mutations were born in April 2025. Editing status of the *FSHB* gene was confirmed by whole-genome sequencing. Ram 1, a heterozygous knockout (KO), was euthanized 24 hours after birth due to medical complications. Ram 2 is a homozygous KO, with both alleles disrupted by a 3 bp deletion at exon 3 cut site and a 59,121 bp insertion at exon 2 site. Ram 3 is a compound heterozygous KO, with a large deletion between the two sgRNA target sites, a 5 bp deletion at exon 2, and a 3 bp insertion at exon 3. Rams 2 and 3 were evaluated for growth and reproductive development between birth and sexual maturity, alongside wild-type (WT; n = 2) half-brothers. Body weights were recorded monthly from birth through December 2025. Scrotal circumference was measured biweekly between September and December 2025 (first fall breeding season). Blood samples were collected monthly between days 0 and 270 of life for serum FSH quantification. Semen was collected via electroejaculation monthly between October and December 2025, and concentration was detected on an automated sperm cell counter. Fluorescent labeling (FITC-PSA/Hoechst 33342) was used to assess sperm viability and morphology. A libido study was conducted by exposing KO and WT rams to ewes in estrus to assess reproductive behavior mid-breeding season. Due to small sample size (n = 2 animals/group) no statistical analyses were performed. Results are descriptive. **Results.** Body weights were similar between WT and KO throughout the period evaluated. In September 2025, KO rams weighed 75.6 and 60.2 kg, and WT rams weighed 68.8 and 64.2 kg. Scrotal circumferences were consistently lower in KO rams, averaging 25.5±1.7 cm compared to 33.75±1.5 cm in WT (25% smaller circumference). Circulating FSH showed the expected rise in WT rams around 90 days of age and remained consistently higher than in KO rams, confirming sustained endocrine disruption. Knockout rams had lower sperm concentration at an average 222±171 million sperm/ml compared with 1,696±1,276 million sperm/ml in WT. FITC-PSA/Hoechst 33342 labeling showed that although the proportion of live sperm was similar between genotypes, KO rams had a higher frequency of sperm defects with an average of 24% sperm with proximal droplets and 11% other defects. In comparison, WT rams averaged 0% proximal droplets and 4.7% other defects. Sexual behavior was similar between KO and WT rams when exposed to ewes in estrus, although when in the presence of females in general, KO rams did not demonstrate copulatory behaviors. Collectively, these findings indicate that a male loss-of-function in *FSHB* does not affect growth or in-season sexual behaviors, but does impact scrotal volume and causes oligospermia accompanied by a higher frequency of sperm defects. This first large animal model of FSH deficiency provides novel insights into the roles of FSH in male reproduction and fertility. Funding: Kraft fellowship to HW; Graham A.E. and Betty A. Gall Faculty Research Endowment in Animal Genetics.

Essential Function of LOTUS Domain in piRNA Biogenesis and Spermatogenesis in Mice

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Abstract Text

piRNAs are a class of small RNAs associating with PIWI proteins in mammalian germ cells that are essential for spermatogenesis and fertility. TDRD5 is a key component of the piRNA pathway that promotes piRNA biogenesis in mice, while the molecular mechanism remains elusive. The LOTUS domain of TDRD5 is a conserved domain known to bind and stimulate Vasa DEAD-box helicases during piRNA biogenesis. Here, we generated a point mutation in a conserved residue within the LOTUS domain of TDRD5 to investigate its functional significance in piRNA regulation in mice. The LOTUS domain mutation resulted in partial mis-localization of TDRD5 to intermitochondrial cement, accompanied by diffuse cytoplasmic distribution. This *Tdrd5^{Lotus}* mutation causes piRNA biogenesis defect and spermatogenesis arrest. Notably, LINE1 transposons remained effectively silenced in *Tdrd5^{Lotus}* mutant, in contrast to their upregulation in *Tdrd5*-null testes. These findings demonstrate that the LOTUS domain of TDRD5 is critical for proper piRNA biogenesis and germ cell development in mice, suggest a protein domain-specific role in regulating distinct aspects of

Flow Cytometric Detection of Live Sperm ROS: Threshold Determination and Association with Sperm Quality

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Abstract Text

Infertility remains a global challenge, with male-factor infertility contributing to approximately 50% of cases. Oxidative stress, characterized by an imbalance between intracellular oxidative and antioxidant activities due to excessive reactive oxygen species (ROS), is a potential cause of male infertility. The application of flow cytometry for the detection of sperm ROS offers several advantages, including speed, stability, and reliability. However, this method presents various complexities. This study aimed to establish a reliable method for detecting ROS in live sperm using flow cytometry and to investigate the relationship between sperm ROS levels and sperm quality. The research included 121 healthy donors and 120 subfertile patients who were referred to the Department of Andrology at West China Second University Hospital for semen analysis. In addition to routine semen analysis and DNA integrity testing, we measured ROS levels in sperm using flow cytometry. The threshold value of ROS in healthy donors was determined to be 25.00%. A negative correlation was observed between ROS levels and sperm concentration, viability, motility, and normal morphology. Conversely, there was a positive correlation between ROS levels and age, as well as the DNA fragmentation index (DFI). Furthermore, subfertile patients exhibited higher ROS levels compared to healthy donors, and elevated ROS had detrimental effects on sperm quality and DNA integrity. In this study, we established a reliable method for detection of sperm ROS, which provides a foundation for the diagnosis and treatment of male infertility associated with reactive oxygen species in clinical settings.

Unraveling Epigenomic Mysteries of the Buck Sperm

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Abstract Text

Buck fertility is vitally important for the reproductive efficiency of goats (*Capra hircus*) to ensure food security. Sperm methylome is critical for genome stability, regulation of gene expression, and a determinant of male fertility and successful embryonic development. Breed-specific variations in the methylome of the buck sperm remain largely uncharacterized. The objective of this study to ascertain comparative dynamics of whole-genome DNA methylation landscapes in sperm cells from three goat breeds: Alpine (ALP), Boer (BR), and Spanish (SPN). Sperm genomic DNA was isolated from 20 bucks (ALP: n=8; BR: n=8; SPN: n=4). High-throughput sequencing was performed on the Illumina NovaSeq 6000 platform using a paired-end, 150 bp read length protocol. Whole-Genome Bisulfite Sequencing (WGBS) was employed to generate single-base resolution methylation maps, with reads aligned to the *Capra hircus* reference genome. Total mapping rates ranged from 86.69% to 90.77% and unique mapping rates exceeded 82%. Genome-wide coverage breadth was across all breeds, with over 99% of the genome covered at least 1X. The percentage of the sites covered at a minimum of 10X depth remained high across all samples. These ranged from 86.89% to 97.34% in the ALP group, 83.72% to 90.46% in the BR group, and 81.09% to 88.27% in the SPN group. At the global level, the methylome remained highly conserved across the three groups: ALP mean CG% ranged from 78.84% to 83.23%, while total cytosine methylation ranged from 4.47% to 5.41%. Non-CpG methylation (CHG/CHH) remained consistently below 0.54%. BR mean CG% exhibited a range of 81.38% to 84.36%, with a total cytosine methylation level of 4.14% to 4.77%. The CHG and CHH methylation levels were stable from approximately 0.48% to 0.51%. The SPN mean CG% ranged from 81.64% to 82.84%, and total cytosine methylation was between 4.5% and 4.94%. Non-CpG levels were consistently between 0.48% and 0.51%. We uncovered that while global methylation patterns are largely conserved among Alpine, Boer, and Spanish buck sperm, the high-resolution maps produced provide a critical reference for livestock epigenetics. The results helped establish a foundational baseline for identifying breed-specific differentially methylated regions (DMRs) that may correlate with male fertility phenotypic traits for precision goat farming.

Poster Session C

Griffin Hall

Wednesday, July 22,

8:00 AM – 9:45 AM

NICHD's Contract Facilities for Contraceptive and Reproductive Health Research Development

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Abstract Text

Priorities of the NICHD include accelerating the development of novel, safe, and effective new products for contraception and other reproductive health-related indications. The NICHD offers researchers free access to two state-of-the-art facilities: 1.) Biological Testing Facility (BTF), which handles later-stage preclinical development, including formulation, ex vivo studies, animal model testing, and toxicology; and 2.) Chemical Screening and Optimization Facility (CSOF), which focuses on early-stage drug discovery, such as high-throughput screening, chemical synthesis, and lead optimization.

To advance contraceptive and reproductive health product development, these facilities have the capabilities and capacity for preclinical and Investigational Device Exemption (IDE)- or Investigational New Drug (IND)-enabling studies. The long-term objective is to enable a preclinical candidate for IND/IDE studies that offers a safe therapeutic option in the field of contraception and/or reproductive health related indications.

NICHD seeks innovative and validated methods for future clinical development. This poster provides potential applicants with information on the mechanism to request up to 12 months of services from these NICHD-contracted research facilities that would advance their development program with an emphasis on facilitating early-stage development research, bridging studies, pivot studies and entry into clinical studies. In vitro and in vivo services are available to support that goal and in pursuit of an IND or IDE application for submission to the US Food and Drug Administration or analogous submissions to other regulatory agencies.

The facilities have the capability to advance multiple projects simultaneously across the entire product development continuum, are open to academic, non-profit, and for-profit organizations, as well as foreign institutions, and are not limited to programs with current NICHD support. Potential applicants with a promising idea are strongly encouraged to contact the PO/COR scientific contacts for a preliminary discussion before applying.

Optimized Preservation Strategies to Prolong the Lifespan and Viability of Boar Spermatozoa During Room Temperature Storage.

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Abstract Text

Spermatozoan health is at the forefront of reproductive studies and of interest to livestock breeders. Due to poor cryo-survival, porcine semen is collected and extended in liquid form for immediate artificial insemination (AI) or experimental use. Subpar storage can expedite the decline in sperm quality reflected in loss of motility and viability within the first 4-5 days of storage. For researchers and swine breeders alike, improving semen storage can decrease collection and processing costs, maximize fertilization success days after collection, and provide more usefulness within a single collection. Sperm mitochondrial health is used as a sentry in optimal storage, as the mitochondria have roles in maintaining cellular homeostasis, ATP synthesis, motility, and fertilization potential. This study aims to increase the understanding of sperm mitochondrial health and its impact on functional lifespan of boar spermatozoa in extended, room temperature (RT) stored liquid semen. As an alternative to commonly used chilling, RT-storage would greatly simplify semen handling and distribution. This includes optimizing sperm concentration and adding mitochondria supporting supplements to extender. While industry commonly stores liquid boar semen at 75-100 million perm mL, we found a concentration of 35-40 million spermatozoa per mL, the boar minimum for satisfactory AI outcome, to be more amenable to sperm survival, keeping motility above 70 % for over two weeks at room temperature, and progressive motility still detectable in some spermatozoa up to 42 days of RT-storage. Diluted samples on days (D) 7 and 14 were significantly different (p value < 0.05) from traditionally extended samples, with average total motilities of: diluted D7 ~79 %, D14 ~72 %; traditional D7 ~20 %, D14 ~5 %. This was further improved by eliminating air from storage containers. A preliminary trial was completed with a 14-day observation to compare traditionally (Tr) and optimally (Op) extended samples, with/without air space. Computer assisted semen analysis (CASA) values of D14 progressively motile spermatozoa (PMS) were: 1.8% Tr/air; 2% Tr/no-air; 1.5% Op/air; 35.6% Op/no-air. Novel semen additives including green tea antioxidant gallic acid and hydrogen sulphate donor NaHS further increased sperm motility and lifespan during storage. Supplemented groups were tested with Tr stored boar semen. Total motility was increased in the treatment groups supplemented with 1 μ M NaHS (N), compared to controls (C). Days 0, 7, 14, 21, 28, and 35 were analyzed for total motility. Average % of total motility was N(0-35): 91.11 %, 71.79 %, 40.19 %, 14.06 %, 11.38 %, 3.52 %; C(0-35): 92.77 %, 68.67 %, 34.21 %, 8.26 %, 8.41 %, 2.39 %. Days 7, 21, and 35 were significantly different (p -value < 0.05), with N having increased total motility. To compare fertilizing potential of Op and Tr semen, in vitro fertilization (IVF) and fluorescent tracers of mitochondrial health, membrane integrity, and ionic composition were assessed via image-based flowcytometry and epifluorescence microscopy. Improved progressive and total motility of the Tr groups was confirmed by CASA. Altogether, we identified key differences between Op and Tr, most notably the lengthened lifespan of extended semen stored and handled at RT, which would greatly simplify AI semen distribution via reduced sperm concentrations, and additives that improve sperm motility and viability over several weeks. Mitochondrion-friendly semen preservation techniques thus mitigate sperm stressors while improving overall cellular health. Study funded by USDA-NIFA grant number 2021-67015-33404 (PS) and seed funding from CAFNR, University of Missouri (PS).

Choline-Induced Alterations in Lipid Biochemistry in the Preimplantation Bovine Embryo

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Abstract Text

Addition of the quaternary amine choline to culture medium of in vitro produced bovine embryos has been associated with increased calf weight at weaning but the mechanisms underlying this effect remain unclear. Choline can be metabolized via biochemical pathways leading to acetylcholine, S-adenosylmethionine, or phosphatidylcholine, the latter being a major component of cell membranes and an important regulator of cellular metabolism. Also, choline can activate AMPK signaling and reduce lipid droplet numbers as seen in other cell types. We hypothesized that addition of choline chloride to culture medium of preimplantation bovine embryos alters embryonic lipid availability by increasing phosphatidylcholine and its metabolites and decreases the amount of stored lipids in the form of lipid droplets. Bovine embryos were cultured with 1.8 mM choline chloride or a vehicle control and evaluated at multiple stages of development. In the first experiment conducted in 8 replicates with 271 embryos, neutral lipid content was assessed using BODIPY 493/503 to determine whether choline treatment affected neutral lipid accumulation. Fluorescent imaging of fixed embryos for BODIPY 493/503 labeling revealed no significant differences in neutral lipid accumulation between choline-treated and vehicle-treated embryos at the 2-cell, 4-cell, 8-cell, 16-cell, or morula stages ($p = 0.583$). However, neutral lipid content varied by developmental stage ($p = 0.015$), with the lowest abundance observed at the 4-cell stage. The second experiment was conducted similarly except only 8-cell embryos were evaluated and neutral lipid content was evaluated by both BODIPY 493/503 and Nile Red labeling. Again, choline treatment did not affect fluorescence for either BODIPY ($p = 0.5241$) or Nile Red ($p = 0.3053$). In the third experiment, pools of 15 blastocysts produced in culture medium $\pm$ 1.8 mM choline were analyzed by liquid chromatography tandem-mass spectrometry for specific lipid types ($n=7$ pools/treatment). Lipidomic data were analyzed using MetaboAnalyst6.0 with default filtering, median normalization, log transformation, Pareto scaling, and partial least squares-discriminant analysis. Choline increased abundance of phosphatidylcholine species ($p = 0.0036$) and decreased abundance of cholesterol esters ($p = 0.0452$). Collectively, these results demonstrate that choline modifies embryonic lipid composition by increasing phosphatidylcholine abundance and decreasing cholesterol ester abundance in blastocysts without significantly altering neutral lipid storage. This shift in blastocyst lipid metabolism may contribute to the long-term developmental programming effects of choline observed in cattle. Funding: USDA AFRI grant 2023-67015-40730.

Post-Fertilization Cumulus Cells Persist, Self-organize, and Activate Luteal-adjacent Secretory Programs

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Abstract Text

Cumulus cells are typically studied for their metabolic and paracrine support of oocyte growth and maturation. However, their functional fate and capacity following cumulus-oocyte complex maturation and fertilization remain poorly defined. We observed that post-IVF cumulus cells retain viability and self-organize into organoid-like structures in extended culture, and when dissociated, continue to proliferate and expand as adherent monolayers over prolonged periods. These cultures also exhibit luteinization-like features, including sustained progesterone production. To define the transcriptional programs underlying these behaviors, cumulus-cell transcriptomes were analyzed across two developmental trajectories: (a) cumulus cells collected after in vitro maturation (MII), following IVF at the 2-cell stage, and at the blastocyst stage during embryo culture; and (b) cumulus cells removed from presumptive zygotes immediately after IVF and maintained as adherent cultures for 7, 14, and 28 days. Across fertilization and extended culture, cumulus cells activate a coordinated secretory program characterized by ECM remodeling, lipid-handling capacity, immune quiescence, and regulated proteolysis, features consistent with a transient, luteal-adjacent niche rather than terminal differentiation or degeneration.

Prominent extracellular matrix and matricellular components included VCAN, LUM, SPARC, POSTN, FN1, COL1A1/1A2, COL3A1, COL4A1, COL5A1, COL12A1, and LAMA1, together with ECM-processing factors PLOD2, LOXL2/3, SERPINH1, and the matrix-associated protease HTRA1, indicating active matrix deposition, stabilization, and turnover. Concurrent expression of stromal growth factors (VEGFA, PDGFC, CCN2, IGF2, FGF2) supported a tissue-remodeling and survival-promoting microenvironment. In parallel, cumulus cells expressed lipid and cholesterol-handling genes associated with sustained steroidogenic capacity, including SCARB1, ABCA1, APOE, and APOA1, alongside mitochondrial cholesterol transfer and progesterone-pathway enzymes (STAR, CYP11A1, HSD3B1). Immune-modulatory and anti-inflammatory features were evident through expression of MFGE8, IL1RN, THBS1, and SPARC, consistent with immune quiescence and non-inflammatory tissue persistence. Proteolytic activity was tightly regulated by coordinated expression of PLAT with multiple inhibitors (SERPINE1, SERPINB2, TIMP1, TIMP3), supporting controlled matrix remodeling rather than invasive or degenerative behavior.

Together, these data indicate that post-IVF cumulus cells adopt a highly regulated secretory and metabolic program that supports structural organization, steroidogenic competence, and immune quiescence over extended periods.

An *in vitro* model of ovarian control of human endometrium decidualization

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Abstract Text

The female reproductive system depends on integrated endocrine and paracrine communication between the ovary and uterus to regulate ovulation, implantation, and pregnancy. Current *in vitro* models, however, fail to reproduce the dynamic hormonal and inter-organ interactions essential for endometrial decidualization. To address this limitation, we established an *ex vivo* female reproductive system that integrates mouse corpus luteum (CL) organoids with human endometrial stromal cells (Endo) organoids in a shared co-culture environment. This multi-tissue co-culture system enables self-regulated ovarian hormone secretion and paracrine signaling to drive real-time uterine responses. During culture, CL-organoids exhibited functional luteinization characterized by increasing progesterone (P4) and decreasing estradiol (E2) levels. When co-cultured with CL-organoids, human Endo-organoids displayed morphological and molecular features of decidualization, including significant upregulation of *IGFBP1*, *PRL*, and *LEFTY2*, comparable to conventional hormonal (E2 + P4 + cAMP, EPC) induction. Transcriptomic profiling revealed that while EPCs and co-culture systems share a core angiogenic program involving vascular development, ECM remodeling, and PI3K-Akt signaling, the co-culture uniquely enriches pathways associated with cell-cell communication (e.g., cell adhesion molecules [CAMs], synapse), complex signal regulation (Ras, cAMP), immune modulation (cytokine activity), and metabolic reprogramming (Type II diabetes). These distinct features demonstrate that co-culture introduces additional layers of complexity through intercellular interactions, thereby better recapitulating the *in vivo* microenvironment to support the decidualization of endometrial stromal cells induced by the EPC cocktail. The following functional testing demonstrated that RU486, a selective progesterone receptor (PR) antagonist, was able to inhibit CL-induced stromal decidualization.

Together, this study introduces a novel, physiologically relevant *ex vivo* ovarian–uterine co-culture model that captures the endocrine regulation of human endometrial decidualization. The system provides a powerful translational platform for investigating ovarian-uterine networks, infertility, and endocrine disrupting effects caused by environmental and pharmaceutical chemicals.

Homeobox Genes Regulate Epithelial Lineage Specification in the Developing Female Reproductive Tract Through Extracellular Matrix Remodeling

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Abstract Text

Homeobox (Hoxa) genes are conserved positional regulators of Müllerian duct (MD) patterning and differentiation into female reproductive tract organs. Genetic studies in mice established that spatiotemporal expression of *Hoxa9* through *Hoxa13* in the mesenchyme is critical for epithelial differentiation of the uterus and cervix. The overall hypothesis is that *Hox* genes in the mesenchyme instruct and direct differentiation of the undetermined epithelium through paracrine factors and alterations in the extracellular matrix (ECM). Single-cell RNA sequencing (scRNA-seq) of the developing mouse cervix and uterus was conducted at embryonic day (ED) 16.5 and postnatal days (PND) 1 and 5. Bioinformatic analysis revealed distinct molecular differences in the mesenchyme of the developing uterus and cervix. In the cervix, *Hoxa13* and *Hoxd13* were co-expressed with several transcription factors (*Isl1*, *Isl2*, *Evx1*, *Evx2*) that are conserved regulators of mesodermal rostrocaudal patterning. To investigate the functional impact of mesenchymal *Hox* genes on epithelial differentiation, we developed a 3D assembloid model. Mesenchymal cells from the PND1–5 uterus were isolated and engineered to express *Hoxa13* or *Hoxd13* using a lentiviral approach. Wild-type control or *Hoxa/d13*-expressing mesenchymal cells (25,000 cells) were co-cultured with PND1–5 uterine epithelial cells (5,000 cells) in 96-well low-attachment plates and maintained under rotation (50 RPM) to promote cellular aggregation for six days. Control assembloids lacked expression of the basal cytokeratin KRT5 within the epithelium. In contrast, epithelial cells expressed KRT5 in assembloids containing *Hoxa13*- or *Hoxd13*-expressing mesenchyme, a phenotype characteristic of cervicovaginal-type epithelium. Transcriptomic analysis identified 530 and 98 genes increased in *Hoxa13*- and *Hoxd13*-expressing mesenchyme, respectively, compared to control mesenchyme. Among the overlapping top upregulated genes were pathways associated with cell migration, angiogenesis, and ECM remodeling, including matrix metalloproteinases (*Mmp3*, *Mmp10*, *Mmp13*) and tenascin C (*Tnc*), while structural collagen genes (*Colgalt2*, *Col22a1*) were decreased in *Hoxa13*- and *Hoxd13*-expressing mesenchymal cells. To determine the impact of increased MMP expression, assembloids were treated with the broad-spectrum MMP inhibitor Ilomastat. This reduced differentiation of the uterine epithelium into *Krt5*-positive epithelium in assembloids containing either *Hoxa13*- or *Hoxd13*-expressing mesenchyme. These findings support a model in which mesenchyme-specific *Hoxa13* and *Hoxd13* direct epithelial differentiation in the cervix through ECM remodeling mediated by MMP activity. Together, these studies provide mechanistic insights into how mesenchymal–epithelial crosstalk regulates female reproductive tract development.

Characterization of Doppler Spectroscopy Associated with Developmental Competence of Pig Embryos Produced *In Vitro*

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Abstract Text

Assessing embryos to predict viability before transfer remains a central challenge in assisted reproductive technologies. Conventional morphological grading is subjective and limited in accuracy, motivating the need to develop non-invasive biophysical screening methods. The Biodynamic Microscope is a non-contact, label-free computational optical imaging system that measures intracellular motility through dynamic light scattering using Doppler spectroscopy. It generates 128-point spectral profiles that capture real-time biophysical activity within intact living embryos quickly without compromising their developmental potential. The objective of this study was to characterize Biodynamic Imaging-derived spectral biomarkers associated with the developmental competence of *in vitro*-produced pig embryos.

Pig oocytes were collected from prepubertal gilt and sow ovaries, matured *in vitro* for 44 hours in TCM-199-based medium at 39 °C in 5% CO₂, and fertilized using liquid stored boar semen in modified Tris-buffered medium. Following fertilization, presumed zygotes were cultured in groups of approximately 10 in 50 µL of Porcine Zygote Medium-3 covered with mineral oil for 24 hours. Each embryo was then individually transferred to a single dish, measured with the Biodynamic Imaging System for approximately three minutes, and returned to an individual microwell in a Well-of-the-Well dish for continued culture and individual developmental monitoring. Developmental outcome was recorded on day 6, at the end of culture, on a four-level scale corresponding to 2-cell stage, 4-cell to morula, unexpanded blastocyst, and expanded to hatched blastocyst, with expanded to hatched blastocyst designated as viable. A total of 138 unique pig embryos were each measured once across 9 independent experimental replicate sessions conducted in separate weeks, comprising 68 viable and 70 non-viable embryos in the primary analysis. Nine spectral biomarkers were computed from each 128-point record, including spectral amplitude at the low-frequency plateau, and high-frequency Nyquist regions, their running-mean equivalents, mid-spectrum knee, dynamic range, back-scatter brightness, mean size of the reconstruction, and normalized standard deviation. Between-group differences were assessed using the Mann-Whitney U test and Cohen's effect sizes.

Seven of the nine biomarkers significantly differentiated viable from non-viable embryos. Spectral amplitude was uniformly higher in viable embryos across the plateau, knee, and Nyquist regions, with Cohen's d values ranging from 0.51 to 0.55, indicating medium-sized biologically meaningful effects. Back-scatter brightness was lower in viable embryos, and the mid-frequency biomarker was one of the strongest individual discriminants with a Cohen's d of 0.55 and a p-value of 0.01. Dynamic range did not reach significance, indicating a proportional upward shift of the entire spectral curve in viable embryos rather than a change in spectral shape. These findings indicate that Biodynamic Imaging-derived spectral biomarkers, including plateau, Nyquist, and mid-spectrum amplitude, are associated with developmental competence in pig embryos. Together, these findings highlight the potential of Biodynamic Imaging as an objective, non-invasive tool for embryo viability assessment in assisted reproduction.

Investigating the Genetic Underpinnings of Aneuploidy in Early Equine Pregnancy Loss

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Abstract Text

Horses experience pregnancy losses at a rate of 15-20%, posing devastating detriment to individual maternal/fetal health and the wider equine industry. Among early pregnancy losses (EPL), prior to 110 gestational days, approximately 50-60% are attributed to aneuploidy (characterized by varying chromosomal numbers). Proposed mechanisms for the development of aneuploidy pinpoint fundamental processes, including mitosis or meiosis, where errors may occur in chromosomal segregation, disjunction, and recombination. These mechanisms involve key regulatory factors providing gene targets for genetic screening of fatal genetic mutations. In an effort to align genotypic and phenotypic attributes of aneuploid EPL, we use the horse as a reproductive model of naturally occurring spontaneous miscarriage. Moreover, beyond reproductive applications in the equine industry, the shared chromosomal synteny between horses and humans also offers potentially translational applications for uncovering important risk loci and mechanisms involved in aneuploid EPL between species. We hypothesize that variants associated with equine aneuploidy will lie within key meiotic genes. To uncover risk loci associated with aneuploidy, a candidate genome-wide association study was performed utilizing PLINK using genotypes generated on Axiom Equine 670K SNP array from 305 collected products of conception (44 aneuploid EPL cases, 261 diploid PL). Following quality control screening to adjust for missing single-nucleotide polymorphisms and individual data, minor allele frequency, heterozygosity, adherence to Hardy-Weinberg equilibrium, and population substructure, a linear mixed modelling involving a genetic relatedness matrix generated with GEMMA was run with covariates of breed, allowing us to account for high inbreeding in equine populations. Currently, fine mapping of strongly associated risk loci in contributing to aneuploidy is being performed using R program SuSiE. The preliminary results highlight loci on chromosomes 2, 5, 18, and 20 that were found to be weakly associated with aneuploidy in EPL arising from meiotic errors. Case numbers are currently being expanded to increase the power of the study and novel diagnostic imaging techniques developed to correlate genotype and ploidy with more precise phenotypic presentations detectable on ultrasound examination. Ultimately, the application of these results will help to inform diagnostic markers of EPL risk among horses, contributing to increasing the rate of successful pregnancies across the species.

Establishment of Canine Endometrial Organoids for Biobanking and Transcriptomic Analysis of Age-Dependent Gene Expression

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Abstract Text

Reproductive aging in dogs differs fundamentally from human menopause, as canines exhibit a gradual, senescent decline in fertility within a unique estrous cycle, highlighting the need for species-specific models of uterine aging. To address this gap, we established a canine endometrial organoid (CEM) biobank using endometrial tissues obtain from diverse clinical cases such as spaying procedure (n=21; age 5–159 months). Uterine horn tissues (~3 cm) were collected from spay surgeries at local veterinary clinics and Seoul National University Veterinary Teaching Hospital. These tissues were either fixed in 4% paraformaldehyde or employed for organoid culture. For culture, diced tissues were dissociated with a non-enzymatic dissociation reagent, filtered (70 µm), subjected to red blood cell lysis, embedded in Matrigel, and cultured as previously described (Kwon et al., 2025). The CEM lines were maintained ≥40 passages, and demonstrated stable cryopreservation and post-thaw regrowth. Histology (H&E, PAS) and immunohistochemistry with glandular epithelial markers (E-cadherin, MUC1), steroid receptors (ERα and PR), stromal marker (vimentin), and proliferation marker (Ki-67) confirmed structural and functional similarity to native tissues. Hormonal responsiveness was validated by estradiol, progesterone, and cAMP stimulation. Subsequent RT-qPCR analysis confirmed the induction of hormone-responsive genes; compared to untreated control, *ALDOB*, *GJB2*, *ESR1*, *PGR*, *RGS9* were upregulated by approximately 2.3-fold, 3.9-fold, 1.7-fold, 15.2-fold, and 2.4-fold, respectively. To investigate the molecular characteristics of endometrial organoids from the aged dogs, RNA-sequencing was performed on organoids categorized into two age groups: a young group (5–12 months, n=4) and an aged group (105–159 months, n=4). As results, pathway enrichment analysis revealed significant activation of the IL-17 signaling pathway and leukocyte transendothelial migration ($P < 0.001$), suggesting a shift toward a chronic inflammatory milieu reflective of the aged canine uterine environment. Consistently, we identified a transcriptomic signature of sterile inflammation in the aged group, characterized by marked upregulation of *CCL2*, *NQO1*, *HMOX1*, *MMP2*, *KDR*, *IL1R2*, *IL1A*, *BMPR1B*, *GSTT2B* and *VCAM1*. The elevated expression of these genes suggests that aged endometrial organoids exhibit a chronic sterile inflammatory state, which may disrupt the receptive microenvironment necessary for implantation. Meanwhile, essential factors for extracellular matrix (ECM) remodeling and structural integrity, such as *EDN1*, *MMP9*, *TNF*, and *CDH5*, were significantly suppressed. The reduced expression of these genes indicates a compromised capacity for structural remodeling, potentially leading to impaired decidual response in older canines. Collectively, these findings demonstrated that endometrial organoids derived from aged dogs recapitulate the phenotype of uterine senescence, specifically chronic inflammatory priming and ECM dysregulation. By elucidating these distinct molecular signatures, this study provided critical insights into pathophysiology of the aging canine endometrium. Furthermore, the CEM biobank can serve as a powerful system for investigating reproductive aging and offer comparative studies in non-menopausal mammals. This study was supported by RS-2021-NR060088 for SRC program: Comparative medicine Disease Research Center, SNU#550-20250132 and SNU#550-20250129.

Untargeted Metabolomics Identifies Changes in the Boar Seminal Plasma Metabolome Following *In Utero* Heat Stress and Genomic Selection for Heat Tolerance

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Abstract Text

In utero heat stress (IUHS) threatens global pork production by reducing sperm output and semen quality in boars. Studies in men have identified candidate infertility biomarkers in the seminal plasma metabolome. However, metabolomic signatures in the seminal plasma of boars with IUHS-induced subfertility remain undefined. In parallel, genomic selection for greater heat tolerance (TOL) represents a potential mitigation strategy for IUHS-induced reproductive consequences. Integrating these approaches may provide insight into the mechanisms underlying subfertility and climatic resilience in boars. The objective of this study was to assess the seminal plasma metabolome and its association with semen quality in IUHS and *in utero* thermoneutral (IUTN) boars whose parents were genomically selected to be either more TOL or heat sensitive (SEN). Landrace X Large White TOL and SEN dams experienced either cyclic heat stress (26–36°C) or thermoneutral (17–20°C) conditions from gestational d 6–70, resulting in four progeny treatment groups: IUHS+SEN (n=8), IUHS+TOL (n=8), IUTN+SEN (n=9), and IUTN+TOL (n=6). After pubertal attainment, semen was collected consecutively for ten weeks to assess semen quality via computer-assisted sperm analysis (CASA). In addition, seminal plasma was isolated from each ejaculate and pooled within boar for untargeted metabolomics using gas chromatography-mass spectrometry. Univariate analyses were performed on metabolome data between treatment groups. Spearman's correlation was used to determine relationships between metabolite abundances and CASA metrics. When considering thermal exposure alone, only 5-oxoproline (amino acid derivative involved in antioxidant metabolism) differed between IUHS and IUTN groups ($P \leq 0.03$). Interestingly, compared with their respective IUTN counterparts, 5-oxoproline abundance was decreased in IUHS+TOL boars ($P=0.03$) but increased in IUHS+SEN boars ($P < 0.01$). Moreover, a lower abundance of several amino acids, including isoleucine ($P=0.03$), tyrosine ($P=0.01$), phenylalanine ($P=0.04$), and serine ($P=0.03$), was detected in seminal plasma from IUHS+SEN versus IUTN+SEN boars. The abundance of proline also tended ($P=0.09$) to be decreased in IUHS+SEN boars versus IUTN+SEN boars. Among organic acids, lactic acid ($P < 0.01$), aminomalonic acid ($P < 0.01$), and succinic acid ($P=0.04$) differed in relative abundance between IUHS+SEN and IUTN+SEN boars. When comparing genotype differences post-IUHS, IUHS+TOL boars demonstrated lower abundances of 5-oxoproline ($P < 0.01$), lactic acid ($P < 0.01$), aminomalonic acid ($P < 0.01$), glutamic acid ($P=0.03$), alanine ($P < 0.01$), inosine ($P=0.03$), and hypotaurine ($P=0.02$) compared to IUHS+SEN boars. Both aconitic acid ($P=0.06$) and glutamine ($P=0.07$) abundance also tended to be decreased in IUHS+TOL versus IUHS+SEN boars. Regarding CASA metrics, sperm concentration was either strongly or moderately correlated with glutamine ($r=0.75$; $P < 0.01$), hypotaurine ($r=0.60$; $P < 0.01$), glutamic acid ($r=0.49$; $P < 0.01$), aconitic acid ($r=0.49$; $P < 0.01$), and inosine ($r=0.64$; $P < 0.01$). Glutamine was also moderately correlated with total sperm count ($r=0.44$; $P=0.01$) and artificial insemination dose number ($r=0.44$; $P=0.01$). Additionally, semen volume was moderately correlated with glutamine ($r=-0.52$; $P < 0.01$), hypotaurine ($r=-0.50$; $P < 0.01$), aconitic acid ($r=-0.50$; $P < 0.01$), and inosine ($r=-0.48$; $P < 0.01$). As for sperm morphology, the percentage of distal droplets was moderately correlated with inosine ($r=-0.44$; $P=0.01$). This study presents the first untargeted analysis of the IUHS boar seminal plasma metabolome. Notably, IUTN boars exhibited higher abundances of several metabolites associated with semen quality. However, genomic selection for TOL may negatively alter metabolite concentrations, as IUHS+SEN boars exhibited higher abundances of metabolites associated with favorable semen quality. Glutamine, inosine, and hypotaurine were consistently associated with sperm production metrics and may serve as biomarkers of boar semen quality. Finally, 5-oxoproline was the only metabolite altered by IUHS alone, suggesting its potential as a marker of IUHS exposure.

A scalable two-step genome editing strategy for generating full-length gene-humanized mice at diverse genomic loci

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Abstract Text

Full-length gene-humanized mice engineered by completely replacing mouse loci with human counterparts, including untranslated and regulatory regions, provide a robust in vivo platform for human gene function studies. However, reliably humanizing large genomic regions remains challenging due to limited DNA insert sizes, complex protocols, and specialized material requirements. This study introduces a streamlined approach that enables full-length gene humanization through two sequential CRISPR-assisted homologous recombination steps in embryonic stem cells. This method supports targeted knock-in of genomic fragments (>200 kbp) and is applicable across multiple mouse strains. Humanized alleles generated using the developed method recapitulate human-like splicing isoforms and organ-specific gene expression while restoring essential functions in hematopoiesis, spermatogenesis, and survival. Furthermore, disease-associated mutations can be engineered into humanized alleles to model human genetic disorders in vivo. This versatile platform enables the creation of physiologically relevant, fully gene-humanized mouse models for broad biomedical research applications, including reproductive biology.

Exploration of a Novel Device for Ovarian Tissue Cryopreservation

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Abstract Text

Ovarian dysfunction and follicle depletion cause primary ovarian insufficiency, which leads to delayed puberty in pediatric patients, as well as early menopause and infertility in adult women. Primary ovarian insufficiency may result from various factors including, but not limited to, genetic disorders, autoimmune diseases, chemotherapy or radiation therapy, and exposure to hazardous substances. Fertility preservation is often recommended to individuals at risk of or experiencing primary ovarian insufficiency, and ovarian tissue cryopreservation can be performed for pediatric patients and patients who require prompt medical treatment. Currently, slow freezing and vitrification have been conducted in clinics to cryopreserve ovarian tissue for patients. However, both procedures require further optimization, and a standardized method is not available. Therefore, experiments were designed to test a novel commercial product for ovarian tissue cryopreservation with a goal of developing an efficient protocol for clinical application. Ovaries were collected from adult female rhesus macaques (*Macaca mulatta*; n = 3) through the Oregon National Primate Research Center Pathology Services Unit tissue distribution program. The outermost ovarian cortical tissue layers (0.5-1 mm thick) were obtained using a Stadie-Riggs tissue slicer and cut into 3 × 3 mm² fragments. For each animal, tissue fragments were randomly divided into two groups: (a) fresh control and (b) cryopreservation using the Fibulas BioFlash Drive. Fresh and frozen-thawed tissues were cultured for 24 hours in alpha minimum essential medium supplemented with glucose, human serum protein supplement, recombinant human follicle-stimulating hormone, bovine fetuin, ascorbic acid phosphate, transferrin, insulin, and sodium selenite. Cultured tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned for hematoxylin and eosin staining to evaluate tissue morphology. Immunohistochemistry was performed to examine granulosa cell viability (anti-Müllerian hormone and phosphohistone H3), oocyte condition (c-Kit), stroma integrity (collagen type I), and apoptosis (caspase 3). After 24 hours of culture, intact follicles were observed in control tissue containing granulosa cells with minimal cell-to-cell space and an oocyte with homogeneous cytoplasm. Morphology of primordial and primary follicles in frozen-thawed tissue was comparable to that of control tissue. Although cryo-induced damage, such as shrunken and vacuolated oocyte, was observed in secondary follicles of frozen-thawed tissue, caspase 3 immunostaining was minimal in primordial and primary follicles. Primary and secondary follicles were positive for anti-Müllerian hormone and phosphohistone H3 in granulosa cells, along with c-Kit in oocytes, in both control and frozen-thawed tissues with similar immunostaining intensity. Collagen type I distribution also showed a similar pattern in both control and frozen-thawed tissues. Data suggest that primordial and primary follicles can be well preserved using the Fibulas product, as indicated by typical morphology, granulosa cell and oocyte protein production, dense and compact stroma, and limited apoptosis. This novel ovarian tissue cryopreservation device can potentially serve as an alternative for female patients seeking fertility preservation. Supported by NIH/NICHD R01HD082208, NIH/OD P51OD011092.

Modified Lipid Extraction Techniques from Bovine Oocytes

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Abstract Text

Lipids serve as key energy substrates during bovine oocyte maturation. Thus, understanding lipid metabolism and distribution within the oocyte is critical for improving in vitro maturation systems and reproductive outcomes in bovine assisted reproductive technologies. We hypothesized that 1) a modified lipid extraction technique would enable LC-MS/MS quantification of linoleic (LA) and α -linolenic (ALA) acids in limited bovine oocyte quantities, and 2) untargeted lipidomic profiling could identify a broad range of lipid species from small oocyte sample sizes comparable to larger sample pools. The objective of this study was to evaluate a modified lipid extraction technique and lipidomic analysis for bovine oocytes in small quantities (< 50 oocytes). In experiment 1, bovine oocytes were aspirated from ovaries obtained from a local abattoir and snap-frozen until lipid extraction. Samples underwent a modified total lipid extraction using ice-cold methanol and chloroform. Extracted lipids were dried, reconstituted, and analyzed at the North Carolina A&T State University Analytical Laboratory, where linoleic and α -linolenic acid standards were used for verification and quantification. In experiment 2, oocytes were collected via syringe and vacuum aspiration, and lipids were extracted using liquid-liquid extraction. Untargeted lipidomic analysis was performed on a C30 column using a Vanquish Horizon UHPLC-Q Exactive HF mass spectrometry system at Cornell University. Samples of 10 and 50 oocytes were compared to determine whether smaller sample sizes yielded comparable lipidomic profiles. In experiment 1, linoleic and α -linolenic acids were successfully extracted and quantified from bovine oocytes using the modified extraction protocol with sample aliquots ranging from 5 to 50 oocytes. ALA

concentrations were similar between the 5-oocyte (0.066 ± 0.063 ppm) and 10-oocyte groups (0.119 ± 0.015 ppm; $P > 0.05$). Likewise, LA concentrations were similar between the 5-oocyte (0.166 ± 0.013 ppm) and 10-oocyte groups (0.300 ± 0.034 ppm; $P > 0.05$). In experiment 2, 449 lipids were identified in positive ion mode and 200 in negative ion mode. In positive ion mode, triglycerides represented 50.33% of identified lipids, while phosphatidylethanolamines accounted for 36% of lipids detected in negative ion mode. Comparable lipid class distributions were observed between the 10- and 50-oocyte groups, indicating that smaller sample sizes produced similar lipidomic profiles. Due to limited sample availability, lipid abundance values were scaled tenfold for normalization, and a third biological replicate was not available for experiment 2; therefore, these results should be interpreted descriptively. Together, these experiments demonstrate the feasibility of quantifying specific fatty acids and performing comprehensive lipidomic profiling from limited oocyte samples. These findings provide a methodological framework for investigating lipid metabolism and its influence on bovine oocyte maturation and developmental potential.

Engineered Endogenous Exosome-Packed Phenyl Boronate, a Biochemical Redox Scavenger as an Effective

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Abstract Text

Cervical cancer is the 2nd largest among all gynecological cancer cases reported worldwide. With more than a million cases globally per year, cervical carcinoma remains a challenge to treat. Immunotherapy remains a widely recognized treatment option but suffers from challenges such as T cell exhaustion, a rapidly evolving tumor microenvironment that blocks effective immune cell cytotoxicity towards cancer cells, and side effects such as those that exist with CAR-T therapy. In a recent study, redox intermediates released by tumor cells were reported to limit the effectiveness of T cell therapy. Based on the above, I devised a bioengineering strategy to construct a T cell prototype involving packing of a peroxynitrite inhibitor in endogenous exosome vehicles and loading them into CD8+ve T cells. Using the construct, I validated each step of my construct and used it for attenuating the proliferation of human cervical cancer cells. My results showed that the PBA-packed CD8+ve T cells decreased peroxynitrite release from cancer cells and induced the cleavage of procaspase-3, an effector caspase in apoptosis. Further, the same PBA packed CD8+ve T cells effectively induced apoptosis as determined by Annexin-V staining with membrane binding of Annexin V and Propidium Iodide staining in the nucleus. The results signified apoptotic cancer cell killing that was significantly higher than non-packed, non-primed CD8+ve T cells. My results provide a new paradigm to overcome the limitations of all forms of immunotherapy that involve T cells and CAR-T cells, especially in reproductive cancers. The research described in this project has huge implications in treating cervical cancer and other forms of carcinomas since immunotherapy has gained prominence as a principal pathway for cancer cure. My novel tool described in this project has far-reaching implications in significantly improving T cell-based immunotherapy in cervical cancer, which has a million cases per year worldwide

Impacts of Combined Exposure to Nanoplastics and Phthalates on Placental and Fetal Development

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Abstract Text

There are more than 400 million tons of plastic produced annually. Less than ten percent of these plastics are recycled. The majority of plastics produced accumulate over time and degrade into small plastic particles known as nanoplastics (NPs), which studies suggest may interfere with female reproductive processes. Additionally, during degradation, plastics leach phthalates, a class of chemical additives used to improve the durability and flexibility of plastics, which are known reproductive toxicants. NPs and phthalates can enter the body via inhalation, ingestion, or dermal contact and can translocate into various organs, including the placenta. The placenta is a vital organ developed during gestation that provides the necessary support for embryonic development. Humans are simultaneously exposed to both NPs and phthalates, yet research on the impacts of co-exposure on placental health remains limited. To test our hypothesis that co-exposure to an environmentally relevant mixture of phthalates and polyethylene terephthalate (PET) nanoplastics will have a greater impact on placental and fetal development compared to exposure to phthalates or nanoplastics individually, pregnant CD-1 mice were orally dosed with either a vehicle control, 200 nm secondary lab-generated PET nanoplastics at 2 mg/kg/day, an environmentally relevant phthalate mixture at 200 µg/kg/day, or a combination of nanoplastics and phthalates from gestational day 7 to gestational day 15. Following dosing, the placentas and fetuses were collected to identify exposure-related modifications. Several maternal and fetal characteristics were also analyzed to identify trends related to exposure. RNA was extracted from the placentas, and gene expression analysis was conducted utilizing quantitative polymerase chain reaction (qPCR) and RNA sequencing. We analyzed the expression of genes that were disrupted by prenatal polystyrene exposure in a previous study from our lab, including genes in the transforming growth factor beta (TGF-β) signaling pathway, which is critical for placental structure development, immune response, and trophoblast invasion, as well as steroid and metabolic regulators. Our results showed no statistically significant differences between the treatment groups among placental and fetal weights, and all observed maternal and fetal characteristics. We similarly observed no significant difference across the various genes analyzed. *Cyp11a1* was borderline significantly decreased in the combined treatment exposure group only, suggesting impacts on steroid hormones from mixture exposures. Next, we will measure the expression of genes under peroxisome proliferator-activated receptor (PPAR) transcriptional control because these are known targets of phthalates. Furthermore, we will assess placental morphology to identify exposure-related differences in placenta layer area. This study provides insight into the potential health impacts of co-exposure to phthalates and nanoplastics.

Development of a Six-Compartment Physiologically Based Kinetic Model of Dibutyl Phthalate and Reproductive Hormones in Female CD-1 Mice (*Mus musculus*)

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Abstract Text

Dibutyl phthalate (DBP) and other phthalates are chemicals widely used in the manufacturing of plastic products. These chemicals have been shown to affect many processes vital to reproductive function in males and females, including steroidogenesis and folliculogenesis. Interruptions to these processes cause a slew of reproductive health effects, ranging from decreased follicle numbers, polycystic ovaries, and stimulation of follicle development. Studies in rats have used physiologically based kinetic (PBK) modeling to characterize DBP's absorption, metabolism, distribution, and elimination (ADME). In addition, many studies have reported the effects of DBP on ovarian function, but its mechanism of action remains unknown and is a current area of research. This presentation focuses on how DBP and MBP move through the body and affect ovarian function and steroid hormone production. The PBK model is based on a six-tissue compartment conceptual design that captures the known ADME processes of the nine molecules being tracked. The six specified tissue compartments are the brain, gut, liver, kidney, ovary, and "other". The nine molecules of interest are DBP, its metabolite monobutyl phthalate (MBP), 17 β -estradiol, testosterone, androstenedione, progesterone, inhibin B, luteinizing hormone, and follicle stimulating hormone. Mass-balance differential equations were formulated for each molecule in each compartment incorporating the known ADME processes for each molecule. Parameter values (e.g., body mass, blood flow rates, kinetic rate constants) were obtained from multiple sources including experimentally measured values for this specific project and published literature. When indicated, parameter values were also allometrically scaled. The resulting PBK model is composed of over 45 ordinary differential equations, which are solved simultaneously in the Complex Pathway Simulator (COPASI). The model predicts the nine molecule concentrations in each compartment as a function of time. Regulatory control of the hypothalamic-pituitary-gonadal axis and steroidogenesis are unique features of this model. Model outputs display predictions showing expected behavior based on the conceptual model designed and mass balance equations formulated. The congruence between the conceptual model, the formulated mass balance equations, and the COPASI model outputs suggests that the model was appropriately built in COPASI. While the mechanism of action by which DBP and/or MBP elicit effects on steroid hormones and folliculogenesis remains unknown, our model is uniquely positioned to test hypothesized mechanisms of action and to evaluate different DBP dosing scenarios. Experimentally measured concentrations of the tracked molecules will be used to calibrate model parameters that are not known through independent measurements, and to evaluate the model's prediction performance. In future work, the calibrated model will be used to extrapolate in vitro follicle culture data to predict in vivo effects as a new approach method for reproductive toxicity testing.

Iodoacetic Acid Causes Oxidative Stress in the Mouse Ovary

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Abstract Text

The incorporation of water disinfectants into the main water supply has significantly decreased the incidence of waterborne diseases such as cholera. However, the interaction between disinfectants and organic material generates water disinfection byproducts (DBPs). Iodoacetic acid (IAA) is an unregulated DBP that has been reported to be a reproductive toxicant. Our previous studies show that IAA causes ovarian toxicity, but the mechanisms underlying IAA-induced ovarian toxicity are unknown. Thus, we tested the hypothesis that IAA exposure causes ovarian toxicity through oxidative stress pathways. Adult CD-1 mice were dosed with vehicle control (reverse osmosis water) or IAA (2.7- 2,695.7 μ M) for 35 days. Following this treatment, ovaries were collected when the mice were in diestrus. Then, both whole ovaries and isolated antral follicles from whole ovaries were subjected to RNA extraction, reverse transcription, and quantitative polymerase chain reaction (qPCR) to analyze the expression of several enzymes that regulate oxidative stress (*Gpx1*, *Gpx2*, *Gsr*, *Gss*, *Gsta1*, *Gstm1*, *Gsto1*, *Gstp1*, *Gstt1*, *Sod1*, *Sod2*, and *Cat*). In other experiments, ovarian antral follicles were collected from CD-1 mice between 40 and 42 days of age. Antral follicles were incubated in individual wells in a 96-well plate in media containing vehicle control $\pm$ the antioxidant Trolox or IAA (2-15 μ M) $\pm$ the antioxidant Trolox for 96 hours. Follicle growth was measured every 24 hours and media were collected for estradiol measurements at 96 hours. IAA decreased *Cat*, *Sod1*, *Gss*, *Gsta1*, *Gstp1*, and *Gstt1* expression and increased *Gstm1* expression in whole ovaries compared to control in vivo. IAA decreased *Sod2* and *Gpx2* expression and increased *Gstp1* expression in isolated antral follicles compared to control in vivo. IAA increased *Gpx1* and *Gsto1* expression in antral follicles compared to control in vitro. IAA inhibited follicle growth and reduced estradiol levels, whereas Trolox rescued follicles from IAA-induced inhibition of follicle growth and IAA-induced low estradiol levels in vitro. Collectively, these data indicate that IAA exposure inhibits follicle growth and causes ovarian toxicity by altering oxidative stress pathways in the mouse ovary. Supported by NIH R21 ES028963.

The Phytoestrogen Equol Dysregulates the Function of the Bovine Corpus Luteum

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Abstract Text

Our lab has shown that consumption of pasture enriched with red clover by dairy cows lowered progesterone production by steroidogenic luteal cells *ex vivo* in response to luteinizing hormone (LH). Red clover forage is used to feed livestock, and it contains abundant phytoestrogens. Equol is one of the more potent metabolites derived from phytoestrogens in red clover, which exerts its actions by binding to endogenous estrogen receptors. Studies have reported the negative impact of phytoestrogens on fertility and reproduction, especially in sheep. However, the impact of these plant-derived estrogens on bovine ovarian function has not been fully elucidated. Thus, the objective of the present study is to determine the effect of equol on luteal cell LH responsiveness, and on effectors associated with luteolysis in the cow corpus luteum. To determine the cellular and temporal changes upon equol exposure, a series of *in vitro* experiments were performed to elucidate the underlying molecular mechanisms. Midcycle corpora lutea (days 8-12, day 0 = estrus) were dissociated and 1×10^6 steroidogenic cells were seeded into 6-well plates. In Experiment 1 (n=4), cells were pre-treated with equol (0.01, 0.1, 1, or $10 \mu\text{M}$) for either 2, 4, 6, or 24 hours before they were treated with LH (10ng/mL) for 4 hours. Afterwards, conditioned medium was collected and subsequently analyzed by radioimmunoassay (RIA) to determine progesterone content. In Experiment 2 (n=5), 1×10^6 steroidogenic luteal cells were seeded into 6-well plates and treated with equol (0.01, 0.1, 1, or $10 \mu\text{M}$) for 2, 4, or 6 hours. Afterwards, total RNA was extracted and used to assess gene expression of oxytocin (OXT), Calcium/Calmodulin-Dependent Protein Kinase 2 (CAMKK2) and Prostaglandin Receptor (PTGFR) via quantitative polymerase chain reaction (qPCR). We found that within 2 hours of exposure to equol, midcycle bovine luteal cells began to lose responsiveness to LH, trending towards significance ($p=0.06$) with the $10 \mu\text{M}$ concentration. At 4, 6, and 24 hours, equol ($10 \mu\text{M}$) treatment resulted in a greater loss of responsiveness to LH, lowering progesterone by 40%-50% at each timepoint ($p \leq 0.001$). At the 6-hour and 24-hour timepoints, equol ($1 \mu\text{M}$ or $0.1 \mu\text{M}$) decreased LH-stimulated progesterone by 35% ($p=0.03$) and 41% ($p=0.01$), respectively. Among all timepoints, equol treatment ($0.01 \mu\text{M}$ and $1 \mu\text{M}$) trended toward an increase in OXT mRNA expression at 2-hours ($p=0.09$, $p=0.1$, respectively). As for CAMKK2, treatment with equol ($0.1 \mu\text{M}$ for 2 hours) and ($1 \mu\text{M}$ for 4 hours) trended toward increased expression ($p=0.08$, $p=0.07$, respectively). PTGFR expression was decreased ($p=0.006$ and $p=0.02$) at 6-hours following treatment with $0.1 \mu\text{M}$ and $1 \mu\text{M}$ equol. In summary, these data suggested to us that equol, a major phytoestrogen metabolite of red clover, lowers the response of the midcycle steroidogenic luteal cell to LH, resulting in decreased progesterone production. At the same time the downstream effectors associated with prostaglandin F2alpha, OXT and CAMKK2 trended toward an increase in expression. Interestingly, treatment with equol for 6 hours lowered expression of PTGFR. The action of equol may lead to the expression of these factors within the midcycle bovine luteal cell, creating a dysregulated environment which lowers luteal steroidogenic capacity. The present results align with our previous finding that consumption of red clover yielded a high ratio of prostaglandin F2alpha to progesterone, shifting the hormonal milieu that compromises CL function.

Mitigating Reproductive Risks of Bisphenols: Evaluation of Novel TMBPF-Based Analogs in *Caenorhabditis elegans*

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Abstract Text

The widespread global use of bisphenol A (BPA) has led to its ubiquitous presence in the environment and continued exposure in the general population. Emerging research demonstrates that even low-level exposure to this plasticizer can trigger meiotic errors, compromising quality of reproductive cells and contributing to infertility. While health concerns have prompted a shift toward alternatives like bisphenol S (BPS) and bisphenol F (BPF), evidence suggests these analogs may pose comparable health risks. Consequently, there is an urgent need for a functionally equivalent substitute that mitigates health risks while maintaining similar physical properties to BPA. To address the need for safer alternatives, we hypothesized that analogs based on a tetramethyl bisphenol F (TMBPF) scaffold with phenolic ring substitutions would have a minimal impact on reproductive health, unlike widely used analogs with central carbon linker substitutions (e.g., BPA, BPS, and BPF). Recent studies indicate that TMBPF, unlike most previously described bisphenols, lacks several hormone-disrupting properties, making it a promising scaffold to use in designing safer bisphenol analogs. To test our hypothesis, we developed five novel bisphenols utilizing the TMBPF scaffold. The novel bisphenols have been evaluated using several *Caenorhabditis elegans*-based reproductive toxicity assays and compared to TMBPF as well as the widely used BPA, BPS, and BPF. By employing exposure levels reflective of human internal concentrations, we have assessed reproductive toxicity through multiple endpoints, including brood size, germ-cell apoptosis, embryonic lethality, and the frequency of male offspring (an indicator of X-chromosome nondisjunction). Preliminary evaluation of two novel analogs, synthesized and purified in quantities sufficient for testing, reveals promising results. While one analog exhibits effects comparable to TMBPF, the other demonstrates significantly lower toxicity. Specifically, TMBPF (C₁₇H₂₀O₂) and the TMBPF A3 analog (C₁₃H₈Br₄O₂) significantly increase germline apoptosis during oogenesis. Conversely, the TMBPF A2 analog (C₂₁H₂₈O₂) showed no such increase, performing comparably to the control. These early findings suggest that modifications to the TMBPF scaffold hold significant potential for mitigating reproductive risks. Testing is currently ongoing to evaluate the remaining analogs and confirm these initial observations and further define the structure-activity relationship. Ultimately, this study will provide critical insights into how bisphenol structure impacts reproductive health and fertility, aiding in the identification of safer chemical alternatives.

The neonicotinoid 6-chloronicotinic acid adversely affects ovarian antral follicles in mice

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Abstract Text

Imidacloprid (IMI) is a neonicotinoid insecticide used in commercial agricultural systems, home gardens, and veterinary pharmaceuticals. People are exposed to IMI via oral ingestion of treated fruits and vegetables and through inhalation or dermal contact during application of the insecticide. When ingested, IMI is readily absorbed through the gastrointestinal tract of mammals where it travels to the liver and undergoes phase I and II biotransformation into terminal metabolites, such as 6-chloronicotinic acid (6CNA). Studies have shown that IMI and its terminal metabolite 6CNA are found in the ovary soon after oral exposure. However, little is known about whether the terminal metabolite 6CNA causes ovotoxicity. Thus, this study tested the hypothesis that 6CNA is ovotoxic to antral follicles. To test this hypothesis, antral follicles were dissected from ovaries of CD-1 mice (31 – 41 days old). The antral follicles were cultured in media (vehicle control) or 6CNA (0.2 µg/ml, 2 µg/ml, 20 µg/ml, and 200 µg/ml) for 96 hours. Follicle growth was measured and morphology was noted every 24 hours for the entire culture period. Follicles and media were collected separately after 96 hours. Media were subjected to enzyme linked immunosorbent assays to measure estradiol and progesterone levels. The results indicate that 6CNA did not significantly affect follicle growth or estradiol and progesterone levels compared to control. However, 6CNA at 0.2 µg/mL and 200 µg/mL caused 35% and 45% more follicles to rupture when compared to control. Collectively, these data indicate that 6CNA may cause follicles to rupture, but it may not affect follicle growth or estradiol and progesterone levels. Supported by NIH R21 ES03650.

Effects of Phthalate Metabolite Mixture Exposure on Mouse Oocyte Development

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Abstract Text

Phthalates are pervasive endocrine-disrupting chemicals widely used in consumer products, resulting in chronic human exposure to complex mixtures rather than single compounds. Despite extensive studies on individual compounds, the combined effects of their phthalate metabolites on oogenesis remain poorly understood. Here, we developed a precise microinjection-based single-oocyte toxicological assay to examine the impact of a defined phthalate metabolite mixture on meiotic progression. Phthalate mixture exposure markedly impaired oocyte maturation, as most oocytes failed to extrude the first polar body. Mechanistic analyses revealed severe meiotic defects, including disrupted spindle morphology, chromosome misalignment, disorganized actin cytoskeleton, and impaired mitochondrial function, accompanied by excessive reactive oxygen species (ROS) accumulation and DNA damage. Single-cell transcriptomic profiling further identified differentially expressed genes enriched in biological processes related to exocytosis, secretory pathway regulation, and cytoskeletal organization, as well as in MAPK, JAK-STAT, cGMP-PKG, and GnRH signaling pathways that are essential for follicular development and oocyte maturation. Together, these findings demonstrated that combined phthalate exposure directly compromises female gamete quality and underscore the importance of evaluating mixture effects when assessing risks to women's reproductive health.

"Dual Effects of Curcumin on Chromium (VI)-Induced Follicular Atresia in F1 Offspring: Protective in Large Follicles, Detrimental in Small Follicles"

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Abstract Text

Hexavalent Chromium (Cr(VI)) is a Group A carcinogen, mutagen, and teratogen. Cr(VI) has been used by more than 50 industries, and its contamination of drinking water is widespread across the United States (U.S.). Epidemiological data of women who lived in Willits, California, U.S., indicate that environmental exposure to Cr(VI) adversely affects pregnancy outcomes and the health of their immediate offspring, resulting in low birth weight, pregnancy loss, and spontaneous abortion, and their children (F1 offspring) experienced birth defects. However, the molecular mechanisms behind Cr(VI)-induced reproductive and developmental toxicity are poorly understood. Cr(VI) enters cells through anion transporters and is rapidly reduced to Cr(III) by endogenous antioxidants within the cell. Cr(III) forms adducts with DNA, which can block DNA replication and transcription; abnormal repair can lead to DNA double-strand breaks, mutations, micronucleus formation, chromosomal abnormalities, and increased genomic instability. Cr(VI) causes oxidative stress via the Fenton reaction, producing free radicals, and depletes antioxidants, promoting apoptosis via p53-dependent and independent pathways, resulting in follicular atresia and accelerated reproductive aging. Antioxidant supplementation with resveratrol, vitamin C, and edaravone mitigates Cr(VI) toxicity in the ovary. However, there is no data on the effects of curcumin in protecting the ovary against Cr(VI) toxicity. For the current study, lactating rats were divided into the following groups and treated as follows: *Group 1* rats (n=5) received regular drinking water *ad libitum*; *Group 2* rats received 50 ppm potassium dichromate dissolved in drinking water from postpartum day 1 to 21; and *Group 3* rats received 50 ppm potassium dichromate dissolved in drinking water from postpartum day 1 to 21, along with curcumin (80 mg/kg body wt. through gavage daily). On the day of birth, male pups were removed, and litters were culled to 4 female pups per mother rat; thus, each treatment group consisted of 5 mothers (F0) and 20 F1 female pups. In each experimental group, the lactating mother rats received respective treatment in drinking water from the day of parturition to day 21 postpartum, and the F1 offspring received the respective treatment through the mother's milk. After weaning on PND 22, F1 female pups were fed regular drinking water and diet until PND 25 and were euthanized under CO2 anesthesia followed by cervical dislocation, and blood and ovaries were collected for further analyses. Data revealed that Cr(VI) increased follicle atresia of the small and large follicles, and curcumin rescued the large follicles from Cr(VI)-induced atresia. In contrast, curcumin exacerbated Cr(VI) effects on smaller follicles. Cr(VI) increased apoptosis-inducing factor (AIF) and caspase-3, and curcumin mitigated the Cr(VI) effects on caspase-3. Cr(VI) downregulated steroidogenic enzymes and receptors, and curcumin mitigated Cr(VI) effects. Cr(VI) decreased antioxidants, cell survival machinery, and HIF1 α in the serum and ovary, and curcumin mitigated it. A mechanistic study using immortalized rat granulosa cells showed that SIGC decreased cytosolic and mitochondrial AOX enzymes, and curcumin mitigated the adverse effects of Cr(VI). Thus, curcumin partially rescues the ovary from Cr(VI) toxicity in the F1 offspring. This work was supported by grants from the National Institute of Environmental Health Sciences, Texas A&M Center for Environmental Health Research (TiCER) (P30ES029067), pilot project award (P30-ES029067 to S.K.B), and 1R21ES034905-01, 1R21ES020561-01, and R01-ES025234 to S.K.B.

Acute Exposure to Perfluorooctanoic Acid (PFOA) Disrupts Steroidogenesis and Mitochondrial Function in Granulosa Cells

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Abstract Text

Perfluorooctanoic acid (PFOA) is a legacy per- and polyfluoroalkyl substance (PFAS) that was historically used in various industrial and commercial applications. Due to its bioaccumulative nature arising from chemical structural characteristics, PFOA has been linked to various toxicological effects, including liver and kidney toxicity. Moreover, recent studies indicate that PFOA exposure contributes to reproductive dysfunction such as hormonal imbalance, reduced birth weight, and infertility. Recent studies also indicate that PFOA exposure induces ovarian dysfunction through disruptions in folliculogenesis, steroidogenesis, and oocyte quality in vivo. Despite these findings, the cellular pathways through which PFOA exerts its effects remain insufficiently characterized. This study investigated how PFOA affects granulosa cell function as a potential mechanism underlying female reproductive toxicity. Specifically, this study tested the hypothesis that PFOA exposure impairs granulosa cell viability and function by inducing oxidative stress, disrupting steroidogenesis, and causing mitochondrial dysfunction. Granulosa cells were collected from the ovaries of adult CD-1 mice and cultured with vehicle control (dimethyl sulfoxide; DMSO) or PFOA (1, 10, 25, 50 and 100 µg/mL) for cell viability assays and vehicle control or PFOA (50 and 100 µg/mL) for oxidative stress and gene expression analyses for up to 72 hours. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Reactive oxygen species (ROS) levels were assessed by using a ROS assay. Gene expression related to steroidogenesis and mitochondrial function were analyzed by quantitative polymerase chain reaction (qPCR). MTT assay results indicated that PFOA exposure significantly reduced granulosa cell viability in a dose-dependent manner compared to control. PFOA also increased ROS levels and decreased the antioxidant superoxide dismutase 1 (Sod1) expression. PFOA also decreased Cyp11a1 expression and increased Cyp19a1 expression, indicating that PFOA may alter steroid hormone synthesis. In addition, PFOA exposure increased Aifm1 expression and decreased Ppargc1a expression, suggesting that PFOA exposure may induce mitochondrial dysfunction and apoptosis activation. Collectively, these results suggest that the PFOA impairs granulosa cell function by inducing oxidative stress, disrupting steroid hormone synthesis, and impairing mitochondrial function. These cellular alterations may contribute to PFOA-induced ovarian dysfunction and reduce female fertility.

Knockout of TLR4 Preserves Gut Microbiome Diversity and Reduces Ovarian Immune Cell Infiltration in a PCOS-like Mouse Model

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Abstract Text

Polycystic Ovary Syndrome (PCOS) is characterized by reproductive and metabolic dysfunction, with emerging evidence implicating gut dysbiosis. Our previous work demonstrated that Toll-like receptor 4 (TLR4) knockout mice were protected against Letrozole (LET)-induced PCOS-like symptoms. Here, we investigate TLR4's mechanistic role in mediating gut microbiome alterations, immune populations, and vagus nerve-dependent gut-reproductive axis communication in PCOS pathophysiology. Peripubertal female C57BL6J (WT) and TLR4^{-/-} mice (n=40 per group) were treated with LET or placebo for five weeks. Using 16S rRNA sequencing, we confirmed microbial dysbiosis in LET-treated WT mice. Strikingly, LET-treated TLR4^{-/-} mice maintained 1-1.5-fold increased Shannon and Chao1 alpha diversity and greater beta diversity by Bray-Curtis dissimilarity versus LET-treated WT mice. The Firmicutes-to-Bacteroidetes ratio in LET-treated TLR4^{-/-} mice resembled placebo-treated animals. Spectral flow cytometric analysis of mesenteric lymph nodes, critical immune sites integrating gut signals, revealed significant increases in T cell, myeloid, B cell, and dendritic cell populations in LET-treated WT mice. Conversely, immune populations in LET-treated TLR4^{-/-} mice remained comparable to placebo controls. This extended to ovaries, where CD45⁺ immune infiltration increased over 2% only in LET-treated WT mice. Parallel dysregulation across gut microbiome, gut-associated lymph nodes, and ovaries suggested coordinated signaling. We hypothesized the vagus nerve, connecting the gastrointestinal tract and hypothalamic-pituitary-gonadal axis, serves as this link. To test, we performed subdiaphragmatic vagotomy (SDV) (n=11 per group). SDV mice treated with LET demonstrated reduced body weight, enhanced glucose tolerance, and decreased luteinizing hormone (LH) levels, with all parameters returning near placebo and sham controls, establishing vagal signaling as a critical mediator. Collectively, our data reveal a novel gut-reproductive axis in PCOS pathogenesis. TLR4 deletion preserves microbiome diversity and gut-immune homeostasis, thereby reducing aberrant vagal signaling that would otherwise propagate reproductive and metabolic disturbances. This work identifies the TLR4-microbiome-vagus nerve pathway as a novel therapeutic target.

Asprosin Modulates Hypothalamic-Pituitary Axis Activity and Reproductive Neuroendocrine Signaling in Female Mice: An In Vivo Study

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Abstract Text

Asprosin (ASP) is a fasting-induced adipokine secreted mainly by white adipose tissue. It regulates glucose and energy homeostasis by stimulating rapid hepatic glucose release. Moreover, studies in mice demonstrated that ASP can cross the blood-brain barrier and activate orexigenic AgRP neurons while suppressing POMC neuronal activity in the hypothalamus, thereby increasing appetite and food intake. Emerging evidence from mouse and rat studies suggests that ASP may also participate in neuroendocrine regulation by influencing hypothalamic GnRH expression and pituitary hormone secretion. Recent studies have demonstrated the expression of ASP and its receptors in murine pituitary tissue and in the mouse gonadotrope cell line LBT2, indicating a potential role in hypothalamic-pituitary axis function. Therefore, the aim of this study was to evaluate, in an *in vivo* mouse model, the involvement of ASP in neuroendocrine regulation by analyzing the effects of both ASP deficiency and administration on hypothalamic and pituitary gene expression, as well as circulating hormone levels, associated with reproductive signaling, neuropeptide activity, steroid hormone signaling, and metabolic regulation.

The studies were conducted using wild-type C57BL/6J mice and mice carrying a targeted mutation leading to reduced ASP expression (mutants, B6.129-*Fbn1*^{tm2Rmz/J}). Sexually mature 8-week-old females (n = 15/group, 60 in total) received daily tail-vein injections of recombinant mouse ASP (1 µg/g body weight) or physiological saline (0.9% NaCl; control) for 14 ± 2 days, covering at least two full estrous cycles, and beginning and ending in the estrus phase (peri-ovulatory and estrogen-dominant phase). Body weight and estrous cyclicity were monitored daily. Following treatment, blood, hypothalamic, and pituitary tissues were collected. RT-qPCR analysis was performed to determine the expression of the ASP-encoding gene (*Fbn1*), the ASP-processing protease (*Furin*), and all known ASP receptors and signaling mediators (*Ptprd*, *Or4m1/Olfr734*, and *Tlr4*) in hypothalamic and pituitary tissues. In the hypothalamus, genes associated with reproductive regulation (*Gnrh1*), appetite control (*Npy*, *Agrp*), metabolic signaling (*Insr*, *Lepr*), and steroid hormone responsiveness (*Esr1*, *Esr2*, *Pgr*) were analyzed. In the pituitary gland, the expression of genes related to gonadotropin signaling (*Gnrhr*), metabolic responsiveness (*Insr*, *Lepr*), and steroid hormone signaling (*Esr1*, *Esr2*) was evaluated. Serum concentrations of gonadotropins (LH, FSH) and reproductive regulators and pituitary hormones (kisspeptin, prolactin) were measured using ELISA assays.

The results confirmed reduced expression of the *Fbn1* gene in mutant mice, while ASP administration further reduced its expression in the hypothalamus and pituitary gland. In both tissues, ASP deficiency and ASP administration altered the expression of *Furin* and all analyzed ASP receptors/mediators. In the hypothalamus, ASP injections increased *Gnrh1* expression in both genotypes, whereas pituitary *Gnrhr* expression was upregulated only in mutant mice. ASP administration reduced elevated LH concentrations in mutant mice and increased FSH levels in both genotypes, resulting in a decreased LH/FSH ratio. Additionally, ASP injections increased circulating kisspeptin and prolactin levels in mutant mice. Moreover, ASP deficiency and administration altered *Esr1* and *Esr2* expression in the hypothalamus and pituitary gland, as well as *Pgr* expression in the hypothalamus, particularly in mutant mice. Additionally, both ASP deficiency and ASP administration modulated *Agrp*, *Npy*, *Insr*, and *Lepr* expression in both analyzed tissues. These findings demonstrate that ASP deficiency and administration modulate hypothalamic-pituitary axis activity, suggesting that ASP may function as a metabolic signal linking energy balance with neuroendocrine and reproductive regulation.

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Neonatal Estradiol Exposure Impairs Pubertal Development and Fertility in Mongolian Gerbils

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Abstract Text

Estrogen is a critical regulator of reproductive development and neuroendocrine organization, and early life represents a sensitive window during which sexual differentiation and hypothalamic-pituitary-gonadal (HPG) axis programming occur. Exogenous estrogen exposure during this period may induce persistent reproductive dysfunction. The Mongolian gerbil (*Meriones unguiculatus*) is a socially monogamous rodent with robust reproductive behaviors, providing a biologically relevant pairing system for evaluating pubertal development and fertility. We hypothesized that neonatal estradiol (E2) exposure disrupts pubertal maturation and impairs adult reproductive function.

Neonatal gerbils received a single subcutaneous slow-release E2 pellet (0.5 or 1.0 mg) on postnatal day (PND) 5, designed to provide sustained hormone release over approximately 2–4 weeks ($n = 5\text{--}6/\text{group/sex}$). Animals were monitored longitudinally for body weight, pubertal onset, and fertility. Pubertal onset was defined as vaginal opening in females and testicular descent in males. Fertility was assessed via mating trials between PND 60–90 with proven fertile intact partners.

Neonatal E2 exposure produced dose-dependent impairments in growth and reproductive maturation. At PND 50, body weight was significantly reduced in males (control: 56.1 ± 3.5 g; 0.5 mg: 50.0 ± 6.8 g; 1.0 mg: 44.6 ± 5.3 g; $P = 0.01$), with a similar but less pronounced trend in females. In males, testicular descent was delayed in 0.5 mg group (102.0 ± 16.5 vs. 53.0 ± 7.8 days in controls; $P = 0.03$), and 50% of males in the 1.0 mg group remained cryptorchid throughout the observation. In females, vaginal opening was delayed or absent after E2 treatment; by PND 100, 60% of 0.5 mg females had not undergone vaginal opening, whereas in the 1.0 mg group, vaginal opening was delayed in 2/6 and absent in 4/6 animals. During a 60-day fertility trial, all control pairs produced two litters/pair averaging 5 pups/litter. In contrast, fertility was markedly reduced in treated animals, with litter-producing rates of 20% in 0.5 mg-treated females, 25% in 0.5 mg-treated males, 16.7% in 1.0 mg-treated females, and 0% in 1.0 mg-treated males.

These findings demonstrate that a single neonatal E2 exposure markedly delays pubertal onset and severely compromises subsequent fertility in both sexes. This model provides a tractable system for investigating the mechanisms by which early-life estrogen exposure programs long-term reproductive dysfunction.

Acute Estradiol Exposure Induces Subtle Transcriptional and Chromatin Responses in Sheep Pituitary Gonadotropes Revealed by Single-Nucleus Multiomics

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Abstract Text

Estradiol (E2) is a key regulator of pituitary gonadotrope function, yet the earliest molecular responses to acute estrogen exposure remain poorly defined, particularly with respect to chromatin regulation. To resolve gonadotrope-specific responses that may be masked in bulk pituitary analyses, we performed same-cell single-nucleus RNA sequencing (snRNA-seq) and single-nucleus ATAC sequencing (snATAC-seq) on four pituitaries from ovariectomized adult sheep treated with vehicle or estradiol for 90 minutes. Analyses were restricted to the gonadotrope population to interrogate cellintrinsic estrogen responses directly. At this early treatment time point, differential gene expression was modest, with a limited number of estradiol-responsive genes detected. In contrast, chromatin accessibility analysis identified 147 differentially accessible regions, the majority of which were distal or intronic, consistent with enhancer-level regulation rather than promoter-driven transcriptional changes. Integration of gene expression and chromatin accessibility using transcription factor–based regulatory modeling (CREMA) reconstructed over 2,000 statistically supported regulatory circuits in gonadotropes, spanning 97 transcription factors and nearly 500 gene targets. Regulatory modules associated with hormone-responsive and signal-dependent transcription factors were detected under estradiol treatment, including selective estradiol-associated genes such as GNRHR. Notably, many inferred regulatory interactions were not directly captured by differentially accessible regions, suggesting that regulatory modeling captures latent or context-specific estrogen signaling beyond peakbased analyses alone. Together, these findings indicate that acute estradiol exposure induces early chromatin remodeling and regulatory circuit engagement in pituitary gonadotropes, preceding widespread transcriptional changes. This study highlights the power of single-nucleus multiomic and regulatory inference approaches for resolving rapid, cell-specific hormone signaling events in a large animal model.

Investigating the Regulation and Function of Transcription Factor OCT4 During Early Primitive Endoderm Development in Mice

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Abstract Text

During early mammalian embryogenesis, cells undergo a series of tightly regulated lineage decisions that lead to the establishment of three foundational embryonic and extraembryonic tissues: (i) the epiblast, which develops into the fetus, (ii) the trophectoderm, which gives rise to the placenta, and (iii) the primitive endoderm, which becomes the yolk sac. The yolk sac is indispensable to the establishment and maintenance of early pregnancy, serving as the first site of nutrient exchange between the mother and fetus, initiating primary hematopoiesis, and guiding axial patterning of the fetus. Defects in yolk sac development have been linked to implantation failure and early pregnancy loss, highlighting the clinical importance of the lineage. However, the molecular mechanisms governing early primitive endoderm development remain incompletely understood. A central unanswered question is the mechanistic role of a key transcription factor known as OCT4 (POU5F1) in the primitive endoderm lineage. OCT4 has been extensively characterized as a master regulator of pluripotency in the epiblast. However, it is also cell-autonomously required for the successful specification and development of the multipotent primitive endoderm, suggesting a novel lineage-specific function. In contrast to its sustained high expression in the epiblast, OCT4 exhibits dynamic and transient expression in the primitive endoderm, where it is highly expressed upon specification but rapidly downregulated and ultimately repressed during subsequent lineage progression. To investigate the function of OCT4 across this transition, we employ two complementary stem cell models: (i) primitive endoderm stem cells and (ii) extraembryonic endoderm stem cells, representing distinct primitive endoderm states before and after OCT4 repression, respectively. Primitive endoderm stem cells closely recapitulate early primitive endoderm identity, maintaining stable OCT4 expression, and exhibiting a naïve stem cell state capable of differentiating into all downstream primitive endoderm derivatives in chimera assays. In contrast, extraembryonic endoderm stem cells represent a later-stage derivative of the primitive endoderm lineage, characterized by a complete loss of OCT4 expression and a substantial reduction in developmental potency. In this study, we investigate the functional role of OCT4 using in vitro differentiation and reversion assays between the two cell states. Most notably, we find that extraembryonic endoderm stem cells can be reverted to a naïve primitive endoderm stem cell-like state accompanied by the reactivation of endogenous OCT4, indicating that OCT4 expression is strongly associated with stem cell state and developmental potency in this lineage. These findings provide novel insights into the molecular mechanisms underlying early primitive endoderm development and plasticity, and establish a foundation for understanding how disruptions to this process can contribute to implantation failure and early pregnancy loss.

OCT4 is required for bovine blastocyst formation

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Abstract Text

Blastocyst formation requires the differentiation of inner cell mass (ICM) and trophectoderm (TE) precursors at the morula stage. The molecular regulation of such cell differentiation event has been well studied in mice, where the mutually exclusive expression pattern of OCT4 (ICM) and CDX2 (TE) plays a major role. However, the ICM-exclusive expression of OCT4 described in mouse blastocysts is not observed in ungulates, rabbits or humans, and CDX2 is not required for initial TE specification in cattle. As functional studies on the role of OCT4 during cattle embryo development have reported conflicting results, the aim of this work has been to test the developmental ability of OCT4 KO embryos. To that aim, bovine in vitro matured oocytes were microinjected with cytosine base editor (CBE)-encoding mRNA and a sgRNA designed to introduce a stop codon at OCT4 (C+G group, containing KO embryos) or with CBE-encoding mRNA alone (C group, formed by wild-type WT embryos) as microinjection control. Microinjected oocytes were fertilized in vitro and allowed to develop to Day 8 (D8) blastocysts in conventional culture, when all embryos were fixed. Fixed specimens were subjected to immunofluorescence to detect OCT4, ICM (SOX2+) and TE (CDX2+) cells. Cleavage rate was similar between microinjection groups (80.8 ± 3.9 vs. 77 ± 2 %; for C and C+G groups), but blastocyst rate was significantly reduced in the group containing KO embryos (24.6 ± 3.1 vs. 7.8 ± 2.1 %; for C and C+G groups, t-test $p > 0.05$). Irrespective of the microinjection group all blastocysts (17/17 for C+G and 42/42 for C) displayed OCT4 expression and a normal pattern of CDX2 (TE) and SOX2 (ICM) expression. In contrast, whereas all arrested embryos analyzed (> 8 cells) in C group were OCT4+ (29/29), about half of the embryos from C+G group (17/37) lacked OCT4 protein. In conclusion, OCT4 is essential for cattle embryo development to the blastocyst stage.

Effect of Porcine Follicular Fluid Supplemented with *In Vitro* Maturation Medium on Porcine Oocyte Maturation, Fertilization and Preimplantation Development.

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Abstract Text

In vitro production of embryos (IVP), consisting of *in vitro* maturation (IVM) of oocytes, *in vitro* fertilization (IVF), and *in vitro* culture (IVC) of embryos, and is widely used in livestock breeding and genetic improvement. IVM process strongly influences the production of high-quality embryos for embryo transfer. Generally, porcine follicular fluid (PFF) was added to IVM medium for pig oocytes. However, no conclusion has been reached regarding the roles and necessity of PFF for porcine IVP system. Therefore, this study aimed to clarify the effects of PFF added to the porcine IVM medium on oocyte maturation, fertilization, and preimplantation development, while also examining the effect of PFF on zygotic gene activation (ZGA) in early embryos. The IVM process was divided into the first (0 - 20 h) and second (20 - 44 h) periods. PFF was added either to the first (PFF+/-) or second period (PFF-/+), and further groups were created where PFF was added (PFF+/+) or not added (PFF-/-) throughout the entire period. The completion of nuclear maturation, that is, the rate of reaching Metaphase-II (M-II) stage in PFF-/- group (38.5%) was significantly ($P < 0.05$) lower than that in PFF+/+ and PFF-/+ (70.3% and 70.2%, respectively). Results of evaluating sperm penetration status and the formation status of both male (MP) and female (FP) pronuclei after IVF, sperm penetration rate (58.2%) in PFF-/- was significantly ($P < 0.05$) lower than that in PFF+/+ and PFF+/- (87.0 and 78.6%, respectively). In addition, both MP and FP formation rate (60.5%) in PFF-/- was lower ($P < 0.05$) than that in other three groups (91.8 - 98.9%). No differences were observed among the four groups in monospermy, normal fertilization (1 MP and 1 FP formation), polyspermy rate and number of sperms per penetrated oocyte. Embryo development status after IVF was assessed from Day 2 to Day 6. The ≥ 2 -cell (45.7%) and $\geq$ morula (22.1%) rates in IVF embryos obtained from PFF+/+ group was higher ($P < 0.05$) than that in PFF+/- and PFF-/- (18.3 - 25.5 and 1.1 - 13.9%, respectively). In addition, the rate of $\geq$ early blastocyst in PFF+/+ (26.0%) and PFF-/+ (26.1%) were higher ($P < 0.05$) than that in PFF-/- (3.2%). ZGA-related gene (EIF1AX, PDHA1 and DPPA2) levels in 4-cell stage embryos were compared among the three groups (PFF+/+, PFF-/+ and PFF+/-). No differences were observed in the expression levels of all three genes. In present study, the presence or absence of PFF and the timing of its supplementation during IVM period altered the status of sperm penetration and pronuclear formation status. These results suggested that the addition of PFF into the IVM medium affected not only nuclear maturation but also the cytoplasmic maturation of porcine oocytes. Furthermore, this study revealed that the addition of PFF at the later stages of porcine IVM may be important for optimizing porcine oocyte and embryo qualities after IVF.

RXFP2 Expression Dynamics and Effect of INSL3 on Bovine Oocyte Maturation and Embryonic Development

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Abstract Text

Insulin-like peptide 3 (INSL3) is a circulating hormone secreted exclusively by testicular Leydig cells in males and by theca interna cells of ovarian follicles and luteal cells in females. The INSL3 receptor (RXFP2) has been localized in bovine ovarian tissues, and RXFP2 mRNA detected in oocytes and early embryos. However, the dynamics of RXFP2 protein levels and the role of INSL3 in bovine oocyte maturation and embryonic development remain unclear. This study investigated RXFP2 protein expression patterns in oocytes and early embryos and examined the effects of INSL3 on nuclear and cytoplasmic maturation of bovine oocytes and subsequent early embryonic development. We collected ovaries from abattoirs and aspirated cumulus-oocyte complexes (COCs) from follicles with diameters ranging between 2 and 8 mm. In Experiment 1, COCs were matured and fertilized *in vitro*, and early embryos (from 2-cell to blastocyst stages) were collected for RXFP2 immunofluorescence analysis. In Experiment 2, COCs underwent *in vitro* maturation (IVM) with 0 (control), 10, or 100 ng/mL bovine INSL3 in IVMD 101 medium. The COC areas were measured both before and after maturation. Nuclear maturation was assessed via first polar body extrusion rate, and cytoplasmic maturation via mtDNA copy number and ATP content. Additionally, COCs were cultured in four maturation media (HPM199, IVMD 101 including TGF α , IVMD 101 without TGF α , and IVMD 101 including TGF α and FSH) each supplemented with 0, 10, or 100 ng/mL INSL3. Cytoplasmic maturation was assessed by quantifying the mRNA expression levels of bone morphogenetic protein 15 (*BMP15*), fibroblast growth factor 2 (*FGF2*), and growth differentiation factor 9 (*GDF9*) in matured oocytes by real-time PCR. To assess the effect of INSL3 on embryo development, COCs matured with 0 and 100 ng/mL INSL3 were fertilized and cultured. At Days 1 and 2, the number of cleaved embryos, and at Days 7 and 8, the number of blastocysts, were assessed. Immunofluorescence revealed RXFP2 protein expression in mature oocytes and early embryos, with levels highest in all mature oocytes ($p < 0.05$), decreasing to the 8-cell stage, then increasing at morula and blastocyst stages ($p < 0.05$). INSL3 supplementation during IVM increased polar body extrusion rate (10 ng/mL INSL3: 83.5%; $p < 0.05$) and mtDNA copy number (10 ng/mL INSL3: 108-fold; $p < 0.001$), with no effects on COC area or ATP content. Gene expression effects were media-dependent: *BMP15* expression increased in IVMD 101 including TGF α (10 ng/mL INSL3; $p < 0.05$) and IVMD 101 including TGF α and FSH (10 and 100 ng/mL INSL3; $p < 0.05$), while *GDF9* increased only in IVMD 101 including TGF α and FSH with 10 ng/mL INSL3 ($P < 0.05$). INSL3 supplementation (100 ng/mL) during oocyte maturation showed no significant effects on subsequent embryo development, with cleavage rates of 92.8% vs. 92.6% (control) and blastocyst rates of 28.8% vs. 24.4% (control). In conclusion, RXFP2 expression was highest in mature oocytes and blastocysts, declining from 2-cell to 8-cell stages. INSL3 promoted nuclear and cytoplasmic maturation of bovine oocytes in a media-dependent manner, with the presence of TGF α and FSH. INSL3 did not affect embryo development, likely due to its absence during culture when RXFP2 expression increases (morula/blastocyst stages), indicating that continuous INSL3 supplementation may be necessary for improved developmental rates.

Investigations of TIAM1 in mouse eggs - a CaMKII substrate that is a candidate mediator of calcium-dependent events in the egg-to-embryo transition

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Abstract Text

Calcium (Ca^{2+}) signaling mediates the egg-to-embryo transition, a series of early developmental events collectively known as "egg activation" that transform the egg into an embryo following fertilization. In mammalian eggs, increased cytosolic Ca^{2+} induces key events including the completion of meiosis, progression to embryonic mitosis, and the establishment of the egg's preventative mechanisms that block fertilization by more than one sperm (known as blocks to polyspermy). Ca^{2+} ions exert their effects through calcium-dependent effector molecules, and Ca^{2+} /Calmodulin-dependent kinase II (CaMKII) is directly involved in egg activation events, particularly exit from metaphase II arrest. In mice, knockout of the gamma isoform of CaMKII (CaMKII γ) causes female infertility due to egg activation defects. CaMKII has numerous downstream effectors, and our study here has examined T-cell lymphoma invasion and metastasis 1 (Tiam1), building on the finding that *Tiam1* transcripts are enriched in mouse oocytes. Tiam1 is a CaMKII substrate in other systems such as neurons, and functions as a guanine nucleotide exchange factor (GEF) that activates small G-proteins Rac1 and Cdc42. Here, we are investigating (a) if Tiam1 is expressed in mouse eggs, and where it is localized; and (b) if Tiam1 is phosphorylated in fertilized eggs and if this phosphorylation is Ca^{2+} -dependent. Oocytes from CF-1 female mice were used for collection. Superovulation was achieved through injections with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) to have ovulated eggs for *in vitro* fertilization (IVF). Immunoblotting detects both Tiam1 and CaMKII γ (control) proteins in lysates of prophase I oocytes, metaphase II eggs, and fertilized eggs. Immunofluorescence shows Tiam1 is present in the cytoplasm and cortex in prophase I, metaphase II, and fertilized eggs. We also detect Tiam1 and CaMKII γ enrichment near the actin-rich area over the paternal DNA in early embryos. This is of interest because the fertilizing sperm induces actin remodeling at the sperm entry point, and Tiam1, as an activator of small GTPases that regulate actin cytoskeleton organization, would be a candidate to mediate this event in early embryos. Ongoing studies are investigating Ca^{2+} -dependent phosphorylation of Tiam1 in early embryos using Phos-tag gel electrophoresis for differential separation of proteins based on their phospho-state. Future studies include: (1) analysis of microscopic images to quantify the co-localization patterns of Tiam1 and CaMKII γ in early embryos, (2) investigating the co-localization of Tiam1 and CaMKII γ under varying Ca^{2+} manipulations using a proximity ligation assay (PLA), and (3) depleting Tiam1 in eggs with a reverse genetics approach known as Trim-Away, to evaluate the potential functions of Tiam1 in oocytes, eggs and early embryos. These studies will elucidate the factors and pathways of Tiam1 and CaMKII γ that contribute to the Ca^{2+} -dependent signaling events in the egg-to-embryo transition.

Testing the Maternal DNMT1o Imprinted Maintenance Methylation Model in Preimplantation Mouse Embryos

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Abstract Text

DNA methyltransferase 1 (DNMT1) is a key protein to maintain imprinted methylation. In preimplantation embryos, the maternally-derived isoform, DNMT1o, is present at all stages, with abundant nuclear localization at the 8-cell stage. A model has been proposed suggesting that DNMT1o is responsible for maintaining methylation exclusively at the 8-cell stage. However, it is unclear, in maternal *Dnmt1o*-deficient embryos, when imprinted methylation loss arises during preimplantation development in individual embryos, whether DNA methylation continues to be lost through subsequent cell cycles, and whether all maternal *Dnmt1o*-deficient embryos experience imprinted methylation loss. In this study, we conducted a comprehensive analysis to test the maternal DNMT1o imprinted maintenance methylation model. Here, we investigated *Snrpn* and *H19* imprinted methylation in single 2-cell, 4-cell, 8-cell, morula and blastocyst embryos using bisulfite mutagenesis and clonal sequencing. At least 8 embryos per stage were analyzed per group. Comparisons between control and maternal *Dnmt1o*-deficient embryos that maintained and lost imprinted methylation were evaluated using Fisher's exact test, at each stage. Our findings challenged several assumptions made in the DNMT1o methylation maintenance model. Specifically, we observed progressive methylation loss of imprinted methylation after the 8-cell stage, with the extent of imprinted methylation loss widely varying among embryos (morulae, 50-68%; blastocysts, 8-70% imprinted methylation). Furthermore, a subset of morulae and blastocysts maintained normal maternal *Snrpn* and paternal *H19* hypermethylation (>70%), bypassing imprinted methylation loss. Interestingly, some cleavage-stage embryos exhibited unexpected methylation loss (12-34%). All together these findings point out potential compensation mechanisms that act to partially or fully restore DNA methylation during early embryo development. Future investigations are needed to understand the mechanisms involved in imprinted maintenance methylation in preimplantation embryos. This work was supported by NIH/NIHCD, 5R01HD101574, to MRWM.

Melatonin-Associated Shifts in the Vaginal Microbiome of Beef Cows During Late Gestation

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Abstract Text

Microbial communities of the bovine reproductive tract are linked to reproductive health, and late gestation represents a period of physiological change that may reshape the vaginal microbiome. Melatonin influences oxidative and immune pathways and may alter the vaginal environment. However, the impact of melatonin on the vaginal microbiome in late-gestation beef cows is not well understood. The objective of this study was to determine whether melatonin treatment during late gestation alters the vaginal microbial community. Pregnant Angus beef cows ($n = 24$) in the third trimester were trained to acquire feed from a Calan gate electronic feeding system and randomly assigned to either the control group (CON) or melatonin group (MEL). Groups were stratified by body weight and sire. Melatonin was dissolved in ethanol, top-dressed onto supplemental feed ($200 \mu\text{g/kg BW}$), and adjusted weekly based on maternal body weight. Control cows received an equivalent ethanol vehicle. Treatments started at approximately day 209 of gestation and continued for 54 days. Vaginal swabs were obtained via a double-guarded equine uterine culture swab on day 0 (prior to dietary treatment) and day 42 of treatment. The vaginal microbial community was determined by sequencing the V3 and V4 regions of the 16s rRNA gene on an Illumina NextSeq platform. Alpha diversity (Chao1, Shannon indices) was analyzed in SAS 9.4 using mixed or generalized linear models as appropriate. Beta diversity was assessed using Bray–Curtis dissimilarity, visualized by PCoA, and tested by PERMANOVA (adonis, R). Differential abundance was evaluated using MaAsLin2 in MicrobiomeAnalyst, adjusting for age, and parity. Alpha and beta diversity did not differ between groups on D0 ($P > 0.7$). At D42 Chao1 richness was higher in the MEL than CON ($P = 0.03$), while Shannon diversity and beta diversity did not differ between groups ($P > 0.2$). Differential abundance analysis identified melatonin-associated shifts in the vaginal microbiota ($P < 0.05$). Several taxa were enriched in MEL, including Micrococcaceae, Staphylococcaceae, Pseudomonadaceae, and Rhodobacteraceae families, as well as *Tessaracoccus*, *Aeromonas*, *Oligella*, and *Jeotgalicoccus* genera. Several enriched taxa are commonly identified in the bovine vaginal microbiome. Together with the increase in Chao1 richness and no differences in beta diversity between groups, these findings point to a subtle shift in community composition without major disruptions in overall community structure. These results suggest melatonin may fine-tune the vaginal bacterial community during late gestation rather than cause dramatic changes.

Integrating Pubertal Classification and Anogenital Distance Measures Identified a Threshold Predictive of Reproductive Longevity and Fertility in Beef Heifers

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Abstract Text

Early pubertal attainment is critical for reproductive efficiency, as females that reach puberty earlier have longer reproductive lifespans and can offset developmental costs through increased lifetime calf production. Despite this advantage, identifying females that should be retained in the herd before reproductive maturation remains challenging due to the lack of inexpensive, noninvasive, predictive tools. Anogenital distance (AGD) has emerged as a potential marker of reproductive longevity and fertility in humans and animal models. In females, AGD is believed to reflect *in utero* androgen exposure, with longer distances being associated with disrupted reproductive development and reduced fertility. Our laboratory previously identified five heritable pubertal heifer classifications: Early, Typical, Start-Stop-Start (SSS), Start-Stop-Discontinuous (SSD), and Non-Cycling (delayed puberty). Early and Typical heifers initiated and maintained ovarian cyclicity while Non-Cycling and SSD heifers failed to establish consistent cyclicity. Furthermore, Non-Cycling and SSD heifers produced the fewest calves conceived via artificial insemination within the first 21 days of the calving season and were the most sexually immature, indicative of delayed pubertal development. Distances from the anus to vulva (AGDv), anus to clitoris (AGDc), and the ratio of these measures (AGDv/AGDc x 100) have each been associated with reproductive performance in dairy heifers. We hypothesized that individual AGD measures and ratios would be associated with fertility traits and development of a receiver operating characteristic (ROC) curve comparing highly heritable categories of Early+Typical vs Non-Cycling+SSD would allow beef producers to more accurately identify heifers to retain in the herd. Anogenital measurements collected from 2020-2023 on 337 heifers revealed that Non-Cycling heifers (45.51 ± 1.0 ; $n=64$) had longer AGDv than Early (41.98 ± 0.87 ; $n=82$; $p < 0.05$). When classifications were combined, Non-Cycling+SSD heifers (45.31 ± 0.88 ; $n=84$) had longer AGDv than Early+Typical (43.14 ± 0.52 ; $n=147$; $p < 0.05$) suggesting that delayed or irregular cyclicity is associated with increased AGDv and reduced reproductive longevity. Conversely, AGDc was reduced in Non-Cycling (104.65 ± 1.36 ; $p < 0.01$), SSS (105.65 ± 1.56 ; $p < 0.01$) and Typical heifers (105.89 ± 0.89 ; $p < 0.001$) relative to Early (111.15 ± 1.18 ; $p < 0.01$), indicating that Early heifers exhibited more advanced external genital development consistent with enhanced reproductive maturity. Reproductive longevity in Early heifers was further supported by analysis of the AGDv/AGDc ratio, as Non-Cycling (43.98 ± 1.08 ; $p < 0.001$) and Typical (41.17 ± 0.71 ; $p < 0.001$) had greater ratios compared to Early (37.59 ± 0.93 ; $p < 0.001$). Receiver operating curve (ROC) analysis conducted in RStudio identified an optimal AGDv/AGDc threshold of 40.43%. Heifers with reduced AGD ratio ($\leq 34.3 \pm 4.21\%$; $n=160$, $p < 0.0001$) were classified as optimal for herd retention (Early+Typical), whereas heifers with a greater AGD ratio (Non-Cycling+SSD; $\geq 46.62 \pm 4.497\%$; $n=190$, $p < 0.0001$) were more likely to belong to delayed or irregular pubertal classifications. Chi-squared analysis demonstrated associations between AGD ratio and key reproductive outcomes. Heifers with a reduced AGD ratio tended to respond more frequently to PGF2a (53.13%) than those with a greater AGD ratio (43.35%; $p=0.07$). Reduced AGD ratio heifers also calved at a younger age ($715.6 \pm 20.88\%$; $n=108$, $p < 0.05$) and were more likely to calve within the first 21 days of the calving season (59.43%, $p < 0.0004$) compared with greater AGD ratio heifers (36.73%, $p < 0.0004$). Overall, integrating pubertal classification data with AGD measurements allowed the establishment of a biologically meaningful AGD threshold capable of predicting reproductive longevity and fertility outcomes. This approach provides producers with a practical, non-invasive tool to improve heifer retention decisions and enhance long-term herd profitability.

Uterine Stromal *ErbB3*-*Igf1* Signaling Is Critical to Gland Development Conducive to Implantation

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Abstract Text

Preimplantation uterine glandular differentiation, accompanied by glandular branching morphogenesis, is a prerequisite for glandular secretion of leukemia inhibitory factor (LIF), thereby establishing uterine receptivity to support embryo implantation. This preimplantation glandular differentiation process is disrupted in the absence of FOXA2, a transcriptional factor specifically present in glands within the uterus right from the glandular genesis. However, it remains unclear whether preimplantation glandular differentiation is regulated exclusively by gland-intrinsic mechanisms or is influenced by the surrounding microenvironment through mesenchymal-epithelial interactions. Here, we show that whole uterus deletion of *ErbB3* leads to impaired preimplantation gland branching and differentiation, and consequent reduced female fertility. In contrast, uterine epithelial specific deletion of *ErbB3* does not compromise female reproduction, indicating a critical role for stromal ERBB3 signaling. Furthermore, transcriptomic profiling of day 3 stromal cells identified *Igf1* as a candidate paracrine factor mediating the stromal-epithelial interaction. Our hypothesis is supported by the observation that the uterine deletion of *Igf1r* (*Igf1r^{fl/fl} Pgr^{Cre/+}*) leads to a reduced gland branching phenotype similar to that observed in *ErbB3^{d/d}* uteri. Collectively, these results uncover a critical *ErbB3*-*Igf1*-*Igf1r* signaling axis mediating stromal-to-epithelial communication essential for uterine gland branching and successful embryo implantation.

Regulation of the Primitive Endoderm by GATA6 and GATA4 Downstream of FGF4/MAPK Signaling in Mouse Embryos

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Abstract Text

During preimplantation development of mouse embryos, the second cell fate decision occurs where the inner cell mass differentiates into the epiblast and primitive endoderm. The epiblast will eventually make up the fetus itself, and the primitive endoderm will become the yolk sac, which provides nutrients to the embryo. The primitive endoderm will differentiate into the visceral and parietal endoderm, and cells of the visceral endoderm will contribute to the gut endoderm. The FGF4/MAPK pathway is essential for the segregation of epiblast and primitive endoderm cells within the inner cell mass. FGF4 that is secreted by epiblast cells, will act on neighboring cells to promote the expression of primitive endoderm marker genes, including GATA6 and GATA4. However, it is not clear whether other pathways act alongside FGF4/MAPK to regulate the second lineage decision. To investigate this possibility, we compared transcriptomes of epiblast and primitive endoderm cells to inner cell masses treated with exogenous FGF4/MAPK or FGFR/MAPK inhibitors. My evidence shows that FGF4/MAPK is likely to be the only signaling regulator of the primitive endoderm in vivo. As a lineage, primitive endoderm differentiation can also be modeled using several stem cell lines, including extraembryonic endoderm stem cells and primitive endoderm stem cells. Extraembryonic endoderm stem cells recapitulate a mature primitive endoderm state, while primitive endoderm stem cells resemble a naïve primitive endoderm. However, the targets of GATA in these cell lines is not known. The goal of my project is to understand the transcriptional targets of GATA6 and GATA4 in stem cell models and in vivo. Prior studies have described redundant roles of GATA4 and GATA6 during pancreas and cardiac development, suggesting that either GATA factor compensates for the loss of the other. I hypothesize that GATA6 and GATA4 share some, but not all, targets during the second cell fate decision, and therefore have semi-redundant functions during primitive endoderm development.

Identifying a Novel Trophoblast Lineage in the Placentome of the Bovine Placenta

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Abstract Text

The placentomes of the bovine placenta are specialized structures composed of cotyledonary villi that invade into aglandular endometrial caruncles to form an extensively branched network of interdigitation between the placental and maternal vasculature. These structures begin to develop around Day 40 of pregnancy and are the primary sites of hemotrophic and gaseous nutrient exchange. The prevailing consensus is that the epithelial cells that line the caruncular crypts of placentomes are endometrial luminal epithelial (LE) cells that proliferate and migrate in coordination with invading cotyledonary villi, in order to accommodate the penetration of the caruncular stroma by the uninucleate trophoblast and trophoblast giant cells (TGCs) of the villi. We hypothesize that these cells may instead be a novel population of trophoblast cells and therefore aim to re-examine the ontogeny of this epithelial cell population that is putatively derived from the LE. We will refer to this cell population as placentomal villous trophoblast (PVT). In this study, we utilized a publicly available single-cell RNA sequencing dataset (Davenport et al., 2024; DOI: 10.1093/biolre/ioad123; GSE234524), consisting of dissected conceptus tissues from Day 17 of pregnancy and chorioallantoic tissues from Days 24, 30, and 50 of pregnancy (n=3-4/day), to characterize the specific cell cluster that corresponds to the proposed PVT. Twenty-three clusters were defined via unsupervised clustering analysis. Among these, nine clusters were identified as trophoblast cells and were further annotated using well-established trophoblast cell-specific markers. One of these trophoblast clusters matched the expected transcriptional profile of the PVT population. This cluster expresses TGC markers (e.g. chorionic somatomammotropin hormone 2 (*CSH2*) and prolactin-related protein 2 (*PRP2*)) but maintains a unique set of expressed genes that differentiate it from other trophoblast clusters (e.g. WW domain-containing transcription regulator 1 (*WWTR1*) and methylenetetrahydrofolate dehydrogenase 2 (*MTHFD2*)). Immunofluorescence analyses, performed on placentomes from Days 21, 31, 40, and 67 of pregnancy, localized *WWTR1* and *MTHFD2* proteins specifically to the cell population that lines the caruncular crypts. Beginning on Day 40 of pregnancy, a small population of these cells are detected forming shallow divots into the caruncular stroma and by Day 67 of pregnancy these cells form the epithelial lining of the caruncular crypts. Further, the PVT cell cluster lacks significant expression of genes for ATP citrate synthase (*ACLY*) and isocitrate dehydrogenase (*IDH1*), in alignment with the lack of detection of these proteins in the proposed PVTs on Day 40 and 67 of pregnancy. While not detected in the PVTs, *ACLY* and *IDH1* are both detected in the LE on Day 21 of pregnancy, and in uninucleate trophoblast and TGCs, respectively, by Day 31 of pregnancy. Here, we provide preliminary evidence suggesting a trophoblastic origin for the cell population that lines the maternal caruncular crypts and propose a re-definition of these cells as placentomal villous trophoblast (PVT). These findings warrant further investigation as the ontogeny of this lineage, that defines the interface between the caruncle and the trophoblasts lining the cotyledonary stroma, carries implications for immunological aspects of pregnancy and offers novel research avenues into early pregnancy loss, placentome insufficiency, and overall reproductive efficiency in cattle. This work was supported by Agriculture and Food Research Initiative Competitive Grant no. 2024-67015-42378 from the USDA National Institute of Food and Agriculture.

Obesity Associated DNMT Downregulation Promotes DNA Hypomethylation of Oncogenic Pathways in Endometrioid Endometrial Cancer

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Abstract Text

Endometrial cancer (EC) is the most prevalent gynecological malignancy, with its incidence rising in parallel with global obesity rates. Obesity plays a crucial role in the development and prognosis of EC yet the molecular mechanisms underlying this association remain incompletely understood. Emerging evidence suggests that diet and body weight can influence DNA methylation, potentially disrupting the expression of genes involved in cell proliferation and differentiation. We hypothesized that obesity promotes hypomethylation in EC by downregulating DNMTs, key enzymes responsible for DNA methylation. To test this hypothesis, we performed genome wide DNA methylation analysis on 299 endometrioid EC tumors from The Cancer Genome Atlas, stratified by body mass index into obese and lean groups. Differential methylation analysis identified 311 obesity associated differentially methylated CpG sites, with more than 70% exhibiting hypomethylation in tumors from obese patients. Hypomethylated genes were enriched in estrogen response, PI3K/AKT/mTOR, TGF- β , and IL-2/STAT5 signaling pathways. Tumors from obese patients showed significantly reduced expression of DNMT1, DNMT3A, and DNMT3B. Stratification by DNMT expression revealed that DNMT3A and DNMT3B low tumors displayed the most extensive methylation changes. To experimentally validate these findings, CRISPR/Cas9-mediated knockout of DNMT3A and DNMT3B was performed in HEC1B endometrial cancer cells followed by genome wide methylation profiling. DNMT3A knockout recapitulated a subset of obesity associated methylation changes, identifying shared hypomethylated loci and convergent pathway enrichment. Integration of tumor and cell line datasets identified INPP5F, a negative regulator of PI3K/AKT signaling and metabolic homeostasis, as a shared hypomethylated and transcriptionally altered target. Reduced INPP5F expression in obese tumors was associated with decreased overall survival. Together, these data demonstrate that obesity is associated with widespread DNA hypomethylation in endometrioid EC, driven in part by reduced DNMT expression that may underlie the aggressive clinical features of EC in obese patients. The identified methylation signatures offer promising potential as biomarkers for prognosis and therapeutic targets in obesity related endometrial cancer.

Advanced Maternal Age Contributes to Sexually Dimorphic Metabolic Outcomes and Placental Differences in a Mouse Model

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Abstract Text

Advanced maternal age (AMA) is associated with adverse pregnancy, birth, and long-term offspring outcomes in humans including altered cardiometabolic function, need for cesarean section, and intrauterine growth restriction. Translating to a mouse model, we hypothesized that AMA offspring would display growth and metabolic changes observed in humans that could be linked to differences in placental development and prenatal growth trajectory. We employed an AMA paradigm for tracking individual offspring outcomes from embryonic age through adulthood with matched placental analyses. Offspring (n=35-48) were generated via naturally cycling young maternal age (YMA) (2-5 months) and AMA (8-14 months) females time-mated to males of proven fertility with postcopulatory plug observance denoted as embryonic day (E)0.5. Development occurred in vivo. Cesarean delivered concepti were tattooed and fostered to young, recently postpartum dams. Mixed effects model analysis with fixed effect of group and random effect of dam were performed to compare weights, ratios, morphology, and metabolic outcomes. Kaplan-Meier curves were performed to assess differences in postnatal development. Placental gene expression data were analyzed by established gene expression pipelines, broadly by evaluation of raw sequencing, genome alignment, limma with empirical Bayes moderation and false discovery rate adjustment, and Ingenuity Analysis software. Analyses were considered statistically significant if p or adjusted p < 0.05. At E18.5, AMA fetuses had reduced fetal weight, while fetal:placental ratio trended towards reduction (p=0.06) and no significant differences in placental weight or morphology compared to YMA. Neonates weighing under 1.1g did not survive the foster procedure, with AMA offspring having 7-fold greater risk of postnatal death. Survivability was essential for conducting postnatal analyses, therefore, AMA dams continued pregnancy an additional day with the intention of obtaining neonates of suitable weight. Provided an additional day of development, AMA concepti displayed increased fetal weight compared to YMA, and importantly, survival rates of AMA offspring greatly increased. Offspring (n=9-25 per sex per group) were assessed for body weight, achievement of specific developmental milestones, and metabolic assays including cumulative food intake, intraperitoneal glucose tolerance tests, and fast-induced refeeding. Tissues were collected at 24+ weeks of age. Female AMA offspring displayed increased cumulative food intake and other altered metabolism, despite no body weight difference between groups. Interestingly, male AMA offspring exhibited increased body weight and enlarged hearts and kidneys by adulthood, but did not display altered metabolic outcomes. To better understand timing of intrauterine growth restriction, additional time-mated pregnancies (n=6-11) were collected at E11.5, E14.5, and E16.5. At E11.5, groups had comparable fetal and placental weight, but by E14.5, fetal:placental ratio was reduced with AMA, and by E16.5, AMA fetuses were of reduced weight. A bulk RNA-sequencing approach was taken to capture altered placenta gene expression at E14.5 (n=6-7 per sex per group). Female AMA placentas exhibited up-regulated cell division pathways, and both sexes exhibited down-regulation of innate immune system genes. Further assessment of fetal macrophages by F4/80 immunohistochemistry indicated no difference in total area of macrophage cells between groups despite differential gene expression. Our data suggests maternal age has a significant impact on placenta development and growth outcomes in a sexually dimorphic manner.

Effects of Zygotic Ablation of Progesterone Receptor Membrane Component-1 on Bovine Peri-implantation Conceptus Survival and Development

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Abstract Text

Previous research has demonstrated that ablation of progesterone receptor membrane component-1 (*PGRMC1*) in bovine zygotes impairs embryo development in vitro and alters cell allocation in resulting blastocysts. The objective of the present study was to determine whether ablation of *PGRMC1* affects peri-implantation conceptus survival. Single guide RNAs (sgRNAs) were designed using Crispor to target exon 1 or 2 (depending on the splice variant) and beginning of exon 3, with each target site located before the predicted protein active site. Selected guides had a specificity score of 96/100, and a cutting frequency score of 96 and 97, respectively. Embryos were produced in vitro using abattoir-derived oocytes. Mature cumulus-oocyte complexes were subjected to in vitro fertilization (IVF) using frozen-thawed semen from a single bull. A different bull was used for each replicate. At 16 - 20 hours post-IVF, putative zygotes were randomly assigned to one of two groups: Cas9 only (*PGRMC1*^{+/+}; n = 389) or sgRNAs plus Cas9 (*PGRMC1*^{-/-}; n = 877). Electroporation was performed using a NEPA21 System with a concentration of 200:100 ng/μl Cas9 mRNA:sgRNA. Blastocyst stage embryos were harvested from culture on day 7 after IVF and loaded in groups of five in holding medium into 0.25 ml French straws for embryo transfer. A group of non-lactating, crossbred beef cows (n = 27) was used as recipients. Cows were subjected to an ovulation synchronization protocol in five separate cohorts (n = 5-6 per cohort) and randomly assigned to receive either *PGRMC1*^{+/+} or *PGRMC1*^{-/-} embryos. Seven days after presumptive ovulation, groups of five embryos of the assigned treatment group were transferred separately into the ipsilateral and contralateral uterine horns of recipients having a corpus luteum confirmed by transrectal ultrasonography (n = 11 and 13 cows for *PGRMC1*^{+/+} and *PGRMC1*^{-/-}, respectively). On day 14 after presumptive ovulation, conceptuses were recovered following collection of the reproductive tract after slaughter (n = 2 cohorts) or via non-surgical embryo recovery procedures (n = 3 cohorts). Regardless of the method of embryo recovery, each uterine horn was flushed separately using commercial embryo flushing medium. Recovered conceptuses were measured to determine conceptus length, and were classified by stage (spherical, ovoid, tubular, or filamentous). Data was analyzed using a generalized linear mixed model. There was a trend (P = 0.097) for conceptus recovery rate to be lower for cows receiving *PGRMC1*^{+/+} embryos as compared to *PGRMC1*^{-/-} embryos (41/90 = 45.6% vs. 52/110 = 47.3%). Conceptus length did not differ (P = 0.343) between *PGRMC1*^{+/+} and *PGRMC1*^{-/-} groups (8.8 ± 6.6 mm vs 16.9 ± 5.9 mm, respectively). There was no effect of *PGRMC1* ablation on the proportion of conceptuses that were either spherical and ovoid (P = 0.474; *PGRMC1*^{+/+}: 50.6 ± 17.4% vs. *PGRMC1*^{-/-}: 32.0 ± 16.1%) or tubular and filamentous (P = 0.453; *PGRMC1*^{+/+}: 49.4 ± 17.4% vs. *PGRMC1*^{-/-}: 68.0 ± 16.1%). None of the endpoints analyzed were affected by uterine horn (ipsilateral vs. contralateral) or the interaction between uterine horn and treatment. Results of the present experiment indicate that peri-implantation conceptus survival in cattle is not impaired by zygotic ablation of *PGRMC1*. While not significant, numeric differences in both conceptus length and stage of development suggest that *PGRMC1*^{-/-} conceptuses may exhibit greater trophoblast proliferation. Further research is necessary to confirm these findings and to assess whether zygotic ablation of *PGRMC1* affects subsequent pregnancy establishment and implantation.

BMP4 Induces Post-Hatching Expansion of Bovine Blastocysts in vitro

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Abstract Text

Bovine embryos exhibit a unique developmental characteristic in which blastocysts remain free-floating and undergo elongation after hatching prior to implantation into the endometrium, a process that cannot yet be fully recapitulated in vitro. Bone morphogenetic protein (BMP) signaling has been shown to regulate the development of extraembryonic lineages, including the trophectoderm and primitive endoderm, during blastocyst formation. However, despite its established role at this stage, the involvement of BMP signaling in post-hatching development of bovine blastocysts remains unclear. In this study, we aimed to investigate the role of BMP signaling in regulating cellular processes following bovine blastocyst formation and hatching in vitro. Cumulus-oocyte complexes were collected from the ovaries of cows of various breeds obtained from a local abattoir and subjected to in vitro maturation and fertilization, followed by removal of cumulus cells and culture to day 8 to obtain blastocysts (day 0 = day of insemination), according to established protocols. Expression profiles of BMP signaling components in bovine blastocysts were analyzed by immunofluorescence for BMP4, BMPR1A, BMPR1B, and BMPR2. The functional role of BMP signaling was examined by culturing blastocysts for 4 days in the presence of recombinant BMP4 or the BMP receptor inhibitor dorsomorphin, followed by blastocyst diameter measurement for size analysis and immunofluorescence analysis of lineage markers, including NANOG (epiblast), CDX2 (trophectoderm), and SOX17 (hypoblast), to assess lineage composition.

Bovine blastocysts expressed BMP4, BMPR1A, BMPR1B, and BMPR2 proteins. BMP4, BMPR1A, and BMPR1B were broadly distributed across all blastocyst lineages, whereas BMPR2 expression was restricted to the trophectoderm. Functionally, modulation of BMP signaling did not affect the hatching rate. BMP4 treatment significantly promoted blastocyst expansion. Lineage composition analysis indicated that the three blastocyst lineages were present without apparent changes in overall lineage proportions following BMP4 treatment. In contrast, inhibition of BMP signaling by dorsomorphin abolished blastocyst expansion and resulted in a marked reduction in the epiblast cell population.

Previous studies have shown that BMP signaling promotes differentiation of embryonic stem cells toward the trophoblast lineage, while studies in mice have demonstrated essential roles for BMP signaling in endoderm formation at the primitive streak and in primordial germ cell specification at the posterior epiblast. However, whether similar functions of BMP signaling are conserved in bovine embryos, particularly during post-hatching development, remains largely unknown. Collectively, these findings highlight a previously unappreciated role of BMP signaling in regulating blastocyst expansion and epiblast maintenance during post-hatching early embryonic development in cattle.

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Influence of pH Control During in vitro Maturation on Bovine Blastocyst Development

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Abstract Text

During in vitro maturation (IVM), cumulus-oocyte complexes (COCs) are typically incubated under 5% CO₂ to maintain a pH of 7.2 to 7.4. The objective was to assess the effect of different IVM buffering systems on embryonic development compared with no external buffering. The maturation medium was TCM-199 supplemented with 10% fetal bovine serum, sodium pyruvate, L-alanyl-L-glutamine, penicillin-streptomycin, FSH and EGF. One ml of maturation medium was added to 1.5-mL polystyrene tubes for each of four treatment groups in a 2 x 2 arrangement with main effects of 0 or 10 mM HEPES, pH 7.4 and with or without gassing the tube with a 5% CO₂ and 5% O₂ mixed gas. Tubes were placed in a portable incubator for maturation and kept at 38.5 °C. Groups were: maturation medium only (O), O + HEPES-TALP (OH), O + gas (OG) and O + H + gas (OHG). Up to 30 COCs were added per tube and matured for 22 h. On average, 112 COCs per group per replicate were used across five replicates. pH was measured 30 min after tubes were placed in the incubator (T1) and at the beginning (T2) and end (T3) of the maturation period in tubes with and without COCs. A pool of three bulls was used for in vitro fertilization (Day 0). After ~17 h, putative zygotes were cultured in groups of up to 30. Cleavage rate (cleaved/cultured) and blastocyst rate (blastocysts/cleaved) were evaluated on Days 3.5 and 7.5, respectively. Data were analyzed using the GLIMMIX procedure in SAS for mixed-effects linear and logistic regression models. There was a significant increase in pH from T1 to T3 ($p < 0.01$). At the end of maturation, a significant treatment effect was observed ($p < 0.01$) for tubes without COCs, with group O exhibiting the highest pH (8.24 ± 0.04) and group OHG the lowest (7.65 ± 0.04). There were no treatment effects in the presence of COCs. Nevertheless, pH differed between tubes containing COCs and those without ($p < 0.01$), with mean values of 7.45 ± 0.02 and 7.93 ± 0.02 , respectively. Treatment did not affect cleavage rate ($p = 0.33$) or blastocyst rate ($p = 0.27$), which ranged from 66-70% and 41-49%, respectively. There was a tendency ($p = 0.06$) for the percent of blastocysts classified as hatching or hatched to be greater for OHG than O ($34.9\% \pm 6.7\%$ vs $19.9\% \pm 5.1\%$). These results demonstrate that COC are capable of maintaining an adequate pH during IVM without the use of external CO₂ or HEPES-TALP. This likely reflects metabolic CO₂ production by the COCs altering the bicarbonate buffering equilibrium and contributing to acidification of the medium. Moreover, there were no major negative impacts on embryonic development by the absence of external buffering during IVM. (Support: BARD).

ATG9A and ATG9B differentially regulate distinct placental zones during development.

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Abstract Text

Autophagy is a highly conserved degradation pathway that maintains cytoplasmic homeostasis by eliminating protein aggregates and damaged organelles. This process requires the coordinated activity of autophagy-related (ATG) proteins and multiple membrane remodeling. ATG9A and ATG9B are vertebrate homologs of yeast Atg9p, the only transmembrane protein of the core autophagy pathway. While ATG9A is ubiquitously expressed and evolutionarily conserved, ATG9B shows tissue-specific expression, predominantly in the placenta, suggesting a specialized role in development of this tissue. By *in situ* hybridization on mouse placentas, we showed high expression of *Atg9b* transcript located in nuclei compared to the ubiquitous cytosolic expression of *Atg9a*. Furthermore, we microdissected the placenta, and found that *Atg9a* is mostly enriched in the labyrinth zone (LZ), the maternal-fetal exchange region, while *Atg9b* is highly expressed in the junctional zone (JZ), the endocrine layer of the placenta. Using a hypomorph mouse model carrying a conditional null/reporter LacZ allele for *Atg9b* (*Atg9b^{TX}*), we observed that male *Atg9b^{TX/TX}* placentas showed disrupted JZ histoarchitecture at E14.5 and significantly reduced placental weight at E18.5. On the other hand, using a CMV-Cre strategy, we generated *Atg9a*-null mice (*Atg9a^{-/-}*). These *Atg9a^{-/-}* mice did not survive after weaning, 70% of fetuses were nonviable at E14.5, and placental efficiency was already decreased at E12.5. Histological evaluation revealed that the LZ was the most affected region. Immunofluorescence for maternal (MCT1) and fetal (MCT4) transporters confirmed LZ structure impairment, particularly in syncytiotrophoblast II cells on the fetal side. Immunoblot analysis revealed no change in LC3B-II/I ratio but a significant accumulation of the autophagy cargo receptor SQSTM1/p62 in both sexes, consistent with impaired autophagic flux in the absence of *Atg9a*. Notably, loss of *Atg9a* was not compensated by *Atg9b* (and vice versa), indicating non-redundant, zone-specific functions in placental development. Our results propose ATG9 as a significant gene for a proper placental development and reveal that *Atg9b* fulfills a distinct and still undefined role in placental biology. Elucidating these unique contributions will improve our understanding of how autophagy orchestrates placental development and function, and how its dysregulation may contribute to adverse pregnancy outcomes.

The Transcription Factor Runx1 in Endometrial Stromal Cells Regulates Critical Pathways that Govern the Trafficking and Differentiation of Uterine Natural Killer Cells during Pregnancy

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Abstract Text

After embryo implantation, the uterine microenvironment undergoes dramatic changes essential for supporting fetal growth until parturition. These adaptations include increased uterine stromal differentiation, angiogenesis, and immune cell trafficking. Uterine natural killer (uNK) cells constitute the majority of immune cells in the pregnant uterus. During pregnancy, tissue-resident NK cells undergo directed expansion into a specific subtype that is phenotypically distinct from circulating NK cells. Multiple studies have highlighted the supportive roles of uNK cells, including spiral artery remodeling, angiogenesis, and trophoblast recognition. However, the specific maternal signals that drive uNK cell transformation during pregnancy remain largely unknown. Our lab has developed a mouse model, Runx1^{d/d}, in which the Runx1 gene is conditionally deleted in uterine endometrial stromal cells. Runx1^{d/d} mice exhibit severe infertility compared with the relevant controls (Runx1^{f/f}). Phenotypic analysis across multiple days of pregnancy shows that Runx1^{d/d} uteri have impaired uterine angiogenesis, trophoblast differentiation, and spiral artery remodeling. To understand the molecular mechanisms underlying the phenotype, histological analysis of uNK cells in these mutant mice was also performed. Our results indicate that Runx1^{d/d} mice have higher numbers of uNK cells accumulating in the decidua. These uNK cells are smaller, less granular, and in a state of sustained proliferation. Bulk RNA sequencing was performed on uNK cells from Runx1^{d/d} and Runx1^{f/f} mice to uncover differences in their functional properties. Preliminary sequencing results indicate that uNK cells from Runx1^{d/d} mice exhibit reduced expression of key factors required for angiogenesis, extracellular matrix remodeling, and interferon- γ signaling, all of which are necessary for uterine adaptations to support pregnancy. Genes involved in NK cell maturation, such as Eomes, are also downregulated in Runx1^{d/d} uNK cells, providing a potential explanation for their functional impairment. Furthermore, stromal signaling molecules, such as Cxcl12, Cxcl14, and Il15, which are known to direct NK cell trafficking and differentiation, are differentially regulated in Runx1^{d/d} decidual tissue. Collectively, these findings reveal a key mechanistic role of stromal Runx1 in directing the uNK cell phenotype during pregnancy. [Supported by the NICHD/NIH R01 HD090066 and R21 HD109726 to ICB and MKB]

Investigating How P-bodies Mediate Trophoblast Differentiation

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Abstract Text

Multinucleated syncytiotrophoblasts (STB) form the outer trophoblast bilayer at the maternal blood interface to support hormone exchange and nutrient absorption. Throughout pregnancy, the inner cytotrophoblast layer fuses into the syncytia to continuously replenish the syncytial layer. Progenitor cytotrophoblasts must transition from a stem-like proliferative state into the high specialized syncytial cell fate. Our preliminary data indicates that processing bodies, or P-bodies, may post-transcriptionally regulate cytotrophoblast differentiation into STB. We hypothesize that P-bodies sequester cell fate transcripts in progenitor cytotrophoblasts as a mechanism of differentiating cytotrophoblasts towards the syncytiotrophoblast lineage. Recent work in P-bodies, demonstrates that P-bodies sequester key developmental transcripts and that P-bodies prevent translation through phase-separated condensate formation. Utilizing an inducible CRISPRi knockdown system against DDX6, a protein required for P-body formation, our preliminary results in BAP differentiation (BMP4, TGF β inhibitor A83-01, and FGF2 inhibitor PD173074) indicate that sustained P-body dissolution increases differentiation into a mature population of STB. Initial qPCR data in *DDX6* knockdown trophoblasts show increased levels of syncytiotrophoblast transcripts *ERVW-1* ($p < 0.05$). Moreover, we observed increased fusion of cells by fusion index ((number of nuclei within syncytial plaques - number of syncytial plaques)/total number of nuclei) ($p < 0.05$), a measure of mature STB. Based on these data, we hypothesize that in trophoblasts, P-bodies sequester RNA transcripts relevant to mature placental cell fate. We further hypothesize that manipulating biomolecular condensate formation will modulate regulation of placental development, causing significant changes to the cell fate decisions in trophoblast subtypes. I aim to further characterize the phenotypic outcomes of P-body dissolution through sequencing of P-body enriched transcripts, quantification of cell fusion and hormone secretion, all hallmarks of differentiated syncytiotrophoblasts.

Maternal Obesity Induces Inflammatory and Signaling Network Expansion in the Junctional Zone and Suppresses Proliferative Programs in the Labyrinth

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Abstract Text

Maternal obesity is strongly associated with placental dysfunction contributing to pregnancy complications including preeclampsia and altered fetal developmental programming. While most studies focus on late-gestation outcomes, there remains a critical gap in understanding how maternal obesity alters the developmental trajectory of key placental cell populations, including trophoblasts, extra-embryonic mesoderm-derived endothelial cells, and maternal immune cells. Therefore, we tested the hypothesis that maternal diet-induced obesity disrupts placental development by reprogramming compartment-specific transcriptional and signaling networks. Female C57BL6/J mice were assigned to normal diet (ND) or high-fat-high-sugar diet (HFHS) before mating. At embryonic day 12.5, placentas were collected and labyrinth (LAB) and junctional zone (JZ) tissues separated by microdissection. Total RNA was extracted, ribosomal RNA depleted, and sequencing libraries prepared using the IDT xGen Broad-Range RNA Library Kit. Libraries were indexed, quality-controlled, and sequenced on the Element Aviti24 platform (paired-end 75 bp) to generate high-depth transcriptomic datasets. Scripts to analyze RNA-seq data were generated using Copilot, and run using codes available at the UNL Holland Computing Center. Reads were adapter-trimmed, aligned to the mouse reference genome, and quantified to generate gene-level count matrices. Differential expression and normalization were performed using DESeq2, including variance stabilizing transformation for downstream analyses. HFHS dams had increased fasting glucose, inguinal fat mass, and body weight ($P < 0.01$) confirming the metabolic phenotype. Total weight of HFHS-derived placentas was modestly decreased, while fetal weight and fetal-to-placental ratio were not different, suggesting early placental adaptations without overt fetal growth restriction. Principal component analysis (PCA) and hierarchical clustering demonstrated robust segregation of JZ and LAB transcriptomes, with tissue identity accounting for approximately 85% of total variance. However, this was attenuated in the HFHS placentas. In ND placentas, fetal sex contributed to transcriptome variation within both compartments, whereas this sex-dependent clustering was lost in HFHS placentas, indicating disruption of sex-specific regulatory programs. Gene ontology (GO) and Ingenuity Pathway Analysis (IPA) confirmed expected tissue biased gene expression in ND placentas, with the JZ enriched for immune-related processes (cytokine signaling and leukocyte activation) and the LAB enriched for cell cycle progression, DNA replication, and metabolic function. In HFHS placentas, this organization was preserved but functionally reprogrammed. The HFHS JZ had marked amplification of inflammatory and stress-responsive pathways, including cytokine signaling, neutrophil degranulation, cytokine storm and phagosome pathways, alongside activation of TLR signaling (*Tlr2/4/7/8*), chemokines (*Ccl2*, *Ccl5*), and macrophage-associated genes (*Nos2*, *Csf2*). This was coupled with strong enrichment of extracellular matrix remodeling and fibrosis pathways (*Mmp2/7/8/13*, collagens, *Tgfb2/Tgfb2*), and expansion of GPCR and PI3K signaling networks, including broad activation of G-protein coupled receptors, cAMP/CREB signaling, and PI3K isoforms, consistent with signaling rewiring and inflammatory amplification. In contrast, the HFHS LAB retained enrichment for cell cycle-associated pathways but there was coordinate suppression of core proliferative and biosynthetic programs. Downregulated pathways included cyclins and CDKs (*Ccna22*, *Ccnb1/2*, *Cdk1/2*), mitotic regulators (*Cdc20*, *Plk1*), DNA replication machinery (MCM complex, *Rfc4*, *Pra2*), and chromatin/epigenetic regulators, alongside loss of stemness-associated genes (*Nanog*, *Sox2*, *Sall4*). These changes reflect attenuation of DNA replication, chromatin organization, and developmental plasticity. Collectively, maternal HFHS exposure drives asymmetric placental reprogramming, with amplified immune, inflammatory, and tissue remodeling pathways in the JZ and suppression of proliferative and biosynthetic programs in the LAB. This reduced compartmental distinction, suggests a shift toward an inflammatory, stress-responsive state. These findings define early molecular mechanisms linking maternal metabolism to impaired placental development.

Exploring the Effect of Progesterone Signaling in Ciliated Epithelial Cells of the Oviduct on the Fertility of Female Mice

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Abstract Text

Motile cilia lining the oviduct epithelium generate directional fluid flow that supports oocyte pickup, fertilization, and embryo movement toward the uterus. Disruption of ciliary beat frequency (CBF) may impair embryo transport and increase infertility risk. Progesterone signaling has been implicated in regulating oviductal physiology, however, its direct role in maintaining ciliated epithelial cell function remains poorly understood. This study investigates the effect of progesterone receptor (Pgr) deletion in oviductal ciliated epithelial cells on fertility and ciliary motility in female mice. A conditional knockout mouse model was generated using *Foxj1^{Cre/+;Pgr^{fl/fl}}* mice to produce ciliated cell-specific progesterone receptor knockout females (ciliated *Pgr^{d/d}*). We hypothesize that deletion of Pgr in ciliated epithelial cells impairs ciliary motility and reduce fertility outcomes compared with controls. Deletion of Pgr was validated using immunohistofluorescent analysis. Fertility was assessed through a six-month breeding trial in which females were paired with proven fertile males, and litter number and pup number were recorded. Embryos were collected at days 3.5 post coitus. Oviduct tissues were collected for histological analysis, while ciliary beat frequency was measured ex vivo using high-resolution live imaging under physiological temperature conditions. Frequency data were quantified using Fast Fourier Transform analysis in RStudio. We found that PGR specifically deleted in the ciliated cells of ciliated *Pgr^{d/d}* oviducts. Ciliated *Pgr^{d/d}* females showed premature reproductive senescence as ciliated *Pgr^{d/d}* females became subfertile starting from the 3rd litter, and eventually sterile around the 6th litter, while the control *Pgr^{fl/fl}* mice still producing pups in their 8th litter. We found blastocysts surrounded by the cumulus cells in ciliated *Pgr^{d/d}* uteri, while all blastocysts from controls were denuded of cumulus cells. The ciliary beat frequency of ciliated epithelial cells from ciliated *Pgr^{d/d}* oviducts were significantly lower than that of controls. We also observed an increased vacuole structures in ciliated epithelial cells in the ciliated *Pgr^{d/d}* mice. These data suggest that proper progesterone signaling is required for the maintenance of ciliary function and reproductive function in vivo.

Impact of Dietary Essential Amino Acid Balance on Pregnancy Success and the Uterine Environment in Lactating Dairy Cows

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Abstract Text

Mammals cannot synthesize essential amino acids (AAs) *de novo* and rely on dietary intake to support critical biological functions, including reproduction. Because AAs support key metabolic pathways during early pregnancy, we hypothesized that balancing essential AAs in the diet of lactating dairy cows would improve pregnancy outcomes and modulate uterine gene expression. Eighty-five multiparous Holstein cows were assigned at calving to either a high rumen degradable protein diet (HRDP; 12% RDP, 3.8% digestible rumen undegradable protein) or an amino acid-balanced diet (BAA; 10.2% RDP with increased digestible rumen undegradable protein, balanced for arginine, histidine, leucine, isoleucine, lysine, and methionine). Diets were isoenergetic, formulated to meet lactation requirements, and fed from calving until 30 days post-artificial insemination. Pregnancy was achieved by using a standard ovulation synchronization protocol followed by timed artificial insemination and confirmed by ultrasonography at day 30. Endometrial cytology samples collected by uterine cytobrush one week before timed artificial insemination were used for transcriptomic analysis. Pregnancy rates did not differ between diets (HRDP: 41.8 ± 7.8% vs BAA: 36.9 ± 7.4%; $P = 0.65$). Transcriptomic analysis revealed 57 differentially expressed genes (DEGs), with 30 upregulated and 27 downregulated in BAA cows. Upregulated genes included immune- and inflammation-related genes (*S100A8*, *DEFB4A*, *IL17A*, *IL36A*), genes involved in lipid metabolism (*ALOX12B*, *CYP2W1*), oxidative stress response (*GSTA1*), and calcium signaling (*TRPA1*, *CAMK2A*). Downregulated genes included *CITED2*, a transcriptional regulator of placental development, as well as reproduction-associated genes *PTGFR*, *CXCL14*, *C4BPA*, and *SALL4*, a key regulator of embryonic stemness. Together, these findings demonstrate that balancing AAs remodeled the uterine transcriptome across immune, metabolic, and reproductive gene networks. These results highlight the sensitivity of the endometrial environment to maternal nutritional status and suggest that further optimization of the timing, duration, and specific AAs targets of dietary balancing strategies may be necessary to translate molecular endometrial responses into improved reproductive outcomes. This work was supported by the Dairy Innovation Hub, University of Wisconsin-Madison.

Oviductal Assembloids: An *in vitro* Model to Study the Pre-implantation Embryo Environment

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Abstract Text

Instances of infertility are increasing at the same rate in low-, middle-, and high-income countries and currently one in six people are estimated to experience infertility at some point in their life. Assisted reproductive technologies (ARTs) are used globally to combat this. However, in the U.S., the success rate of ARTs is only 30-40%. While most babies conceived via ARTs, like *in vitro* fertilization (IVF), are healthy, ART-derived singletons have higher rates of adverse perinatal outcomes and epigenetic modifications related to imprinting disorders compared to those naturally conceived. This indicates a need for optimizing the current culture conditions employed in the *in vitro* production of embryos. The oviduct (Fallopian tube) is the site of fertilization and pre-implantation embryo development. During ARTs, like IVF, the oviduct is completely bypassed and in culture, embryos often take longer to develop to blastocyst stage than *in vivo*. Oviductal epithelial organoids are 3D structures that mimic their tissue of origin, providing a biomimetic approach to model oviductal epithelial-stromal interactions. We aim to develop an *in vitro* model of the oviduct to investigate the impact of the microenvironment on embryo development and determine factors that could be added to culture media. Here we present a novel method that generates apical-out oviduct assembloids, consisting of ciliated and secretory epithelial cells on the exterior and stromal cells inside the structures. The inclusion of oviductal stromal cells into this 3D co-culture approach not only facilitates paracrine signaling between both cell types but also effectively models intrinsic cell-cell communication, which may be necessary for establishing a microenvironment conducive to optimal embryo development. By using low-attachment culture plates, free-floating assembloid structures can be generated from both human and mouse primary cells. With the apical side facing outward, secretions are present in the media, allowing direct access to the apical cellular surface. Cell types in assembloids were confirmed by immunofluorescence for Acetylated- α -Tubulin (ciliated epithelial cells), Oviductal Glycoprotein 1 (secretory epithelial cells), and Vimentin (stromal cells). After treatment with estrogen, mouse and human oviduct assembloids did not show an increase in cell death or proliferation, similar to what has been observed *in vivo* in mice. Cilia on the exterior of the mouse and human assembloids have similar length to *in vivo* counterparts. Importantly, functional ciliary beat activities are retained in both mouse and human assembloids. Our immediate goal is to use human and mouse oviductal assembloids to assess the impact on mouse embryo developmental potential. The findings from this study will lead to a better understanding of the oviduct microenvironment required for normal preimplantation embryo development and can be clinically translated to ART applications.

Investigating the Role of Hedgehog Signaling on Myometrial Physiology During Gestation

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Abstract Text

Preterm birth, defined as delivery before 37-weeks of gestation, affects ~10% of pregnancies in the U.S. and increases the risk of maternal morbidity. While treatments have advanced in supporting premature neonatal growth, there is still a critical barrier in developing effective treatments to prevent pre-term birth. As premature contractility is the primary driver of pre-term labor, it has been an active area of research; however, fewer studies have investigated the molecular regulators of myometrial quiescence in earlier pregnancy. Although we know that the human uterus experiences a 24-fold increase in mass during pregnancy, the regulation of uterine smooth muscle hyperplasia, hypertrophy, and differentiation during gestation remains unclear. In the cycling uterus, Hedgehog (HH) signaling regulates myometrial homeostasis. When HH signaling ablated in the uterus (*Smoothed* cKO), myometrial fibers are enlarged, suggesting disrupted myometrial homeostasis. Data from other fields suggest that HH signaling has an important role in regulating visceral and vascular smooth muscle differentiation and homeostasis. However, the role of HH signaling on myometrial function during gestation has yet to be studied. Additionally, the ligand driving myometrial signaling in the cycling and pregnant uterus is completely unknown. While epithelial-derived IHH drives endometrial remodeling, preliminary spatial-transcriptomic data from our lab shows that DHH was expressed near myometrial blood vessels in the cycling uterus. We hypothesized that DHH drives HH signaling in the myometrium during early gestation, promoting differentiation and quiescence. To first assess when HH signaling is active during gestation, we collected myometrial samples throughout gestation (daily timepoints between embryonic day (E) 4.5-18.5). Overall, we found that mRNA and protein expression of HH targets (*Gli1*, *Gli2*, *Gli3*) were downregulated by E12.5. Additionally, LacZ staining (using the *Gli1-LacZ* mouse model) confirmed that *Gli1* expression became sparse in myometrial cells by E12.5. Next, we wanted to determine if *Dhh* is required for HH signaling. To investigate this, we collected uterus and myometrial samples from *Dhh* KO; *Gli1-LacZ* mice during diestrus, when HH signaling is upregulated. Interestingly, while we found that HH signaling is reduced in the mesometrial myometrium, the remainder of the myometrium remains *Gli1*+ in *Dhh* KO mice. However, further work is required to determine if DHH is required to drive HH signaling in the myometrium during proestrus/estrus, when HH signaling is downregulated in the endometrium but not in the myometrium. Overall, we conclude that HH signaling may have an important role in regulating myometrial quiescence early in gestation. Future studies will focus on elucidating the role of HH signaling on smooth muscle cell proliferation, fiber growth, and quiescence, in addition to investigating the role of DHH/other HH ligands on myometrial quiescence during gestation.

Neonatal Outcomes of Biological Extremes in Porcine Uterine Crowding

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Abstract Text

Ongoing and intensive selection for greater litter sizes without a corresponding change in uterine capacity has increased intrauterine crowding in modern production sows. Such overcrowding restricts placental development, limits nutrient availability, and increases the incidence of intrauterine growth restriction (IUGR). The canonical IUGR phenotype is a byproduct of non-allometric growth, where the growth of the brain is favored at the expense of other organs. The present study aimed to assess the sex specific phenotypic and morphometric consequences of biological extremes in uterine crowding. To this end, commercial crossbred gilts were allocated to one of two surgical interventions. Unilateral hysterectomy-ovariectomy (overcrowded, n=7) decreased available uterine space by half while maintaining effective ovulation rate, thereby exacerbating overcrowding. In contrast, unilateral oviductal-ligation (uncrowded, n=6) decreased the effective ovulation rate by approximately half to minimize crowding. Following a recovery period, dams were artificially inseminated using pooled semen from Duroc sires, and following confirmation of pregnancy, gestated to term and farrowed. At 18 h after birth, the resulting piglets (n=104) were photographed for morphometric evaluation, then euthanized to collect detailed body and organ weights. Morphometric assessment included evaluation of left and right lateral profile images and an image of the dorsal view of the head. These images were analyzed through a semi-automated pipeline in ImageJ. Phenotypic data was analyzed in R using Pearson's correlation and linear mixed-effects models with surgical intervention and sex as fixed effects. In overcrowded litters, piglet body weight was significantly reduced ($p < 0.01$), while brain-to-liver weight ratio (BRN:LVR) was elevated ($p < 0.01$). These findings are consistent with conventional definitions of IUGR in pigs. Within-sex comparisons indicated a significant difference between overcrowded and uncrowded male piglets ($p < 0.05$), whereas this was muted in females ($p > 0.05$). However, the interaction term was not significant. Similarly, when evaluating overall brain weight compared to body weight, restricted piglets had a larger brain-to-bodyweight ratio compared to unrestricted piglets ($p < 0.05$), and although the interaction of surgery and sex was not significant, overcrowded males were more affected ($p < 0.05$) than overcrowded females ($p > 0.05$) than their uncrowded counterparts. Further morphological analyses revealed a significant increase in the ratio of skull length to body length ($p < 0.01$) in piglets from crowded uteri, suggesting non-allometric growth. Although the interaction of surgery and sex was not significant, pairwise comparisons suggest a significant increase in overcrowded male piglets ($p < 0.05$) that was lost in overcrowded females ($p > 0.05$). Unsurprisingly, many other phenotypic ratios were negatively correlated with both bodyweight (BW) and BRN:LVR, including height (BW, $R^2 = 0.72$; BRN:LVR, $R^2 = 0.68$, $p < 0.05$), girth (BW, $R^2 = 0.91$; BRN:LVR, $R^2 = 0.81$, $p < 0.01$), and crown-rump-length (BW, $R^2 = 0.79$; BRN:LVR, $R^2 = 0.60$, $p < 0.001$). Collectively, the results of this study demonstrate that biological extremes in porcine uterine crowding will drastically alter neonatal phenotypes. This work is supported by the Foundational and Applied Science Program, project award no. 2026-67015-45761, from the U.S. Department of Agriculture's National Institute of Food and Agriculture.

Characterization of *NRP2* Expression in Human Endometriosis, Adenomyosis, and Ovarian Cancer Cell Lines

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Abstract Text

Endometriosis is a gynecological disease affecting 10% of women and adolescent girls worldwide. Endometriosis is defined by the presence of endometrial-like tissue outside the uterine cavity. Adenomyosis is a type of endometriosis in which endometrial-like tissue grows within the uterine myometrium. Ovarian endometriomas, deep infiltrating cystic lesions of the ovary, represent the most common typology of endometriosis, affecting up to 50% of women with endometriosis. Women with endometriomas exhibit a 19-fold increased risk of type I ovarian cancer, such as clear-cell or endometrioid. Our previous work (GSE23339) showed neuropilin-2 (*NRP2*) was 2.31-fold upregulated in endometriomas compared to endometrium from women without endometriosis (Student's t-test, $p=9.96 \times 10^{-6}$, $n=20$). *NRP2* is a transmembrane co-receptor that integrates growth factor and microenvironmental signals, including vascular endothelial growth factor C- and D-mediated pathways, to regulate key cellular processes such as survival, migration, and angiogenesis. The role of *NRP2* in endometriosis, adenomyosis, and ovarian cancer remains unknown. **Objective:** 1) To evaluate *NRP2* expression in publicly available transcriptomic datasets for human tissues and cell lines; 2) To determine immortalized cell lines for future studies. Expression of *NRP2* was queried across publicly available datasets. *NRP2* was compared between endometriomas and endometrium. Using the QIAGEN OmicSoft platform, *NRP2* was evaluated using the Cancer Cell Line Encyclopedia RNA-seq datasets, restricted to ovarian cancer cell lines. *NRP2* was quantified across human cell lines, including endometriosis (12Z), adenomyosis (tAEC21), and available ovarian cancer cell lines, by qPCR. Analysis of the GSE7305 dataset demonstrated a 3.42-fold increase in *NRP2* in endometrioma compared to matched eutopic endometrium (limma-moderated t-test, $p=2.63 \times 10^{-10}$, $n=20$). Analysis of the GSE37837 dataset demonstrated a 2.2-fold increase in *NRP2* expression in endometrioma compared to matched eutopic endometrium (limma-moderated t-test, $p=0.03$, $n=18$). Evaluation of *NRP2* within the EndometDB (GSE141549) showed higher median *NRP2* expression in endometrioma ($n=24$, log2fold-change median: 7.96 (interquartile range (IQR) 7.85-8.23) than control endometrium ($n=36$, log2fold-change median: 6.57 (IQR: 6.45-6.82) or endometriosis endometrium ($n=88$, log2fold-change median: 6.70 (IQR 6.5-6.91). OmicSoft analyses of *NRP2* across ovarian cancer cell lines demonstrated variable *NRP2* expression ($n=47$, 0.05 to 75.87). Independent validation by qPCR showed no statistical difference between the endometriosis, 12Z (1.06 ± 0.45), and adenomyosis-derived, tAEC21 (0.88 ± 0.46), cells (unpaired, two-tailed t-test; $n \geq 3$). Undifferentiated ovarian cancer cell line, A2780, showed decreased expression (0.30 ± 0.13 ; $p < 0.0001$; $n \geq 3$). Compared to 12Z. The ovarian clear-cell carcinoma cell lines showed markedly higher *NRP2* levels (OVTOKO: 3.82 ± 1.63 ; $p < 0.01$; KK: 19.33 ± 6.65 $p < 0.001$; ES2: 26.54 ± 3.77 ; TOV21G: 34.75 ± 4.27 ; and OVISE: 40.56 ± 15.21 ; $p < 0.0001$; $n \geq 3$). In contrast, the OCCC cell line RMG-1 showed lower *NRP2* expression (0.26 ± 0.11 , $p < 0.0001$, $n \geq 3$) relative to 12Z. The endometrioid ovarian cancer cell line, IGROV1, displayed intermediate expression (5.40 ± 0.53 ; $p < 0.0001$; $n \geq 3$). Interestingly, the non-serous, endometrioid-like line SKOV3 also showed low *NRP2* expression (0.03 ± 0.01 ; $p < 0.0001$; $n \geq 3$), whereas its more aggressive derivative SKOV3ip1 demonstrated substantially higher expression (86.36 ± 6.00 , $p < 0.0001$, $n \geq 3$). In summary, analyses of multiple endometrioma datasets revealed increased *NRP2* expression. By independent qPCR, *NRP2* expression was markedly higher in ovarian clear-cell lines than in endometriosis-derived cell lines. *NRP2* expression was higher in the more aggressive SKOV3ip1 cell line, suggesting a functional role. Future studies will focus on independent validation of *NRP2* in control, endometrioma, and ovarian cancer samples and on the molecular and cellular functions of *NRP2* in both endometriosis and ovarian cancer.

Multimodal ultrasound imaging in the evaluation of endometrial receptivity and prediction of pregnancy outcome

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Abstract Text

Purpose: Endometrial receptivity is crucial for the establishment of successful implantation in assisted reproductive technology (ART). This study is to identify multiple endometrial receptivity related factors by applying non-invasive, repeatable multimodal ultrasound methods to predict pregnancy outcome in ART.

Material and Methods: Forty-one women scheduled for hormonally prepared frozen embryo transfer (FET) in Wuhan Union Hospital between August 2024 and October 2024, in whom three-dimensional transvaginal sonography and real-time shear wave elastography were performed in the receptive phase of the endometrium before embryo transfer. According to whether the clinical pregnancy was successful or not, the participants were divided into the pregnancy group (n = 28) and non-pregnancy group (n = 13), and the differences in endometrial thickness, endometrial echo pattern, endometrial volume, endometrial elasticity, uterine peristaltic wave frequency, endometrial flow index, uterine spiral artery, the endometrial-myometrial interface thickness and elasticity, as well as clinical information including age and embryo quality were compared between the two groups. Furthermore, the multivariate logistic regression model to predict pregnancy outcome was constructed based on endometrial features.

Results: Pregnant women had higher endometrial volume, endometrial elasticity, and endometrial-myometrial interface elasticity, but lower uterine spiral artery resistance and endometrial-myometrial interface thickness, and younger age compared with nonpregnant volunteers ($p < 0.05$). Consequently, the pregnancy outcome prediction model was developed with its value of area under the receiver operating curve up to 0.85 for predicting pregnancy by using endometrial volume, uterine spiral artery, and endometrial-myometrial interface thickness and elasticity.

Conclusions: Multimodal ultrasound imaging is a promising non-invasive tool for evaluating the condition of endometrium. The pregnancy prediction model established on endometrial factors can give clinicians a quantitative evaluation and a real-time screen of the uterus condition as well as optimal treatment recommendations for infertility-related patients.

Intermediate Conductance Calcium Activated Potassium Channel is Critical to Balance Trophoblast Development During Human Placentation.

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Abstract Text

Successful outcome of human pregnancy relies on proper placentation that depends on precisely controlled cytotrophoblast (CTB) proliferation and differentiation to syncytiotrophoblasts (STB). The STB cells are crucial to ensure transfer of nutrients between the mother and the fetus. Along with nutrient transport, another major function of STB cells is to regulate ion transport. Ion channels are important in proliferation and differentiation of cells. The importance of potassium ion channels that regulate potassium ion transportation in trophoblast cells during human placentation is poorly defined. In this study, we focused on the importance of the intermediate conductance calcium activated potassium channel (IK) in CTB proliferation and STB differentiation processes. We found that *KCCN4* gene that encodes for IK channel is expressed in both CTBs and STBs within a 1st trimester human placenta with a higher expression in STBs. In the cell culture model system, the expression of *KCNN4* recapitulates the in-vivo expression, supporting the relevance to do functional studies in the cell culture system. Genetic depletion of IK channel using lentiviral mediated shRNA delivery as well as pharmacological inhibition of IK channel reduces human trophoblast stem cell (hTSC) proliferation analyzed by 5-bromo-2'-deoxyuridine (BrdU) incorporation assay. Genetic depletion reduces expression of key hTSC proliferation regulating genes *ELF5*, *ITGA6*, *GATA2*, *GATA3*, *TEAD4* and *TP63* and impair self-renewal capability as seen in organoid formation, indicating the importance of this channel in stem cell maintenance. Interestingly, gain-of-function of IK channel also reduces hTSC proliferation and it accelerates STB differentiation process. In hTSCs, gain of function causes downregulation in expression of key stem markers – *ELF5*, *ITGA6*, *GATA2*, *GATA3*, and *TP63*. Mechanistic analyses revealed that the activation of IK channel in hTSCs reduces expression of several WNT ligands. FLUX-OR potassium ion channel assay confirms that the gain of function of IK channel using an agonist opens a potassium channel. Furthermore, patch clamp electrophysiology shows membrane potential reduction with the agonist in hTSCs, indicating the expected activity of the agonist. In STB differentiation process the activation of IK channel promotes expressions of STB -specific genes, retroviral genes *ERVW-1*, *ERVFRD-1*, transcription factor *GCM1* and pregnancy hormone hCG-beta, highlighting a direct link between IK channel activity and the molecular program of syncytialization. As STBs are fused cells, using split GFP that self-reassociates during trophoblast fusion confirms that the gain of function of IK channel enhances the fusion index. Furthermore, using genetically encoded calcium indicator – GCaMP we show that STBs have higher intracellular calcium level compared to hTSCs and the level increases even more due to IK channel gain of function. Overall, our results provide a novel insight regarding human trophoblast development in which balanced function of the IK channel provides critical balance of CTB proliferation and activation of the channel promotes STB differentiation process.

Nitric Oxide Metabolic Enzymes: Expression and Regulation at the Maternal-Conceptus Interface in Pigs

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Abstract Text

Nitric oxide (NO) is an important regulator in physiological processes in mammals. NO is generally synthesized by nitric oxide synthase (NOS) isoforms (NOS1, NOS2, NOS3) and the activity of NOS isoforms depends on the availability of L-arginine, oxygen, and NADPH. Under hypoxic conditions, NO can also be generated via the reduction of nitrite, which is catalyzed by xanthine dehydrogenase (XDH), whereas ceruloplasmin (CP) facilitates the reverse oxidation of NO. In the uterine lumen, the presence of oxygen-consuming conceptuses creates localized hypoxia in the endometrium during early pregnancy. Thus, this study investigated the expression, localization, and regulation of enzymes related to NO metabolism in porcine endometrium during pregnancy. Realtime RT-PCR showed that the expression of NOS isoforms, *NOS1*, *NOS2*, *NOS3*, and *CP* mRNAs in the endometrium was pregnancy stage-specific with lower levels during the implantation period than the later stages of gestation. The expression of *XDH* mRNA was greater during the implantation period than the estrous cycle and the later stages of gestation. Localization of enzymes related to NO synthesis and metabolism was cell-type specific within the endometrium. Enzymes related synthesis and metabolism to NO were also expressed in conceptuses during early pregnancy and in chorioallantoic tissues during mid- to late pregnancy. In endometrial explant cultures, *NOS2* and *XDH* expression was upregulated by interleukin-1 β , and *XDH* expression was increased by interferon- γ . These results showed that NO metabolic enzymes are expressed at the maternal-conceptus interface in a stage- and cell-type specific manner and the endometrial expression of *XDH* is induced by conceptus-derived signals, suggesting that stage-specific expression of NO metabolic enzymes and appropriate production of NO may play an important role in the establishment and maintenance of pregnancy in pigs.

18 β -Glycyrrhetic Acid Induces Apoptotic Cell Death in Cervical Cancer Cells with Diverse HPV-Associated Backgrounds

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Abstract Text

18 β -Glycyrrhetic acid (18 β -GA), a bioactive triterpenoid derived from licorice root, has been reported to possess anti-inflammatory and anticancer properties. However, its anticancer activity in reproductive tract malignancies, particularly cervical cancer cells with distinct human papillomavirus (HPV) status and cellular backgrounds, remains insufficiently characterized. Cervical cancer is closely associated with persistent high-risk HPV infection and represents a major malignancy of the female reproductive tract. Therefore, identifying compounds that exert consistent antitumor effects across heterogeneous cervical cancer models may provide useful insight into reproductive cancer biology and therapeutic development. In this study, we evaluated whether 18 β -GA exhibits broad cytotoxic activity and induces apoptotic responses in cervical cancer cells with diverse HPV-related and cellular characteristics. Preliminary screening was performed in multiple human cancer cell lines, including gastric, pancreatic, colorectal, lung, and cervical cancer cells, to assess the general anticancer potential of 18 β -GA. Based on these observations, detailed analyses were conducted using four cervical cancer cell lines, HeLa, SNU-17, CaSki, and SiHa, which differ in HPV status, histological origin, and genetic background. Cell viability was assessed using MTT assays, and apoptosis-related changes were examined through apoptotic population analysis, nuclear morphology, and cell cycle profiling. 18 β -GA reduced cell viability in all cervical cancer cell lines examined. Although the degree of sensitivity varied among cell types, all four cell lines showed susceptibility to 18 β -GA treatment, suggesting that its cytotoxic activity is not restricted to a single HPV-associated or cellular background. In addition, 18 β -GA increased apoptotic cell populations and induced nuclear morphological alterations consistent with apoptosis. Treatment also altered cell cycle distribution and signaling responses associated with cell survival and apoptosis, indicating that 18 β -GA may suppress cervical cancer cell growth through coordinated regulation of apoptotic signaling and cell cycle progression. Collectively, these findings suggest that 18 β -GA exerts antitumor effects across multiple cancer types, including cervical cancer cells with diverse genetic and phenotypic backgrounds. These results highlight its potential as a broad-spectrum anticancer agent and warrant further investigation into its molecular mechanisms and therapeutic applicability.

Insight Into Placenta Autophagy Regulation After Exercise and Implication to Placental Development and Fetal Growth

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Abstract Text

Physical activity stress promotes tissue remodeling and nutrient flux, allowing cells to recycle resources through the autophagy pathway. Previous human studies showed that sustained, moderate to high intensity exercise during pregnancy stimulates placental growth, increased vascularity, and morphological changes in which autophagy is involved. Given the homeostatic role of this pathway during stress and the adaptability of the placenta, we hypothesize that autophagy in this organ is upregulated in response to physical activity, resulting in altered levels of cargo and autophagosome biogenesis proteins. To test this hypothesis, adult female mice were exposed to a voluntary running wheel protocol before and during pregnancy; this group was compared to sedentary animals at the same gestational age. After monitoring running wheel activity daily, we observed that physical activity declined as pregnancy progressed, indicating a prepartum behavior guided towards ensuring offspring safety. Immunoblot analysis from placental tissue showed increased LC3II/LC3I protein ratio only in female exercised placentas when compared to their control, suggesting a sex dimorphic autophagy response. A decrease in BNIP3 was also observed, suggesting more mitophagy in the placenta. Morphological analysis of the mitochondria from the labyrinth zone revealed a decrease in mitochondria shape suggesting an increase in fission dynamics in response to exercise stress. Histological analysis revealed an area reduction of the junctional zone in male placentas exposed to exercise, and a higher deposition of glycogen positive cells compared to their controls and female placentas had higher glycogen content. To further analyze the autophagy flux, pregnant mice were dosed with chloroquine, a known autophagy inhibitor that disrupts the lysosome-autophagosome fusion, resulting in autophagosome accumulation. Expression of LC3II/LC3I protein was significantly higher only in male placentas in response to chloroquine, suggesting again a sex dimorphic response where more LC3 protein is being conjugated to the autophagosome membrane in response to exercise. Altogether, our study shows a significant role of placenta-specific autophagy in regulating fetal development during exercise. Targeting autophagy through exercise may offer therapeutic strategies to prevent placental dysfunction in pregnancy.

Adenomyosis: A case of uterine unREST

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Abstract Text

Adenomyosis is commonly seen in 20-30 % of women who are of reproductive age. However, it is not accurately known what percentage of women are affected and the number is likely higher. Adenomyosis is the presence of endometrial glands and stroma in the myometrium, however, the exact cause is not well understood. It has been proposed that endometrial invasion, tissue injury and repair, and endometrial stem/progenitor cells are involved in the pathogenesis. It is also thought that increased angiogenesis, altered cell proliferation, decreased apoptosis, impaired immune function, and altered hormone levels contribute to the pathogenesis and symptomatology. The symptoms of Adenomyosis can include abnormal uterine bleeding, pelvic pain, and infertility although some women can be asymptomatic. Hormonal management for Adenomyosis such as Selective Progesterone Receptor Modulators (SPRMs) and Gonadotropin-releasing hormone (GnRH) antagonists are available. However, these treatments only help manage symptoms, do not preserve fertility, and can only be used for a limited time. Unfortunately, one of the most frequent treatment options for adenomyosis is a hysterectomy, or full removal of the uterus. For women who wish to maintain fertility an adenomyomectomy can be performed, which is the removal of the adenomyotic tissue. However, not only are both procedures invasive in many cases, after an adenomyomectomy there is a high rate of recurrence of the disease. Therefore, adenomyosis needs to be better understood to produce new and improved treatments. RE1-silencing transcription factor (REST) is an epigenetic silencer of neuronal genes in the periphery but has been shown to act as a tumor suppressor in epithelial cells. In the uterus REST functions as a tumor suppressor and in a uterus specific knockout of murine Rest, a model for uterine fibroids, also results in an adenomyosis phenotype. We hypothesize that the loss of REST promotes adenomyosis through two of its targets, α -disintegrin and metalloproteinase-12 (ADAM 12) and matrix metalloproteinase 24 (MMP24). Our results indicate that when Rest is ablated in the uterus Adam 12 and Mmp24 are overexpressed. ADAM 12 has important roles in cell adhesion, cell migration, ECM remodeling, ectodomain shedding of cell surface receptors and in activation of signaling molecules such as Notch and EGFR. MMP24 has a role in cell-cell interactions, cell invasion, and extracellular matrix degradation. Our lab has previously shown that human uterine leiomyoma samples have down-regulation of REST and overexpression of its downstream target, ADAM 12. In addition, siRNA knock down of REST in primary myometrial cells leads to overexpression of ADAM 12. The Restfl/fl CaBP9K-icre (Restfl/fl M-iCre) mouse model, developed to study uterine leiomyomas, produces both a uterine leiomyoma phenotype and disruption of myometrial integrity. Furthermore, overexpression of ADAM12 using adenovirus in myometrial cells promotes endometrial cell migration and invasion in vitro. In addition, our lab has shown that knockdown of REST in primary myometrial cells lead to overexpression of MMP24. The Restfl/fl Ltf+/iCre mouse model produced to study the effect of REST downregulation in the endometrial epithelium produces an adenomyosis phenotype. This mouse model also produces an extreme phenotype where epithelium is seen outside of the uterus confirmed by e-cadherin immunofluorescence. Together these results and further investigation of the Restfl/fl M-iCre and Restfl/fl Ltf+/iCre mouse models can help us elucidate the molecular mechanisms responsible for Adenomyosis.

Bovine Trophoblast Organoids as a Source of Extracellular Vesicles for the Study of Placentation

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Abstract Text

The culture of trophoblasts provides physiologically relevant platforms for studying placentation. Traditional two-dimensional models often fail to reproduce the complex architecture and cellular interactions of the trophoblast. Organoid cultures are particularly promising because they allow long-term maintenance of three-dimensional structure and may provide a tractable system to investigate trophoblastic signaling. An important characteristic of trophoblast in vivo is the secretion of extracellular vesicles (EVs), nanoscale particles recognized as critical mediators of intercellular communication during early development, carrying proteins, lipids, and regulatory nucleic acids reflective of their tissue of origin. Based on these considerations, we hypothesized that bovine trophoblast organoids secrete EVs that retain trophoblastic molecular characteristics and can serve as a model for in vitro studies of trophoblast-derived signaling. This study aimed to characterize extracellular vesicles secreted by bovine trophoblast organoids while confirming organoid identity and phenotypic stability over long-term culture. Organoids were established from Angus cows synchronized with OvSynch + CIDR, and artificially inseminated. Conceptuses were recovered transcervically on days 15 and 25 post-insemination, and trophoblastic tissue was briefly digested with 0.25% trypsin. Cultures were maintained in trophoblast organoid medium at 37 °C and 5% CO₂, with sampling at 0, 3, 6, and 12 months. Conditioned media were clarified through sequential centrifugation steps to remove cells, debris, and apoptotic bodies. EVs were then isolated via ultracentrifugation. Organoid identity was assessed by morphology and gene expression, while EV characterization included nanoparticle tracking analysis (NTA) and flow cytometry. Morphological assessment confirmed trophoblastic identity. Gene expression analyses showed stable levels of PD-L1, PRP-VII, PAG11, PAG12, and PTGS2 for up to 12 months, indicating maintenance of a trophoblastic phenotype. Qualitative time-lapse microscopy revealed cellular dynamics and the formation of a three-dimensional organoid structure. NTA detected particles within the expected EV mode size (137.3 ± 10.2 nm), with an estimated concentration of 13.92 × 10⁸ particles/mL in the conditioned culture medium prior to isolation. In contrast, post-ultracentrifugation supernatants contained negligible particle counts, confirming efficient recovery of vesicles in the isolated pellet. Flow cytometry confirmed enrichment of EVs and detected canonical markers, including CD9, CD63, and ALIX, while calnexin was absent, indicating minimal contamination by cellular material. Taken together, these preliminary results indicate that bovine trophoblast organoids maintain phenotypic stability over extended culture, actively secrete extracellular vesicles that can be reliably isolated and molecularly validated, and demonstrate sustained organoid viability, preservation of three-dimensional architecture, and proliferative activity throughout long-term culture. Organoid-derived EVs provide a promising, tractable system to investigate trophoblastic communication in vitro, opening avenues to dissect molecular pathways involved in maternal-fetal signaling and potentially to inform strategies to improve reproductive outcomes in livestock.

Elucidating Senescence-Mediated Cell Death in Primary Oocytes

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Abstract Text

In females, ovarian function is sustained by a pool of dormant primordial follicles enclosing primary oocytes, known as the ovarian reserve. Depletion of this reserve occurs with age, leading to menopause. In addition, ovarian reserve loss can also be triggered by genetic, environmental, and idiopathic factors. Because of this, understanding the mechanisms underlying primordial follicle loss is imperative. The most studied type of cell death that contributes to ovarian depletion is apoptosis. Notably, this programmed cell death goes largely undetected unless the oocytes experience severe damage, such as double-strand DNA breaks. Our recent study revealed that primary oocytes undergo cellular senescence during aging in mice, evidenced by the presence of multiple well-established markers of somatic senescence and senescence-associated secretory phenotype (SASP) proteins in primary oocytes. To investigate the cellular mechanisms underlying transitions from quiescence to senescence and to cell death in primary oocytes, we employed genetic mutant mouse models and an in vitro culture model of postnatal day 4 mouse ovaries, which allows for induced oocyte senescence in a temporally controlled and synchronized manner. We found that a 24-hour treatment with low-dose Doxorubicin led to approximately 25% of primary oocytes exhibiting senescent features. These findings were further supported by enrichment of senescence-related pathways identified through Ingenuity Pathway Analysis (IPA) of single-cell RNA sequencing data from in vitro-induced ovaries. Furthermore, we found that senescence-mediated oocyte death may contribute to primordial follicle loss across multiple mouse models of primary ovarian insufficiency (POI). In summary, our study identifies a previously unrecognized mechanism of primordial follicle loss mediated via senescence in quiescent primary oocytes, providing new insight into ovarian aging and POI pathophysiology.

First Evidence of Progranulin and its Receptors Expression in the Porcine Ovarian Follicle and its Direct Effect on Granulosa Cell Proliferation

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Abstract Text

Progranulin (PGRN) is an anti-inflammatory adipokine that is known as growth factor involved in cell proliferation, survival and repair in different tissues. Besides, elevated level of PGRN is observed in the serum of women with Polycystic Ovary Syndrome (PCOS) and during obesity. However, there is no data about its presence and function in ovarian cells. This study aimed to investigate serum and follicular fluid (FF) PGRN levels, PGRN and potential receptors: *SORT1*, *EphA2* and *TNFR1/2* expression in porcine ovarian follicle during oestrous cycle as well as PGRN *in vitro* effect on granulosa cell proliferation.

Blood, FF and ovarian follicles were collected from sexually matured pigs at three phases of the oestrous cycle (days: 4-6, 10-12 and 14-18). Then PGRN level in FF and blood serum (ELISA) and both mRNA and protein expression of PGRN and mRNA expression of *SORT1*, *EphA2* and *TNFR1/2* were analyzed (RT-qPCR, Western blot) in ovarian follicles across the oestrous. Additionally, the mRNA expression of *PGRN*, *SORT1*, *EphA2* and *TNFR1/2* were compared between whole follicle, granulosa (Gc) and theca (Tc) cells at days 10-12 of oestrous, while PGRN level was assessed in the medium after culture of structures described above. Next, Gc from mid-luteal phase (days 10-12) were isolated and cultured in control medium or in medium supplemented with PGRN (0.1, 1, 10, 100 ng/ml for 24h, 48h and 72h). Viability analysis was conducted by alamarBlue assay, proliferation by BrdU assay and completed with mRNA expression analysis of proliferation marker (*PCNA*) and cyclins A, B, D, E. Statistical analysis was conducted by One-way ANOVA followed by Tukey post hoc test (GraphPad Prism) at $p < 0.05$, $n=5$.

The results documented the highest level of PGRN in FF at the mid-luteal phase of the oestrous cycle and this pattern was confirmed at the protein level and partly at mRNA, whereas PGRN level in serum was constant. *TNFR2* decreased with the oestrous progression, while *TNFR1* and *EphA2* had the lowest expression in mid-luteal phase. Moreover, PGRN and its receptors were detected in whole ovarian follicles as well as in their compartments, with highest expression of *PGRN* in Gc, suggesting a possible local model of action. However, the secretion of PGRN to the culture medium was the highest by whole follicle and the lowest by Tc. Despite a decrease in cell viability at higher doses (10, 100 ng/ml at 24h and 48h), PGRN consistently enhanced proliferative activity of Gc after 48h of culture in all investigated doses. This outcome was accompanied by increased mRNA expression of cell cycle-related markers, including cyclins (A, B, D, E) and *PCNA* especially at dose 10 ng/ml.

This research provides the first evidence of presence and activity of PGRN in porcine ovarian follicles demonstrating cycle-dependent expression, as well as stimulatory role in Gc proliferation. This suggests that PGRN may be involved in the control of follicular dynamics especially folliculogenesis and ovulation.

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Dynamics of the Bromodomain-Containing Protein BRD2 During Meiotic Resumption in the Domestic Cat Model

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Abstract Text

With the growing threat of extinction facing many wild felid species, gamete preservation and assisted reproductive technologies (ARTs) are increasingly important tools for conservation. Key ART applications include in vitro maturation (IVM) and in vitro fertilization (IVF), in which oocytes are matured and fertilized to produce embryos. However, developmental efficiency in feline oocyte culture remains low, with IVM rates of ~50–60% and blastocyst development of only 10–20%. These challenges highlight the need for a deeper understanding of the complex factors governing efficient culture and identification of competent oocytes. A critical step toward this goal is improving our basic understanding of what makes meiotically competent, high-quality oocytes. As such, identifying proteins essential for meiosis may enable their use as biomarkers of meiotic competence and inform improvements in culture conditions. One such protein is the transcriptional regulator, bromodomain-containing protein 2 (BRD2). BRD2 is a member of the BET family of proteins, which has been shown to be involved in mammalian embryogenesis and spermatogenesis. Moreover, BRD2 has recently been identified as playing a key role in oogenesis of feline oocytes, as transfection of BRD2 antibodies into oocytes results in oocyte arrest at the germinal vesicle (GV) stage of development. As a result, we hypothesized that BRD2 plays a role in the transition from the GV to the GVBD stage of oogenesis. To test this hypothesis, domestic cat oocytes were cultured using a 3-hour IVM protocol or collected without IVM. Guided by previously established in vitro meiotic timelines from our group (unpublished results), we tested our hypothesis by subjecting oocytes to a 3-hour IVM cycle to collect primarily GVBD-stage cells, or by omitting IVM to collect GV-stage cells. These cells were then stained with anti-BRD2 and DAPI for DNA and visualized via fluorescence microscopy. We observed 2 classifications of BRD2 localization patterns: punctate distribution throughout the GV and concentrated spherical domains (~1-2 μm) in the GV. Concentrated spherical BRD2 domains were observed exclusively in $24.5\% \pm 6.22$ of IVM-treated oocytes ($n = 23$) and were absent from all non-IVM controls ($n = 14$), a statistically significant difference ($p = 0.028$). Notably, these spherical concentrations did not co-localize with chromatin, suggesting they represent a distinct nuclear structure. Future co-localization studies with additional nuclear markers will help determine the identity of these structures. These results suggest that BRD2 localization shifts after, or coincident with, meiotic resumption and further support a role for BRD2 in regulating oocyte maturation. By improving our understanding of BRD2 dynamics during oocyte development, this work contributes to efforts to identify biomarkers of oocyte quality and to optimize ARTs for wild felid conservation. Future mechanistic studies, including targeted protein degradation approaches, will further define the functional role of BRD2 during feline oogenesis.

Effect of Form of Selenium on Transcripts Regulating Inflammatory Responses in the Early Luteal Phase Corpus Luteum of Beef Cattle

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Abstract Text

Selenium- (Se-) deficient soils are widespread across the southeast, northeast and northwest US, necessitating supplementation of this trace mineral to the diet of forage-grazing cattle. To achieve a Se-adequate status, Se is typically supplemented in a free-choice vitamin mineral mix in an inorganic form (ISe). Our laboratory has previously reported that supplementation with an isomolar 1:1 mix (MIX) of ISe and organic Se (OSe), versus ISe alone, affects luteal progesterone synthesis, endometrial function, and conceptus development at maternal recognition of pregnancy. The form of Se supplemented to cattle to provide a Se-adequate status has also been shown to affect the complement of selenoproteins in the early luteal phase CL. Selenoproteins act as important antioxidants and are key to the maintenance of cellular integrity and function. The objective of this experiment was to determine the effect of form of supplemental Se on the expression of transcripts regulating inflammatory, immune, and apoptotic responses in the early luteal phase CL.

Cycling, Angus-cross beef cows were maintained on ISe or MIX for at least 120 days prior to synchronization of estrous and were in a stable, Se-adequate status (n=5 per treatment). Corpora lutea were collected on Day 7 post-estrus by lutectomy (n=5 per treatment). Following extraction of RNA and reverse transcription, real-time PCR was performed on all luteal samples in triplicate, with the relative expression of each transcript assessed against the geometric mean of 3 housekeeping genes (*Actb*, *Hprt1*, and *Sdha*).

In CL retrieved from MIX versus ISe treated cows, the abundance of mRNA encoding *p105/Nf-kb1*, a member of the nuclear factor kappa beta family and transcription factor involved in regulation of innate or adaptive immune and inflammatory response, was decreased ($P < 0.05$). In contrast, the abundance of mRNA encoding *Ifng*, *Il1b*, *Ilf2*, *Ptk2* and *Tnfsf13* were all increased ($P < 0.05$) in CL from MIX vs ISe treated cows. Interferon gamma (*Ifng*) is a cytokine regulating apoptosis and local immune responses, with a well-established role in luteal regression. Interleukin 1B (*Il1b*) is a potent modulator of steroidogenesis and the synthesis of progesterone by the CL. Interleukin enhancer-binding factor 2 (*Ilf2*) is involved in mRNA stabilization within luteal cells. Protein Tyrosine Kinase 2 (*Ptk2*) has been described to effect cell adhesion, migration, and survival, whereas *Tnfsf13* is a member of the TNF superfamily involved in immune-mediated cell remodeling. *Il12b*, *Il13ra1*, *Il18*, *Il27ra*, *Pappa2*, *Tlr4*, *Tlr8*, *Tnfa*, and *Tnfsf13b* were not affected by the treatment ($P < 0.05$).

Collectively, our results indicate that the form of Se provided to cows to achieve a Se-adequate status coordinates active regulation of immune and inflammatory responses within luteal cells. In addition, these transcripts can interact with the Liver-X-Receptor – Retinoid-X-Receptor (LXR/RXR) activation pathway that is central to cholesterol biosynthesis, trafficking, and progesterone secretion by the early luteal phase CL.

Follicle-Stimulating Hormone Stimulation Regulates Granulosa Cell Metabolism and Follicular Fluid Composition in Cattle

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Abstract Text

Follicle-stimulating hormone (FSH) is widely used to enhance follicular development and embryo production in cattle. However, the mechanisms by which FSH regulates the follicular metabolic environment, which ultimately influences oocyte competence, remain poorly defined. It was hypothesized that FSH induces coordinated metabolic reprogramming in granulosa cells, thereby altering the composition of follicular fluid. Pregnant Holstein heifers (n=6) were subjected to a 3x3 crossover design using a traditional superstimulatory protocol (four intramuscular administrations at 12h intervals) with 0, 280, or 525 IU of FSH, respectively. Transvaginal follicle aspirations were performed 40 h after the last FSH injection. Granulosa cells and corresponding follicular fluid were collected from individual follicles for transcriptomic (RNAseq) and metabolomic (LC-MS) analyses. For the granulosa cell transcriptome analyses, clean reads were aligned to the bovine reference genome (Bos_taurus ARS-UCD.1.2.104) using STAR, and gene-level counts were generated using featureCounts. Differentially expressed genes (DEGs) between treatments were identified with edgeR, applying a false discovery rate (FDR) threshold of 0.05 for statistical significance. For the follicular fluid metabolome, intensities were log₂-transformed before statistical analysis, and metabolite abundance was analyzed using linear mixed-effects models with FDR correction (significance threshold: FDR < 0.10). Stimulation with FSH induced a robust dose-dependent transcriptional remodeling in granulosa cells, with 1,715 downregulated and 1,701 upregulated genes in cells from heifers stimulated with 280 IU FSH compared to controls (non-stimulated). Granulosa cells from heifers stimulated with 525 IU FSH resulted in 2,393 downregulated and 2,324 upregulated genes compared to controls. Enriched pathways included lipid metabolism, cholesterol biosynthesis, and carbohydrate metabolism. Key regulators of fatty acid activation and β -oxidation (e.g., *ACSL3*, *ACADVL*) and cholesterol synthesis (e.g., *HMGCR*, *SQLE*) were increased, alongside glycolysis markers (e.g., *ALDOA*, *PGD*), indicating increased metabolic capacity in stimulated cells. In contrast, expression of glucose transporters (*SLC2A1*) and select extracellular matrix-related genes was reduced. Consistent with transcriptomic changes, follicular fluid metabolomics revealed decreased concentrations of free fatty acids, including FFA (18:2) and FFA (22:6) (FDR < 0.05), alongside increased lipid oxidation intermediates such as 3-oxohexadecanoic acid (FDR = 0.01), indicating enhanced lipid turnover. Collectively, these findings indicate that FSH drives coordinated metabolic reprogramming in granulosa cells, promoting lipid utilization and intracellular metabolic processing, which is reflected in the follicular fluid metabolome. These results support a hierarchical model in which endocrine regulation of granulosa cells establishes a metabolically active follicular environment that can subsequently regulate downstream cumulus and oocyte competence. Funding: Hatch Act and SciMed Graduate Research Scholars.

Age-Related Shifts in the Reproductive Microbiome of Layer Hens – Implications for Enhancing Reproductive Performance

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Abstract Text

Eggs are a globally important food source and integral to optimal poultry production. Understanding the microbial ecology of the hen reproductive tract is essential for improving both food safety and reproductive efficiency. While the oviduct has been shown to harbor a continuous microbial community, this study is the first to demonstrate the presence of microbiota on the hen ovary surface, suggesting that the ovary is an extension of the oviductal microbial continuum. In this study, the ovarian and oviductal microbiomes of white-leghorn hens from mid-lay (high laying) and post-lay (lower laying) cohorts were analyzed.

Using 16S rRNA sequencing, we identified significant shifts in reproductive tract microbiota between 9- and 18-month-old hens, coinciding with changes in lay performance. Several differentially abundant genera, including *Acinetobacter*, *Ligilactobacillus*, *Bacillus*, and *Akkermansia*, are known to modulate steroid hormone metabolism, with age-related abundance changes suggesting potential effects on hormone-driven reproductive processes. Other genera such as *Ruminococcus_torques_group*, *Mucispirillum*, and *Fusobacterium* not traditionally associated with reproductive hormone pathways may influence laying efficiency through their roles in mucin degradation, immune modulation, and inflammation. Notably, *Turicibacter*, newly identified on the ovary, increased with age and negatively correlated with lay performance, raising questions about its role in bile acid metabolism and stress response within the hen reproductive tract.

Collectively, these findings highlight the ovary as an active microbial niche influenced by age and suggest that both hormone-associated and mucosal-interactive microbes contribute to lay dynamics. This work opens new avenues for probiotic strategies targeting key genera to support hen fertility and egg production across the productive lifespan.

Myoinositol Supplementation in Cows Increased Myoinositol Concentration Within Preovulatory Follicular Fluid

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Abstract Text

The follicular microenvironment plays a vital role in supporting oocyte growth, maturation, and the acquisition of developmental competence. Myoinositol, a fertility promoting supplement that has been used extensively in women's studies, has strong potential to positively impact bovine reproductive success. Myoinositol is a B-vitamin-like compound that is a component of cell membranes and has a role in intracellular calcium signaling pathways that are required for follicle recruitment, growth, and ovulation. Myoinositol also contributes to insulin signaling and increases the cellular uptake of glucose, which are key functions required by a healthy follicle. Physiologically relevant outcomes of myoinositol availability on the bovine follicle and fertility has yet to be confirmed and leaves a critical gap in understanding. To fill this gap, the appropriate dosage of myoinositol must first be determined in cattle. We hypothesized that daily myoinositol supplementation at an average body weight adjusted equivalent to the dose identified to improve oocyte competence in women (30 g/d) would reflect elevated metabolite levels in bovine preovulatory follicular fluid. Our objective was to determine if oral supplementation of 30 g/d of myoinositol for 90 days would increase myoinositol levels in the preovulatory follicular fluid. For this study, mature beef cows were provided an identical ration either non-supplemented (control; n=4) or supplemented with 30 g/d of myoinositol (myoinositol; n=5). At study onset (baseline) and on days 60 and 90, preovulatory follicular fluid samples were collected. Development of a preovulatory follicle was achieved at each timepoint using a 7-day controlled internal drug release (CIDR) protocol. Follicular fluid was collected ~6 hours post gonadotropin-releasing hormone to induce the preovulatory luteinizing hormone surge. Follicular fluid samples were deproteinized and myoinositol concentration was measured using the Megazyme® myo-Inositol assay kit (Neogen, Lansing, MI, United States). Statistical analyses were conducted in R Studio, and Analysis of Variance was used to determine differences in myoinositol level between treatment groups and collection days. Significance was determined at $P < 0.05$. There were no significant differences between the myoinositol and control group on days 0 and 60 ($P > 0.4$). There were no significant differences in the myoinositol levels of the control group between days 0, 60, and 90 ($P < 0.17$). The myoinositol supplemented group, however, exhibited greater concentrations of myoinositol in preovulatory follicular fluid on day 90 compared to day 0 ($P < 0.005$) and day 60 ($P < 0.05$). Interestingly, on day 90, preovulatory follicular fluid myoinositol concentrations in the myoinositol supplemented group ($13.56 \pm 1.47 \mu\text{g/mL}$) were approximately double that of the control group ($7.02 \pm 0.98 \mu\text{g/mL}$; $P < 0.02$). These results verify that an oral myoinositol dose of 30 g/d for 90 days successfully elevated myoinositol levels in preovulatory follicular fluid compared to baseline and the control group. We speculate that this treatment dose and timeline could lead to improved follicular function, oocyte competence, and fertility in cattle. We would like to thank the USDA MultiState project NE2227, the University of Tennessee Animal Science Department, and the University of Tennessee AgResearch for their funding and support of this research.

The Roles of Dynein, Kinesin-5 and Kinesin-12 in Stabilizing the Mouse Metaphase II Spindle

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Abstract Text

Oocyte aneuploidy is one of the leading causes of infertility, miscarriage, and congenital diseases. Mammalian oocytes, particularly human oocytes, are highly prone to aneuploidy. Notably, oocytes undergo meiosis in the absence of centrosomes, which help with spindle assembly in somatic cells. Instead, oocytes rely on the coordinated action of motor proteins, acentrosomal microtubule-organizing centers (aMTOCs), microtubule-associated proteins, and tightly regulated microtubule dynamics to assemble and maintain a functional bipolar spindle. However, these acentrosomal spindles are inherently unstable, particularly during prolonged Metaphase II arrest. The molecular mechanisms that regulate spindle integrity during this stage remain poorly understood. In this study, we investigate the contributions of three key motor proteins, dynein, kinesin-5, and kinesin-12, to spindle stabilization in murine oocytes.

Using small molecule inhibitors, we acutely inhibited these motor proteins during Metaphase II arrest, allowing us to assess their roles in maintaining spindle structure independently of earlier assembly defects. Our preliminary data reveal a critical role for kinesin-12 in spindle stabilization. Inhibition of kinesin-12 results in severe spindle abnormalities, including widened spindle poles, distorted spindle architecture, chromosome misalignment, and the formation of dual spindles. These defects ultimately disrupt the progression of meiosis underscoring the essential function of kinesin-12 during meiotic arrest.

We also examined the role of dynein in spindle stabilization. While dynein's roles in spindle assembly have been extensively studied, its function during prolonged Metaphase II arrest is less well defined. Our preliminary findings indicate that dynein inhibition leads to unfocused spindle poles and that aMTOCs, usually concentrated at the spindle poles, are delocalized. These results suggest that dynein plays a continuous role in maintaining spindle pole integrity and aMTOC organization during meiotic arrest.

Finally, dual inhibition of dynein and kinesin-5, as well as triple inhibition of dynein, kinesin-5, and kinesin-12, revealed a compensatory mechanism where kinesin-12 partially preserves spindle bipolarity in the absence of both dynein and kinesin-5. This unexpected finding highlights kinesin-12 as a key stabilizing force within the acentrosomal spindle and suggests that mammalian oocytes use compensatory mechanisms to maintain spindle integrity during prolonged arrest.

Together, these findings provide new insights into the mechanisms that stabilize the meiotic spindle in mammalian oocytes. Understanding how these motor proteins cooperate to prevent spindle collapse may help explain the intrinsic vulnerability of oocyte meiosis and inform future strategies to reduce aneuploidy and improve reproductive outcomes.

Cortical Biomechanics of Human Germinal Vesicle Oocytes May Predict In Vitro Maturation Potential

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Abstract Text

Immature germinal vesicle (GV) oocytes are frequently discarded during *in vitro* fertilization (IVF) cycles despite being able to undergo *in vitro* maturation (IVM), representing a potentially valuable resource for patients with limited oocyte yield. This is, in part, because methods to assess GV developmental competence rely primarily on morphology and do not reliably predict whether they will undergo IVM to yield high-quality MII (metaphase II) oocytes. Given that oocyte biomechanical properties are important for spindle migration and chromosome segregation during maturation, we hypothesized that cortical biomechanical properties are associated with the ability of GV oocytes to undergo IVM. To test our hypothesis, we obtained GV oocytes donated from patients undergoing controlled ovarian stimulation for IVF, used micropipette aspiration to measure cortical tension and stiffness, cultured them in IVM conditions, and maturation status was determined by the presence of a polar body. Importantly, aspirated and non-aspirated GV oocytes had similar IVM rates (50.6% vs. 47.8%; $n = 41$ patients; $p = 0.8182$), indicating that micropipette aspiration does not impair maturation potential. Next, we correlated the biomechanical properties of the aspirated GV oocytes with their maturation rates ($n=10$ patients). Mean geometry-corrected cortical tension was significantly lower in GV oocytes that matured than in those that failed to mature (1.48×10^5 pN/ μ m vs. 2.35×10^5 pN/ μ m; $n = 30$; $p = 0.0062$). Mean stiffness was similar between GV oocytes that did and did not mature (1210 Pa vs. 1515 Pa; $n = 30$; $p = 0.2959$). Cortical tension and stiffness were linearly related in GV oocytes, and GV oocytes that matured exhibited a steeper cortical tension/stiffness slope than those that failed to mature (0.00704 vs. 0.004119). In addition to intrinsic biomechanical differences between oocytes, we examined whether patient-level clinical factors may influence oocyte mechanics and maturation outcomes. In the United States alone, over 100 million adults are obese, and obesity is well-known to be associated with reduced oocyte quality. Therefore, we analyzed our data according to patient body mass index (BMI). A lower percentage of MII oocytes were retrieved from patients with BMI ≥ 30 than from patients with BMI < 30 (61.95% vs. 75.06%; $n = 10$; 18 cycles; $p = 0.0394$). Higher BMI patients also demonstrated a trend toward reduced IVM rates (29.25% vs. 66.67%; $n = 10$ patients; $p = 0.2068$). Finally, GV oocytes from patients with BMI ≥ 30 had similar cortical tension but increased inter-sample variability ($1.99 \times 10^5 \pm 1.12 \times 10^5$ pN/ μ m vs. $1.51 \times 10^5 \pm 6.81 \times 10^4$ pN/ μ m; $n=10$; $p=0.176$) and higher stiffness ($1.56 \times 10^3 \pm 8.62 \times 10^2$ Pa vs. $1.01 \times 10^3 \pm 3.86 \times 10^2$ Pa; $n=10$; $p = .0352$) than GV oocytes from patients with BMI < 30 . Together, these results suggest that cortical biomechanics can be used to predict GV oocyte maturation potential. In future studies, we will expand sample sizes and perform cytoskeletal imaging to link mechanical phenotypes of GV oocytes with cortical actin organization as a marker of MII oocyte quality. Additionally, combining biomechanical parameters with patient clinical variables may enable development of a time- and cost-effective method to identify GV oocytes with the highest likelihood of successful maturation, facilitating the use of GV oocytes in assisted reproductive technologies.

Effects of Follicle Stimulating Hormone on Preantral Follicle Apoptosis

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Abstract Text

Follicle stimulating hormone (FSH) is required for antral folliculogenesis. Studies examining the potential roles of FSH in preantral folliculogenesis, however, have yielded conflicting results, and questions related to positive and negative effects of the hormone in early follicles remain unanswered. The objective of this study was to determine whether FSH regulates apoptosis in preantral follicles in cattle. Eighteen crossbred beef heifers were enrolled in a crossover experiment containing three treatment groups ($n=6/\text{group}$) with a 98-day washout period between three experimental rounds, to reflect the estimated 100-day length of preantral folliculogenesis in cattle. Heifers were randomly assigned to receive either exogenous FSH [300mg of Folltropin V IM over 7 treatments (FSH; 43mg, 2.2mL per treatment)], saline (VEH; 2.2mL 0.9% NaCl IM), or charcoal-extracted, sterile follicular fluid (FF; 3 doses of 15mL of bovine follicular fluid IV followed by 2.2mL of 0.9% NaCl IM), to create a supraphysiologic, physiologic, and sub-physiologic FSH environment, respectively. Follicular fluid was used as a source of inhibin and pooled from follicular aspirates from live cows and abattoir-derived ovaries. The follicular fluid underwent two rounds of charcoal extraction to remove steroid hormones and leave a protein-rich fraction containing inhibins, and sterile-filtered before injection. Heifers were presynchronized with a modified double ovynch to ensure all animals were in proestrus or early estrus at the start of the experiment. Follicular ablation was conducted to remove all antral follicles $>2\text{mm}$ and trigger recruitment of a new follicular wave (T0). Starting at T0, serum was collected immediately prior to heifers receiving assigned treatment every 12hr for 3.5 days (84hr). Twelve hours after the final treatment (96hr) ovarian biopsies were taken transvaginally using a 16G needle. Ovarian biopsies were fixed in 10% formalin and processed for cryosectioning or paraffin embedding. Cryosections were processed for immunolocalization of cleaved Caspase-3 to quantify apoptosis in preantral follicles. Cleaved Caspase-3 activity was inferred utilizing arbitrary fluorescence intensity units from the signal intensity measured using ImageJ software (NIH). Three signal intensity measurements were taken and averaged for each follicle and then divided by average of three area measurements of the follicle to correct for effect of follicle size on total intensity. Negative controls were produced by omitting the primary antibody. Statistical analysis was performed by Analysis of Variance with treatment and cow within treatment as independent variables using the SAS software version 9.4. Tukey Results are presented as least square means $\pm$ standard error of the mean. Sample processing and data analysis are ongoing. To date, a total of 97 follicles (35 follicles from FSH, 51 from VEH and 11 from FF group) from biopsies of four animals have been analyzed. Cleaved caspase-3 intensity was greater in follicles from FSH-treated cows ($17.58 \times 10^{-3} \pm 1.2 \times 10^{-3}$ arbitrary units) compared to saline ($10.47 \times 10^{-3} \pm 2.1 \times 10^{-3}$ arbitrary units, $P < 0.0001$) and follicular fluid ($9.94 \times 10^{-3} \pm 0.9 \times 10^{-3}$ arbitrary units, $P < 0.01$) treated cows. Intensity was not different between saline and follicular fluid treated animals ($P=0.83$). The current findings could indicate that exposure to supraphysiological levels of FSH leads to increased apoptosis in preantral follicles when compared to physiological FSH. In contrast, sub-physiological FSH levels does not seem to induce apoptosis in preantral follicles. Funding: USDA-NIFA award 2023-67015-40941.

Ex Vivo Ovulation Model to Investigate Mechanisms of Ovulation and Environmental Influences on Ovarian Function

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Abstract Text

Ovulation of an oocyte from the ovary into the oviduct for fertilization is a tightly regulated process, engaging multiple cellular signalling pathways to ensure the timely release of high-quality oocytes. Our goal is to identify molecular regulators of ovulation particularly hormonally controlled effectors that are downstream of progesterone receptor (PGR) transcription factor. In addition, we are focused on understanding how environmental factors such as obesity, aging and toxicant exposure affect the intrafollicular microenvironment with consequences on ovulation and the acquisition of oocyte developmental competence.

To address these knowledge gaps in our understanding of ovulation mechanisms and intrafollicular control of oocyte quality, we are utilizing the *ex vivo* follicle culture system. Antral follicles are dissected from ovaries of mice at ~24d of age and cultured for 24h to complete folliculogenesis. Follicles are then treated with hCG and monitored for ovulation within 12-15h. In some experiments ovulated eggs undergo IVF and are cultured to the blastocyst stage. During the establishment of this system, we compared FSH and PMSG for efficacy to support optimal follicle growth and ovulation. We found no statistical difference between the established oFSH dose (0.06IU/mL) and PMSG (0.4IU/mL) to stimulate follicle growth and ovulation. Importantly, both hormonal stimulation protocols produced ovulated eggs that fertilized and developed to blastocyst with normal morphology.

To investigate steroid hormone regulation of follicular rupture we examined the relative role of glucocorticoid receptor (GR), which is constitutively expressed in granulosa cells, compared to that of PGR which is induced in response to ovulatory hCG. Follicles were treated with either CORT125281 antagonist of GR transcriptional activity or ulipristal acetate (UPA), a PGR antagonist. UPA (50uM) completely blocked ovulation as expected and interestingly, CORT (100nM) caused a ~40% decrease in follicle rupture ($P < 0.01$). This observation indicates that GR plays a role in the regulation of ovulatory gene expression.

Because environmental stressors such as obesity and aging are known to cause mitochondrial dysfunction in granulosa cells, we investigated whether direct inhibition of mitochondrial activity had consequences for ovulation. Follicles were treated with rotenone, an inhibitor of Complex I, at doses between 0.04uM and 5uM and in each case there were zero ovulations, compared to typically 65% in controls. These results indicate that mitochondrial energy production is essential for follicle rupture.

Overall, these studies provide evidence that *ex vivo* follicle culture followed by IVF provides a platform for 1) measuring hormonal regulation of folliculogenesis, 2) identifying new intrafollicular regulators of ovulation, such as GR, and 3) uncovering how stressors, such as those that impinge on mitochondrial function, impact ovulation and oocyte developmental competence.

Electrospun fibers regulate compartment-specific assembly, growth, and steroidogenesis in long-term culture of human ovarian organoids.

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Abstract Text

Ovarian organoids represent a transformative tool for reproductive and gynecological research, offering a unique platform to model ovarian physiology and pathology with unprecedented fidelity in a controlled, *in vitro* setting. To recapitulate the distinct layers of native ovarian tissue, we mechanically and enzymatically dissociated human ovarian cortex and medulla to create ovarian compartment-specific stromal cell organoids. Cortical ovarian organoids (cOOs) and medullary ovarian organoids (mOOs) were seeded at 120,000 cells per well and maintained for eight weeks in suspension culture, either as self-assembled structures or with microfiber reinforcement. Electrospun microfibers consisting of dextran vinyl sulfone were functionalized with basement membrane binder peptide or arginyl-glycyl-aspartic acid (RGD) to mimic the fibrillar structure and biological function of the native ovarian extracellular matrix (ECM). These biomimetics enabled the investigation of the effects of cell-fiber interactions on organoid assembly, growth, and hormone production. Organoid area and morphology changed drastically over time and varied significantly across conditions. The area of cOOs increased by 16% ($235,833\mu\text{m}^2$ - $273,663\mu\text{m}^2$) and exhibited dynamic, asymmetric cell growth (range of circularity between 0.7472-0.8332), while mOOs decreased in area by 51% ($246,586\mu\text{m}^2$ - $125,783\mu\text{m}^2$) and maintained a consistently round structure (circularity >0.9 at all timepoints). These differences were altered by the incorporation of electrospun fibers on Day 0 of cell seeding, resulting in a significant decrease in area with a significant increase in circularity for both the cOOs and mOOs over time, with no differences between conditions. Both cOOs and mOOs self-assembled a basement membrane surrounding the organoid via cell-secreted ECM proteins, with metabolic labeling confirming robust cell-associated ECM production and fiber-guided deposition in reinforced organoids. Excitingly, cOOs produced measurable levels of estradiol, starting at 286 pg/mL after one week and gradually declining to undetectable levels by week 6, before a small increase again at week 8. In contrast, mOOs did not produce measurable levels of estradiol at any timepoint. Finally, progesterone was secreted by both cOOs and mOOs throughout the eight-week study (cOOs ranged from 153 pg/mL to 700 pg/mL while mOOs ranged from 85 pg/mL to 388 pg/mL). These findings establish a viable, long-term human ovarian organoid platform. Ongoing work is focused on targeted characterization of organoid cell composition to refine our understanding of cellular contributions to ECM remodeling and hormone production. Ultimately, this organoid model will be applied to co-culture with early-stage human ovarian follicles to support *in vitro* activation and growth as a novel fertility preservation method.

Asprosin Role in Human Granulosa Cell Function in Obesity: Transcriptomic Insights and Endocrine Regulation

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Abstract Text

Asprosin is an orexigenic adipokine involved in glucose homeostasis and inflammation, yet its role in human ovarian physiology remains poorly understood. Given that its production depends on the proprotein convertase furin and its action has been linked to the olfactory receptor OR4M1, we aimed to investigate the expression and functional effects of asprosin in human granulosa cells (hGCs) under normal weight (NW) and obese (OB; BMI >30) conditions, integrating transcriptomic profiling with functional analyses.

hGCs were obtained from patients undergoing *in vitro* fertilization procedures and cultured under control conditions or treated with asprosin (0.1–100 nM) for 24–72 h. Expression of asprosin, furin and OR4M1 mRNA was assessed by qRT-PCR (n=10, p< 0.05). Immunolocalization of studied proteins was examined in human ovarian tissue. Plasma and follicular fluid asprosin levels were measured by ELISA (n=10, p< 0.05), and correlation analyses with hormonal and metabolic parameters were performed. RNA isolated from NW and OB hGCs was subjected to high-throughput sequencing using the Illumina NovaSeq X platform (n=5, p< 0.05). Differentially expressed genes were identified following data processing and gene ontology and functional analyses were performed. Functional studies were conducted in hGCs treated with asprosin (0.1–100 nM) for 24–72 h. Cell viability (alamarBlue assay), proliferation (BrdU assay) and secretion of estradiol (E2) and progesterone (P4) (ELISA) were evaluated (n=6, p< 0.05). Statistical analysis was conducted by one-way ANOVA followed by Tukey post hoc test (GraphPad Prism).

Expression of all analyzed genes was evaluated, with increased asprosin mRNA levels in OB compared to NW, while furin and OR4M1 remained unchanged. Immunolocalization confirmed the presence of all studied proteins predominantly in hGCs, but also in theca cells. ELISA measurements showed elevated circulating asprosin levels in OB women, with no differences in follicular fluid levels. Notably, serum asprosin levels negatively correlated with FSH concentrations (p< 0.05).

Transcriptomic profiling revealed extensive gene networks associated with steroidogenesis, cholesterol and lipid metabolism in both groups, with more frequent and stronger interactions in OB. Exposure to 10 nM asprosin (48 h) induced more pronounced transcriptomic changes in OB compared to NW. In NW, asprosin downregulated genes involved in follicular signaling (e.g. GDF1) and oxytocin receptor expression, whereas in OB it reduced expression of IGF receptor and adipokine-related genes (e.g. apelin, adiponectin receptor). Additionally, asprosin ↓cyclin A expression in OB. In terms of steroidogenesis, asprosin ↓STAR, ↓CYP11A1 and ↓3βHSD expression, while ↑CYP19A1. It also modulated metabolic pathways by ↑SLC2A6, ↓INSR and ↑PNPLA2. Functionally, asprosin ↓hGCs viability in NW at 10 and 100 nM after 48 and 72 h and in OB at the same doses after 72 h. No significant effects were observed for proliferation. ELISA analyses demonstrated decreased secretion of E2 and P4 in response to asprosin.

Asprosin is expressed in hGCs and its circulating levels are elevated in obesity, where it may contribute to reproductive dysfunction. Its stronger impact on the OB transcriptome and hGCs function suggests increased ovarian sensitivity under metabolic imbalance. In conclusion, asprosin emerges as a potential link between metabolic status and ovarian physiology, modulating steroidogenesis and cellular viability.

Oocyte-Derived Rac1 is Dispensable for Fertility

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Abstract Text

One key determinant of the female reproductive lifespan is the ovarian reserve. The ovarian reserve, defined as the number of primordial follicles present at birth, is established in early development in both humans and mice. Rho GTPases are well-characterized regulators of numerous cellular processes including cellular asymmetry, cell movement, and cytoskeletal modulation which are essential processes for proper primordial follicle formation and thus fertility. Rho GTPase *Rac1* is ubiquitously expressed throughout the ovary. Previous studies have demonstrated that pharmacological inhibition of *Rac1* in *ex-vivo* ovary culture during primordial follicle formation resulted in a significant reduction in the number of primordial follicles formed. Despite this, the contributions of *Rac1* within specific ovary cell types remain unknown, and the downstream impact on fertility has not been evaluated. We hypothesize that oocyte-specific loss of *Rac1* will lead to a decrease in fertility. To test this, we generated a mouse model with an oocyte-specific conditional knockout of *Rac1* using the novel *Stra8-Cre (Rac1cKO)*. Results showed that compared to wild-type controls, *Rac1cKO* dams produced a comparable number of pups per litter over time and had similar time intervals between litters. Additionally, histological analyses of *Rac1cKO* ovaries across developmental stages failed to exhibit any deficits. Transcriptomic analysis verified *Rac1* downregulation in post-natal day 0 (P0) and P21 ovaries consistent with the conditional nature of the knockout. Further, RNA-seq results at P0 showed a total of 311 upregulated genes and 16 downregulated genes in *Rac1cKO* ovaries compared to wild-type controls. Of note, *Rac2* was one of the upregulated genes in *Rac1cKO* ovaries, suggesting a potential compensatory mechanism (p=0.003). In total, our results suggest that oocyte-specific elimination of the single Rho GTPase family member *Rac1* does not impair fertility.

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Ovarian Cyst–Associated Disruption of the Ovarian–Oviductal Axis in the Dromedary Camel: Biochemical, Endocrine, Histopathological, and Molecular Evidence

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Abstract Text

Ovarian follicular and ovulatory cysts are an important cause of reduced fertility in the dromedary camel; however, the mechanisms by which cystic ovaries influence the ovarian–oviductal functional axis remain poorly defined. This study investigated whether ovarian cysts are associated with coordinated biochemical, endocrine, histopathological, and molecular alterations extending from the ovary to the oviductal isthmus. Ovarian cystic fluid was analyzed for biochemical composition and oxidative stress indicators, while circulating progesterone, estradiol, FSH, and LH were evaluated. Histopathological assessment of the oviductal isthmus was combined with targeted gene expression analysis of inflammatory and stress-response markers in cyst wall, ovarian, and oviductal tissue. Biochemical analysis demonstrated that cystic fluid was characterized by elevated oxidative stress markers compared with non-cystic follicular fluid. Endocrine profiling revealed increased progesterone concentrations in animals bearing ovarian cysts, accompanied by reduced estradiol and luteinizing hormone levels, consistent with altered folliculo-ovulatory signaling. Histopathological examination of the oviductal isthmus revealed structural alterations associated with ovarian cysts, including epithelial thickening and stromal remodeling. Gene expression analysis showed a consistent up-regulation of inflammatory mediators (TNF, IL1B, IL6, and IL8) in cystic tissues, with corresponding but less pronounced expression patterns observed in the ovary and oviduct. In conclusion, ovarian cysts in the dromedary camel are associated with coordinated biochemical, endocrine, and molecular disturbances that extend beyond the ovary to the oviduct, suggesting functional disruption of the ovarian–oviductal axis. These findings provide applied mechanistic insight into cyst-associated subfertility and identify potential biomarkers relevant to reproductive management in camels.

Promoter analysis of the SDF2L1 and SERPINE2 genes reveals potential convergence between activin/SMAD and FSH/cAMP signaling pathways in bovine granulosa cells.

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Abstract Text

Oocyte competence relies on a well-coordinated environment in the follicle, where the oocyte and its surrounding cumulus and granulosa cells communicate through direct contact, projections, gap junctions, and paracrine signals. Activin A, a member of the TGF- β superfamily, plays a key role in follicle function by activating SMAD2, SMAD3, and SMAD4, which influence gene expression associated with granulosa cell growth, differentiation, and oocyte support. Earlier studies found that INBHA, SDF2L1, and SERPINE2 are part of a co-regulated group in the bovine cumulus-oocyte complex, suggesting they are involved in pathways important for oocyte competence. INBHA codes for the β A subunit of activin and inhibin proteins. SERPINE2 is involved in remodeling the extracellular matrix, and SDF2L1 codes for a protein in the endoplasmic reticulum that helps with protein folding and cell balance. In this study, we examined the promoter regions of the bovine SDF2L1 and SERPINE2 genes and created reporter constructs for future testing in primary bovine granulosa cells. We defined promoter regions as the -1000 to +100 base pair range from the transcription start site, using the bovine ARS-UCD2.0 genome. We used FIMO/MEME Suite v5.5.9 and JASPAR matrices to find possible transcription factor binding sites. We looked for motifs linked to activin/TGF- β -SMAD and FSH/cAMP signaling, including SMAD2, SMAD3, SMAD4, ATF2, NR5A1/SF-1, FOXL2, FOS, JUN, JUNB, AP-1, CREM, CREB1, and SP1. Sites with $p \leq 1 \times 10^{-3}$ were considered significant. For experimental validation, we extracted genomic DNA from the blood of a Holstein cow. We amplified a 982-bp fragment of the SDF2L1 promoter by PCR using specific primers and cloned it into pcDNA3.1/NT-GFP-TOPO. We also synthesized an SDF2L1 promoter region rich in FSH/cAMP-related binding sites, adding KpnI and HindIII restriction sites for cloning into pGL4.10[luc2]. We established primary bovine granulosa cell cultures from 4–8 mm antral follicles for future promoter activity tests. Our computer analysis confirmed that both promoters have possible SMAD2, SMAD3, and SMAD4 binding sites, supporting the idea that the activin/TGF- β pathway may regulate them. Both promoters also have several motifs linked to the FSH/cAMP pathway. The SDF2L1 promoter has motifs for CREB, CREM, ATF, and AP-1, while the SERPINE2 promoter has motifs for CREB, FOXL2, AP-1, and NR5A1/SF-1. These results suggest that SDF2L1 and SERPINE2 may be regulated by both paracrine activin/SMAD and endocrine FSH/cAMP signaling in bovine granulosa cells.

In summary, this study identified regulatory elements in the SDF2L1 and SERPINE2 promoters that may play a role in gene programs involved in remodeling the extracellular matrix, maintaining endoplasmic reticulum homeostasis, and communication between oocytes and somatic cells. Developing luciferase reporter vectors and setting up primary granulosa cell cultures are important next steps to test how these promoters respond to activin, FSH, or both.

Estrous Cycle-Dependent Transcriptional Remodeling of Ovarian Tissue-Resident Macrophages

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Abstract Text

The ovary is one of the most dynamic organs in the body and is also among the first to show signs of aging, with functional decline occurring long before complete reproductive failure. Ovulation represents a highly regulated inflammatory event requiring immune activation and tissue remodeling. Tissue-resident macrophages help regulate this inflammation and tissue remodeling in many organs, but their role in supporting normal ovarian cycles is still not well understood. This limits our understanding of how immune activity helps maintain ovarian function and how disruption of these processes may contribute to ovarian aging. To address this, we tested the hypothesis that ovarian macrophage activation is cyclic and hormonally regulated rather than static, predicting peak activation during estrus when ovulatory remodeling is maximal. Using a tamoxifen-inducible Cx3cr1^{CRE/ERT2}NuTRAP mouse model, we defined phase-specific transcriptional changes in ovarian tissue-resident macrophages across the estrous cycle. Estrous stage was determined by daily vaginal cytology, and Translating Ribosome Affinity Purification (TRAP) was used to isolate macrophage-specific translating RNA for transcriptomic profiling. The inducible nature of this model minimizes labeling of short-lived circulating monocytes, enabling selective profiling of long-lived tissue-resident macrophages. We found that ovarian macrophages adopt flexible, phase-specific transcriptional programs across the estrous cycle, with peak activation occurring during estrus. This phase was characterized by increased expression of phagolysosomal and remodeling-associated genes including (*Lamp1*, *Gba1*, and *Hexb*), alongside elevated inflammatory mediators such as (*Il1b* and *Il18bp*). These findings are consistent with the heightened inflammatory remodeling and debris clearance required for ovulation and post-ovulatory repair. Together, these data identify the estrous cycle as a physiological model of immune plasticity within the ovary and demonstrate that ovarian macrophage function is dynamically tuned to reproductive state. We propose that loss of this tightly regulated immune plasticity may contribute to the chronic pro-inflammatory environment associated with ovarian aging. Future studies will define the hormonal drivers of these cyclic immune programs and determine how disruption of macrophage-regulated remodeling impacts reproductive longevity and female health.

Impact of Yoga-Based Lifestyle-Intervention on Metabolites, Transcript Dynamics, and Microbiota Composition for Personalized Interventions in Polycystic Ovary Syndrome

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Abstract Text

Introduction: Polycystic ovarian syndrome (PCOS) is a prevalent, heterogeneous metabolic and endocrine disorder affecting women from intra-uterine life to post-reproductive age, with a global prevalence of approximately 11–13%. It is characterized by ovulatory dysfunction, hyperandrogenism, and metabolic abnormalities such as dyslipidaemia, insulin resistance, and chronic low-grade inflammation. The multisystem involvement of PCOS underscores the need for integrative therapeutic approaches that extend beyond symptom-based pharmacotherapy.

Materials and methods: This Pilot study involved 30 women (20 diagnosed with PCOS, 10 age and BMI-matched healthy controls). PCOS women underwent a 12-week Yoga practice (5 days a week, 1 hour a day) that included physical postures (Asanas), regulated breathing practice (Pranayama), and meditation (Dhyana) under the guidance of a trained therapist. Clinical characteristics were recorded following LC-MS for metabolome and hormonal profile, RT-qPCR for transcripts, and Shotgun Metagenomic Sequencing on a Fecal sample. $P < 0.05$ is considered significant.

Results: We observed significant improvements in anthropometric, hormonal, and lipid profiles following the yoga intervention. Molecular analyses revealed enhanced mitochondrial health, improved metabolic regulation, and reduced expression of inflammatory genes ($FC < 2$, $FC > 2$), indicating attenuation of core pathogenic pathways. Metabolomic profiling demonstrated significant post-intervention downregulation of key metabolites, including glucose, fructose, taurine, and methylmalonate, alongside upregulation of succinate and ascorbate, suggesting improved energy metabolism and redox balance. Furthermore, metagenomic analysis revealed partial restoration of gut microbial richness and diversity after 12 weeks of yoga, with species-level alterations in microbial composition and associated metabolic and signalling pathways, indicating a trend toward normalization of dysbiosis following intervention.

Conclusion: Yoga intervention for 12 weeks led to favourable modulation of the gut microbiota in women with PCOS. In addition, metabolic shifts address key factors underlying PCOS pathogenesis.

Deciphering the Role of the Actin-Binding Protein Nexilin in Asymmetric Division of Meiosis I in Mouse Oocytes

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Abstract Text

Asymmetric cell division in mammalian oocytes is essential for retaining maternal cytoplasmic components required to support early embryonic development. This asymmetry is established by migration of the metaphase I spindle from the center of the oocyte to a subcortical position, where it remains anchored until fertilization, resulting in the emission of small polar bodies. Defects in spindle migration and asymmetric division compromise oocyte quality and increase the risk of aneuploidy, highlighting the importance of defining the molecular mechanisms that regulate these processes. Although the actin cytoskeleton and associated actin-binding proteins are known to drive spindle migration and asymmetric division, the specific functions of many actin-binding proteins in this context remain poorly understood.

We have data to demonstrate that nexilin, a novel F-actin-binding protein, as a regulator of spindle positioning and asymmetric division in mouse oocytes, specifically for metaphase I spindle positioning near the cortex. Acute depletion of nexilin using Trim-Away leads to defects in asymmetric division. Nexilin-deficient oocytes underwent symmetric divisions, or progressed to metaphase II with normal size polar bodies but significantly increased spindle-to-cortex distance. To confirm that nexilin depletion is directly responsible for these abnormalities, we are performing rescue experiments by reintroducing nexilin into nexilin-deficient prophase I oocytes, followed by in vitro maturation to assess whether the rescued oocytes are able to mature normally. Our preliminary rescue experiments show that reintroducing nexilin decreases the number of abnormally dividing oocytes and increases the number of normally divided metaphase II eggs with normal-sized polar bodies. Additionally, we have performed further experiments to determine nexilin's functions during meiosis I. In our work to date, we identified abnormal symmetric division and increased spindle-to-cortex distance from immunofluorescence analyses of nexilin-deficient oocytes fixed at 8 and 16 hours after initiation of in vitro maturation. To understand how these abnormalities arise, we have used live-cell imaging to observe the behavior of nexilin-depleted oocytes during meiotic maturation. Live-cell imaging revealed that nexilin-depleted oocytes exhibit a higher percentage of abnormal divisions. Around 11 hours after the start of in vitro maturation, many oocytes begin to divide symmetrically and display abnormal membrane blebbing. Some oocytes complete cytokinesis and divide symmetrically, whereas others, around 13.5 hours, merge their segregated DNA back to a metaphase plate, and then remain arrested at metaphase I instead of dividing. Our previous immunofluorescence results also showed abnormal cytoplasmic F-actin localization in the form of an actin cloud surrounding the spindle, suggesting altered actin dynamics. We therefore hypothesized that nexilin depletion alters the F-actin/G-actin ratio. In preliminary assessment of these hypothesized effects of nexilin depletion on actin, we have detected a slight decrease of the F-actin/G-actin ratio in nexilin-depleted prophase I oocytes compared to controls (non-immune antibody-injected and uninjected).

Together, this work defines a previously unrecognized role for nexilin in oocyte asymmetric cell division, providing new insight into the actin cytoskeletal mechanisms that govern the production of normally fertilizable oocytes capable of supporting embryonic development.

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Acute LDH Inhibition Disrupts Sperm Energetic Metabolism and Reduces Motility

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Abstract Text

Lactate dehydrogenase C (LDHC) is a testis-specific isoform of lactate dehydrogenase that plays a central role in maintaining ATP production and supporting the motility required for fertilization. Sperm rely heavily on glycolysis to fuel motility, capacitation, and fertilization. However, the metabolic and functional consequences of acute LDH inhibition on sperm energetic metabolism and motility remain poorly defined.

In this study, we evaluated the impact of a small-molecule LDH inhibitor on mouse sperm LDH activity and motility parameters. Treatment with the inhibitor produced a clear, dose-dependent decrease in enzyme activity in mouse sperm lysates, confirming the inhibitor's efficacy in sperm.

Computer-assisted sperm analysis (CASA) demonstrated that inhibitor-treated sperm exhibited marked reductions in total motility and progressive motility. These functional deficits emerged rapidly, supporting the concept that sperm motility depends on a functional LDH enzyme.

Together, these findings highlight LDH as a key regulator of sperm glycolysis and motility.

As a next step, future work will pair LDH inhibition with stable-isotope metabolic tracing to give insight into how carbon moves through central metabolic pathways when LDH activity is suppressed. These experiments will help determine whether sperm attempt to reroute flux through alternative pathways or experience a true metabolic bottleneck when LDH is inhibited.

Proteomic Consequences of *Zfy* Loss in Mixed Testicular Germ Cells

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Abstract Text

The mouse Y-linked zinc-finger gene *Zfy*, present as two homologues, *Zfy1* and *Zfy2*, is an essential regulator of spermatogenesis, with genetic studies demonstrating roles in chromatin remodeling, post-meiotic differentiation, and sperm function. Our lab generated *Zfy* knockout (KO) mice and reported that *Zfy1* KO males remain fertile, *Zfy2* KO males exhibit mild fertility defects, and *Zfy* double knockout (*Zfy* DKO) males are completely infertile with severe testicular and sperm abnormalities. Although loss of *Zfy* disrupts germ cell development, the molecular pathways controlled by ZFY remain poorly characterized. Transcriptomic analyses revealed extensive gene deregulation associated with *Zfy* loss, but the proteomic consequences remain undefined. Because protein-level changes ultimately drive spermatogenic phenotypes, characterizing the proteome of germ cells from *Zfy* KO males is critical for understanding how ZFY1 and ZFY2 support male fertility. Mixed germ cells were isolated from adult XY B6, *Zfy1* KO, *Zfy2* KO, and *Zfy* DKO males ($n = 5/\text{genotype}$) using enzymatic dissociation and filtration, followed by centrifugation and snap-freezing. Samples were analyzed by data-independent acquisition (DIA) mass spectrometry (MS), and data analysis was performed to identify differentially expressed proteins (DEPs). Selected candidates were validated by Western blotting (WB). Proteomic analysis showed minimal disruption in single knockouts (*Zfy1* KO: 2 DEPs; *Zfy2* KO: 6 DEPs), consistent with possible functional redundancy between ZFY1 and ZFY2. In contrast, *Zfy* DKO males exhibited 237 DEPs (77 downregulated and 160 upregulated), aligning with the severe spermiogenic phenotype of these mice. Downregulated proteins were enriched in pathways essential for sperm development, including glycolysis, mitochondrial function, acrosomal activity, and flagellar motility. Two proteins with known functions in spermatogenesis, PGK2 and CRISP2, were among the most strongly downregulated candidates. Western blot analysis of germ cells from *Zfy* KO males was performed to validate MS data. Band intensity was quantified and analyzed with one-way ANOVA, which showed a significant effect of genotype ($P < 0.0001$). Post hoc multiple comparison tests revealed significant reduction of PGK2 and CRISP2 in *Zfy* DKO ($P < 0.0001$) and reduction of CRISP2 in *Zfy2* KO ($P < 0.05$), compared to XY males. The findings demonstrate that ZFY proteins maintain the post-meiotic protein network required for sperm maturation, with substantial disruption occurring only when both proteins are absent. The depletion of proteins critical for metabolism and sperm motility highlights the central role of ZFY in supporting sperm function. Future work will include immunofluorescence (IF) to assess spatial localization and further confirm deregulation of PGK2 and CRISP2 in germ cells. Since our lab has recently developed mice with tagged ZFY1 and ZFY2, future work will also include testing how ZFY regulates expression of PGK2 and CRISP2 using co-immunoprecipitation (Co-IP) to assess potential protein-protein interactions and chromatin immunoprecipitation followed by PCR (ChIP-PCR) to determine whether ZFY binds to promoter regions of these genes, consistent with its proposed role as a transcriptional regulator. This research was funded by the NIH R01HD114645 grant to M.A.W. Ryan Lum was supported by an NIH R01HD114645 minority supplement.

Male Germ Cell-Specific Deletion of *Tfrc* Disrupts Spermatogenesis and Causes Infertility in Mice

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Abstract Text

Iron is the most abundant essential trace element in the human body and plays a critical role in numerous physiological processes. Although iron deficiency has been associated with impaired spermatogenesis, its specific role in male reproductive function remains largely unclear. Transferrin receptor 1 (TFRC) mediates cellular iron uptake via transferrin-bound iron and is expressed on the plasma membrane. To investigate the role of iron in male germ cells, we generated germ cell-specific TFRC conditional knockout (cKO) mice and analyzed their reproductive phenotype.

TFRC expression was examined by immunofluorescence (IF). Fertility was assessed by mating cKO and control males with wild-type females. Testicular histology, sperm morphology (scanning electron microscopy), and sperm motility (computer-assisted sperm analysis; CASA) were evaluated. To assess fertilizing ability, in vivo-fertilized oocytes were collected to determine fertilization rates. In vitro fertilization (IVF) was performed under cumulus cell-intact, cumulus-free, and zona-free conditions. Intracytoplasmic sperm injection (ICSI), followed by embryo transfer, was conducted to evaluate developmental potential.

TFRC expression began at the differentiating spermatogonia stage. cKO males exhibited reduced testis size and atrophic seminiferous tubules and were infertile. Abnormal sperm morphology was observed in approximately 90% of cKO sperm compared with 30% in controls, and motility was significantly decreased (approximately 40% vs. 90%, $p < 0.05$). IVF revealed markedly reduced fertilization rates in cKO sperm under cumulus cell-intact, cumulus-free, and zona-free conditions (approximately 0%) compared with controls (approximately 60%, 40%, and 70%, respectively). However, ICSI successfully produced viable embryos and offspring from cKO sperm. To clarify the mechanisms underlying defective spermatogenesis, spermatogonia were isolated by FACS and subjected to mRNA sequencing. Transcriptome analysis identified approximately 800 differentially expressed genes between control and *Tfrc* cKO mice. Gene Ontology analysis indicated that many of these genes were associated with germ cell development, sperm motility, and fertilization. Pathway analysis further revealed downregulation of genes involved in glycolysis and gluconeogenesis.

These findings demonstrate that TFRC-mediated iron uptake in spermatogonia is essential for normal spermatogenesis and sperm morphology and plays a critical role in male fertility and successful fertilization in mice.

Lumicrine Signaling from The Mesonephric Tubule Mediates Androgen Actions in Promoting Wolffian Duct Development

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Abstract Text

Androgen signaling is essential for male reproductive tract development, and its disruption is a major cause of congenital abnormalities associated with infertility. During embryogenesis, testis-derived androgens act through its androgen receptor (AR) to support the maintenance and differentiation of the mesonephric tubules (MT) and Wolffian duct (WD), which ultimately form the efferent duct and the epididymis. These structures are required for post-testicular sperm transport, maturation and storage. Classic models attribute WD maintenance to local epithelial-mesenchymal crosstalk via autocrine and paracrine factors. However, here we report a new cellular communication paradigm underlying androgen action in WD development. We discovered that MTs, positioned upstream of the WD, secrete AR-regulated luminal signals that travel distally to sustain WDs. This transluminal signal transduction in the male reproductive tract is known as lumicrine signaling, distinct from paracrine and autocrine signaling. Our first evidence for this new mechanism came from the degeneration of WDs following the concurrent deletion of *Ar* in MT and WD epithelia using Pax8-Cre. Because deletion of *Ar* in WD epithelium alone does not disrupt fetal WD development, the WD degeneration in our model was supposed to result from the loss of *Ar* in MT epithelia. Two *ex vivo* culture experiments further supported this notion: (1) removal of MTs followed by recombination of testis and WD, and (2) severing the MT-WD connection to block luminal flow. Both manipulations led to impaired WD growth, demonstrating that MTs promote WD development through AR-dependent, MT-derived lumicrine signals. To identify the underlying mechanisms, we performed RNA-seq on control and *Ar* conditional knockout (*AR^{CKO}*) testes, MTs, and WDs at the early stage of androgen-driven differentiation. MTs displayed numerous differentially expressed genes (DEGs, 2626), in contrast to minimal changes in the testes (10) and the WDs (30), indicating that MTs were the principal site of AR action at this stage. Because lumicrine signaling depends on luminal fluid composition and homeostasis, we focused on dysregulated transmembrane transporters among DEGs. The most downregulated transporter in *AR^{CKO}* MTs was *Slc26a3*, which acts as an important electroneutral exchanger of $\text{Cl}^-/\text{HCO}_3^-$ in regulating luminal fluid absorption and pH. MTs play major roles in controlling luminal pH, which is important determinant of epithelial survival. *Slc26a3* is mainly expressed in MT epithelia, and pharmacological inhibition of SLC26A3 recapitulated WD degeneration in *AR^{CKO}* embryos. Mutations in *Slc26a3* have been associated with male infertility in humans. These observations indicate that SLC26A3 mediates MT-derived lumicrine signaling in promoting WD maintenance. Taken together, our work provides a new conceptual framework for androgen action in male reproductive tract development and potentially offers mechanistic insights into male reproductive tract defects.

Regulation and rewiring of epididymal fluid rheology: insights from a *Gata2* conditional knockout model.

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Abstract Text

The luminal microenvironment of the epididymis is critical for sperm maturation, storage, and transport. A highly-regulated key feature of the microenvironment is the rheological properties of epididymal fluid, including viscosity and elasticity. As sperm transit from the caput (head) to the cauda (tail) epididymis, luminal viscosity normally increases, maintaining sperm in a quiescent, energy-saving state suitable for long-term storage. Disruption of epididymal fluid rheology has been implicated in male infertility. However, the mechanisms by which the cauda epididymis coordinates these biophysical properties remain unknown. We previously reported a mouse model with mesenchyme-specific deletion of *Gata2* (*Gata2^{ckO}*) that exhibits male infertility despite normal testis weight, daily sperm production, and normal-appearing sperm in the caput epididymis. By contrast, the cauda epididymis of *Gata2^{ckO}* males shows markedly reduced sperm count and motility, as well as increased morphological abnormalities. Trichrome staining revealed that the lumen of the *Gata2^{ckO}* cauda epididymis contains an accumulation of smooth muscle fibers and/or collagens, forming a cohesive, elastic mass rather than the viscous luminal fluid observed in control. This phenotype provides a unique opportunity to investigate how the cauda epididymis establishes and maintains optimal viscoelastic conditions for sperm storage. To identify components that contribute to altered luminal rheology, we performed proteomic analysis of luminal contents from the control and *Gata2^{ckO}* cauda epididymides. The *Gata2^{ckO}* lumen content was enriched with proteins normally restricted to the seminal vesicles, including prostate and testis expressed 4 protein (PATE4) and seminal vesicle secretion 2 protein (SVS2). Both proteins facilitate the formation of copulatory plugs, which are solid-like, viscoelastic materials for preventing female remating. These findings indicate a shift of the cauda epididymis toward a seminal vesicle-like luminal microenvironment. Ongoing studies will validate the presence and source of seminal vesicle-associated proteins, including PATE4 and SVS2, and test whether local production or retrograde flow from seminal vesicle fluid can contribute to their accumulation using fluorescent tracers. We will also define the viscoelastic properties of the luminal mass with rheometric analysis. Together, our studies highlight the critical role of fluid rheology in shaping luminal microenvironment and establish a unique mouse model for exploring male contraceptive strategies that target epididymal fluid rheology.

Intracellular Crosstalk Between Ca^{2+} and Metabolism in Mouse Sperm

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Abstract Text

Calcium ions (Ca^{2+}) serve as secondary messengers essential for regulating many cellular processes. In somatic cells, Ca^{2+} modulates metabolic pathways through allosteric regulation of enzyme activity. Ca^{2+} could be performing a similar function in sperm as, in the absence of Ca^{2+} , sperm exhibit a loss of motility and altered ATP levels. Ca^{2+} is required for capacitation where sperm continue to increase their Ca^{2+} uptake through the sperm-specific Ca^{2+} channel CatSper. Using a combination of mass spectrometry, stable isotope labeling, pharmacological inhibition, and flow cytometry, we investigated the relationship between Ca^{2+} uptake and metabolism. Sperm incubated in nominally Ca^{2+} -free conditions failed to exhibit the capacitation-induced increase in ^{13}C -label accumulation as observed in the presence of Ca^{2+} . However, surprisingly, pharmacological CatSper inhibition resulted in increased abundance of many endogenous glycolytic intermediates and an overall increase in metabolic activity. During capacitation, sperm progressively increased Ca^{2+} uptake over time, a response that was similar under both standard and physiological concentrations of glucose and fructose. Ca^{2+} uptake was diminished but not fully absent in the presence of CatSper inhibitor. Together, these findings reveal a functional interplay between Ca^{2+} signaling and sperm metabolism, advancing our understanding of the metabolic regulation of capacitation, which may have implications for non-hormonal male contraception and infertility treatment.

Evaluation of Andrological Development and the Impact of Hemicastration Timing in Crossbred Angus Bulls from Pre-Puberty to Sexual Maturity

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Abstract Text

Andrological development is a key determinant of bull reproductive potential. However, existing reference thresholds are based on studies conducted 40-50 years ago in genetically distinct populations under outdated management systems. Modern beef genetics, nutrition, and production demand updated longitudinal benchmarks. This study characterized common reproductive development benchmarks in crossbred Angus bulls that which included intact controls, hemicastrated prepubertally, and hemicastrated immediately after puberty. We hypothesized hemicastration would result in faster testis growth but have similar age at sexual maturity.

Twenty-three healthy crossbred *Bos taurus* bulls underwent weekly Breeding Soundness Examinations from 7–17 months of age, randomized to prepubertal hemicastration (n=8), peripubertal hemicastration (n=7, performed immediately after first pubertal ejaculation), or intact controls (n=8). Assessments included body weight and body condition score, scrotal circumference (SC), testicular volume, Computer-Assisted Sperm Analysis parameters, and sperm morphology. Puberty was defined as $\geq 10\%$ total motility; sexual maturity as $\geq 60\%$ total motility and $\geq 70\%$ normal morphology. Descriptive statistics, Pearson correlation coefficients, and linear regression modeling were conducted in R Studio (v. 2026.1.0) with significance set at $p \leq 0.05$.

In controls, puberty occurred at 357 ± 13 days and sexual maturity at 427 ± 14 days. Scrotal circumference followed sigmoid growth (32.0 ± 1.5 cm at puberty to 35.0 ± 1.0 cm at maturity) while testicular volume increased linearly (682 ± 188 cm³ at puberty to 863 ± 159 cm³ at maturity). Prepubertal cumulative SC negatively correlated with maturity age ($r = -0.71$, $p = 0.047$). Hemicastration did not alter age at puberty or sexual maturity, nor semen quality versus controls ($p > 0.05$). Prepubertal hemicastration induced superior compensatory hypertrophy versus peripubertal timing (remaining testis length growth $+0.33 \pm 0.13$ mm/week faster, $p = 0.01$).

Preliminary outcomes suggest that prepubertal SC predicts maturation rate, testicular volume reflects changes in spermatogenesis, and prepubertal hemicastration affects testicular plasticity. Overall, contemporary crossbred bulls show similar or later puberty, similar or reduced time from puberty to sexual maturity, and larger testes than historical published norms.

Melatonin Alters Gene Networks Associated with Spermatogenesis and Testicular Function in Yearling Angus Bulls

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Abstract Text

Melatonin is a key regulator of circadian and seasonal reproductive physiology, yet its direct effects on testicular molecular function in cattle remain poorly defined. This study investigated the impact of oral melatonin supplementation on the testis transcriptomic profile in bulls. Yearling Angus bulls ($n = 10$) were randomly allocated into two groups, either melatonin fed (MEL; $n = 5$) or control fed (CON; $n = 5$) for 90 days of treatment from October 2024 to January 2025. MEL bulls were supplemented with 200 mg/kg of body weight of melatonin dissolved in ethanol, while CON bulls were supplemented with an equivalent ethanol vehicle control. After 90 days of treatment, bulls were castrated, and testicular parenchyma was collected. Samples underwent homogenization for quantification of testicular testosterone and melatonin concentrations. Additionally, RNA was extracted, and samples underwent sequencing via Illumina platforms. Raw reads were mapped to the *Bos taurus* ARS-UCD2.0 reference genome. Differentially expressed genes (DEGs; false discovery rate (FDR) ≤ 0.05) were identified using a quasi-likelihood negative binomial generalized log-linear model from edgeR's QL framework. The DAVID bioinformatics database (<https://davidbioinformatics.nih.gov/home.jsp>) was used to identify significantly enriched (FDR ≤ 0.05) Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. R Studio Version 4.3.1 was used to identify significant (FDR ≤ 0.05) correlations between DEG expression and testicular melatonin and testosterone concentrations. There were 2,798 DEGs (FDR ≤ 0.05) in MEL compared to CON testis with 1,263 DEGs being upregulated and 1,535 DEGs being downregulated. DEGs primarily belong to KEGG pathways related to intracellular signaling, hormone response, cell-matrix interaction, and cytoskeletal remodeling, all of which play important roles in testicular physiology. Consistent with these findings, GO term enrichment analysis displayed similar themes. Enriched GO terms include focal adhesion, cell junction, receptor complex, and sperm head plasma membrane components. Melatonin treatment resulted in moderate down regulation of DEGs within these pathways. Notably, melatonin reduced expression of the pro-inflammatory cytokine *IL6* ($\log_2FC = -1.27$) while increasing expression of *LHB* ($\log_2FC = 0.55$) and *PRL* ($\log_2FC = 0.96$) suggesting coordinated modulation of inflammatory and endocrine signals that support testosterone production and germ cell development. The expression of several genes within the testicular testosterone production cascade were influenced by melatonin administration. The expression of *TSPO*, *STARD7*, and *HSD17B14* were increased while *CYP11A1*, *STAR*, *HSD3B1*, and *CYP19A1* were decreased. These differing expression patterns suggest that melatonin may promote steroid homeostasis within the testis. Interestingly, melatonin treatment resulted in divergent expression patterns in members of the fibroblast growth factor family (*FGF17*, *FGF9*, *FGF2*, and *FGFR1*), which regulate spermatogonial stem cell homeostasis. No DEGs were correlated with testicular testosterone concentrations and only two DEGs (*PICK1*: $r = 0.95$, FDR = 0.04, $\log_2FC = 0.19$; *GARS1*: $r = -0.96$, FDR = 0.03, $\log_2FC = -0.25$) were correlated with testicular melatonin concentrations. *PICK1* is essential for normal sperm acrosome formation and function while overexpression of *GARS1* has been identified as a testicular tumor marker. These results indicate that oral melatonin supplementation influences gene networks governing testicular structure, endocrine function, and spermatogenesis in bulls. However, further investigation is needed to determine the extent to which these changes in transcript abundance translate to differences in sperm concentration, morphology, and motility.

Flow Cytometric Sorting Modifies Boar Sperm Phenotype and PLC ζ Head Signal Heterogeneity

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Abstract Text

Flow cytometric sperm sorting is widely used in animal agriculture to enrich defined subpopulations, including sex-sorted sperm, yet the procedure can adversely affect sperm physiology. Post-sort boar sperm often exhibit reduced motility and viability, capacitation-like changes, and altered membrane and mitochondrial function. Notably, individual sires differ in their tolerance to sorting, with some maintaining post-sort fertility while others experience substantial functional decline. The objective of this study was to identify cellular features that distinguish sperm populations affected by sorting and to evaluate phospholipase C zeta (PLC ζ), a sperm-borne oocyte activating factor, as a candidate marker of sorting susceptibility. To assess broad phenotypic differences, pooled semen from three boars was used to generate unsorted and fluorescence-activated cell sorted populations using a BD FACSMelody. A mock-sorted group underwent identical media handling without actual sorting. Image-based flow cytometry (IBFC; ImageStream^x Mk II) was performed at 40x magnification, and 12,000 sperm per treatment group were used to train a convolutional neural network model (VGG16 architecture) within Amnis AI software. Models were trained to discriminate sorted versus unsorted sperm using brightfield (BF), Hoechst 33342 (DNA morphology), side scatter (SSC), and channel combinations. F1 classification scores demonstrated that deep learning could reliably distinguish sorted from unsorted sperm, with F1 values of 86.06% (sorted) and 86.23% (unsorted) using BF alone, 84.57% and 86.20% using Hoechst 33342, and 80.19% and 75.01% using SSC. Performance improved with channel combinations, reaching 92.81% (sorted) and 92.88% (unsorted) when BF, Hoechst 33342, and SSC were combined. These findings indicate that sorting induces detectable morphological and structural alterations at the single-cell level. Given the known reduction in porcine IVF developmental competence (reduced cleavage) following sorting, we next evaluated PLC ζ localization as a functionally relevant marker. Using IDEAS software, PLC ζ -FITC intensity was quantified within the sperm head to exclude tail labeling. Mean head-localized PLC ζ intensity did not differ between unsorted and mock-sorted groups ($2,511 \pm 223$ SEM vs $1892 \pm 1,892$; $p = 0.765$) but was significantly altered in sorted sperm compared to control ($8,371 \pm 912$ vs unsorted $2,511 \pm 223$; $p < 0.0001$). Additionally, a subpopulation of sperm exhibiting increased PLC ζ head localization was identified in sorted sperm. This population represented $10.7 \pm 3.2\%$ of unsorted controls, $4.8 \pm 3.2\%$ of mock-sorted samples ($p = 0.529$ vs unsorted), and $84.7 \pm 0.98\%$ of sorted samples ($p < 0.0001$ vs unsorted). Data was generated from three biological replicates, each with two technical replicates, 10,000 sperm/replicate. Collectively, these results demonstrate that deep learning can detect sorting-induced phenotypic shifts in porcine sperm and that PLC ζ head localization is significantly altered following sorting. Identification of markers associated with sort susceptibility may enable pre-screening of sires for sorting tolerance, reducing reliance on costly fertility trials and facilitating broader adoption of sperm sorting technologies within the swine industry.

Urethral-derived cells are found in erectile tissues of the mouse penis

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Abstract Text

The penis, the male organ critical for reproduction, is particularly susceptible to birth defects and adult disorders. Hypospadias, a birth defect of the penis, is the second most common congenital defect in the world. Hypospadias is defined as incomplete closure of the urethra along the midline of the penis. The developmental mechanisms that drive normal urethra closure remain largely unknown. We do know that prior to urethra closure, the urethral epithelium starts out as two bilateral sheets. As the urethra closes, the bilateral urethra epithelium meet at the midline of penis. After fusion, the urethral epithelium remains connected to the skin of the penis, so there is extensive reconstruction of epithelium to form a urethral tube. The developmental mechanisms involved in the urethra reconstruction is debated, with no experimental data clearly identifying the process. To investigate this process, we generated a urethra-specific reporter mouse, *Foxa2cre x Rosa-nTnG*. After urethra formation was completed (post-natal day 10), we found the urethra epithelium was labeled with GFP, however FOXA2+ cells were also found in the urethral sphincter and the mouse analog of the corpus spongiosum. These findings led us to hypothesize that FOXA2+ cells were undergoing epithelial to mesenchymal transition (EMT). To test this hypothesis, we used live imaging during urethra closure. We found FOXA2+ cells to leave the urethral epithelium and enter the surrounding mesenchyme throughout the process of urethra closure. To determine if FOXA2+ cells exiting the urethra epithelium was associated with basement membrane breakdown, we used immunofluorescence against laminin. When the urethra begins to close, laminin becomes perforated and FOXA2+ cells were found passing through the holes in laminin. When investigating single cell mRNA sequencing of the urethral epithelium, we found a subpopulation of cells to have strong expression of EMT markers, *Zeb1*, *Zeb2*, *Vim*, *Cdh2*, and others. Overall, these data demonstrate that cells from the urethral epithelium undergo EMT and enter surrounding erectile tissues. Results from this study provide entry points to study how EMT drives urethra closure and how urethra-derived cells contribute to normal penis formation and function.

The KH Domain RNA-Binding Activity of TDRKH Regulates piRNA Trimming and Safeguards Spermatogenesis

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Abstract Text

PIWI-interacting RNAs (piRNAs) are a class of small non-coding RNAs that play a central role in transposon silencing and post-transcriptional gene regulation in the germline. TDRKH (Tudor and KH domain-containing protein) is a conserved, mitochondria-anchored scaffold protein required for piRNA biogenesis and male germline development. Loss of TDRKH results in the accumulation of extended, untrimmed pre-piRNA intermediates. However, TDRKH lacks an intrinsic 3'-5' exonuclease domain required for pre-piRNA trimming activity. How it contributes to 3' end maturation of piRNAs has remained unclear. Here we provide genetic and biochemical evidence that the KH domains provide the RNA-binding activity that positions pre-piRNA precursors for 3'-5' trimming by the exonuclease PNLDC1. In vitro disruption of KH-mediated RNA binding abolishes pre-piRNA trimming while leaving TDRKH's interactions with MIWI and PNLDC1 intact. By mutating the KH domain RNA-binding site in mice, we reveal that this activity is required for both fetal and postnatal pre-piRNA 3' end processing. KH domain mutant males exhibit defective piRNA biogenesis characterized by accumulation of extended pre-piRNA intermediates, failure to silence LINE1 retrotransposon, impaired spermatogenesis, and complete infertility. Together, these data define a mechanistic model in which TDRKH acts as a mitochondrial tether that recruits PIWI-pre-piRNA complexes and, via its KH domains, directly engages the RNA substrates to present them to PNLDC1 for exonucleolytic trimming. This RNA-positioning function explains how a non-enzymatic scaffold can be essential for precise piRNA maturation and ensures the fidelity of transposon repression during germ cell development.

A Framework for Evaluating Predicted Sperm Trajectories in Crowded Microscopy Videos

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Abstract Text

Common laboratory methods for assessing sperm motility rely on short-term measurements collected under manual observation or practical imaging conditions. These approaches work well for routine analysis, but they provide a limited grasp into how sperm navigate space, change motility patterns, or persist over time in complex environments. During natural conception, sperm must search through a maze-like structure in the female reproductive tract to reach the oocyte. In this context, egg contact depends less on brief motion patterns and more on heterogeneity in single-sperm behavior and variation across the population. This suggests that how sperm explore space over time may better reflect fertility than short-term motility metrics alone. Measuring sperm search behavior presents several challenges that are specific to sperm and not shared by most cell types. Sperm are fast-moving, visually closer to debris, and frequently enter and exit the microscope's field of view. Their trajectories can change direction rapidly, overlap in projection, or momentarily disappear, making long-term tracking difficult. These characteristics complicate trajectory reconstruction over extended recordings and limit the use of metrics that depend on continuous paths. As a result, many existing analyses favor short trajectories that overlook longer-term search behavior. Here, we develop and refine a computational framework for sperm-specific detection, tracking, and trajectory analysis that accounts for these biological and imaging constraints. Tracking parameters are optimized based on sperm morphology and imaging conditions, with an emphasis on preserving trajectory continuity over longer time scales. Using search-relevant metrics such as root mean square displacement (RMSD), we quantify how sperm explore space over time. These trajectory-based measures support predictions of sperm search efficiency in complex environments and offer a behaviorally grounded perspective on sperm performance and fertility potential.

Is Normal Fertilization Optimized When Using Fewer Sperm for Swine AI?

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Abstract Text

The use of artificial insemination (AI) in swine is a management tool that is practiced at a global scale such that greater than 90% of the world's pigs are bred using AI with fresh semen. Following industry practices associated with bovine AI, there is a continued movement in the swine industry to produce and utilize AI doses possessing fewer sperm. Historically, AI doses were produced with multiple billions of sperm per AI providing satisfactory farrow rates accompanied by double-digit numbers of total pigs born per litter. Albeit fertility in the pig is associated with a significant embryonic wastage near the level of 30% and this is understood to be partly due to polyspermic fertilization. Presently and unexpectedly, more than a few swine producers have observed an increase in total pigs born when switching to using AI doses possessing fewer sperm than previously used. The objective of this report is to provide retrospective data based on thousands of services across dozens of farms showing a slight increase in number of pigs born when a production system chooses to use an AI dose with less total sperm. A secondary objective is to propose a possible explanation for the trending results. Data from a particular sow farm is provided as an example. We compared farrow rates (FAR) and total number of pigs born (TBN) per litter for sows receiving 2.5 billion total cells/AI dose (n=758 matings) versus 2.25 billion total cells/AI dose (n=721 matings) when bred over a 12-week period within farm. Matings were performed consecutively (i.e. 6 weeks at 2.5 billion and 6 weeks at 2.25 billion) using 70mL dose volume as conventional AI with technician unaware of the cell number change. The mean FAR and TBN trends were 85.9% and 15.9 versus 90.0% and 16.5 for the 2.5 and 2.25B, respectively. We propose that a reduction in polyspermy may contribute to the upward trend occurring because of fewer sperm at the sperm reservoir leading to an increase in fertility.

Fertility-Associated Proteins Are Newly Synthesized During Bovine Sperm Capacitation

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Abstract Text

Ejaculated mammalian sperm were considered translationally quiescent due to inherently fragmented ribosomal RNA and limited cytoplasm, suggesting that sperm functions rely solely on pre-existing proteins and post-translational modifications. However, we previously reported a capacitation-associated increase in testis-specific isoform of Na/K-ATPase (ATP1A4) in bull sperm, suggesting *de novo* protein synthesis. We performed Surface Sensing of Translation (SUnSET) assay in frozen-thawed bull sperm to identify *de novo* synthesized proteins during capacitation and investigate their potential roles in sperm function. Frozen-thawed sperm were Percoll-washed, motile fractions were collected and in vitro capacitated for 45 min and then exposed to puromycin (7.5 µg/mL, 15 min) to label nascent peptides. Control received no puromycin. Sperm lysates (RIPA buffer) were analyzed via western blotting (n = 5) using anti-puromycin antibody, with Ponceau S as a loading control. Puromycin-incorporated peptides were immunoprecipitated (IP) using Protein A/G Agarose beads conjugated with anti-puromycin antibody and analyzed via silver-stained SDS-PAGE (n = 3) followed by tandem mass spectrometry (MS/MS). Differentially synthesized proteins were identified using unpaired t-tests ($p \leq 0.05$) with Benjamini–Hochberg correction ($p \leq 0.01$). Gene Ontology and KEGG pathway analyses were performed with ShinyGO (0.85). Localization of puromycin-labelled peptides was examined through immunocytochemistry and confocal microscopy. Additionally, fertility-associated differences in puromycin incorporation (n = 10, frozen-thawed semen) was evaluated with flowcytometry, alongside sperm kinematics assessed through Computer-Assisted Semen Analysis (CASA). Western blot demonstrated several sperm proteins (250-10 kDa) incorporating puromycin. IP coupled with MS/MS identified 158 proteins exclusively bound to anti-puromycin-conjugated beads, but not to control beads (bead alone, no antibody). ATP1A4 was one of the puromycin-labelled proteins, validating our previous findings. Functional enrichment showed activities related to endopeptidase inhibition, translation regulation, ribonucleotide binding and glycosaminoglycan binding. These puromycin-labelled peptides were a component of sperm mid-piece, flagellum, acrosomal vesicle, plasma membrane and ribosomes. KEGG analysis highlighted enrichment in complement and coagulation cascades, metabolic pathways, cGMP–PKG signalling, and HIF-1 signalling pathways. Immunocytochemistry demonstrated strong puromycin labelling in the post-acrosomal region and mid-piece, with weaker signals in the principal piece and acrosomal region. High-fertility group exhibited significantly greater total motility, curvilinear distance (DCL) and curvilinear velocity (VCL) values, whereas low-fertility group had a significantly higher straightness (STR) and linearity (LIN) values. High-fertility bulls demonstrated right-shifted peaks compared to low fertility bulls, indicating higher global protein synthesis, which could influence fertility outcomes. We conclude that sperm synthesize new proteins during capacitation, which could support biochemical events during capacitation, acrosome reaction, and fertilization. We also demonstrated a fertility-associated difference in sperm kinematics and protein synthesis. Furthermore, our previous work suggests that some of these newly synthesized proteins associate with mRNAs during capacitation, supporting a potential translational regulatory mechanism in sperm. Reconceptualizing the dogma of translational quiescence could transform approaches to fertility evaluations and management.