Vaginal healing research: from bench to bedside and beyond

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Introduction: For women and girls undergoing pelvic radiation or any surgical intervention within the pelvic area, they can unfortunately expect debilitating vaginal tissue fibrosis. Additionally, every woman will reach menopause which wreaks havoc on the vagina's elasticity and lubrication and can cause immense associated pain. The resulting lifelong sequelae can interfere with their daily lives, including retained menstruation, painful vaginal exams, and dyspareunia. While some strategies such as vaginal stents are known to aid in healing, we do not fully understand the underlying mechanisms behind varying degrees of vaginal fibrosis. It is our goal to elucidate the vaginal healing process so that we can refine therapeutic strategies in the clinic for improved quality of life.

Methods: We developed both in vitro and in vivo methods to elucidate fundamental mechanisms of vaginal healing. In vitro, we developed a relatively simply protocol to isolate primary murine vaginal fibroblasts. We are also designing a vagina-on-chip to model the interplay between vaginal layers thus better mimicking a human vaginal environment. Murine models, including full thickness wounds and bleomycin instillation plus vaginal trauma, were developed to study regenerative vaginal healing and fibrosis, respectively. We assessed histologic and gene expression changes at various time points after insults in combination with oophorectomy. Clinically, vaginal stents were designed and validated in a porcine model and review panels. Lastly, implemented virtual reality (VR) technology and 3D imaging facilitated and continues to improve pre-operative training.

Results: We have successfully isolated and rapidly expanded murine primary vaginal fibroblasts, which has allowed us to study the response to estrogen and mechanical stretch. Initial prototypes for the vagina-on-chip effectively integrate fluidic components with distinct multilayer hydrogels. Full thickness murine vaginal wounds result in complete epithelial layer closure and resolution of inflammatory changes by 72 hours, which is altered in low estrogen-oopherectomized mice. Bleomycin instillation alone did not lead to fibrosis, but in combination with wire brush trauma led to disrupted collagen organization. Physical vaginal stent designs to improve retention and patient comfort were validated and two designs were chosen for further benchtop testing in a novel pressurized 3D-printed model and a funded Phase I clinical trial. Resorbable stents were designed with polycaprolactone-based shape memory foams to provide sufficient radial force to maintain vaginal caliber over a target healing time of 4 weeks. Lastly, we are improving clinical training with 3D imaging and VR modeling.

Conclusion: We are advancing vaginal healing research by translating clinical problems into basic science questions and closing the loop ultimately back to bedside improvements. By parallelizing in vitro and in vivo methods of studying vaginal healing, we are driving advancements with multiple platforms within an underappreciated organ.

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Dietary coconut oil mitigates hyperandrogenemia in obese female pigs due to suppression of androgen steroidogenesis in the adrenal cortex and theca externa

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Introduction: An increasing number of obese reproductive-aged women experience hyperandrogenemia and infertility. A sedentary lifestyle in combination with a western-style diet (WSD) may predispose women to these reproductive co-morbidities and metabolic syndrome. Although a healthy diet and exercise are the first line of treatment for obesity, many patients struggle to improve their health with these approaches. Coconut oil, which is primarily comprised of medium chain saturated fatty acids and modulates both glucose homeostasis and cholesterol levels in animal models, may be a promising nutraceutical to improve metabolic health and fertility in obese women. We hypothesized that dietary coconut oil as a part of a high fat diet would mitigate obesity-associated hyperandrogenemia via suppression of androgen steroidogenesis in the theca externa and adrenal cortex.

Methods: Seventeen sexually mature female Ossabaw pigs were divided into three dietary groups: control diet (n = 6; C; 2200 kcal/pig/day), high fat western-style diet (n = 5; WSD; 5000 kcal/pig/day), or high fat coconut oil diet (n = 6; COC; 5000 kcal/pig/day). The ingredients in WSD and COC diets were the same except 9% of calories in the WSD group were from lard and 9% of calories in the high fat COC dietary group were from virgin coconut oil. Pigs were fed for nine estrous cycles (~ 8 months) with fasting blood collected and trans-rectal ovarian ultrasound imaging performed at estrous cycles 0, 7, and 9. After estrous cycle 7, ovarian folliculogenesis and steroidogenesis were suppressed by daily oral administration of 45 mg of synthetic progestogen per pig for 18 days starting at estrus (D1) of estrous cycle 8. On day 18, while ovarian steroidogenesis was suppressed, adrenocorticotropic hormone (ACTH) stimulation of adrenal androstenedione (A4) steroidogenesis was performed. On day 19, the oral progestogen was withdrawn, and 7.5 µg/kg IM dexamethasone was given every 12 hours to suppress adrenal steroidogenesis. On day 20, 3000 IU/m2 human Chorionic Gonadotropin (hCG) was given intravenously and fasting blood was collected at: 12, 24, 36, 48, 72, and 96 hours post-hCG for serum A4. In estrous cycle 9 ovaries with dominant ovarian follicles (5-7 mm) and the adrenal glands were collected. Follicular fluid was assessed by LC-MS/MS for steroid hormones. Theca externa and adrenal cortical cells were cultured for 48 hours in the following treatments: 1) theca: basal, insulin (I; 100 ng/ml), LH (10 ng/ml), or LH+I; 2) adrenal: basal or 1 µM ACTH.

Results: WSD pigs had a protracted estrous cycle length and increased FF testosterone, A4, androstenediol, and allo-pregnanolone compared to C pigs. COC pigs had a normal estrous cycle length and increased FF testosterone, dihydrotestosterone, and allo-pregnanolone compared to C pigs. Both serum and cell culture media A4 concentrations were highest in response to ACTH in WSD as compared to C or COC pigs. By 72 hours post-hCG, COC pigs had higher total serum A4 than C or WSD pigs but cell culture LH and LH+I groups yielded higher media A4 concentrations in WSD as compared to COC pigs. COC pigs developed significantly more follicles at 24, 48 and 72 hours post-hCG administration. Interestingly, in contrast to total serum A4, A4 per follicle was lower in COC and WSD as compared to C pigs at 72 hours post-hCG administration.

Conclusions: These results suggest that inclusion of dietary coconut oil in a high fat diet may suppress excessive androgen steroidogenesis by adrenal cortex but may only partially mitigate excessive androgen production by theca externa in obese females. Additionally, coconut oil dietary treatment may improve ovarian follicular development via LH action in obese females.

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SARS CoV2 nonstructural proteins reprogram placental autophagy and also impair lipid metabolism

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Introduction: COVID-19, caused by SARS COV2, is mainly considered a respiratory disease, but emerging evidence points to a high rate of pregnancy complications in infected women including premature birth and preeclampsia, stillbirth, and developmental defects. The viral mechanisms driving these responses and the impact of viral infection in the placenta and the developing fetus remain to be elucidated. Like other RNA viruses including ZIKV, SARS CoV2 has been reported to modulate intracellular vesicular trafficking pathways for its replication and propagation. We have previously shown that basal autophagic activity is a crucial component of placental syncytiotrophoblast barrier function and plays a key role in vertical transmission of ZIKV and limits fetal damage. However, whether SARS COV2 alters intracellular trafficking and regulates autophagy in the placenta remains unknown.

Methods: Placental tissues from deliveries in women are positive for SARS COV2 were investigated for modulation of autophagic activity via monitoring the autophagy flux markers, LC3B and p62 by immunofluorescence. Frozen placental tissues from COVID19+ and negative patients were investigated for accumulation of lipid droplets by BODIPY staining. Trophoblast cell line, JEG-3, was transfected with mammalian expression plasmids encoding SARS CoV2 non-structural proteins (NSPs), which are critical elements of the viral replication and transcription complex. The transfected cells were analyzed for autophagy flux markers by western blots and immunofluorescence. The expression levels of autophagy proteins were correlated with lipid droplet area and number.

Results: We show that overexpression of SARS COV2 non-structural proteins, ORF3a and NSP6 modulates autophagic response and upregulates lipid droplet biogenesis respectively. NSP6 in particular associates with upregulation of lipid droplets whereas ORF3a shows a stronger response in blocking the fusion of autophagosome with lysosomes. The blocking of autophagy which is an essential pathway in normal functioning of trophoblasts could lead to poor placentation. These altered pathways may trigger inflammatory responses during pregnancy which could drive adverse outcomes.

Conclusion: Our findings suggest that SARS COV2 modulates autophagy machinery for its own replication and likely has an inhibitory effect on the autophagy process via blocking autophagosome/lysosome fusion and affecting lipid droplet metabolsim. Ongoing studies are investigating the mechanism of action of SARS COV2 including modulation of host processes via its nonstructural proteins. Studies of these NSPs will shed light on their specific role in COVID19 pathogenesis in the placenta and may also provide attractive drug targets for intervention.

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Cycle characteristics and treatment outcomes among BRCA mutation carriers undergoing in-vitro fertilization

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Introduction: Female carriers of *BRCA1/2* gene mutations often undergo in-vitro fertilization (IVF) for fertility preservation, infertility, and/or preimplantation genetic technology (PGT). However, recently there has been concern regarding the reproductive potential of *BRCA1/2* carriers. Specifically, some authors suggest carriers of *BRCA1/2* have poorer ovarian response with ovarian stimulation and reduced parity, perhaps secondary to the role of *BRCA1/2* in maintaining telomere length in embryogenesis. To assess if *BRCA1/2* carriers may be at risk for suboptimal outcomes after IVF, we seek to evaluate the differences in IVF utilization patterns and outcomes between *BRCA1/2* and non-*BRCA1/2* mutation carriers.

Methods: This was a single-center retrospective cohort study of *BRCA1/2* mutation carriers who presented for fertility preservation and/or preimplantation genetic technology (PGT). The electronic medical record was queried using the ICD 10 code Z84.81 to identify all female patients with *BRCA1/2* mutations who presented to our clinic over the past 10 years. Patient demographics, medical history, and treatment characteristics such as cycle type, number of oocytes retrieved, fertilization rates, number of embryos, and pregnancy outcomes were abstracted from the chart. IVF cycle type was compared among all *BRCA1/2* carriers. Additionally, *BRCA1/2* carriers who underwent IVF were compared to age and BMI-matched controls. The primary outcome of interest was fertilization rate. Welch's two sample T-tests were performed to compare interval data, and a p-value less than 0.05 was considered statistically significant.

Results: Out of 27 BRCA1/2 patients who presented for evaluation, 15 (55%) *BRCA1/2* mutations carriers elected to undergo IVF for embryo banking and preimplantation genetic testing (PGT), whereas 4 (7%) underwent oocyte cryopreservation. Of the BRCA mutation carriers undergoing IVF, 74%, 19%, 4%, and 4% were White, Hispanic, Asian, and Black, respectfully. *BRCA1/2* mutations carriers had similar mean AMH (4.14 ± SD 2.53 vs 4.62 SD ± 4.33; p=0.718), mean number of oocytes retrieved (19 ± SD 9.68 vs 18.93 ± SD 10.27; p=0.986), mean number of mature oocytes (15.5 ± SD 8.42 vs 14.92 ± SD 8.81; p=0.867), fertilization rate (73.95% ± SD 18.89 vs 75.61% ± SD 13.92; p=0.786), mean number of blastocysts (5.67 ± SD 3.62 vs 5.87 ± SD 3.52); p=0.879, clinical pregnancy rate (53.3% vs 60%; p=0.713), and live birth rate (46.7 vs 60%; p=0.715) to age and BMI-matched controls. Although not statistically significant, *BRCA1/2* patients had fewer mean euploid embryos per cycle (2.15 ± SD 1.34 vs 3.67 ± SD 2.58). The euploid rate (euploid per 2 pronuclei embryos) was 42% in BRCA1/2 carriers vs 59% in controls (p=0.1312).

Conclusion: Despite their *BRCA1/2* mutations carrier status, patients with *BRCA1/2* or *BRCA1/2* have similar IVF outcomes including fertilization, pregnancy, and live birth rates to non- carriers. *BRCA1/2* mutations carriers demonstrate normal response and outcomes after IVF.

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Placental NRF2 May Serve a Key Role in Maternal-Fetal Tolerance during Pregnancy

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Introduction: A fundamental unanswered question in reproductive biology is: What protects the hemi-allogeneic fetus from rejection by the maternal immune system? We are testing the hypothesis that the multinucleated syncytiotrophoblast (SynT), formed by fusion of proliferative cytotrophoblasts (CytT) and covering the chorionic villi, may serve a critical role through production of immune modulators that act on immune cells within the maternal decidua to protect the hemiallogeneic fetus from rejection by the maternal immune system.

Methods: Human primary CytT and human trophoblast stem cells (hTSCs; provided by Drs. Okae and Arima, Sendai, Japan) cultured under conditions to promote CytT to SynT differentiation in 20% O2, or in a hypoxic (2% O2) environment, were analyzed at different times using RT-qPCR and Western blotting. We also analyzed placentae of global *Nrf2* knockout (KO) vs. wild-type (WT) mice at 12.5 days post-coitum (dpc) for effects on immune modulator expression.

Results: We observed that genes involved in the induction and maintenance of immune tolerance were markedly upregulated upon CytT to SynT differentiation of mid-gestation human trophoblasts in primary culture and of hTSCs. These immune modulators include HMOX1, PD-L1, GDF15, and the kynurenine receptor, AhR. Intriguingly, we discovered that the redox-regulated transcription factor NRF2 and co-regulated C/EBPβ and PPARγ, which serve critical roles in mouse labyrinthine trophoblast development, were also markedly induced during SynT differentiation. Notably, NRF2 knockdown prevented induction of C/EBPβ, PPARγ and the immune modulators, as well as induction of aromatase (CYP19A1), a key marker of human SynT differentiation. ChIP-qPCR revealed that temporal induction of aromatase and immune modulators was associated with increased binding of endogenous NRF2 to its putative response elements within their promoters, indicating that these transcription factors and immune modulators are direct downstream targets of NRF2. Consistent with our cell culture studies, placentas of global *Nrf2* KO mice at 12.5 dpc manifested a significant decrease in *C/ebpβ, Pparγ, Hmox1* and *Ahr* mRNA, compared to those of WT mice. Importantly, NRF2 deficiency has been implicated in preeclampsia, a hypertensive disorder of pregnancy, associated with shallow implantation, inflammation and placental hypoxia. Notably, when hTSCs were cultured in a hypoxic environment, the differentiation-associated induction of NRF2, C/EBPβ, aromatase and immune modulators was prevented.

Conclusion: Our compelling findings suggest that the O2-regulated transcription factors, NRF2, C/EBPβ and/or PPARγ, serve as key regulators of immune modulator expression during SynT differentiation. We propose that the immune modulators are secreted into maternal blood, or directly into the decidua, where they act on immune cells to maintain maternal tolerance to the hemi-allogeneic fetus during pregnancy.

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Novel concepts of oocyte dynamics during oviductal transport

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Introduction: Traditionally, the oviduct is a neglected organ in research due to the success of in vitro fertilization where the oviduct is bypassed. In recent years however, attention has turned to the oviduct due to the importance of the oviductal microenviroment in creating the optimal conditions for fertilization as well as gamete/embryo survival and transport. Transport in the oviduct is governed by three main factors: ciliary beating, smooth muscle contraction and the flow of tubal fluid. Little is known regarding the individual roles of these factors, but previous experiments involving targeted smooth muscle inhibition primarily relied on evaluating the reproductive outcome - whether or not oocytes reached the fertilization site or if embryos implanted successfully. The dynamics involved in oocyte and embryo transport before fertilization and implantation are unknown due to a lack of imaging techniques capable of visualizing intraoviductal events in vivo. Furthermore, it is not clear if such dynamics are conserved between species. Therefore, the aim of this study was to investigate the dynamics of oocyte transport in the murine and bovine ampulla.

Methods: In the bovine model, gameto-maternal interactions were investigated using segments of the ampulla which were excised, opened longitudinally and visualized with a digital live cell imaging system equipped with immersion objectives. In the murine model, cumulus oocyte complex (COC) dynamics were investigated in vivo using a house-built spectral domain optical coherence tomography (OCT) system which utilizes a broadband laser centered at ~810 nm with a spectral wavelength of ~100 µm. It employs low coherence interferometry which provides an axial resolution of ~5 µm in tissue and an imaging depth covering the entire oviductal lumen. The spatial resolution clearly distinguishes individual oocytes, embryos, and cumulus cells, making OCT a highly unique tool to study reproductive processes in vivo. In order to bypass skin and muscle layers, intravital imaging windows were implanted on the dorsal surface of female mice through a single surgical procedure. This approach was used to investigate the dynamics of unfertilized oocytes in virgin females. 3D and 4D data sets were reconstructed and rendered using Imaris software. Specifically, the spot tracking and cell tracking functions were used to quantify COC trajectories and speed.

Results: Live cell imaging of explanted sections of bovine ampulla revealed that the COC displayed circular movements as if rolling along the ciliated epithelium. Similarly, in the mouse ampulla, in vivo volumetric OCT imaging revealed highly mobile COCs that displayed continuous and fast circular movements, completing a circle within a few minutes and remaining intact throughout movement. In the mouse model, COC tracking showed that the 3-dimensional paths formed by each COC were synchronized despite being spatially distinct from each other and having different trajectory lengths (COCs proximal to the ciliated epithelium had a longer trajectory than COCs located more distally). As a result, the average speed of circling COCs ranged from 7.0 to 18.4 um/sec. COC speed was affected by path perimeter, such that COCs with longer trajectories displayed higher speeds ($p<0.001$, linear regression). It is likely that COCs located adjacent to the epithelium are influenced by the beating cilia. A previously unknown inter-ampullary gate was found in mouse oviducts which restricted the progression of COCs from the upper ampulla into the mid ampulla. Remarkably, this luminal constriction opened over time and was fully dilated at 6 hours, when COCs were finally permitted to progress to the lower ampulla. This luminal constriction is likely to function in the same way as other junctions in the oviduct such as the ampullary-isthmus and uterine junction, and regulate the timely transport of COCs.

Conclusion: These results suggest that oviductal transport of oocytes is controlled by spatially dependent regulatory processes and is much more complex than originally thought. The circular movements of COCs in the bovine and murine ampulla suggests that this process might be conserved across species, despite significant reproductive differences such as length of pregnancy and ovulation rate, and raises the question if cilia might be physiologically important in maintaining this process. Future work will focus on disrupting ciliary dynamics to quantitatively investigate the role of ciliary activity in orchestrating COC dynamics in the ampulla.

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Functional similarity between TGF-beta type 2 and type 1 receptors in the female reproductive tract

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Introduction: Transforming growth factor β (TGFβ) signaling plays critical roles in reproductive development and function. TGFβ ligands signal through the TGFβ receptor type 2 (TGFBR2)/TGFBR1 complex. As TGFBR2 and TGFBR1 form a signaling complex upon ligand stimulation, they are expected to be equally important for propagating TGFβ signaling that elicits cellular responses. However, several genetic studies challenge this concept and indicate that disruption of TGFBR2 or TGFBR1 may lead to contrasting phenotypic outcomes. Thus, it is critical to assess whether TGFBR2 and TGFBR1 function similarly in a given experimental system to understand their contextual interactions. In a previous study, it was found that conditional deletion of *Tgfbr1* using anti-Mullerian hormone receptor type 2 (*Amhr2*)-Cre causes oviductal and myometrial defects. However, the role of TGFBR2 in the female reproductive tract and the potential phenotypic divergence/similarity resulting from conditional ablation of either receptors remain unknown.

Methods: Mice harboring *Tgfbr2* conditional deletion and *Tgfbr1/2* double deletion using *Amhr2*-Cre were generated in the current study. Immunohistochemistry and indirect immunofluorescence were utilized to analyze histological features of the reproductive tract, while qRT-PCR was conducted to determine mRNA levels of candidate genes.

Results: Tgfbr2 conditional knockout (cKO) mice showed disrupted myometrial formation and developed oviductal diverticula. These mice also developed age-dependent endometrial abnormalities similar to those observed in *Tgfbr1* cKO mice. In addition, reduced expression of potassium channel associated gene potassium channel subfamily K, member2 (Kcnk2) and stroma cell differentiation marker membrane metallo endopeptidase (*Mme/Cd10*) was found in the uteri of *Tgfbr2* cKO mice at PD10, suggesting potential alteration of ion channel function and endometrial differentiation. Furthermore, genetic removal of *Tgfbr1* in the *Tgfbr2*-deleted uterus had minimal impact on the phenotype of *Tgfbr2* conditional knockout mice. Thus, conditional deletion of *Tgfbr2* led to a similar phenotype to that of *Tgfbr1* deletion in the female reproductive tract.

Conclusion: Our results reveal the functional similarity between TGFBR2 and TGFBR1 in maintaining the structural integrity of the female reproductive tract. The *Tgfbr2* cKO mice may be exploited to understand the pathogenesis of oviduct-, myometrium-, and uterine epithelium-associated disorders and diseases that adversely affect pregnancy outcomes. The *Tgfbr2* cKO mice may also serve as a reference model in dissecting the contextually dependent function of TGFBR2 and TGFBR1, in terms of the development of compensatory signaling branches and/or interactions between one receptor with available signaling components upon inactivation of the other.

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Exogenous Progesterone in Early Pregnancy has Programming Effects on Phosphate, Calcium, and Vitamin D Signaling in the Ovine Endometrium and Placenta in Late Pregnancy

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Introduction: Calcium and phosphorous are two of the most abundant minerals in the body, with critical roles postnatally in the skeletal and renal systems, and in the regulation of many processes including cellular proliferation, protein synthesis, and cellular metabolism. Despite the wide appreciation that the fetus is hypercalcemic and hyperphosphatemic to ensure appropriate fetal skeletal mineralization, the mechanisms governing transplacental transport of calcium and phosphate remain poorly investigated. Progesterone (P4) is the hormone of pregnancy, critical for regulation of the establishment and maintenance of pregnancy across mammalian species. Administration of P4 to ewes during the first 9 to 12 days of pregnancy accelerates blastocyst development by Day 12 of pregnancy, likely due to P4-induced up-regulation of key genes in uterine epithelia responsible for secretion and transport of components of histotroph into the uterine lumen. This study aimed to determine if acceleration of blastocyst development induced by exogenous P4 during the pre-implantation period affects conceptus (fetus and associated placental membranes) growth, as well as calcium, phosphate, and vitamin D signaling in the endometria and placentae in late gestation.

Methods: Suffolk ewes (n=48) were mated to fertile rams and received daily intramuscular injections of either corn oil vehicle (CO, n=28) or 25 mg P4 in CO (P4, n=20) for the first 8 days of pregnancy. Ewes were hysterectomized on Days 9 (CO, n=5; P4, n=6), 12 (CO, n=9; P4, n=4), and 125 (CO, n=14; P4, n=10) of pregnancy, and endometrial and placental samples collected for analyses. The expression of mRNAs involved in phosphate (ADAM1*0, ADAM17, KL, FGFR1, FGFR2, SLC20A1,* and *SLC20A2*), calcium (*S100G, TRPV6*, and *ATP2B4*), and vitamin D signaling (*VDR, CYP2R1*, and *CYP11A1*) was quantified using qPCR analyses. KL protein was immunolocalized in Day 9, 12, and 125 endometria and Day 125 placentomes. Additionally, the calcium binding proteins S100G and S100A9 were immunolocalized in Day 9, 12, and 125 endometria.

Results: While conceptus development was enhanced in P4-treated ewes compared to CO-treated ewes at Days 9 and 12 of gestation, treatment of ewes with P4 did not alter fetal or placental growth at Day 125 of gestation. No significant differences in endometrial expression of mRNAs for the candidate genes involved in phosphate, calcium, or vitamin D signaling were detected at Day 9. At Day 12, the expression of *FGFR2* mRNA was decreased in endometria from ewes treated with P4 in early pregnancy compared to CO-treated ewes ($P<0.01$). Expression of *ADAM10* ($P=0.08$), FGFR2 ($P=0.07$), *ATP2B4* ($P<0.05$), and *CYP2R1* ($P=0.09$) mRNAs was greater in endometria from ewes treated with P4 in early pregnancy compared to CO-treated ewes at Day 125 of gestation. Similarly, the expression of *ADAM10* ($P<0.01$), *FGFR2* ($P<0.05$), *ATP2B4* ($P=0.1$), *TRPV6* ($P<0.05$), and *VDR* ($P<0.05$) mRNAs was greater in placentae from P4-treated compared to CO-treated ewes at Day 125 of gestation. In contrast, expression of *KL* ($P<0.05$) mRNA was less in placentae from P4-treated compared to CO-treated ewes at Day 125 of gestation. Interestingly, expression of *ADAM17* ($P<0.05$), *ATP2B4* ($P<0.01$), *S100G* ($P<0.05$), *SLC20A2* ($P=0.07$), and *VDR* ($P<0.05$) mRNAs was greater in placentae of ewes bearing twin conceptuses and treated with P4 compared to other treatment groups at Day 125 of gestation. KL protein was localized to the myometrium, uterine superficial glandular epithelium (sGE), and luminal epithelium (LE), but not the glandular epithelium (GE). P4 treatment did not influence the endometrial localization of KL protein at Days 9, 12, or 125 of gestation. Interestingly, KL protein was less abundant in uterine LE and sGE of P4-treated ewes compared to CO-treated ewes at Day 9 of gestation. KL protein was localized to the caruncle, cotyledon, and syncytium of placentomes at Day 125 of gestation, with no expression detected in stromal cells of the chorioallantois. S100A9 protein was localized to the uterine LE, sGE, and GE throughout gestation, and to the uterine stratum compactum at Day 125 of gestation. The intensity of S100A9 staining was less on Day 12 compared to Days 9 and 125 of gestation. P4 treatment did not alter the localization of S100A9 in the uterine endometrium at Days 9, 12, or 125 of gestation. In contrast, the calcium binding protein S100G localized to uterine GE and sGE, but not LE at Days 9, 12, and 125 of gestation. S100G protein expression

was less in uterine GE of P4-treated compared to CO-treated ewes at Day 12 of gestation. At Day 125 of gestation, there was downregulation of S100G protein expression in uterine sGE of P4-treated compared to CO-treated ewes.

Conclusion: Exogenous P4 administered in early pregnancy regulates the expression of key genes for calcium, phosphate, and vitamin D regulatory molecules on Day 125 of pregnancy, suggesting a ‘programming’ effect of P4 on mineral transport at the maternal-conceptus interface. Improving the understanding of how sex steroids regulate placental transport of fundamental minerals has the potential to improve pregnancy outcomes and fetal growth in both humans and livestock species.

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Oocyte-specific deletion of the SUMO E2 ligase, *Ubc9*, results in chromosome abnormalities and meiotic arrest

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Introduction: The ovarian reserve consists of a pool of non-growing, prophase I arrested oocytes that are formed perinatally. These oocytes remain meiotically arrested until a surge of luteinizing hormone results in the resumption of meiosis immediately prior to ovulation. Though meiotically arrested, oocytes within follicles are transcriptionally active in preparation for the resumption of meiosis and early stages of embryogenesis. Once oocytes reach the antral stage of folliculogenesis, their chromosomes condense, and they become transcriptionally silent. Defects in the maturation process lead to meiotic defects, aneuploid oocytes, follicular atresia, or non-viable embryos. SUMOylation, a dynamic posttranslational modification (PTM), has been shown in mature germinal vesicle (GV) stage oocytes in vitro to be required for proper formation of the meiotic spindle and chromosome segregation.

Methods: Our lab previously showed that SUMOylation is essential for female fertility and proper meiotic maturation in mouse oocytes by generation of an oocyte conditional deletion of *Ubc9*, the only E2 ligase in the SUMOylation pathway. *Ubc9* was deleted in oocytes within primordial oocytes by crossing *Ubc9*$^{flox/flox}$ mice to *Gdf9*-iCre. To further characterize the role of SUMOylation during folliculogenesis and meiosis, we have crossed the *Ubc9*$^{flox/flox}$ mice to *Zp3*-cre to delete *Ubc9* in oocytes within primary follicles, after the oocytes have been recruited for folliculogenesis.

Results: Similar to the previous model, loss of *Ubc9* at the primary follicle stage results in female sterility. However, unlike oocyte-specific deletion of Ubc9 in primordial follicles, deletion at the primary follicle stage does not affect stability of the ovarian reserve or cause a significant decrease in the proportion of oocytes that resume meiosis. Loss of Ubc9 in oocytes of growing follicles results in a decrease in the number of oocytes containing surrounded nucleoli (SN) and an increase in oocytes with non-surrounded nucleoli (NSN), indicating defects in chromosomal condensation and transcriptional silencing. Additionally, these oocytes arrest at metaphase I, with intact spindles and poorly organized chromosomes.

Conclusion: We hypothesize that SUMOylation regulates key changes in chromosome structure as well as meiotic maturation and progression of oocytes. Further investigation is necessary to understand the complex roles of SUMOylation during oocyte meiotic maturation. These studies were supported by NIH/NICHD RO1 HD085994 (to S.A.P.).

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Maternal nutrition and regulation of oocyte number and quality

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Introduction: Maternal nutrition impacts the health of the progeny. However, it is uncertain how maternal nutrition regulates the number and quality of the oocytes. In mammals, oocytes are formed in the fetal ovary. Thus, understanding the signaling pathways or factors that determine oocyte number or quality *in vivo* is challenging. To overcome this, we use the worm *Caenorhabditis elegans* as a model, where oocyte development during meiosis I occurs in the adult gonad. In *C. elegans*, insulin-like signaling couples the production of viable gametes with the availability of nutrients. In the presence of food, insulin-like receptor signals through the conserved RAS-ERK pathway to drive meiotic prophase I progression and oogenesis. In the absence of food, inactivation of the signaling pathway halts oogenesis. Thus, availability of nutrients is directly linked with oocyte numbers and quality; the mechanisms remain unknown. Similar to nutrient deprived animals, genetic mutants with loss of ERK activation [ERK(loss)] also display lower number of oocytes even in the presence of nutrition. Conversely, increased ERK activation, through a RAS gain of function allele [RAS(act)], results in production of increased oocyte number. In both cases, the oocytes formed are low in quality, as upon fertilization they lead to embryonic lethality. Using these mutants, here we discovered that how the RAS/ERK pathway regulates oocyte number and quality in *C. elegans* gonad.

Methods: Given the advantage of transparent body of the worms and transgenic worm lines, we used several imaging techniques including fluorescent and confocal microscopy for counting oocyte number, germ cell proliferation, apoptosis and detection of synaptonemal complex (SC) in either live worms or immuno-stained germlines. To determine ERK substrate we bacterially expressed proteins and performed in vitro kinase assay. Following the detection of the ERK substrate (HTP-1) and its phospho-acceptor site (S325), we made phosphodead and phospho-mimetic *htp-1* mutant lines using CRISPR/Cas9-mediated genome editing technique. To detect the localization of p-HTP-1(S325) in the germline, we made phosphosite-specific antibody and performed immunostaining assay.

Results: We found that neither germline stem cell proliferation nor pachytene germ cell apoptosis is involved in RAS/ERK-mediated oocyte number regulation. Rather, precocious activation of ERK in RAS(act) mutant triggers the early transition of pachytene germ cells to the diplotene stage to form oocytes. To control these events of cell-stage transition, RAS/ERK pathway regulates meiotic chromosome axis protein HTP-1 by phosphorylation at serine-325. Phosphorylated HTP-1(S325) accumulates in early-mid pachytene stage of germ cells in an ERK-dependent manner, which is necessary for synaptonemal complex (SC) extension and/or maintenance. Lack of ERK activation, and hence HTP-1(S325) phosphorylation, results in asynapsis of the homologus chromosomes, slower meiotic progression, persistence of meiotic DNA double strand breaks, aneuploidy and overall reduced oocyte number and quality. Restoring HTP-1 phosphorylation in the ERK(loss) mutants rescues the reduced oocyte number, through correction of the SC defects. Removal of HTP-1 phosphorylation reverses the increased oocyte number in RAS(act) mutants.

Conclusion: Together, these data suggest that threshold ERK activation coordinates chromosomal behavior via HTP-1 phosphorylation, with meiotic progression to produce healthy oocytes in favorable nutrient enriched environment. Given the conserved nature of meiosis and RAS/ERK pathway we propose that maternal nutrition might play a critical role in regulating the number and the quality of the oocytes.

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Identifying pre-pubertal primate spermatogonial stem cells and their niche

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Introduction: Advances in cancer treatments have improved the life expectancy of childhood cancer patients, but may also result in a lifetime of side-effects, including male infertility. Spermatogonial stem cell (SSC) transplantation into the testis of infertile recipients is a promising approach for restoring fertility. However, utilizing this technology in humans is limited by the lack of exclusive biomarkers which can unequivocally identify therapeutic SSCs. In addition, the scarcity of SSCs which can be recovered from cryopreserved tissue may necessitate their ex vivo expansion. We reasoned that conserved primate testicular transcriptome signatures will help refine the identity of the most likely pre-pubertal human SSCs and identify unique testicular somatic cell populations that may comprise the primate SSC niche to advance their propagation *in vitro*.

Methods: We performed single-cell RNA-seq (10x Genomics) on unselected testis cells from 2 immature baboons (P0, 26mo) and 2 rhesus monkeys (15mo, 20mo) from the Southwest National Primate Research Center. Using Seurat, we analyzed single-cell transcriptomes of 23,219 baboon and 25,342 rhesus monkey testis cells, respectively. Unbiased clustering and subsequent differential expression analysis identified transcriptional signatures of major spermatogonial populations in both non-human primate species. Using Monocle3, we inferred the trajectory pseudo-time for each species independently. To identify a conserved primate SSC gene expression signature diagnostic of the immature human SSC population, we compared our primate data with published pre-pubertal human datasets and matched with markers of functionally-defined mouse SSCs.

Results: Unbiased clustering of baboon germ cells indicated there were 4 subgroups which displayed a gradient of expression of undifferentiated spermatogonial markers (*ID4, ETV5, UTF1, FGFR3*) and some differentiated spermatogonial markers (*DMRT1, SOHLH1*). Similar analysis of rhesus monkey germ cells defined 5 subsets which were characterized by a gradient of expression of *ID4*, but also species- specific features including absence of *UTF1* and *FGFR3* expression. In contrast, unbiased clustering revealed seven distinct groups in human spermatogonia. Cells in the putative human SSC cluster were characterized by the high expression of undifferentiated spermatogonial markers (*FGFR3, ID4, UTF1*). Interestingly, expression of known SSC genes (*ETV5, GFRA1, ZBTB16*) was coincident with expression of markers of spermatogonial differentiation (*DMRT1, NANOS3, SOHLHL1*) in all three species. In addition, *KIT* and *STRA8* mRNAs were essentially absent in all three species, indicating an absence of retinoic acid (RA) signaling. Surprisingly, we detected a subset of cells expressing multiple meiosis-related genes (*SYCP2, SYCP3, HORMAD, MEIOB*) among human pre-pubertal germ cells. Meiotic cells were not observed in baboon or rhesus cells. We next compared the differentially expressed genes among germ cells from each species and found 82 genes which were enriched in the putative human SSCs and conserved in Baboon and Rhesus spermatogonia. Pathway analyses indicated that these genes were involved in Transforming growth factor beta signaling, apoptosis modulation and mTOR signaling, and may represent the conserved primate SSC signature. Among these genes, 19 were conserved in transplant-validated immature mouse SSCs and involved in regulation of aldehyde catabolic processes (*HAGH, AKR1A1*) and translation (*RPSA, RPL32, RPL18, RPL22L1*).

Conclusion: These results suggest that a conserved primate SSC signature further resolves the identity of the most likely pre-pubertal human SSCs. Future work examining testicular somatic cell populations will help define the primate SSC niche. Collectively, these data can arm future studies to identify therapeutic human SSCs and advance their propagation *in vitro*.

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Sertoli cell induced Tregs: Guardians of foreign tissue

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Introduction: Testicular Sertoli cells (SC) are important in protecting late-stage germ cells from immune destruction through physical and immunological barriers. Germ cells develop during puberty, long after the body has established central tolerance and are thus viewed as 'foreign' by the immune system. Any exposure of germ cell antigens to immune cells should result in a strong inflammatory immune response and autoimmune orchitis. However, germ cell antigens have been shown to be present outside blood-testes-barrier without eliciting inflammation; the reason for this is currently unknown. Interestingly, we have shown that SC protect allogeneic and xenogeneic grafts under the kidney capsule without immune suppression. Previous research has linked transplant survival to the activity of T regulatory cells (Tregs), a lymphocyte that can suppress immune response. The goal of this study was to determine if the mechanism by which SC protect foreign tissue is dependent on Tregs. By further illuminating the mechanism through which SC protect transplants, we can better understand how SC protect 'foreign' germ cells in the testes.

Methods: Two separate transplant models were utilized: In allogeneic transplant experiments, B6 mouse SC and rejecting control cells (mouse SC line 1; MSC-1) were transplanted under the kidney capsule of a recipient BALB/c mouse. In xenogeneic transplant experiments, neonatal pig SC and rejecting control neonatal pig islets were transplanted under the kidney capsule of Lewis rats. Transplant recipients did not receive immune suppression and grafts were collected at varying timepoints until day 20 for analysis of Tregs though flow cytometry and immunohistochemistry. Tregs were knocked down in the mouse model using anti-CD25 monoclonal antibody.

Results: SC allografts and xenografts survived 100% to day 20, while control rejecting grafts were 100% rejected by day 20. Analysis of the grafts through immunohistochemistry and flow cytometry revealed that CD4+ Tregs were present in the SC grafts at early timepoints, but not in the rejecting control grafts. To determine if Tregs were critical for SC graft survival, SC were allografted into Treg depleted mice before transplantation. SC grafts experienced 57% survival; flow cytometry showed that the only grafts to survive were able to induce Tregs after depletion. The presence of CD4+ Tregs in surviving SC grafts was also shown in a xenogeneic model.

Conclusion: SC induction of Tregs is essential to their survival. This data, combined with previous research, strongly suggests that SC promote the same tolerance in the testes as is seen in the grafts through the induction of Tregs, thus preventing autoimmune orchitis.

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Emerging Roles of Melanoma Antigens in Male Germ Cells Stress Protection

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Introduction: Genetically modified mice with loss-of-function of melanoma antigen (*Mage*) genes provided new insights into the enigmatic connection between spermatogenesis and cancer. MAGEs are proteins that are selectively expressed in the testis in healthy males but get often aberrantly activated in diverse types of cancer. They have been intensively studied for cancer therapy, however, the more fundamental questions about their physiological roles in male germline remained neglected. We have determined the expression profiles of MAGEs at discrete stages of male germ cell differentiation, which suggested that Mages function throughout spermatogenesis.

Methods: To address the physiological role of Mage proteins, we used primary cultures of spermatogonia and CRISPR Cas9-generated mouse knock-out models of genes expressed at different stages of spermatogenesis, including undifferentiated spermatogonia, primary spermatocytes, and haploid spermatids. Initial analysis of male fertility suggested that Mages are dispensable for mouse fertility. Next, we exposed animals and germ cells to diverse stressors, e.g., DNA damage, testis over-heating, and calorie restriction, mimicking potential hazardous insults to male germline in a natural environment.

Results: Intriguing, in an un-optimal environment, Mages protected germ cells against damage and provided a reproductive advantage to males. Given the recent evolutionary expansion of the MAGE gene family in placental mammals, the phenotypes of *Magea* and *Mageb4* KOs imply that distinct MAGE genes evolved as an adaptation to specific stress of a subset male germ cell population, including *Magea* against metabolic stress in differentiating spermatogonia and *Mageb4* against heat in undifferentiated spermatogonia. Furthermore, Mages contribute to the stemness of spermatogonial stem cells and enable faster regeneration of spermatogenesis after stress-induced damage. Intriguingly, these protective Mage functions get coopted in cancer cells.

Conclusion: Our results suggest that mammalian germ cells evolved MAGE-regulated mechanisms that enable faster adaptation to changes in the environment and protection against stress. In our future work, we want to uncover molecular behind MAGE protective functions, which may catalyze novel strategies for male fertility preservation and cancer therapy.

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Porcine conceptus IFNG is post-transcriptionally regulated and released from the trophectoderm via extracellular vesicles to recruit metabolically activated T cells

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Introduction: Elongating and implanting porcine conceptuses secrete large amounts of pro-inflammatory cytokines, including IFNG, into the uterine lumen. Our published results demonstrated that conceptus IFNG recruits T helper cells to the endometrium, and suggests that these cells contribute to a tightly controlled inflammatory environment that supports the active breakdown and restructuring of the endometrium in response to implantation of the conceptus. Further, initial characterization of pregnancies in which porcine conceptuses lack IFNG, due to CRISPR-Cas9 gene editing, suggest an inflammatory response within the intrauterine environment characterized by conceptus fragmentation, endometrial hyperemia, edema, and inflammation. Accumulating evidence suggests that extracellular vesicles (EVs) mediate paracrine actions of conceptus-secretory proteins, including IFNG, on the endometrium during the peri-implantation period. The synthesis of cytokines, including IFNG, can be controlled through post-transcriptional regulation by glyceraldehyde-3- phosphate dehydrogenase (GAPDH) and epigenetic modification by metabolites including alpha ketoglutarate (α-KG) and succinate. GAPDH, resulting from active glycolysis, can increase translation of IFNG mRNA, and glutaminolysis increases α-KG and succinate that can alter epigenetic modifications to affect IFNG expression. Oxygen levels are key drivers of metabolism. Normoxia supports the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, whereas hypoxia leads to the activation of hypoxia-inducible factor 1 alpha (HIF1α) and enhanced glycolysis. It has become apparent that the functions and fate of immune cells are dependent on metabolic reprogramming. A hallmark of proliferating lymphocytes undergoing rapid activation is their ability to enhance aerobic glycolysis.

Methods: We collected porcine conceptus tissues on Days 11, 13, 15, 16, and 20 of pregnancy and performed real-time PCR, Western blotting, glutamine oxidation analyses, and immunofluorescence staining for IFNG and various genes associated with immunometabolism. Day 15 conceptus tissues were cultured with [U-14C]glutamine in a culture medium that contained 4 mM glutamine with or without 4 mM glucose and 14CO2 produced by the conceptuses was determined.

Results: Our results demonstrated that: 1) porcine conceptus IFNG protein decreased sharply on Day 16 while the mRNA remained elevated, and conceptuses expressed enzymes involved in glutaminolysis; 2) the cytoplasm of uterine luminal epithelial (LE) cells at sites of implantation contained IFNG protein, but when conceptuses lacked IFNG there was no IFNG within the LE and these conceptuses became fragmented; and 3) the endometrial LE expressed enzymes that convert glucose to serine and some proliferating T cells within the endometrial stroma expressed enzymes associated with the pentose phosphate pathway and one-carbon metabolism.

Conclusion: We propose that: 1) enhanced glycolysis and glutaminolysis-derived alterations of TCA cycle metabolites in porcine conceptuses regulate the expression of genes that encode inflammatory cytokines such as IFNG; 2) IFNG protein is synthesized and released from the conceptus trophectoderm via EVs on Days 13 and 15 of gestation, and taken up by the LE directly adjacent to the implanting conceptus; and 3) the mTOR-HIF1α pathway induces glycolysis to provide substrates for metabolism via pathways including the pentose phosphate pathway and one-carbon metabolism in T cells within the endometrium to support proliferation and the functions of T cells. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2016-67015-24955 from the USDA National Institute of Food and Agriculture.

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Tracking sperm movement toward the oocyte in vitro and in vivo

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Introduction: Fertilization is one of the most important biological events to convey our genetic information to next generation via sperm and oocyte. However, due to the inability to visualize in vivo fertilization processes and the development of in vitro fertilization (IVF) technique, most of the current knowledge is derived from in vitro experiments. If this technical limitation was overcome, we would better understand mammalian reproduction leading to the development of infertility treatments, contraception, and assisted reproductive technologies. This project is focused on the development of functional imaging technology which would allow for capturing fertilization in the moue oviduct in vivo. We have recently developed a new approach for live imaging of the mouse oviduct by functional optical coherence tomography (OCT), imaging oocytes and preimplantation embryos dynamics, and tracking individual sperm trajectories at the site of fertilization. This approach enables precise spatio-temporal analysis of sperm tracks in the female reproductive tract. In this study, we describe new functional measures to detect sperm movements through the cumulus matrix based on intensity fluctuations in OCT images, which is currently being optimized in vitro. Potentially, it will be combined with intravital OCT imaging to investigate sperm movement toward the oocyte and the fertilization process in vivo in its native state.

Methods: To capture reproductive events in vivo, we combined intravital microscopy with OCT imaging. The focus of this study was on the development of a new functional approach for tracking sperm movements through the optically dense cumulus cell mass. This method was first tested by capturing sperm movement during in vitro fertilization (IVF). Female CD1 mice were super-ovulated by the injection of gonadotropins. The cumulus-oocyte-complexes (COCs) were collected from the oviduct ampulla and incubated in human tubal fluid (HTF) medium until IVF. Sperm were collected from the epididymis of male CD1 mice, and incubated in HTF medium for 1h to induce sperm capacitation. The sperm suspension was added to the HTF medium drop including COCs at a final sperm concentration of approximately500-1000 cells/drop to begin IVF. We performed time-lapse 3D OCT imaging at different settings for IVF as well as a COC-only control group. Data processing was performed using custom MatLab codes. The obtained 4D (3D plus time) data were rendered by using Imaris software for visualization.

Results: In vivo OCT imaging in combination with intravital microscopy allowed us to capture oocytes and sperm at the fertilization site. However, tracking sperm movements through the cumulus matrix was not feasible through this method. To address this limitation, the new functional approach was developed and tested by IVF. We confirmed that the COCs were clearly visualized, and that sperm cells swimming nearby COCs were detected as bright spots and their trajectories were tracked volumetrically. To track sperm movements within the cumulus matrix, we calculated speckle variance (SV) of individual pixels, which resulted in higher contrast for spatial locations, where the cumulus cells were pushed by sperm, indicating the location and progression of sperm in the cumulus cell mass. Several bright spots for SV in the cumulus matrix were detected after a few minutes after the addition of sperm, which were not found in the COC-only group. We are now optimizing this approach and characterizing sperm movements within cumulus matrix by using SV in IVF.

Conclusion: We introduced a new functional measure, which has the potential to distinguish sperm movements through the cumulus matrix. Once optimized, this method will be used in vivo to characterize sperm behaviors and fertilization in its native state.

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Nonrandom population of the nascent SSC pool by dividing fetal prospermatogonia

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Introduction: Spermatogenesis is maintained throughout adulthood by spermatogonial stem cells (SSCs). One theory describing the mechanism of SSC specification during testis development holds that SSCs arise from a distinct subpopulation of prospermatogonia that is determined to the SSC fate. Published single-cell RNA-seq data describing embryonic day (E) 16.5 fetal prospermatogonia demonstrated that a subpopulation exhibits a SSC-like signature, which is consistent with the determination theory, but fails to prove that this subpopulation actually gives rise to the foundational SSCs. Subsequently, we found that the subpopulation of fetal prospermatogonia bearing SSC-like transcriptomes was also proliferative based on RNA signatures at E15.5-E16.5, and therefore, hypothesized that this subpopulation could be lineage-traced by S-phase labeling to determine whether they become putative SSCs.

Methods: Pregnant *Id4-Egf* transgenic dams received a single injection of 5–ethynyl–2’–deoxyuridine (EdU) at E14.5 (M-prospermatogonia), or at E15.5, E16.5 or E17.5 (T1-prospermatogonia) and testes were collected from male pups at postnatal day (P) 0. P0 testis sections were used to detect DDX4 (marking germ cells), EdU incorporation (marking cells that were proliferative at the time of EdU injection), and ID4-EGFP+ (marking nascent SSCs).

Results: At P0, DDX4+/ID4-EGFP+ male germ cells are presumed to be nascent SSCs, while DDX4+/ID4-EGFP- are considered to be non-SSC prospermatogonia. When labeled at E14.5 (when M-prospermatogonia are known to be proliferative), EdU labeling was detected in both ID4-EGFP- and ID4-EGFP+ P0 prospermatogonia (20.98% of DDX4+/EGFP+ and 5.31% of DDX4+/EGFP-), but significantly more of the EGFP+ germ cells were EdU+ ($p<0.05$). Similarly, when labeled at E15.5 (when T1 prospermatogonia are thought to be largely quiescent but the supposed SSC-determined subpopulation might be proliferative), we also observed EdU labeling among both ID4- EGFP- and ID4-EGFP+ P0 prospermatogonia (4.96% of DDX4+/EGFP+ and 5.55% of DDX4+/EGFP-), but the labeling indices were similar. Finally, no P0 prospermatogonia were EdU-labeled when dams were treated at either E16.5 or E17.5, confirming the dogma that male germ cells are quiescent during late fetal stages.

Conclusion: These lineage tracing results indicate that nascent SSCs preferentially arise from prospermatogonia that are dividing during a narrow temporal window at E14.5. Overall, these results suggest that the mechanism of foundational SSC specification is at least in part based on a determination mechanism that is characterized by differential proliferative activity during a narrow window in mid-fetal development.

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RNA-seq reveals transcriptome changes in *Nlrp2* null mice oocytes

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Introduction: NLRP2 is a member of subcortical maternal complex (SCMC), a multiprotein complex with a role in early development. Inactivation of genes encoding SCMC proteins cause different types of reproductive failure including early embryonic lethality and biparental molar pregnancy associated with widespread epigenetic defects. In the biparental molar pregnancies, the primary epigenetic change is loss of DNA methylation at the differentially methylated regions (DMRs) of maternally imprinted genes. These observations indicate that the source of defect may be the female germline. To further characterize causes and consequences of these epigenetic changes, the aim of this study was to define the oocyte transcriptome of *Nlrp2*-null (KO) female mice.

Methods: We performed deep RNA-Seq on pooled oocytes retrieved from 26 day-old superovulated mice. Each mouse was defined as a sample and accordingly we had four KO and three wild-type (WT) samples. Library preparation and sequencing was done at the genomic and RNA profiling core of Baylor College of Medicine. Raw reads were mapped to mouse genome (GRcm38, p6.VM24). A previously published oocyte transcriptome annotation general transfer format (GTF) file was used as reference annotation to obtain read counts. Analysis of transcript abundance in KO versus WT oocytes was performed using DESeq2 (version 1.22.2) in R.

Results: The final analysis was done after removal of one of KO samples that was not clustered with other KO samples when analyzed by principal component analysis (PCA). Therefore, we had three WT and three KO samples. From the differential transcript analysis, the abundance of 228 transcripts was different (FDR< 0.05) in oocytes of WT and KO samples. Of these, 90 transcripts were less abundant and 138 transcripts were more abundant in oocytes of KO compared to those of WT samples. Transcript annotation to the closest genes identified 159 genes. No imprinted gene or genes involved in epigenetic regulation were found among these 159 genes. In parallel, we performed a targeted analysis of imprinted genes as well as genes that encode important epigenetic modulators. We found that transcript abundance of the following genes was significantly changed between WT and KO: Kdm1b, Nlrp2, Peg10, Sgce, Slc38a4. Kdm1b is a histone demethylase that is enrolled in the methylation establishment process of GV. Peg10, Sgce and Slc38a4 are imprinted genes.

Conclusion: This is the first report of transcriptome profiling in ovulated oocytes from mice with inactivation of a gene that encodes a protein of the SCMC. We found that maternal loss of *Nlrp2* impacts the transcript abundance in oocyte. In oocytes, transcriptional activity and methylation establishment are tightly connected and a deficit in one may effectively disturb the other one. Therefore, unraveling the methylome changes in oocytes and embryos of *Nlrp2*-null mice is of the particular interest and the next goal of this project.

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The Epididymal Amyloid Matrix Displays Key Properties of Bacterial Biofilms

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Introduction: We previously established that the epididymal lumen contains a nonpathological amyloid matrix with likely roles in sperm maturation and protection. This matrix contains the amyloid structures of four members of the CRES subgroup (CRES, CRES2, CRES3, and cystatin E2), a reproductive subgroup within the family 2 cystatins of cysteine protease inhibitors, implying CRES subgroup amyloids have functional roles. Indeed, our studies show that CRES amyloid and the endogenous epididymal amyloid matrix have potent antimicrobial activity in vitro, suggesting a role in host defense. Several host defense structures such as bacterial biofilms have common components including an amyloid infrastructure, extracellular DNA (eDNA), and a proteoglycan-carbohydrate rich extracellular matrix (ECM). Based on its functional properties, we hypothesize the epididymal amyloid matrix is a novel host defense structure. The objective of our current study is to determine if the epididymal amyloid matrix also shares the structural features of biofilms and contains eDNA and ECM.

Methods: Epididymal amyloid matrix was isolated from the caput and cauda epididymal fluid from CD1 mice by high speed centrifugation. Samples were spread on slides and stained with Thioflavin S (ThS), an amyloid specific dye, and DNA stains Hoechst, Sytox Green, and TOTO-3 before and after exposure of the matrix to denaturants (SDS, urea, and formic acid) or DNAse I. Images were captured using a Zeiss microscope XX equipped with epifluoresence. Immunoblot analysis was used to identify ECM proteins and other amyloidogenic precursors in the amyloid matrix.

Results: Staining with all three DNA stains showed that eDNA was present in the epididymal amyloid matrix from both the caput and cauda epididymis. Partially disassembling/unwinding the matrix with denaturants further exposed the eDNA, as well as the amyloid core, suggesting eDNA was part of the amyloid matrix infrastructure. Exposure to DNase I caused the matrix to disassemble further indicating eDNA contributes to amyloid matrix structure. Neurocan, a chondroitin sulfate proteoglycan enriched in ECM surrounding unique populations of neurons, and amyloid precursor protein (APP) were both found in the epididymal amyloid matrix.

Conclusion: Our findings suggest the epididymal amyloid matrix is a unique host defense structure that structurally, and perhaps functionally, resembles bacterial biofilms.

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Effect of Pre and Post-AI Nutrition on Ovarian Dynamics, Steroidogenesis, and Expression of Estrus in Beef Heifers

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Introduction: The objective of this study was to evaluate nutrition before and after artificial insemination (AI) on follicular dynamics, expression of estrus, and steroidogenesis.

Methods: Seventy-nine beef heifers were randomly assigned to one of two dietary treatments (High;155% and Low;86% of maintenance energy) 30d prior to AI. Estrus was synchronized (PG 6-d CIDR protocol) and heifers received AI (d0) 8 to 12h following onset of estrus. On d0, heifers were randomly reassigned diets generating four Pre-x-Post-AI nutritional treatments; High-High (HH, n=20), High-Low (HL, n=20), Low-High (LH, n=19), and Low-Low (LL, n=20). Heifers remained on new diet treatments until embryo collection (flush; d7-8). Blood samples were collected daily from d-3 to d0, and on d1, 3, 5, 7 and 8 for plasma concentrations of estradiol (E2) and progesterone (P4). Dominant follicle diameter was evaluated on d-3 and d0, and CL diameter was recorded at flush. Statistical analyses were completed in SAS using the MIXED (body weight, E2, P4), GLIMMIX (expression of estrus), and GLM (ovarian parameters, interval to estrus) procedures.

Results: There were Pre-AI ($P<0.0001$) and Post-AI by time ($P<0.0001$) interactions on body weight; heifers in high and low treatments gained and lost weight, respectively, during both periods. Estradiol concentrations increased ($P<0.0001$) from d-3 (2.18±0.15pg/mL) to d0 (6.05±0.04pg/mL). Pre-AI diet increased the proportion of heifers in estrus (80±6.3% vs. 59±7.9%; $P=0.05$), and dominant follicle size (11.7±1.42mm vs. 10.68±1.33mm; $P=0.0016$) in High vs. Low, respectively. Additionally, High Pre-AI heifers had greater P4 after AI compared to Low Pre-AI heifers (4.85±0.37ng/mL vs. 3.53±0.38ng/mL; $P=0.015$). Post-AI treatment did not influence concentrations of P4 ($P=0.88$). There was no effect of Pre, Post or Pre-x-Post-AI treatment on initial follicle size, follicle growth rate, E2 concentrations, interval to estrus, or CL size at flush ($P>0.10$).

Conclusion: In conclusion, nutrient restriction before AI negatively impacted ovarian function, steroidogenesis, and expression of estrus.

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Immunohistological Profiling of Spermatogenic Cell Types in Testes from Men with Non-Obstructive Azoospermia

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Introduction: Non-Obstructive azoospermia (NOA) is a form of male infertility that affects 10-15% of men. Patients with NOA exhibit an absence of sperm in the semen without any physical blockage or barrier in the male reproductive tract. One cause of NOA is primary spermatogenic failure which manifests as Sertoli cell only syndrome (SCO) in which germ cells are essentially absent or maturational arrest (MA) in which spermatogenesis is blocked prior to spermiation. As part of a larger study investigating gene expression changes as a tool to precisely diagnose spermatogenic defects, immunofluorescence analysis (IF) were performed on human testis samples. The objective was to confirm cell types present in patients with NOA in comparison with normal testis samples.

Methods: Patient tissues were recovered during microscopic vasectomy reversal (MVR) or open testicular sperm extraction (TESE) procedures and excess tissue were provided for research. After overnight shipping, testis tissue was fixed in 4% PFA at 4ºC overnight washed thoroughly with DPBS, paraffinized and sectioned (5µm). Sections were deparraffinized using xylene in a graded ethanol series and subjected to EDTA antigen retrieval (1mM EDTA pH 8.0, 0.05% Tween-20) in a 97.5ºC water bath for 30 minutes. Slides were rinsed with PBST, blocked in donkey (Dk) blocking buffer (DPBS + 0.1% Triton X-100, 5% normal serum from donkey, 3% BSA) for 30 minutes in a humidified chamber at room temperature and incubated with antibodies overnight at 4ºC in a humid chamber. Antibodies were goat (Gt) anti-DDX4 (0.2mg/mL R&D Systems, AF2030) and rabbit (Rb) anti-SOX9 (1.23mg/ml Abcam, ab185966). Primary antibodies were omitted as a negative control. Sections were washed and incubated with Invitrogen fluorescent secondary antibodies Dk anti-Gt 488 (2µg/ml, A21208), Dk anti-Rb 647 (2µg/ml, A31573) and fluorophores DAPI and PNA-AF568 (1mg/ml Thermofisher Scientific, L32458) for 45 minutes at room temperature. Negative controls were not labeled with PNA. After subsequent PBST and PBS washes, slides were mounted a 50% mixture of glycerol and 1X PBS and sealed with nail polish before imaging at 20X using an AxioImager M1 (Zeiss) microscope with an AxioCam MRm (Zeiss). Slides were stored in a sealed container at 4ºC after mounting.

Results: Samples from normal human testis exhibited positive cytoplasmic staining DDX4 in spermatogenic cells, nuclear SOX9 in Sertoli cells and PNA labeling of acrosomes in spermatids. DDX4 and PNA labeling in NOA patients varied with most consistent with SCO and evidenced by negative DDX4 and PNA staining despite positive SOX9 labeling. Some samples were consistent with MA indicated by presence of some DDX4 labeling without PNA staining with only two patients having some PNA positive tubules despite being diagnosed with maturational arrest.

Conclusion: Despite the tedious and time consuming effort required for IF analysis it provides an effective and reliable readout of spermatogenic deficiencies. The ultimate goal of this study is to compare the IF staining with comprehensive gene expression measurements (qRT-PCR) to precisely diagnose spermatogenic deficiencies in patients with NOA. Here, our results already show a higher diagnostic precision with IF staining over traditional histological analyses and we expect this will be far exceeded by parallel mRNA analyses. Among samples exhibiting maturational arrest, additional labeling with markers of spermatocytes, differentiating spermatogonia and undifferentiated spermatogonia will help to more precisely define which spermatogenic cell types are present.

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Antimicrobial Functions of The Epididymal Amyloid Matrix

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Introduction: The epididymis plays a critical role in protecting sperm from invading pathogens that can ascend the male tract, causing inflammation and infertility for years following an infection. The epididymis relies heavily on antimicrobial proteins (AMPs) to defend against these pathogens, but it remains unclear mechanistically how these AMPs function. In other organs, some AMPs require an amyloid conformation for antimicrobial function. We previously established a nonpathological, functional amyloid matrix is in the epididymal lumen and contains the amyloid forms of four CRES subgroup members (CRES, CRES2, CRES3, cystatin E2), a reproductive subgroup within the family 2 cystatins of cysteine protease inhibitors. However, the function of this epididymal amyloid matrix has not been defined. We hypothesize the epididymal amyloid matrix plays a role in host defense by using its amyloid structure to create a protective net around sperm that traps and kills pathogens.

Methods: CRES monomer, various maturational states of CRES amyloid, and epididymal amyloid matrix were incubated with wildtype and uropathogenic *E. coli* and *S. aureus* strains in a colony forming unit (CFU) assay to assess killing of planktonic bacteria. Amyloid matrix was also incubated with established biofilms to examine the disruption of biofilms. Transmission electron microscopy (TEM) was used to visualize interactions between amyloid and bacteria, while a Live/Dead assay was performed to determine if amyloids permeabilize bacterial membranes.

Results: CRES amyloid, rather than monomer, showed the most pronounced antimicrobial activity against multiple strains of planktonic bacteria. Similarly, the epididymal amyloid matrix caused a profound decrease in the survival of *E. coli.* In addition to killing planktonic bacteria, the endogenous structure disrupted established biofilms, suggesting it has multiple antimicrobial functions. TEM revealed that CRES amyloid and the epididymal amyloid matrix surrounded and trapped bacteria. In addition, TEM showed that amyloid disrupted the bacterial membranes. A Live/Dead assay confirmed that bacterial membranes were permeabilized.

Conclusion: Together, our results show that the epididymal amyloid matrix has potent antimicrobial activity against both planktonic bacteria and biofilms and uses its highly ordered amyloid structure to trap and kill. These findings suggest the epididymal amyloid matrix is a host defense structure which plays critical roles in protecting sperm.

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26TH ANNUAL TFRS - ABSTRACTS

June 17 – 18, 2021

SUMOylation Directly Modifies Transcription Factors Necessary for Early Folliculogenesis

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Introduction: 12% of U.S. women struggle with infertility caused by pathology or aging. There is a growing need to understand the mechanisms regulating intraovarian oocyte development and ovarian aging in order to develop clinically-relevant testing and treatment modalities. Two oocyte-specific transcription factors, NOBOX and SOHLH1, are both necessary to maintain the ovarian reserve. In addition, *NOBOX* is one of the most mutated genes (6%) in women diagnosed with primary ovarian insufficiency (POI). SUMOylation, a ubiquitin-like posttranslational modification, regulates protein function within the oocyte and *in silico* analysis indicated that NOBOX and SOHLH1 are potential substrates.

Methods: Epitope tagged *Nobox* and *Sohlh1* expression plasmids were transfected alongside uncleavable SUMO1 or SUMO2/3 into HEK293T cells. Immunoprecipitation for the tag followed by immunoblotting validated the SUMOylation of each protein. Mutant expression plasmids were created for each protein and its respective predicted SUMOylation sites and immunoprecipitation was performed. Localization studies were done in HeLa cells via immunofluorescent staining following transfection of the appropriate transcription factor and mutant. Luciferase assays were performed in HEK293T cells using Renilla as an internal control.

Results: *In vitro* analysis showed that SOHLH1 was modified by SUMO2/3. Mutation of the predicted SUMOylation site, lysine 345, led to near total loss of SOHLH1 SUMOylation. In transfected HeLa cells, wild-type and SOHLH1^{K345R} mutant were localized to both the nucleus and cytoplasm. 3D structural modeling revealed predicted SUMOylation sites near the DNA binding domain of mouse and human NOBOX. In vitro analysis revealed NOBOX is modified by both SUMO1 and SUMO2/3. Mutation of NOBOX lysine 97, a nonconsensus predicted SUMO site, led to loss of SUMO2/3 conjugation while retaining modification by SUMO1. Localization studies in transfected HeLa cells showed NOBOX is retained in the nucleus for both wild-type and the K97R mutant. *Gdf9* and *Oct4* promoters, two known NOBOX target genes, were cloned into luciferase plasmids. Transfection with each promoter and NOBOX or NOBOX^{K97R} revealed that NOBOX^{K97R} had significantly increased transcriptional activity relative to wild-type NOBOX.

Conclusion: SUMOylation is an important regulator of NOBOX transcriptional activity while also directly modifying SOHLH1. Further studies will more clearly elucidate the effects of SUMOylation on SOHLH1 function. These studies were supported by NIH/NICHD R01 HD085994 (to S.A.P.) and NIH/NICHD T32 HD098069

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Prenatal transportation stress does not impact ovarian follicle count in Brahman offspring

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Introduction: Calves from transported dams had greater concentrations of plasma cortisol when restrained and cleared plasma cortisol at a slower rate than calves from non-transported dams. Considering this hypothalamic-pituitary-adrenal axis effect, investigation of other parameters influencing reproduction is warranted in offspring exposed to prenatal transportation stress. The purpose was to determine impact of prenatal transportation stress on offspring ovarian follicle count.

Methods: Brahman cows were transported for 2 h on d 60, 80, 120, and 140 (± 5 d) of gestation. Offspring from transported (Stressed, n = 19) or non-transported (Control, n = 15) dams were slaughtered at 5 yr (Replication 1, n = 14) or ovariectomized at 8 yr (Replication 2, n = 20). A cross-section of ovary was collected, serially sectioned, and stained. Numbers of total, primordial, primary, secondary, and antral follicles were determined per section. Total ovarian follicle count for each stage was calculated using ovary dimensions. The MIXED procedure of SAS was used to analyze ovarian follicle count with treatment, replicate, and the interaction as fixed effects.

Results: Total ovarian follicle count decreased with age ($P < 0.01$; R1 = 383,663, R2 = 154,560); however, there was no difference in total ovarian follicle count between offspring due to treatment ($P = 0.17$; S = 221,813, C = 316,409). Similarly, there was no difference in primordial ($P = 0.22$; S = 127,300, C = 188,304), primary ($P = 0.28$; S = 58,610, C = 77,237), or antral ($P = 0.48$; S = 23,202, C = 28,695) follicle count between offspring due to treatment. Fewer secondary follicles were observed in Stressed offspring compared to Control offspring ($P = 0.03$).

Conclusion: These results suggest that the ovarian follicular reserve, AFC, and potential fertility of cows may not be impacted by exposure to prenatal transportation stress.

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Expression of CD46 and CD55 are critical to testicular Sertoli cell survival of hyperacute rejection

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Introduction: Seminiferous tubules are lined with immunoregulvatory Sertoli cells, which nurture and protect germ cells from immune destruction by creating an immune privileged environment. However, this immune privileged environment is broken down in autoimmune orchitis, a disease where anti-germ cell antibodies bind to the germ cells and activate the complement system, leading to their destruction and causing infertility. The complement system is an enzymatic protein cascade that culminates with destruction of the target cells through insertion of the membrane attack complex (MAC) and a pro-inflammatory response activating leukocytes for further immune destruction. Many complement inhibitory proteins (CIPs) have been identified on germ cells and in seminal plasma to prevent undesired complement activation. Similarly, antibody-binding to xenografts, an alternative to traditional human tissue transplants that allows for an unlimited supply of transplantable tissue and organs, also activates the complement system to destroy the xenotissue through means of hyperacute rejection. To prolong the viability of traditional transplants and xenografts, chronic immunosuppression is required, which can cause many harsh side effects including cancer and increased infections. Interestingly, neonatal porcine Sertoli cells (NPSCs) survive as xenografts long-term—over 90 days—without the requirement of immunosuppressive drugs, suggesting that study of the mechanism(s) NPSCs use to survive hyperacute rejection could improve protection of transplants and germ cells. Therefore, we investigated whether NPSCs survive complement and express CIPs.

Methods: To measure cell viability after complement exposure, NPSCs were subjected to a human serum + complement cytotoxicity assay. NPSCs or control cells were cultured for 1.5 hours in the following conditions: human serum containing complement, heat-inactivated human serum (antibody control), media (positive control), or detergent (negative control). Cell viability was assessed by a subsequent MTT assay. Immunocytochemistry (IHC) of complement fragments was performed on cultured NPSCs or control cells to determine deposition of C3, C4, factor B, and MAC. PCR and western blot analyses were performed on NPSCs or control cells to quantify and compare mRNA and protein production of the CIPs CD46 and CD55. Using a lentiviral vector, NPSCs were transduced with shRNA to knockdown expression of CD46 or CD55 to evaluate their importance in protection of NPSCs from complement-mediated destruction.

Results: NPSCs survive human complement while control cell survival was below 30%. IHC of NPSCs and control cells exposed to human antibodies and complement showed that the complement fragments C3 and C4 were deposited on both cell types, but factor B and MAC were not deposited on NPSCs. This indicates that NPSCs are inhibiting the complement cascade before insertion of the MAC, and are also inhibiting the alternative pathway and amplification loop. One mechanisms NPSCs may use to inhibit complement is through expression of CIPs. NPSCs express mRNA and protein for the CIPs CD46 and CD55 at significantly higher levels than control cells. When CD46 and CD55 were knocked down in NPSCs and the transduced cells were exposed to the human serum + complement assay, their survival was severely diminished, dropping below 10%.

Conclusion: These data suggest that CD46 and CD55 are critical to NPSC survival of complement. NPSC survival of complement is important, not only in transplantation, but also in regard to male fertility as inhibiting the complement system is important in protection of maturing germ cells from autoimmune destruction. Continued study of NPSC CIP expression will further understanding in prolonging xenograft survival without immune suppressing drugs and of immune regulation in male fertility.

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26TH ANNUAL TFRS - ABSTRACTS

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In vivo measurement of cilia function in mouse fallopian tube

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Introduction: Cilia are hair-like microtubule-based organelles located on the surface of various cells. Cilia in fallopian tube play important roles in transporting fertilized oocytes to uterus for implantation. Due to their small size (~5–10µm in length and ~300nm in diameter) and deep location within the female body, analysis of cilia function in their native state is a major challenge. To fill in that gap, our lab has established a functional low-coherence optical imaging technique that allows in-vivo depth-resolved mapping of the cilia location and cilia beat frequency (CBF) in the intact mouse oviduct with micro-scale spatial resolution. Coordination of cilia is highly important for their function. Cilia metachronal wave is from the coordinated movement of cilia beating, which are believed to help transporting the oocytes. In this project, we are developing a novel method to visualize and quantify the cilia metachronal wave within the mouse fallopian tube. By extracting the phase of cilia beating in the neighboring pixels through Fourier transform and calculate the phase delay of neighboring pixels, we will be able to reconstruct the cilia metachronal wave velocity map of entire mouse fallopian tube. Potentially this method will be used to study the role and regulation of cilia dynamics in the oviduct. It will contribute to understanding the mechanistic link between ciliopathies and reproductive disorders.

Methods:
OCT in vivo imaging. We use a house-built spectral-domain Optical Coherence Tomography (OCT) system in this study. The light source of this system is a broadband laser centered at ~810 nm, with a spectral bandwidth of ~100 um. A fiber-based Michelson interferometer will be employed for the system, providing an axial and transverse resolution of ~5 µm, and an imaging depth enough to cover the diameter of fallopian tube lumen.
Metachronal wave quantification and CBF mapping based on OCT imaging. To quantitatively assess the metachronal wave in the mouse fallopian tube, phase information for each pixel on a certain frequency is needed. To achieve that, a fast Fourier transform will be performed to the time-lapse temporal intensity profile to obtain the amplitude spectrum of all the pixels. And using that amplitude spectrum, we will eliminate the noise and select frequency with highest amplitude as cilia beat frequency for each pixel. Then dominant frequency can be determined for all the pixels in specific region, followed by the phase extracting under that dominant frequency. Ideally, the phase shift will be visualized across the entire image with a recurring rainbow-like pattern because of gradual shift of phase and -pi to pi loop. The velocity of the metachronal wave is equal to the product of its wavelength and frequency. Since multiple wavelengths can be picked by counting the pixels of one entire loop cover from -pi to pi, multiple metachronal wave velocities will be picked up in one image, which will be used for statistical analysis.

Results: *In-vivo* CBF measurements from mouse oviducts range from 1.5Hz to 15Hz and vary between mice. There is no significant difference of cilia beat frequency in different regions of oviduct. We have visualized and quantified cilia metachronal wave in mouse fallopian tube ex vivo on bright field microscope. The time-lapse video of mouse fallopian tube was acquired at 153 frames per second. The cilia beat frequency analysis shows a dominant frequency at 6.6 Hz. Phase map at dominant frequency showed a gradual shift (rainbow-like pattern) of phase along the oviduct epithelium corresponding to the metachronal wave. Statistical analysis has shown that the wavelength of cilia metachronal wave in fallopian tube is 9.1 ±2.1um, while the velocity is 59.9 ±13.9 um/s.

Conclusion: Through this study, we defined spatial and temporal scales toward OCT analysis of cilia metachronal wave. Our next step will expand the analysis on OCT imaging data and will be used for quantification of cilia function *in vivo*.

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New in vivo functional imaging approach for analysis of sperm hyperactivation in the mouse oviduct

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Introduction: Sperm dynamics within female reproductive tract are largely a mystery due to restricted imaging access. Majority of what we know about sperm behaviors is extrapolated from in vitro and ex vivo analyses. Specifically, hyperactivation producing a change in sperm motility, which is essential for successful fertilization, is not studied in its native state due to limited access to the tissue. Toward investigation of reproductive processes in vivo, our group recently established an innovative volumetric imaging approach, which allows tracking of individual sperm trajectories within mouse oviduct. The method is based on optical coherence tomography (OCT) imaging through intravital imaging window for access to female reproductive organs. Our current efforts go toward defining the hyperactivation state of sperm from these analyses based on sperm trajectories.

Methods: The study consists of three major components: analysis of sperm trajectories acquired (1) in vitro with bright field microscopy, (2) in vitro with 3-D OCT, and (3) in vivo within the oviduct with OCT. To induce hyperactivation in vitro, freshly extracted sperm from CD-1 males was split into two parts, one of which was incubated with HTF medium (Sigma-Aldrich) at 37oC for 1.5 hours; the other part was incubated with M2 medium (Sigma-Aldrich) to serve as non-hyperactivated control. The trajectories of sperm were recorded using bright field microscopy at 60 frames per second. Different computational measures have been extracted from the trajectories to distinguish hyperactivated sperm based on their trajectories. To account for potential variation in viscosity within female oviduct, the viscosity of the media was altered in vitro by adding methylcellulose (Sigma-Aldrich). This allowed varying viscosity in the range from 1.0 mPa•s to 29.4 mPa•s which cover natural viscosity range within the mouse oviduct. OCT was performed using house-built spectroscopic OCT imaging system operating at central wavelength of 800 nm providing isotropic spatial resolution of ~4 um. In vivo OCT analysis was performed in anesthetized females after natural mating through intravital implantable windows.

Results: Following quantitative parameters have been extracted from sperm trajectories: standard deviation of direction variation (SDofDV), curve-line velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), and wobble (WOB). The parameters, which provided the highest statistically significant difference between hyperactivated and non-hyperactivated sperm are SDofDV, linearity, and wobble. These parameters did not change significantly over variation in media viscosity, suggesting that they could provide a quantitative measure of hyperactivation status based on the trajectory in vivo.

Conclusion: This work revealed a potential quantitative approach for differentiating sperm hyperactivation status based on trajectories in vitro. Next, we will integrate this quantification with previously established in vivo imaging methods to define, whether it could be used in vivo on OCT acquired sperm trajectories. Upon the validation, this method will be implemented to study the regulation of hyperactivation in vivo within the oviduct in its native state.

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Investigation of collagen degradative pathways in the cervix during pregnancy

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Introduction: Dynamic changes in processing, assembly, and composition of the extracellular matrix (ECM) dictate the structural and mechanical function of the cervix. Transcriptome studies show constant expression of genes encoding fibrillar collagens and other ECM molecules required for collagen assembly. Relatively few collagen-degrading proteases were expressed or induced in pregnancy. Notable exceptions were MMP14 (constant expression at all time points) and fibroblast activation protein 1 (FAP1-induced expression on gestation days 15 and 18). Recent proteomics studies demonstrate a remarkably high turnover rate for fibrillar collagens in the cervix of both nonpregnant (NP) and pregnant mice. Collectively, these findings suggest that during cervical remodeling collagen degradative pathways are required to ensure the replacement of collagen in the ECM. We hypothesize that both extracellular and intracellular collagen degradative pathways are required to ensure collagen homeostasis during cervical remodeling. Elucidating the relative contribution of these pathways to ensure a successful pregnancy is essential to understand how perturbations in ECM remodeling can cause premature birth.

Methods: Experiments were conducted in the wild-type mouse cervix. Immunohistochemistry (IHC) was used to evaluate the expression of the proteases MMP14 and FAP in the non-pregnant (NP) and pregnant (d6-d18) cervix. MMP14 collagenolytic activity was determined by in situ-zymography (ISZ). UPARAP-Endo180 receptor protein levels were assessed by Western blot in the NP and pregnant cervix. Assessment of collagen internalization was conducted by immunofluorescence (IF) in human cervical fibroblasts.

Results: Cell-type specific expression of MMP14 in the cervix was detected by IHC. Strong immunostaining for MMP14 was observed in the epithelial compartment in the NP and D6 pregnant cervix with a decline in late pregnancy. Conversely, in the stromal compartment a strong immunostaining was observed in the D15 and D18 pregnant cervix compared to early gestational time points. ISZ experiments demonstrate MMP14-dependent collagenolytic activity that can be pharmacologically inhibited with a MMP14 inhibitor. Consistent with the transcriptomic profiles, FAP1 protein expression was increased in the stromal ECM in the late pregnant cervix (d15 and d18). UPARAP-Endo180 receptor was expressed in the NP and pregnant (days 6-18) cervix. IF revealed internalization of collagen by the cervical fibroblasts. Inhibition of the lysosomal proteases resulted in an increase in intracellular collagen. Blockage of the phagocytic pathway did not perturb the cell-mediated internalization of collagen, implying the necessity of the UPARAP receptor endocytic pathway for the uptake and intracellular degradation of collagen.

Conclusion: Under physiological conditions constant turnover of collagen in the cervical ECM is critical to maintain the structural and mechanical changes that takes place during pregnancy. Our animal and cell studies showed expression and activity of molecules required for collagen degradation in the cervix. Importantly, these results may support the continuous use of both the extracellular and intracellular degradative pathways of collagen to facilitate cervical ECM homeostasis in pregnancy and parturition.

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Progesterone regulates the biosynthesis and interconversion of polyamines in the sheep endometrium during the pre-implantation period of pregnancy

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Introduction: Agmatine and polyamines (putrescine, spermidine, and spermine) are products of arginine, methionine, and proline metabolism that have a wide variety of roles in eukaryotic cells. They are essential for cell survival, growth, and proliferation, and the amounts of these amines must be tightly regulated for normal cellular functions. Importantly, agmatine and polyamines play crucial roles in the survival and growth of conceptuses in mammalian species. Studies suggest that steroid hormones and other paracrine signaling mechanisms may affect the activity of the pathways responsible for production of polyamines in the female reproductive system. However, the regulatory mechanisms governing their biosynthesis, interconversion, and degradation in the sheep uterus are not well characterized. In addition, it has been demonstrated that exogenously administered progesterone to sheep during the pre-implantation period of pregnancy accelerates conceptus development. This is due to the exposure of the conceptus to an advanced uterine environment that results in an early downregulation of progesterone receptor (PGR), and early secretion of histotroph. This study tested the hypothesis that progesterone, the hormone of pregnancy, regulates the expression of genes involved in the metabolism, interconversion, and transport of polyamines that are important for conceptus development.

Methods: Mature Suffolk ewes (n = 24) were synchronized to estrus (Day 0) and bred to rams of proven fertility. Ewes were assigned randomly to be treated with daily intramuscular injections of either 1 ml corn oil alone (CO; n = 14) or 25 mg progesterone in 1 ml of corn oil vehicle (P4; n = 10) from 36 h after breeding (Day 1.5) through Day 8 of gestation. Five CO-treated ewes and six P4-treated ewes were euthanized and hysterectomized on Day 9 of pregnancy, and nine CO-treated ewes and four P4-treated ewes were euthanized and hysterectomized on Day 12 of pregnancy. Endometrial tissue from the uterine horn ipsilateral to the corpus luteum was collected, and we then quantified the following mRNAs using qPCR analysis: 1) mRNAs for proteins involved in metabolism of amino acids to polyamines including ASL (argininosuccinate lyase), MAT2B (methionine adenosyltransferase 2B), AMD1 (adenosylmethionine decarboxylase 1), and ARG2 (arginase 2); mRNAs for proteins involved in the interconversion of polyamines including SRM (spermidine synthase), SMS (spermine synthase), SAT1 (spermidine/spermine N-acetyltransferase), and PAOX (polyamine oxidase); and 3) mRNAs for proteins involved in transport into and out of cells including SLC3A2 (solute carrier family 3 member 2) and SLC12A8 (solute carrier family 12 member 8).

Results: Exogenous progesterone administered to ewes from Day 1.5 through Day 8 accelerated conceptus development, as conceptuses from P4-treated ewes were larger than those from CO-treated ewes on Day 9 of pregnancy. Additionally, on Day 12 of pregnancy the conceptuses from P4-treated ewes were elongated while conceptuses from CO-treated ewes were spherical or tubular. Immunolocalization of progesterone receptors (PGR) confirmed that PGR were downregulated in the uterine luminal (LE) and superficial glandular (sGE) epithelia of control ewes on Day 12 compared to Day 9 of pregnancy, and down-regulation occurred earlier in the P4-treated ewes. On Day 9 of pregnancy, the expression of mRNAs for AMD1 ($P<0.01$) and SLC12A8 ($P<0.05$) were increased in the endometria of P4-treated compared to CO-treated ewes. The expression of ASL mRNA was affected by gestational day, with increased expression in the endometria of CO-treated ewes on Day 12 compared to CO-treated ewes on Day 9 ($P<0.01$). Further, the expression of ASL mRNA in P4-treated ewes on Day 9 was increased compared to CO-treated ewes on Day 9, and similar to that in ewes treated with CO on Day 12 ($P<0.01$). The expression of SAT1 mRNA was influenced by day ($P<0.05$), with greater expression in endometria from CO-treated ewes on Day 12 than CO-treated ewes on Day 9. The expression for MAT2B mRNA was greater ($P<0.001$) for P4-treated compared to CO-treated ewes on both Days 9 and 12 of pregnancy. In addition, expression of MAT2B mRNA was greater in ewes on Day 12 compared to Day 9 of pregnancy ($P<0.001$). Interestingly, the expression of SMS mRNA was less ($P<0.001$) in the endometria of ewes treated with P4 on Day 12 compared to that for P4-treated ewes on Day 9 of pregnancy.

Conclusion: Early administration of progesterone influenced the expression of enzymes and transporters involved in the biosynthesis and interconversion of agmatine and polyamines in the sheep uterus during the pre-implantation period of pregnancy. These results suggest that progesterone signaling affects the enzymatic activity of polyamine synthesis and interconversion pathways, and that putrescine and/or spermidine may be the preferred polyamine for utilization by endometria and/or conceptuses in sheep. Identifying and characterizing the relative significance of each polyamine will advance understanding of the importance and utilization of these molecules during the critical period of early conceptus development that lays the foundation for subsequent placental development required for reproductive success in livestock species and humans. This research was supported by Agriculture and Food Research Initiative Competitive Grant 2016-67015-24958 from the USDA National Institute of Food and Agriculture. **Presenter:** Katherine Halloran **E-mail:** kittyhalloran15@tamu.edu

Generating a *Phf21a*-Floxed Mouse With a Novel DECAI CRISPR System to Study the Function of BHC80 in Development and Disease

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Introduction and objectives: Deletions in the *PHF21A* gene located in the 11p11.2 region have been associated with intellectual disability and craniofacial anomalies. In addition, overexpression of splice variants of this gene promotes proliferation of treatment-induced neuroendocrine prostate cancer (t-NEPC). Our preliminary data also show that high expression of *PHF21A* occurs in normal testicular somatic cells, and aberrant high expression levels are found in testicular cancers of germ cell origin (seminoma). *PHF21A* encodes the protein BHC80, which is an epigenetic reader often associated with the demethylase KDM1A and H3K4Me0, and therefore with gene silencing. However, the specific function, gene targets, and regulation of BHC80 in each of these tissues is not known. As a floxed *Phf21a* mouse was not available, we decided to establish this model using a novel CRISPR protocol.

Methods: We adapted a new method called DECAI (DEgradation based on Cre-regulated-Artificial Intron), by inserting a small artificial intron via CRIPSR into an exon found in all isoforms of the *Phf21a* mouse gene. This intron has loxP sites on each side of its branch point. Without Cre, it splices itself out of the mRNA, leading to normal protein production. The intron also has 3 STOP codons in different frames so when the branch point sequence is removed by Cre, one of the STOP codons is put in-frame, which leads to degradation of the mRNA and protein knock out. We first tested how efficiently the artificial DECAI intron was inserted using blastocysts. In this procedure, we PCRed, then Sanger sequenced DNA from mouse blastocysts previously injected as zygotes with the same CRISPR components intended to produce the engineered mice. As insertion appeared successful, we then made mice.

Results: 31% of the blastocysts and ~20% of the founder mice showed evidence of perfect insertion of the artificial DECAI intron leading to the floxed gene. ~50% of the pups from founder mice crossed with wildtype mice contained the insert.

Discussion: Several methods for generating floxed mice by CRISPR have been developed to improve the low efficiency of correct dual loxP insertion but are either technically challenging or do not work much better than the standard CRISPR method. Our adapted method for making floxed mice appears to be easier, faster and more reliable, and the *Phf21a* floxed mouse will help us understand the possibly different functions of BHC80 in different tissues.

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Increased Preovulatory Estradiol Improves Pregnancy Success of Embryo Transfer in Beef Cows

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Introduction: Preovulatory estradiol concentrations and expression of estrus have been associated with greater pregnancy rates in beef cattle following AI, thus an experiment was designed to determine if supplemental estradiol (E2) at GnRH-induced ovulation would improve pregnancy retention in postpartum beef cows after embryo transfer.

Methods: Cows were synchronized with the 7-d CO-Synch + CIDR® protocol. On d0 (48h postprostaglandin) cows were grouped by estrual status (Estrual; n=198, nonestrual; n=406). Nonestrual cows were administered GnRH and randomly assigned to either no treatment (Control; n=204) or administration of 0.1 mg (IM) estradiol 17-β (Estradiol; n=202). In a preliminary study, 0.1 mg (IM) estradiol 17-β increased plasma estradiol (17.11±3.4 pg/mL; P<0.01) and remained elevated above baseline for approximately 8h compared to control (1.70±0.58 pg/mL). All cows received an in vivo produced embryo on d7. Embryos were matched by grade, stage, and flush across treatments. Pregnancy was classified on d30 by either plasma pregnancy-associated glycoproteins analysis (Year 1) or ultrasonography (Year 2). Plasma estradiol concentrations (d-2, d0h0, d0h2) were analyzed by PROC MIXED as repeated measures in SAS(9.4), while GLIMMIX procedures were used to analyze differences in pregnancy rates with treatment, group, year, and their interactions as fixed effects.

Results: There was a treatment by time interaction (P<0.001) in E2 concentration. There was no difference in plasma E2 concentrations on d0h0 (P>0.30) between treatments. At d0h2, Estradiol cows (15.7±0.25 pg/mL) had greater plasma E2 compared to Estrual (4.0±0.13 pg/mL) or Control (3.4±0.25 pg/mL) cows (P<0.001), and Estrual cows had greater plasma E2 than Control cows (P=0.02). Control cows had decreased (26%) pregnancy rates compared to both Estradiol (37%; P=0.03) and Estrual (40%; P=0.01) cows. Pregnancy rates did not differ (P=0.51) between the Estrual and Estradiol cows.

Conclusion: In conclusion, elevated preovulatory concentrations of estradiol are critical for improved pregnancy retention following embryo transfer.

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Porcine Conceptuses Utilize the Pentose Phosphate Pathway to Support Development during the Peri-Implantation Period of Pregnancy

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Introduction: Implantation and early placentation in mammals involve rapid growth and remodeling of the conceptus (embryo and associated placental membranes) that requires extensive cell proliferation and migration – processes that rapidly consume and deplete available O2 and nutrients. Metabolism of glucose primarily occurs through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, which is an efficient way to produce ATPs. However, a metabolic switch from oxidative phosphorylation to aerobic glycolysis occurs in proliferating and migrating cells such as cancer cells and activated lymphocytes, and this enhanced glycolysis provides glycolytic intermediates that can be shunted into biosynthetic pathways such as pentose phosphate pathway (PPP). Our preliminary studies showed that the hexose sugars, glucose and fructose, are abundant in the uterine lumen of pigs, and utilization of glucose and fructose by conceptuses increases as they elongate during the peri-implantation period. We hypothesized that aerobic glycolysis occurs in proliferating and elongating conceptuses to provide biosynthetic intermediates required for rapid proliferation and migration of conceptus trophectoderm (Tr) cells. In this study, we determined if elongating conceptuses metabolize glucose and fructose via the PPP to generate ribose 5-phosphate, a precursor for the synthesis of nucleotides.

Methods: We collected porcine conceptus tissues on Days 11, 13, 15, and 16 of pregnancy and performed real-time PCR, Western blot, and immunofluorescence staining to examine temporal and spatial expression of G6PDH, an enzyme required for the PPP, during the peri-implantation period. We also collected elongating conceptus tissues from Days 15 and 16 of pregnancy, and incubated these tissues with [1-14C]glucose, [6-14C]glucose, [1-14C]fructose, or [6-14C]fructose, and measured 14CO2 released from the conceptuses to determine whether carbons from glucose and fructose enter the PPP.

Results: Our results demonstrate that: 1) G6PDH mRNA significantly increases on Day 13 of pregnancy; 2) G6PDH protein remains high through Day 15 of and then sharply decreases on Day 16 of pregnancy; 3) G6PDH protein localized to the conceptus Tr cells; and 4) carbons derived from glucose, but not fructose, entered the PPP with maximum levels of utilization on Day 15 of pregnancy.

Conclusion: These results suggest that the elongating and proliferating conceptus Tr cells of pigs preferentially metabolize glucose via the pentose phosphate pathway in order to synthesize ribose-5-phosphate for the *de novo* synthesis of nucleotides during the peri-implantation period of pigs. This project was supported by Agriculture and Food Research Initiative Competitive Grant no's. 2018-67015-28093 and 2020-67015-31136 from the USDA National Institute of Food and Agriculture.

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Pathologic lesions in placentas of fetuses with left ventricular outflow tract obstruction (LVOTO) compared to non-LVOTO congenital heart disease

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Introduction: The placenta and heart are both vascular organs of fetal origin that develop concurrently in early pregnancy. Hemodynamic disturbances to the extra-embryonic circulation can result in cardiac anomalies, particularly lesions characterized by left ventricular outflow tract obstruction (LVOTO). Placental microstructure is known to be disrupted in pregnancies complicated by congenital heart disease (CHD), but studies using accepted classification systems of placental histopathology in LVOTO and non-LVOTO CHD are lacking. In this study, we sought to test the hypothesis that fetuses with LVOTO have higher rates of placental pathology and lower placenta-to-birth weight ratios compared to fetuses with non-LVOTO CHD.

Methods: In this single-center cohort study of neonates with CHD, we determined the prevalence of pathologic lesions of the placenta in those with LVOTO and non-LVOTO CHD. We used the Redline classification system to characterize placental lesions into four main categories: 1) maternal stromalvascular lesions, 2) fetal stromal-vascular lesions, 3) acute inflammatory/ immune processes, and 4) chronic inflammatory/ immune processes. Additionally, we quantified placentas with umbilical cord abnormalities, large for gestational age (>90th percentile), and small for gestational age (<10th percentile). Placentas in each group with any pathology and those with multiple pathologies were also determined. Prevalence of each lesion type, presence of any pathology, and presence of multiple pathologies were compared using Chi square analysis. We determined differences in placental weight to birth weight ratios between the two groups using linear regression analysis.

Results: A total of 272 pregnancies complicated by CHD were assessed for inclusion and 196 had placental pathologic examination performed (LVOTO n=60, non-LVOTO n=136). Pathologic lesions were present in 82% of placentas from fetuses with CHD (LVOTO 88% vs. non-LVOTO 79%; p=0.13) and multiple pathologies were present in 55% (LVOTO 52% vs. non-LVOTO 56%; p=0.58). No specific pathology was significantly more common in LVOTO compared to non-LVOTO fetal CHD. Specifically of interest were fetal stromal-vascular lesions which were found in 30% and 19% of fetuses with LVOTO and non-LVOTO CHD, respectively (p=0.09) and umbilical cord abnormalities (LVOTO 22% vs. non-LVOTO 17%; p= 0.43). There was no significant difference in placenta-to-birth weight ratios between groups (p=0.99).

Conclusions: Pathologic lesions of the placenta are highly prevalent in fetal CHD irrespective of type of structural heart disease and no specific category of placental pathology predominates. Pathologic examination of the placenta should be considered in all pregnancies complicated by fetal CHD.

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Absence of Steroid Receptor Coactivator 3 in the Murine Decidua Results in Pre-Placental Pregnancy Loss

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Introduction: Early pregnancy loss or miscarriage continues to be a significant reproductive health concern for women worldwide. While the etiology of early pregnancy failure within the first trimester is multifactorial, signaling miscues between the developing embryo and the maternal endometrium is thought to be a primary etiologic factor. Although we previously demonstrated that members of the Steroid Receptor Coactivator (SRC) family in the murine uterus are critical for embryo implantation and endometrial decidualization, the role of SRC-3 was not evaluated due to the lack of a suitable mouse model. Using a recently generated SRC-3 conditional knockout mouse model (*SRC-3* $^{d/d}$) in which SRC-3 is ablated in cells that express the progesterone receptor (PGR), we demonstrate that decidual-derived SRC-3 is dispensable for embryo implantation but is required for pregnancy progression to placentation. Therefore, while not required for initial decidual cell formation from stromal fibroblasts, SRC-3 is required for maintenance of decidual function and survival until placentation. This proposal is further supported by an observed compromised ability of cultured human endometrial stromal cells (HESCs) to fully decidualize when SRC-3 levels are significantly decreased.

Methods: We recently generated a conditional knockout bigenic mouse (*SRC-3* $^{d/d}$) in which SRC-3 is selectively ablated in PGR positive cells. Crossing our Pgr-cre driver mouse with a mouse engineered with a SRC-3 floxed allele *SRC-3*$^{f/f}$, we generated the SRC-3 d/d mouse. Female *SRC-3* $^{d/d}$ mice along with *SRC-3* $^{f/f}$ control females were mated with wild-type proven stud males over a 6-month breeding period. Standard immunohistochemical, cellular and molecular analysis were conducted to phenotype the infertility defect displayed by the *SRC-3* $^{d/d}$ female. Using an established HESC line in culture, a standard *in vitro* decidual cell response assay was used in conjunction with siRNA-mediated knockdown of SRC-3 to determine the importance of SRC-3 expression in HESC decidualization.

Results: Female *SRC-3* $^{d/d}$ mice failed to produce litters during the 6-month fertility trial. Timed pregnancy experiments revealed that normal embryo implantation occurs in *SRC-3* $^{d/d}$ mice; however, pregnancy failure manifests by gestation day 7 (GD-7) just before placentation. While initial endometrial decidualization appears to occur in the *SRC-3* $^{d/d}$ mouse during GD 5-6, histological analysis reveals extensive decidual cell death and a compromised vascularization of the decidua in the *SRC-3* $^{d/d}$ by GD-7.5, with concomitant demise of the embryo, as evident by active caspase3 and CD31 immunostaining analysis. Based on the mouse phenotype results, we propose that endometrial SRC-3 is critical for maintaining the functionality and survival of the decidual stromal cell, and, by extension, embryo developmental progression. Translational support for a similarly important role for SRC-3 in HESC decidualization is provided by our recent *in vitro* decidualization assay using an established HESC line. Knockdown of SRC-3 alone in HESCs resulted in significant reduction in the ability of HESCs to decidualize, particularly during later time points of cell culture. Together, these results underscore a critical role for SRC-3 in pregnancy success.

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Fructose metabolism by the sheep conceptus as a mechanism for adapting to hypoxia

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Introduction: Appropriate development of the mammalian embryo and associated extraembryonic membranes (hereafter referred to as the conceptus) prior to implantation to the endometrium is critical for establishment of a successful pregnancy. The mother provides nutritional support during this early developmental period via histotrophic secretions, which include various growth factors, proteins, nutrients (e.g., glucose, fructose, and glutamine), and other macromolecules. Glucose and fructose are two important hexose sugars that, when metabolized, yield products that are required as building blocks for other molecules or to provide substrates for other metabolic pathways. It is well established that fructose is the most abundant hexose sugar present in fetal fluids and fetal blood throughout gestation in the sheep; however, the metabolic roles of fructose remain under-investigated. Previous results from our laboratory indicated that the sheep conceptus at Day (D) 16 of pregnancy can utilize fructose via the pentose phosphate pathway (PPP) and tricarboxylic acid cycle (TCA) in the absence of glucose under both hypoxic and normoxic conditions. The present study aimed to investigate the metabolic roles of fructose in comparison to glucose at physiologically relevant concentrations by the D17 ovine conceptus.

Methods: Reproductively mature Suffolk ewes (n=24) were bred to fertile rams in six groups of four ewes each upon detection of estrus (D0). On D17 of gestation, ewes were euthanized and hysterectomized. The conceptus was flushed from the uterus with 15 ml sterile phosphate buffered saline (pH 7.2). Conceptuses from the ewes within a group (n=5 groups of 4 ewes each) were finely minced together to ensure homogeneity among conceptuses and cell types. Conceptus tissue (approximately 20 mg) was cultured in Krebs-Henseleit Buffer + 1 mM glutamate (an amino group donor), with unlabeled 2 mM glucose, 15 mM fructose, both alone and in combination, as well as with a specifically labeled isotope: [1-14C]-glucose, [6-14C]-glucose, [1-14C]-fructose, or [6-14C]-fructose. Cultures were incubated in oxygenated (95% O2/5% CO2) or hypoxic (90% N2/5% O2/5% CO2) conditions for 4 h to ensure equilibration of the medium. 14CO2 produced during these cultures was captured and measured to determine the carbon contribution of glucose or fructose to the PPP by subtracting the 14CO2 produced from the [6-14C] isotopes from the [1-14C] isotopes for each unlabeled hexose sugar combination. After the tracer experiment and subsequent homogenization, the medium was analyzed for lactate, pyruvate, and 14C-lipids, while the remaining conceptus pellet was analyzed for 14C-labled glycoproteins.

Results: The contribution of carbons from glucose or fructose into 14CO2 by way of the PPP was not different due to treatment or atmosphere (P>0.05). Incorporation of the carbons from radiolabeled glucose and radiolabeled fructose into glycoproteins was significant when all treatments were compared (P<0.05). Within treatments with a fructose tracer, there were no differences between hypoxic and normoxic conditions, with the exception of the 15mM fructose only treatment labeled with [6-14C]-fructose wherein hypoxic conditions reduced its incorporation into glycoproteins. Incorporation of labeled glucose and fructose into lipids was below the limit of detection of the assay. Lactate was more abundant in medium in which conceptuses were cultured under hypoxic conditions as compared to normoxic conditions (P<0.05) and this was an effect across all treatments (P<0.0001), but the interaction between atmospheric conditions and treatment was not significant. The amount of pyruvate in culture medium overall was significantly different due to treatment (P<0.05).The ratio of lactate to pyruvate was greater in medium in which conceptuses were cultured under hypoxic compared to normoxic conditions (P<0.05) and different across treatments (P<0.0001), however the interaction between atmospheric condition and treatment was not significant.

Conclusion: Previous results from culturing sheep conceptuses with 4 mM glucose and 4 mM fructose indicated that they metabolized fructose via the PPP and that glucose was the preferred substrate. Findings from this study suggest that at physiological levels of 2 mM glucose and 15 mM fructose, the sheep conceptus can use either glucose or fructose as an initial substrate for the PPP at similar rates. The conceptus may also use fructose, particularly under hypoxic

conditions, in the synthesis of carbohydrate moieties of glycoproteins, such as those synthesized via the hexosamine biosynthesis pathway. These findings, in conjunction with finding significant amounts of lactate in medium of conceptuses cultured under hypoxic conditions, suggest that fructose may have a role in facilitating adaptation of the conceptus to hypoxic conditions of the uterus during the pre-implantation period of pregnancy. Better understanding of the metabolism of fructose by the conceptus will elucidate its roles and related mechanisms affecting development, growth, and survival of the ovine conceptus. This research was supported by Agriculture and Food Research Initiative Competitive Grant no. 2018-67015-28093 from the USDA National Institute of Food and Agriculture.

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Compartmentalized glucose oxidation supports midgestation mammalian development

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Mammalian embryogenesis requires rapid growth and proper metabolic regulation. Midgestation features rising oxygen and nutrient availability concomitant with fetal organ development. Understanding how metabolism supports development requires approaches to observe metabolism directly in model organisms in utero. We used isotope tracing and metabolomics to identify evolving metabolic programs in the placenta and embryo during midgestation in mice. We pinpointed gestational days (gd) 10.5-11.5 as a period of metabolic divergence between placenta and embryo. Isotope tracing revealed compartmentalized metabolism including extensive recycling of glycolytic intermediates in the placenta, and rapid glucose-dependent purine synthesis in the embryo. Glucose's contribution to the tricarboxylic acid (TCA) cycle rises throughout midgestation in the embryo but not the placenta. By gd12.5, the embryo contains organ-specific metabolic programs including prominent pyruvate oxidation in some but not all organs. To use this approach to contextualize developmental anomalies in light of embryonic metabolism, we used mice defective in LIPT1, the enzyme that activates 2-ketoacid dehydrogenases in the TCA cycle. LIPT1 deficiency reduces glucose oxidation in embryo and placenta just prior to the gd10.5-gd11.5 transition and leads to embryonic demise at gd11.5 despite ongoing placental development and function. These data document individualized metabolic programs in developing organs in utero.

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Epithelial-specific deletion of SMAD1/5 in the mouse endometrium disrupts early pregnancy

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Introduction: The endometrium is the mucosal inner lining of the uterine wall and plays vital roles during the menstrual cycle and during pregnancy. The endometrium is a highly regenerative tissue that is under the cyclical control of estrogen and progesterone and is composed of two major cell types, the epithelium and stroma. The bone morphogenetic signaling (BMP) pathway is a highly conserved signaling pathway that controls key cellular processes, such as cell regeneration and differentiation. The SMAD1/5 proteins are the downstream effectors of the BMP signaling pathway that upon activation by phosphorylation, translocate to the nucleus and control gene expression. We recently identified that conditional deletion of *Smad1* and *Smad5* from uterine epithelium and stroma using progesterone receptor (PR)-cre resulted in endometrial glandular defects and infertility. However, how intact stromal BMP signaling affects epithelial cell function remains unknown. Lactoferrin (Ltf) is a non-heme iron-binding glycoprotein, which is highly responsive to estrogen in mouse uterus and mostly expressed in uterine epithelium of adult mice. Conditional deletion of *Smad 1* and *Smad 5* in the mouse uterine epithelium by lactoferrin (*Ltf)*-cre allows us to study SMAD1 and SMAD5 signaling in uterine epithelium in the adult uterus and during pregnancy.

Methods: We generated Smad1 and Smad5 conditional knockout mice using lactoferrin (*Ltf*) cre (*Smad1$^{flox/flox}$;Smad5$^{flox/flox}$;Ltf-cre+/-, or "Smad1/5* cKO") and performed timed mating studies to evaluate fertility in this mouse model. To evaluate fertility, control and *Smad1/5* cKO mice were mated to fertile WT males. We collected mouse uterus at 3.5 days post coitum (dpc), 4.5 dpc, 6.5 dpc or from virgin aged female mice and compared gene changes by qPCR.

Results: During a 3-month mating trial starting at 6 weeks of age, *Smad1/5* cKO female mice (n=4) only delivered 13 pups from a total of 3 litters, while control female mice (n=5) gave birth to 132 pups from a total of 20 litters, indicating a striking decrease of fertility in *Smad1/5* cKO mice. To investigate how the pregnancy failed at early stages, we performed timed mating studies at 6.5, 4.5, and 3.5 dpc. At 6.5 dpc, only 1 out of 8 cKO mice was pregnant compared to 8 out of 9 control mice and the weight of implantation site significantly decreased in cKO mice (13.4 ±0 mg/implantation site) compared with control mice (19.4 ±0.7 mg/implantation site), suggesting a severe defect in the decidua of cKO mice. At 4.5 dpc, 5 out of 5 control mice had implantation sites; 5 out of 8 cKO mice had implantation sites, however, 3 out 8 cKO mice had no implantation sites and flushed unattached blastocysts were recovered from the uterine lumen. Normally, *Hand2* regulates the antiproliferative action of progesterone and its mRNA level increases in the mouse uterus as it undergoes decidualization following implantation. Our qPCR results showed *Hand2* mRNA level significantly decreased in cKO mouse uterus at 4.5 dpc. At 3.5 dpc, there were similar numbers of blastocysts observed in control and cKO mice. qPCR results also showed a significant reduction of mRNA levels of the two progesterone response genes, *Ihh* and *Lifr*. These results demonstrate that uterine epithelial *Smad1* and *Smad5* are essential for implantation and decidualization.

Conclusion: We found that compared to controls, *Smad1/5* cKO mice had fewer offspring over 3 months, lower pregnancy rate at 6.5 dpc and variable implantation defects at 4.5 dpc with decreased *Hand2* expression in the uterus. Although similar numbers of blastocysts were recovered at 3.5dpc, we identified decreased *Lifr* and *Ihh* expression in the uterine epithelium. Our results reveal that deletion of *Smad1* and *Smad5* from the uterine epithelium leads to severe subfertility in the mouse.

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Impact of p53 gain-of-function mutants in ovarian cancer cell metastasis

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Introduction: High-grade serous ovarian cancer (HGSOC) is a heterogeneous disease for which there is currently no cure. Because p53 is mutated in >90% of all ovarian cancers, we sought to determine the impact of wild-type (WT), null and specific gain-of-function (GOF) p53 mutant proteins on tumor morphology and metastasis *in vivo*.

Methods: For this, we selected HGSOC cell lines, ALST (WT p53), SKOV3 (p53 null), TYK-NU (p53-R175H), OVCAR3 (p53-R248Q) and OVCA420 (p53-R273H), to generate *in vivo* xenografts in *Foxn1-/-* mice. Lesions were fixed in 4% PFA to assess morphology, differentiation, and gene expression. To assess the effects of GOF mutations within the same genetic background, ALST (WT) and SK-OV-3 (null) cells were engineered to express p53-R175H or p53-R273H. A panel of ~30 antibodies recognizing diverse phosphorylation marks was used to evaluate pathway dependencies impacting cell proliferation, inflammatory status, or apoptosis in OVCAR3 tumors. Cell proliferation was determined *in vitro* using the sulforhodamine B (SRB) assay. Proliferation assays coupled to targeted and chemotherapeutic treatments were used to determine the impact of inhibited pathways identified in the phospho-protein screen stains.

Results: ALST cells failed to metastasize *in vivo*, likely due to the known apoptotic effects of WT p53 in response to stress. SK-OV-3 and the p53-GOF cell lines preferentially metastasized to the omentum and exhibited distinct morphologies: SK-OV-3, epithelial-like, TYK-Nu, vascular-like, OVCAR3, epithelial/mesenchymal-like and OVCA420 epithelial exclusive. Despite different morphologies and p53 status, each tumor type contained large, polyploid giant cancer cells (PGCCs) that are stem-like cells undergoing endoreplication and are considered markers of drug resistance. A specific phosphorylated, active form of β-catenin (pCTNNB1-S31/S37/T41; pCTNNB1), the mitotic stress regulatory kinase pMSK1-T581, and activating phosphorylation of mTOR-S2448 colocalized with p53 to centrosomes and microtubules of mitotic cancer cells and PGCCs, in a screen of ~30 phospho-antibodies. This indicates a specific role of these factors in HGSOC tumor progression. Remarkably, the ALST (WT/R273H) cells, but not the ALST (WT/R175H) cells, formed solid tumors on the ovary, visceral fat, and uterus; and not on the omentum as do the OVCA420 p53 mutant cells harboring the same R273H p53 mutation. In culture, ALST (WT/R273H) cells proliferate more rapidly and are less sensitive to paclitaxel than the ALST (WT) or ALST (WT/R175H) cells; possibly related to PGCCs. When the p53-R175H or -R273H GOF mutants were stably expressed in SKOV3 (p53 null) cells, tumor progression *in vivo* was enhanced, likely through increased flux through the cell cycle (with either R175H or R273H) as measured by KI67 and pMSK1 stains, as well as enhancement of PGCCs. In culture, both R175H and R273H introduced into the SK-OV-3 (p53 null) background resulted in increased proliferation, in contrast to the ALST (p53 WT) background. Inhibitors of MTOR (vistusertib, sapanisertib), MSK1 (SB747651A), NEK2 (NCL 00017509) with and without paclitaxel were used to determine whether the pathways found to be highly active in mitotic and PGCC tumor cells by IF analyses could be targeted pharmacologically *in vitro*. In fact, MTOR inhibition using sapanisertib was similar in efficacy to paclitaxel at similar concentrations, and was more broad spectrum across all cell lines; synergy with paclitaxel was weak or absent in most cases. Inhibition of MSK1 and NEK2 with their respective inhibitors did not strongly impact growth *in vitro*, except at very high doses (>5 µM).

Conclusion: The HGSOC mutant and engineered cell lines allowed for the measurement of proliferation rates in culture and *in vivo*, which is a strong determinant of outcomes. However, because recurrence and resistance to current taxol mediated chemotherapies are common in ovarian cancer, identifying and targeting specific stress response(s) underlying polyploidy and endoreplication should be considered in future. It was also noted that R273H was the stronger mutation, establishing a hierarchy of mutations: R273H > R175H/null > WT. These observations are reflected not only in our models, but in patient samples as well. Collectively, 1) elevated levels of specific CTNNB1, MSK1, and MTOR phosphorylation marks identify mitotic cells and PGCCs that may provide new targets for therapeutic approaches; 2) knowing the status of p53, determining the impacts of steroid hormone receptors in both tumor and stromal cells, as well as the presence of pCTNNB1, pMSK1, and pMTOR indicate combinatorial approaches may also be useful for reducing tumor growth and preventing recurrence. NIH-CA181808; NIH-HD097321

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Real-time quantitative PCR versus Droplet Digital PCR for quantification of peripheral blood leukocytes interferon-tau stimulated genes in beef cattle

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Introduction: Quantitative Reverse Transcription PCR (qPCR) consists of the real-time amplification and quantification of specific complementary DNA targets. This technique had become the gold standard for many diagnostic purposes, but the resulting data is often variable and non-reproducible. Droplet Digital PCR (ddPCR) is a recently developed technology for absolute nucleic acid quantification based on sample partitioning in nanodroplets. This results in the measurement of multiple amplification events within a single sample. Therefore, ddPCR has some advantages over qPCR, such as increased sensitivity and precision. We aimed to compare the abundance of interferon-tau stimulated genes (ISG) transcripts in peripheral blood leukocytes of artificially inseminated beef cows using qPCR versus ddPCR.

Methods: Multiparous *Bos taurus* beef cows (n = 7) were subjected to timed artificial insemination (TAI) on day 0. Pregnancy was determined by transrectal ultrasonography on days 26 and 30 post-TAI, and cows were classified as: pregnant (n=4; embryo detected on days 26 and 30) or non-pregnant (n=3; no embryo detected). Coccygeal vein blood samples were collected on days 0, 15, 17, 19, 20 and 24 post-TAI. Leukocyte RNA was extracted from the buffy-coat fraction using Trizol (Thermo Fisher Scientific) associated with the DirectZol-RNA kit (Zymo Research) and transcribed to cDNA. The abundance of ISG (ISG15 and MX2) was assessed by relative quantification to a reference gene (RPS9) using RT-qPCR and by absolute quantification using the QX100TM Droplet DigitalTM PCR System (Bio-rad Laboratories) according to manufacturer's recommendations. Data was analyzed using PROC MIXED on SAS 9.4.

Results: For the RT-qPCR, pregnant cows had greater ($P<0.05$) ISG15 and MX2 abundance compared to non-pregnant cows on days 20 (ISG15:0.11±0.1 vs. 0.01±0.001 and MX2:0.73±0.4 vs. 0.06±0.06) and 24 (ISG15:0.34±0.2 vs. 0.01±0.001 and MX2:0.77±0.2 vs. 0.13±0.04). For ddPCR, a greater ISG15 and MX2 copy numbers in pregnant cows was observed on days 15 (ISG15:129 vs. 44 copies/µl and MX2:33 vs. 10 copies/µl) and 20 (ISG15: 216 vs. 30 copies/µl and MX2: 44 vs. 7 copies/µl), and on day 24 for ISG15 (32 vs. 7 copies/µl) compared to non-pregnant cows.

Conclusion: Digital Droplet PCR was able to detect an earlier expression of ISG in pregnant cows. With future studies to establish a suitable cutoff value, this could be a less subjective approach for pregnancy diagnosis as it does not require the use of a reference gene.

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Obese PCOS mouse model has increased body mass with fat mass greater than lean mass

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Introduction: Polycystic ovarian syndrome (PCOS) is a common reproductive disorder affecting 12-20% of women. Metabolic derangements, such as insulin resistance, hyperinsulinemia, glucose intolerance, and glucose intolerance, are an important feature of the disease and have lifelong implications. Changes in fat distribution and body composition are one of the manifestations of metabolic derangements. While studies have shown differences in fat distribution in obese patients with PCOS, lean patients with PCOS have not had clear results. Mouse models of PCOS have been used as a means to study different metabolic effects of PCOS and can be used to further evaluate differences in body composition; however, data regarding body composition in PCOS murine models is lacking. In our study we have sought to further characterize lean and obese PCOS mouse models by evaluating body mass, including total body lean mass and total body fat mass.

Methods: We utilized two different PCOS mouse models using C57/B6J strain. A total of 8 obese PCOS, 8 obese PCOS controls, 9 lean PCOS, and 7 lean PCOS controls were used. The obese model was created by placing a 2.5 mg dihydrotestosterone (DHT) 90-day controlled-release pellet in 6-week-old female mice. Controls received a placebo pellet. The lean model was created by administering 250 µg of DHT in sesame oil vehicle to pregnant dams on days 16.5, 17.5, and 18.5 post-coitus. Lean controls were administered sesame oil vehicle only. QMR measurements were taken on postabsorptive, unanesthetized mice at 2 months of age in lean model and 4 months of age in obese model. Statistical analysis was performed using Minitab software. The lean and obese groups were compared to their respective controls using Student's T-test.

Results: In the lean PCOS group, average body weight (18.5 ± 0.4 (SEM) g versus 18.7 ± 0.4 g) average lean mass (15.6 ± 0.3 g versus 15.6 ± 0.3 g) and fat mass percentage (10.9% ± 1.1 versus 11% ± 0.9) were similar between the control group (n=7) and treatment group (n=9). Compared with obese PCOS controls (n=8), the obese PCOS group had increased average body weight (21.5 ± 0.3 g versus 25 ± 0.3 g, $p<0.05$), lean mass (17.7 ± 0.2 g versus 20.5 ± 0.2 g, $p<0.05$), and fat mass percentage (11.5 ± 0.7% versus 13.9 ± 0.7%, $p<0.05$).

Conclusion: The obese PCOS group had a higher body weight attributable to a greater increase in fat (42%) than lean mass (16%) compared to controls, thereby resulting in greater percentage fat than in controls. There was no difference between lean or fat mass between the lean PCOS group and its respective control.

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Validation of DAPI/Anti-lamin A/C Immunofluorescent Labeling Technique for Improved Classification of Meiotic Maturation Stage in Porcine Oocytes

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Introduction: In oocyte maturation protocols, aceto-orcein stain does not easily differentiate between the germinal vesicle (GV) in the diplotene stage of meiosis from the germinal vesicle breakdown (GVBD) stage. An immunofluorescent technique to highlight the nuclear membrane and chromatin would improve the ability to accurately discern the diplotene (GV intact) versus post-diplotene (GVBD) stages of meiotic maturation. We tested a fluorophore-bound antibody to lamin proteins and 4',6-diamidino-2-phenylindole (DAPI) as an alternative approach to aceto-orcein stain. The objectives of this study were: 1) validation of a fluorophore-bound antilamin antibody in porcine oocytes; 2) comparison of the percentage of oocytes classified as GVBD with each staining technique; 3) comparison of procedural losses with each staining technique.

Methods: Immature porcine oocytes were aspirated from slaughter-house gilt ovarian follicles (3-8 mm in size) using vacuum aspiration, after which those oocytes with at least two layers of cumulus cells and homogenous cytoplasm were selected for maturation. Oocytes (n = 180) were matured in groups of 40-50 per well in Medium 199 in 4-well IVF plates (NUNC, ThermoFisher). Replicates (n = 5-6) of oocytes were divided into two groups: aceto-orcein and DAPI/Anti-lamin A/C. Aceto-orcein stained oocytes were mounted on slides, fixed for 48 hours in an aceto-alcohol fixative, followed by staining with aceto-orcein and meiotic maturation stage was immediately determined with bright field microscopy at 400x magnification. DAPI/Anti-lamin A/C oocytes were fixed in 4% paraformaldehyde for 30 minutes followed by two permeabilizations with 0.5% Triton-X and 0.05% Tween 20 detergents for 30 minutes each. Next, these oocytes were blocked with 2% BSA for 60 minutes. Next oocytes were exposed to mouse anti-lamin A/C Alexa Fluor 488 antibody (Cell Signaling Technology) diluted 1:50 in phosphate-buffered saline with 1% BSA and 0.3% Triton-X for 60 minutes, and mounted on a slide with ProLong Antifade mountant with DAPI (Cell Signaling Technology). For the DAPI/Anti-lamin A/C technique, meiotic stage was classified from morphologic observation of chromatin material (DAPI, blue) and germinal vesicle (anti-lamin, green) under a fluorescent confocal microscope at 200x magnification. Procedural losses for each technique were calculated by dividing the initial number of oocytes allocated to a given technique by the final number of oocytes found on the slide.

Results: The DAPI/Anti-lamin A/C technique tended to result in less procedural loss of oocytes than the aceto-orcein staining technique (3.2 ± 2.3% vs. 17.1 ± 9.4% oocytes lost during staining, $p = 0.10$). Additionally, the DAPI/Anti-lamin A/C technique had fewer oocytes whose meiotic stage was unable to be classified (3.0 ± 1.5% vs. 28.7 ± 11.2% oocytes unable to be classified, $p = 0.04$). It was easier to qualitatively identify the GVBD using DAPI/Anti-lamin A/C than aceto-orcein stain. In two of the replicates, the anti-lamin A/C antibody appeared to cross-react with a protein in the cell membrane. This cross-reactivity of the anti-lamin A/C antibody occurred in the condensed chromatin and telophase stages of meiotic maturation.

Conclusion: This mouse anti-lamin A/C Alex Flour 488 antibody can be used for identification of the GVBD stage in porcine oocytes although it demonstrate some non-specific binding. The DAPI/Anti-lamin A/C technique is more efficient than aceto-orcein staining with greater numbers of oocytes retained during the staining process. Performing additional replicates to compare these techniques will boost numbers of oocytes tested and likely decrease the variability within each technique.

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A potential role for calcitonin gene related peptide in regulating mitochondrial function in endothelial cells

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Introduction: Elevated level of soluble tyrosine kinase-1 (sFLT-1) is a suggested cause of oxidative stress and endothelial dysfunction in preeclampsia (PE). Calcitonin gene related peptide (CGRP) is a potent vasodilator known to support vascular adaptation during pregnancy. Circulatory levels of CGRP are lower in PE and blocking function of CGRP results in fetal growth restriction. Therefore, this study was designed to assess if CGRP regulates mitochondrial function in endothelial cells and identify if CGRP effects are altered by sFLT-1 in endothelial cells.

Methods: Endothelial cells (HUVEC from ATCC) were cultured in complete endothelial cell growth medium as per manufacturer's instructions. Cells were starved in growth medium containing 2% charcoal stripped FBS for 6 hours, treated with or without peptides (10-8M) in presence or absence of sFLT-1 for 24 hours and harvested for mRNA extraction using QIAGEN RNA extraction kit. Expression of mRNA for mitochondrial enzyme complexes was assessed by qRT-PCR using gene specific primers and analyzed relative to the expression of GAPDH and 18s. Data was analyzed with Prism GraphPad Software using 1-way ANOVA or unpaired t test. $P \leq 0.05$ was considered statistically significant.

Results: 1)CGRP dose-dependently increases: a) ND1 and ND2 mRNA encoding proteins in mitochondrial complex 1, b) Cytochrome b (CYTB) in complex 3,c) Cytochrome c oxidase subunit I (CO1) mRNA in complex 4, and d) ATPase V mRNA in complex 5 ($P<0.05$); 2) sFLT- 1 decreases the expression of ND1 and these decreases are inhibited in presence of CGRP; 3) sFLT-1 shows a tendency to decrease the expression of ND2 in HUVEC which becomes significant in presence of CGRP ($P<0.05$) and 4) sFLT-1 has no effect on the expression of CYTB, CO1 and ATPase5 in HUVEC cells in presence or absence of CGRP.

Conclusion: CGRP mediated increase in mitochondrial enzyme complexes in endothelial cell and rescue of sFLT-1-mediated decreases in ND1, suggest a potential role for CGRP in regulating oxidative stress and ameliorating sFLT-1 induced endothelial dysfunction.

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Ovarian dynamics of Brahman cows submitted to five-day or twelve-day progesterone-based estrus synchronization protocol

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Introduction: The aim of this study was to investigate the ovarian response of Brahman cows submitted to a five-day or twelve-day progesterone-based estrus synchronization protocols and to determine the estrus response and ovulation time.

Methods: A total of 153 Brahman cows were randomly assigned to receive one of the following estrus synchronization protocols: 1) CIDR insert and an injection of prostaglandin F2α (25 mg, PGF) on Day -5 and CIDR removal with PGF on Day 0 (CIDR5; n= 77); 2) CIDR insert on Day -12 and an injection of PGF on Day -12, Day -5 and Day 0 upon CIDR removal (CIDR12; n= 76). Estrotect breeding indicator patches were placed on Day 0 and cows were artificially inseminated (AI) 12h after estrus detection; cows not detected in estrus after 96h received TAI coupled with 100 µg of GnRH. Ovarian dynamics were observed during the protocol and follicular diameter were measured every 12h after AI until ovulation.

Results: Cows synchronized with CIDR12 had increased follicular diameter (CIDR5, 10.19 ± 0.51 vs. CIDR12, 13.12 ± 0.52 mm; $P = 0.01$) and decreased serum concentrations of progesterone (CIDR5, 1.68 ± 0.12 vs. CIDR12, 0.81 ± 0.12 ng/ml; $P < 0.01$) at CIDR removal. The percentage of cows exhibiting estrus did not differ between treatments (CIDR5, 66.2 vs. CIDR12, 64.5%; $P = 0.25$). No difference was observed in ovulation rate (CIDR5, 94.1 vs. CIDR12, 94.1%; $P = 0.6$). Time of estrus expression and ovulation time after CIDR removal did not differ (CIDR5, 59.9 ± 2.3 vs. CIDR12, 61.5 ± 2.3h; $P = 0.71$ and CIDR5, 88.1 ± 2.6 vs. CIDR12, 90.9 ± 2.6h; $P = 0.75$; respectively)

Conclusion: In conclusion, extended length of CIDR with additional PGF increased follicular diameter and decreased concentrations of progesterone but did not influence estrus response and ovulation time in Brahman cows.

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Choriocarcinoma Presenting as Lower Extremity Weakness: A Case Report and Literature Review of Spinal Cord Metastatic Disease

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Introduction: Gestational trophoblastic disease (GTD) is a range of disorders characterized by the proliferation of cells originating from placental villous trophoblasts.[1] When GTDs persist, they are considered gestational trophoblastic neoplasia (GTN) which include choriocarcinoma, invasive mole tumors, placental site trophoblastic tumors and epithelioid trophoblastic tumors, all of which are characterized by persistently elevated HCG levels[2]. Choriocarcinomas, which are known to be perfused by fragile blood vessels and occupied with necrosis, arise after molar pregnancy (50%), after previous abortion (25%), in normal pregnancy (25%) and consequent to an ectopic pregnancy (3%).[3, 4] The incidence of spinal metastasis of a choriocarcinoma is extremely rare – it most commonly metastasizes to lungs (94%), vagina (44%), liver (28%), brain (28%), followed by skin, GIT, kidney, breast, and bone.[5] We present a case of a patient who initially developed symptoms of spinal compression caused by epidural space metastasis.

Methods: In order to find past case reports about this specific presentation of choriocarcinoma, the NIH National Library of Medicine was chosen as data base for this search. A total of 21 papers were read and evaluated. 15 of these were discarded on the basis that no case report was presented. Finally, 6 papers were identified, 2 of which contained past reviews of spinal cord metastases cases. Combining the data in these 2 with the data in the 4 additional case reports yielded 19 cases of spinal cord metastasis, with 12 of them involving the epidural space.

Case Report: A 24-year-old female G4P3013 presented October 3, 2018 with vaginal bleeding at 10 4/7 weeks of gestation with a 15 cm sized uterus and a quantitative serum HCG of 769,419 mIU/mL. She was treated with dilation and suction curettage (D&C). Her hospitalization was complicated by diffuse intravascular coagulation and atypical preeclampsia. Final pathology showed hydatidiform mole. In March 11, 2019, she visited our obstetric evaluation room due to vaginal bleeding and a positive home pregnancy test. She was found with a quantitative serum HCG of 2,387 mIU/mL. Chest X-ray was without abnormalities. Follow up HCG 48 hrs after 2,781 mIU/mL. Patient was lost to follow up for four months. In July 2019, patient was admitted at periphery hospital due to progressive lower extremity weakness resulting in a fall and inability to move lower extremities. Labs done showed HCG >908,170.00. Patient was transferred to University District Hospital due to suspected Gestational Trophoblastic Neoplasia (GTN). Staging work up was notable for Thoracic MRI with extensive abnormal posterior and bilateral lateral epidural expansion from T3 through T9. Chest CT with innumerable, bilateral, randomly- distributed, soft tissue pulmonary nodules consistent with metastatic lung disease. Multidisciplinary reunion with Gynecology Oncology, Neurosurgery and Hematology Oncology services determined that the risks of neurosurgical intervention were too high without assurance of neurological deficit improvement. It was determined that the best option for the patient was starting a chemotherapy regimen. Patient completed 10 cycles of EMA-CO in December 2019 and currently has no evidence of disease. She was unable to recover from paraplegia and now has limited lower extremity control and mobility.

Results: This case report presents a rare case of metastasis to the epidural space associated with spinal cord compression causing a clinical presentation of bilateral paraplegia. A patient with this kind of metastasis is classified as "high-risk" and is unlikely to respond to single drug chemotherapy.[2] To our knowledge from the published literature, there have been 19 reported cases of spinal metastases with 10 of them involving the epidural space. Presentations included back pain, headache, visual field defects and paraplegia.[1] Imaging such as myelography, CT scan or MRI is useful in diagnosis and planning of surgery. To date, it has been agreed that patients with high-risk GTN should be treated initially with multiagent chemotherapy, particularly EMA-CO, with or without adjuvant therapy or radiotherapy.
However, the best treatment for spinal metastases has not been established because of its rarity. Surgery has a limited role with this form of disease but could be considered for progressive neurologic decline or when a mass effect is present.[6] Despite improvements in treatment modality, the prognosis for these choriocarcinoma cases with spinal metastases is unfavorable.[1]

Conclusion: This rare case of choriocarcinoma with epidural metastases causing extremity weakness followed by paraplegia illustrates the importance of having a multidisciplinary approach for managing gynecologic malignancies. In our case, patient had an adequate response to multi-agent chemotherapy, without requiring radiation or surgery. Earlier diagnosis and multimodality treatment is crucial for significant reduction in mortality. Further research is required to determine the best approach for GTN spinal cord metastasis.

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The Creatine Metabolic Pathway in Ovine Utero-Placental Tissues During the Peri-Implantation Period and in Late-Stage of Pregnancy

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Introduction: Creatine (Cr) is synthesized de novo from arginine, glycine, and methionine in two successive steps: first conversion of arginine and glycine to guanidinoacetate (GA) by arginine-glycine amidinotransferase in the kidneys, followed by methylation of GA to Cr by S-adenosylmethionine-dependent guanidinoacetate N-methyltransferase (GAMT) in the liver. The synthesized Cr is then transported by solute carrier family 6 member 8 (SLC6A8) to target tissues. Cells with high rates of ATP turnover, such as skeletal muscle, maintain ATP homeostasis by utilizing an intracellular pool of phosphocreatine (PCr) generated from free cellular Cr and ATP via a reversible reaction catalyzed by creatine kinase (CK). The mammalian conceptus is metabolically active and requires significant amounts of ATP for growth, development, implantation, placentation, and fetal development. Components of the Cr-CK-PCr system have been identified in utero-placental tissues in humans and rodents. However, roles of the Cr-CK-PCr system are poorly understood during pregnancy in sheep. In this study, we determined the temporal and cell-specific expression of proteins involved in the synthesis and transport of Cr and PCr during the periimplantation period and in late-stage of pregnancy in sheep.

Methods: Paraffin embedded sections of uteri from pregnant ewes at Days 9, 12, 17, and 125 (n=3-5 per Day), conceptuses on Days 16, 17, 18, and 20 (n=2-3 per day); and placentomes on Day 125 (n=5) were used for immunolocalization of proteins involved in the synthesis, and transport of Cr and PCr.

Results: Immunofluorescence staining showed that GAMT, CK brain-type (CKB), CK muscle-type (CKM), and SLC6A8 proteins were all expressed in uterine epithelia, whereas only CKM was localized in endometrial stromal cells, on Days 9, 12, 17, and 125 of pregnancy. In the ovine conceptuses, GAMT, CKB, and CKM were localized in trophectoderm (Tr) cells at Days 16, 17, 18, and 20 of pregnancy. Interestingly, the expression of CKB was more abundant in trophoblast giant cells compared to mononuclear Tr cells of the conceptuses, whereas uniform expression of CKM was observed in all Tr and endodermal cells of conceptuses. In Day 125 placentomes, GAMT, CKB, and CKM were localized only in the syncytial plaques, whereas SLC6A8 was localized in the caruncular stroma adjacent to the syncytial plaques. Results of this study suggest that Cr is synthesized from GA by uterine endometria and converted in a cell-specific manner to PCr or be secreted and/or transported directly into the uterine lumen during the peri-implantation period. The conceptus Tr cells can also synthesize Cr from GA or may utilize Cr from uterine luminal fluid to store high energy phosphate groups as PCr. Synthesis of Cr and PCr and their transport also occurs in placental tissues at late-stage of pregnancy.

Conclusion: The components of Cr-CK-PCr system are expressed in uterine, conceptus, and placental tissues during the peri-implantation period and in late-stage of pregnancy suggesting an important role of the Cr-CK-PCr system in energy homeostasis for conceptus development, implantation, placentation, and fetal development during ovine pregnancy.

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Identification of MASTL and TTK kinases as therapeutic targets for endometrial cancer

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Introduction: Endometrial cancer is characterized by malignant growth of uterine cells and is the 5th most common cancer affecting over 60,000 women in the United States. Treatment of endometrial cancer often requires surgical intervention (hysterectomy), radiation, chemotherapy and hormone therapy. Prognosis for patients with early detection is favorable. However, recurrent disease is characterized by poor prognosis due to invasion of other organs and treatment resistance. Our objective is to identify novel drivers of aggressive disease by performing proteomic and transcriptomic analysis of clinical samples. Our goals are: (1) To identify new targets (proteins) that are markers and drivers of aggressive cancers. (2) To test if the expression of these proteins is necessary and sufficient to control cancer cell growth and migration. (3) To develop drugs that control the activity of these driver proteins to prevent cancer cell growth.

Methods: Kinases and phosphatases with significant correlation with aggressive endometrial cancers and poor prognosis were identified by integrating clinical proteomic and transcriptomic data. The following panel of human cell lines, representative of low- and high-grade endometrial cancer, were employed to functionally characterize targets of interest: HEC-1-A, SK-UT-1B, KLE, AN3 CA, EI, ETN1, MFE-319, and HEC-1-B. Basal expression of targets was evaluated by RT-qPCR and Western Blot. Loss-of-function assays were performed with siRNAs targeting kinases of interest to evaluate their role in proliferation (CellTiter-Glo® Luminescent Cell Viability Assay, Promega) and 2-D migration (wound-healing assay) of endometrial cell lines.

Results: At the gene transcript level, we detected expression of *MASTL, TTK, MAP3K2*, and *SCYL1* to *GAPDH* by qRTPCR in all the cell lines. Similarly, we detected the protein products of these gene transcripts in whole cell lysates of the cell lines by Western blot using commercially-available antibodies. Knock-down of all kinases evaluated was successfully achieved by siRNA-mediated silencing as determined by qRT-PCR and Western blot. Cell viability and 2-D migration (wound-healing) following siRNA-mediated depletion was evaluated in Ishikawa and HEC-1-A cells. Ishikawa cells treated with siSCYL1 had increased viability relative to control cells treated with non-targeting siRNA (siNT). In contrast, treatment with siMASTL had a small, yet significant, suppression of cell viability. siTTK treatment completely abolished viability in Ishikawa cells. In HEC-1-A cells, both siTTK and siMASTL treatment robustly and significantly abolished cell viability. siMAP3K2 had a modest effect on HEC-1-A cell viability on the last day of analysis. Compared to siNT-treated Ishikawa cells, cells treated with siTTK demonstrated a significant reduction in wound closure. HEC-1-A cells on the other hand, showed a significant reduction in wound closure capacity compared to siNT after treatment with siMAP3K2, siMASTL, and siTTK. Notably, although cell viability was not affected by siMAP3K2 treatment in HEC-1-A cells, wound closure was significantly reduced.

Conclusion: The proteomic signatures associated with clinical measures of more aggressive endometrial cancers yielded novel molecular clues of disease drivers. Expression analysis in endometrial cancer cells lines allowed us to establish cell line models in which to functionally characterize our target candidates *in vitro*. With this method, we identified protein kinases having a functional impact on viability and/or migration of endometrial cancer cells, including MAP3K2, MASTL, and TTK. We will pursue the functional characterization of these targets and others with more mechanistic studies in combination with chemotherapeutic agents and xenograft mouse models. In parallel, we will submit our validated targets to our drug discovery pipeline using our DNA-encoded library of small molecules. Ultimately, our findings will provide novel therapeutic alternatives for the treatment of this devastating gynecological malignancy.

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SARS-CoV-2 colonization in human placenta: infection and impact on Renin Angiotensin System and Preeclampsia

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Introduction: The COVID-19 pandemic caused by the Beta-coronavirus, SARS-CoV-2 has affected people of all ages. Alarmingly, SARS-CoV-2 infection appears to increase the risk of adverse pregnancy outcomes such as preeclampsia in pregnant women. The mechanism(s) by which this occurs remains unclear.

Methods: We investigated the pathophysiology of SARS-CoV-2 at maternal-fetal interface in pregnant women who tested positive for the virus using RNA in situ hybridization (viral RNA), immunohistochemistry, and hematoxylin and eosin staining. To investigate whether viral infection alters the renin angiotensin system (RAS) in placenta, which controls blood pressure, we treated human trophoblasts with recombinant spike protein or a live modified virus with a vesicular stomatitis viral backbone expressing spike protein (VSV-S).

Results: Viral colonization was highest in maternal decidua, fetal trophoblasts, Hofbauer cells, and in placentas delivered prematurely. We localized SARS-CoV-2 to cells expressing angiotensinconverting enzyme 2 (ACE2) and demonstrate that infected placentas had significantly reduced ACE2. In response to both spike protein and VSV-S, cellular ACE2 decreased although angiotensin II receptor type 1 (AT1R) increased with concomitant increase in soluble fms-like tyrosine kinase-1 (sFlt1), an antagonist of angiogenesis. Furthermore, viral infection decreased pro-angiogenic factors, AT2R and Placental growth factor, which competitively binds to sFlt1. Sera from infected pregnant women had elevated levels of sFlt1 and angiotensin II type 1-receptor autoantibodies (AT1-AA) prior to delivery, both signatory markers of pre-eclampsia.

Conclusion: In sum, we demonstrate that SARS-CoV-2 colonizes ACE2-expressing fetal trophoblasts, stromal cells, and macrophages in the placenta and SARS-CoV-2 infection in pregnant women correlates with alteration of normal placental RAS. As RAS regulates blood pressure, SARSCoV-2 may thus increase adverse hemodynamic outcomes such as preeclampsia in pregnant women.

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Sex dependent mitochondrial dysfunction in the skeletal muscle of low protein programmed lean type 2 diabetic rats

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Introduction: We had previously developed a low protein (LP) programmed lean type 2 diabetic rat model and showed that the progression and severity of the disease is sex dependent. We had identified that males have low testosterone and females have low estradiol levels. Further, our data showed that supplementation of sex steroids prevented the onset of the disease in both sexes. In this study, our aim was to investigate the role of mitochondrial function in skeletal muscle in order to identify the LP programming effects on the mitochondrial function.

Methods: Pregnant rats were fed with a control (20% protein) or an iso-caloric LP (6%) diet from day 4 of pregnancy until delivery. Normal diet was given to mothers after delivery until weaning, and pups were given a normal diet after weaning. Three month old LP programmed offspring were implanted with 90 days slow release sex steroid pellets (Males with testosterone (0.5mg/day) and females with estradiol (1µg/day) or placebo subcutaneously. Controls were implanted with placebo pellets. Skeletal muscle fibers were isolated from 6 month old animals, and mitochondrial function was assessed by Cell Mito Stress test using Seahorse XF 96 analyzer.

Results: Preliminary results show that mitochondrial spare respiratory capacity and oxygen consumption rates at maximum respiration were markedly lower in the skeletal muscle fibers of the LP programed female offspring compared to the controls skeletal muscle fibers. However, treatment with estradiol rescued the spare respiratory capacity and maximum respiration. In contrast, there are no significant differences in male skeletal muscle fiber mitochondrial spare respiratory capacity between LP programmed offspring and the control.

Conclusion: Our results suggests that gestational LP programming leads to mitochondrial dysfunction, and sex steroid treatment may improve the mitochondrial health in a sex dependent way. Further investigations and analyses are in progress to understand the relationship between mitochondria, LP programming, steroid hormones and type 2 diabetes.

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Transcriptional heterogeneity of epithelial in non-pregnant and pregnant cervix

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Introduction: The cervix is a metabolically active and vital organ critical for maintaining pregnancy and timely parturition. In preparation for birth, the cervix, which throughout gestation remains tightly closed and rigid, undergoes remodeling to a soft, distensible structure to allow passage of the fetus. Thus, proper cervical function is essential for successful pregnancy outcomes. The mouse cervix is composed of stratified squamous epithelia surrounded by stroma. The epithelia serve as a protective barrier from ascension of pathogens. Moreover, disruption in these barriers could results in entry of pathogens into the lower reproductive tract leading to preterm birth. This cervical epithelium undergoes extensive proliferation and differentiation during pregnancy. Pregnancy causes cellular and molecular changes in the cervical epithelia which is not fully understood. We address this significant knowledge gap by using SCRNAseq. From these data, gene expression patterns reflecting the distinct cell states changes during pregnancy can provide valuable insights into the epithelial cell population in cervix.

Methods: Single cell libraries were made from mouse cervix from non-pregnant (NP) and four pregnancy timepoints early (day 6 and 12); late (day 15 and 18) and in labor (IL) using the 10x Genomics Single cell platform. A range of 5000-10,000 cells per library were sequenced. After normalization, cell clustering was performed using the Seurat software package

Results: To identify the epithelial cellular subsets in the mouse cervix we performed scRNA seq of isolated cells from at all timepoints from NP, early, late and IL samples. The generated data from all timepoints were combined to perform unsupervised clustering and to characterize the epithelial heterogeneity. Based on the distinct cytokeratin (intermediate filament family of protein) transcriptional profiles we were able to identify two major epithelial cell types: cycling and proliferating basal cells (TRP63, KRT5) and non-cycling differentiating luminal cells (DMKN, TPRG). The luminal cells were further divided into two transcriptionally distinct secretory cell types that are differentiated (Pigr and Rbp2) at all gestational time points. These secretory cells were enriched for mucin genes and expressed Spdef (transcription factor for goblet cell differentiation), MUC1, MUC 4, MUC5ac and MUC5b.The transcriptional profile of secretory cells in the NP cervix showed higher expression of defense barrier function genes (MUC1, MUC4, IFITM1, LCN2, SLPI, DEFB1, WFDC18) and cornified envelope proteins (SPRRLA, SPRR2D, SPRR2F) compared to secretory cells in pregnancy. By contrast to NP, secretory cells in pregnancy displayed higher levels of innate immune defense genes MUC5B, MUC5AC, complement protein (C3), protease inhibitors (WFDC2, WFDC15B) suggesting pregnant cervix is functionally distinct from NP cervix. Further in situ hybridization has identified distinct spatial and temporal pattern in the secretory epithelial populations between endocervix and ectocervix. Together, cervical epithelia are a critical player that provides physical and immunological protection as the cervix undergo remodeling during pregnancy, in preparation of parturition and in the NP cervix.

Conclusion: Our scRNA seq has allowed us to readily identify diverse epithelial cell types lining the mouse cervix by differential gene and protein expression pattern. Our results demonstrate distinct set of genes are regulated in the NP and pregnancy cervix suggesting that epithelia have unique functions depending on the environmental clue. Further findings on epithelial diversity might allow us to dissect the transcriptional heterogeneity in pathogenesis such as preterm birth.

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