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50 Years of Male Reproduction

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50 Years of Australian Leadership in Ovarian / Oocyte biology

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Since its inception the SRB (or ASRB) membership has been a world leader in contributions at the cutting edge of ovarian and oocyte biology. Discoveries by Australian researchers that have changed the field over the last 50 years, too numerous to list in detail, cover the hormonal actions of the ovarian follicle, the signalling interactions between ovarian somatic cells and the oocyte, the importance of nutrition and metabolism to oocyte quality and many others. These achievements to date are substantial, but more importantly, this culture of excellence is continuing. Exciting current research building on the example and training of our SRB founders promises new heights in understanding of ovary and oocyte biology and translation into important reproductive medicine and biotechnology applications.

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THE UTERUS: from near obscurity to frontline of reproductive success over 50 years

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50 years ago, the uterus was a little understood organ, beyond that it was where the embryo attached, the placenta was formed and the fetus nurtured until birth. Most information available related to the considerable differences in uterine type between species, the morphology, particularly of the endometrium, and its responsiveness to ovarian hormones resulting in cyclical tissue remodelling in all species and menstruation in women. Since the 1980's, the cyclical molecular and cellular changes in the endometrium, their importance for establishment of healthy pregnancy in all species, and the particular differences between species have become better understood. Australian scientists have been world leaders in research in marsupial and human endometrial function, and contributed to the field of ruminant uterine function. In reproductive medicine, endometrial dysfunction is responsible for major women's health issues, including endometriosis and abnormal uterine bleeding, and contributes significantly to infertility. Yet this complex tissue has been largely ignored by the IVF community and by funding bodies. Looking ahead, further focused investigation of endometrial function offers strong potential for translational outcomes for conservation, food production and women's health.

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Reproductive Immunology

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The immune response is centrally involved in all aspects of the reproductive process, from generation of gametes, conception and implantation, to fetal and placental development and birth. In contrast to early theories about immune suppression, immune cells and cytokines play an active role, resulting either in immune tolerance and permissive effects on development, or immune effector function which is important in sensing and selecting healthy gametes and embryos, and mediating parturition and birth. Many forms of infertility and pregnancy disorders have an immune aetiology. Australian reproductive biologists have been instrumental in defining the fundamental biology of how immune cells and cytokines can act to both facilitate or impede reproductive events and processes, and impact developmental programming and offspring health. Their work has led to new understanding of how the reproductive and immune systems work in collaboration to ensure quality control and maximise genetic diversity and fitness in outbred populations, and is now contributing to development of new clinical treatments involving targeted modulation of the immune response.

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Lactation versus placentation: diverse reproductive strategies of mammals

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Viviparity is widespread across the animal kingdom, and many groups evolved a means of nurturing the young via a placenta that forms an intimate contact with the uterus of the mother. This required many adaptations to allow the genetically foreign offspring remain in the uterus for the duration of pregnancy. However, only mammals extended the period of maternal

protection longer, dependent on the amazing secretion that is milk. In eutherian mammals, this ranges from days and months to several years (elephant lactation 3-5years, !Kung Hunter Gatherers of the Kalahari, 4 years, in whom it acts as “Nature’s contraceptive”, and even up to 3-4 years in breastfeeding mothers in Melbourne. In contrast, marsupials deliver highly altricial young that complete their development during an extended lactation, producing the most complex and changing milk composition known for any mammal. In most mammals, apart from the immediate post-natal colostrum, the mature milk does not change in composition. In marsupials and also in monotremes, milk composition is an ever-changing process, and the milk is tailor made for each particular stages of the development of the young. Growth of the young is under maternal control via the milk, much as the placenta does in eutherians. Mismatching the stage of lactation with the age of the young can growth accelerate development, and maturation - and cause obesity. We now know that a number of genes expressed in the mammary glands in the tammar are the same as those that are functionally important in the placenta in eutherians. In marsupials lactation therefore controls post-natal development of the exteriorised fetus until it reaches independence with at least some of the same genes and molecular pathways as the eutherian placenta. Thus marsupials have truly exchanged the umbilical cord for the teat.

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Legacies of the revolutions and evolutions in animal production

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Over the past 50 years, the production of animals for agricultural and biomedical research purposes has advanced radically through the use of breakthrough technologies that involve the transfer or manipulation of gametes, embryos, somatic cells, and the genome itself. The dissemination of valuable male and female genetics by artificial insemination and embryo transfer revolutionised animal breeding programs, particularly in livestock species. Continued genetic improvement of livestock using advanced reproductive technologies (ARTs) and quantitative selection schemes will be vital to meet the future global demand for food. The development of associated ARTs, including oestrus synchronisation and superovulation, embryo in vitro production and culture, and sperm and embryo cryopreservation, have not only enabled wider dissemination of valuable genetics, but also facilitated the detailed study of gamete and embryo biology. Without the knowledge gained from animal studies, the treatment of infertility in humans could not have advanced to the stage it is today. To date, an estimated 6 million children have been born worldwide as a result of in vitro fertilisation, an incredible legacy indeed. The birth of Dolly the sheep, the first animal cloned by somatic cell nuclear transfer, heralded a new era in the generation of genetically engineered (GE) animals. Also, recent advances in genome editing techniques have been stunning, making it much easier to achieve the desired genetic manipulations with greater precision. Consequently, the number of GE animals produced to provide models of human diseases is increasing dramatically, advancing research into major public health problems, such as neurodegenerative disorders, heart disease, and diabetes. It remains to be seen whether meat from GE livestock will be approved for human consumption, but given the undeniable benefits to production and continuous world population growth, it seems certain that such animals will one day be used to help meet the global demand for food.

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A video guide to research success, mentoring, collaboration and happiness. Lessons from the SRB Founding Mother on how to create a successful research career. "You can do it if you put your heart into it!".

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A perspective of the contribution of my past research to current reproductive biology and medicine

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Major advances in scientific research do not arise as a quantum event but are built upon the efforts and findings of many earlier workers in a particular field of endeavour. In mammalian reproductive physiology and embryology: in vitro fertilization, embryo culture, manipulation, embryo transfer and cryopreservation are now commonplace for research in the laboratory and for clinical application in both human and veterinary medicine. Over time the accumulation of small technical discoveries has culminated in translation to the benefit of human infertility and genetics, agriculture and our basic understanding of the genetic control of embryonic development. Early studies of fertilisation and embryonic development were focused on obtaining a basic understanding of these events and the knowledge obtained was not directed towards a practical application – this came later. Important early contributors will be discussed. When I obtained IVF in the mouse (1968) this was only the second mammal in which this had been achieved with the birth of live young. Ten years earlier MC Chang had achieved this in the rabbit. It took another 10 years before a human baby was born from IVF – Edwards & Steptoe. Why did it take so long? At the time further information was required for culture media components for ova and spermatozoa, fertilisation in vitro, culture of embryos and the stage of the cycle for transfer of the embryos. My research model was the mouse; studies involved the maturation of the ovum, parthenogenesis, fertilization in vitro, preimplantation development and the cryopreservation of mammalian gametes and embryos. This research has contributed to the development of techniques used in IVF, embryo culture, cryopreservation and

stem cell production. The ethical and moral impact of research in these areas on society today is continually being debated as new developments are reported in assisted conception and human genetics. Discussion of these issues will be included in my presentation.

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Pushing the boundaries of biology and technology: A non-linear model with rewards and tensions

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My research career has been primarily disruptive and rewarding in a model where collaborations with individuals from different fields has worked well in opening new opportunities in animal biotechnology and human medicine. Generally the new studies were not supported by conventional granting agencies because there was little preliminary data or evidence that it would succeed. There were also ethical issues raised by some of the work because the subject had strong connections to religious beliefs that were not supportive. At other times the collaboration with industry was seen as a negative. These issues created tension between the desire to continue the research and institutional or community concerns. Generally the science won out because it provided highly desirable outcomes.

The subject of my research moved from large animal reproductive technology – developing embryo transfer, freezing and transport for the animal industries (cattle, sheep, horses, pigs) to human IVF where there was little knowledge and a belief that IVF should not be developed. The tensions were very strong over the ethics of embryo research, involvement of commercialisation and the pace of discovery. Over 8 million babies have now been born using basically the technologies we developed at Monash. Cloning of animals after “Dolly the sheep” again created tensions but also resulted in a new farm animal industry. Human embryonic stem cells were developed again with considerable criticism and a lack grant funding. This field moved quickly with the early discoveries and was strongly funded as Australia’s Biotechnology Centre of Excellence by the ARC ($110million). However, this was a disappointment when governance issues and structure led to key scientists leaving. In a very different structure the state bond funded California Institute of Regenerative Medicine (CIRM) (US$3billion) demonstrated how to drive stem cell discovery to clinical trials and eventually patient benefits. The cell therapy that has arisen around chimeric antigen receptor technology (CAR-T) shows also incredible potential as an effective cancer therapy and when combined with stem cells may revolutionise cancer treatment.

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Significant Advances in ART – under the “The clinical picture"

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IVF is part of the reproductive revolution that became visible in the 1960’s with the introduction of the oral contraceptive pill. This was followed by legalised termination of pregnancy, the use of donor sperm and attempts to facilitate improved fertility for couples where tubal blockage had prevented them from having children. The first clinical pregnancy was reported in Melbourne in 1973 but was an ectopic pregnancy. A second failed pregnancy was reported in the United Kingdom several years later, but with the birth of Louise Brown in 1978, the processes for IVF in humans became researched much more. Initially, natural cycles were used but subsequently the introduction of Clomiphene Citrate and Gonadotropins made a major impact on the efficiency of the process by enabling embryologists to obtain more eggs. Multiple pregnancies were common and unpredictable but were a side effect of the need to put back several embryos to obtain good quality pregnancy rates. In time, single embryo transfer became the norm in Australia, ICSI was incorporated for poor quality or absent sperm and genetic testing of embryos for chromosomal abnormalities became wide spread. There are many challenges remaining in IVF, including the woman in advanced reproductive age who produces poor quality eggs, unexplained implantation failure, recurrent unexplained miscarriage and ongoing questions about the health of children conceived from assisted reproductive technology. While 4% of all babies born in Australia come from IVF pregnancies, this is likely to increase with an aging population, more acceptance of assisted reproductive technology by the general public and by more competition in the market place for pricing. Clinic guidelines are required to enable that only the patients who really need it will get IVF, quality management processes for clinicians and scientific staff need to improve and much more research needs to be done into the causes of infertility and the personalisation of treatment, so that pregnancy rates can continue to increase. Australia was at the forefront in the early days of assisted reproductive technology and then significantly lost its place, largely due to commercialisation of the industry and the retreat of researchers and universities from the arena. It is hoped that in the next decade more funding for research and the contribution of clinical scientists will grow.

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Accelerated ovarian reserve depletion has no effect on fertility in female *Amh*$^{-/-}$ mice.

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The size of the ovarian reserve in each female is largely determined by events prior to puberty. However, the rate of ovarian reserve depletion is thought to influence fertility later in life. Depletion of the ovarian reserve by primordial follicle recruitment is modulated by multiple processes including anti-Müllerian hormone (AMH) signalling. AMH produced by granulosa cells inhibits primordial follicle activation, leading to an accelerated loss of ovarian reserve in AMH-/- mice. The assumed reduction of the

fertility of female AMH-/- mice has not been tested. The aim of this experiment was to determine the age that reproductive output diverges in AMH+/+ and AMH-/- females and to examine the quality of AMH-/- mouse embryos at this phase of life. AMH+/+ males were paired with AMH-/- and AMH+/+ females and birth and litter size data were collected up until the age of 11 months. Unexpectedly, no differences were observed in litter size, parturition intervals or cumulative number of offspring at any age. Litter size declined in AMH+/+ and AMH-/- mice from around the 9th litter onwards. Few females remained fertile at 11 months of age but corpora lutea were present in the ovaries and cumulus-oocyte complexes were observed in the oviducts on the day of estrus in both strains, indicating ovulation was still occurring. This bears similarity to the age-related infertility that affects women approximately 10 years before menopause. The present data might indicate AMH has minor effects on ovarian reserve depletion, with little effect on fertility. Alternatively, the determinants of age-related infertility may be independent of the ovarian reserve. Ultimately, this study questions the current hypothesis that the role of AMH is to conserve the ovarian reserve to prolong the reproductive lifespan.

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The oocyte-secreted factors GDF9, BMP15 and cumulin regulate inhibin and activin production in human granulosa cells

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The oocyte-secreted factors bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) regulate folliculogenesis, oocyte quality and fecundity. They are comprised of a pro-domain and a mature domain and it is known the pro-proteins are biologically active on cumulus cells. In addition, there is evidence that GDF9 and BMP15 can form a potent heterodimer called cumulin. This study utilized various recombinant forms of GDF9, BMP15 and cumulin to assess the effect of; 1) homo- versus hetero-dimer proteins and, 2) pro-mature versus mature forms of proteins, on primary human granulosa-lutein cell function. Cells were treated in vitro with GDF9, BMP15, or cumulin ± their pro-domains; mRNA expression and protein secretion of inhibin A and B and activin B were measured. Regardless of whether cells were treated with mature- or pro-forms, GDF9 or BMP15 alone exhibited minimal effects on inhibin/activin production, whereas the addition of both factors elicited a marked synergistic increase in *INHβB* mRNA and protein production of inhibin B and activin B, but not inhibin A. Consistent with the hypothesis GDF9-BMP15 synergism is due to the actions of cumulin, both mature cumulin and pro-cumulin dose-dependently increased *INHβB* mRNA expression and inhibin B and activin B production, but not inhibin A. FSH induced expression of *INHα* mRNA expression leading to a 2- to 3-fold increase in inhibin B production when co-treated with GDF9+BMP15 or cumulin, and a corresponding decrease in activin B production. In general proteins in the pro-form and those lacking the pro-domain elicited the same effect on inhibin/ activin production, suggesting that the pro-domain may have a minimal role in the actions of these growth factors on granulosa cells. In conclusion, oocyte-secreted GDF9 and BMP15, likely in the form of the heterodimer cumulin, exert paracrine control of gonadotrophin-induced production of inhibin B/activin B in follicular granulosa cells.

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The role of Semaphorin 7a during ovulation

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Reproductive success is dependent on ovulation of a developmentally competent oocyte. However, the molecular mechanisms underlying ovulation are ill-defined. Following the ovulatory luteinising hormone (LH)-surge, cumulus cells become transiently migratory and invasive, predicted to be required for ovulation. What regulates this migratory phenotype is not clear. Interestingly, *Sema7a*, known to alter the migratory phenotype in other cells, is dysregulated in granulosa cells (GCs) of the anovulatory progesterone receptor null mouse (PRKO; microarray data). We hypothesised that SEMA7a abundance in the periovulatory follicle plays a role in regulating the migratory phenotype of the cumulus oocyte complex (COC) during ovulation. We confirmed that *Sema7a* was 2.3-fold higher in GCs from infertile PRKO mice than GCs from fertile heterozygous littermates at 8 h post-hCG ($P<0.05$). During the ovulatory period *Sema7a* was significantly reduced by 13- and 6.8-fold at 4 h post hCG-administration in COCs and GCs, respectively. In both cell types *Sema7a* increased following ovulation (16 h post-hCG). In antral follicles SEMA7A protein was predominantly localised to the granulosa cells on the apical side. This specific pattern was lost following post hCG. Localisation of SEMA7a in a particular spatiotemporal pattern prior to hCG may be due to interacting effects of hypoxia and FSH. We found both hypoxia ($2\%O_2$) and FSH significantly increased *Sema7a* during in vitro culture of GCs ($P<0.05$). SEMA7A receptors Plexin C1 and β1 integrins are known to decrease and increase migration, respectively. *Plxnc1* was significantly downregulated in both the COC and GCs post-hCG while *Itgb1* remained constant prior to ovulation and significantly upregulated following ovulation in both cell types. Interestingly expression of *Itga5* whose gene product forms a heterodimer with β1 integrin and promotes migration following SEMA7A binding was significantly upregulated in both the COC and GCs following hCG. Together these data show that Sema7a may be important during ovulation.

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Effects of different wavelengths of light at the same energy on the development of preimplantation embryos

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Aim:

In-vivo development of embryos occurs in the absence of light. Time-lapse imaging enables observation of embryo development and uses low energy wavelengths. However, more informative photonic assays like hyperspectral analysis use wavelengths of higher energy light, which can cause DNA damage, mitochondrial degeneration, increased reactive oxygen species production and blastomere apoptosis, thus lowering implantation potential of embryos. We hypothesised that exposing embryos to discrete wavelengths of light delivered at the same energy (1Joule/cm^2/day) causes differences in embryo development rates. We assessed the toxicity of these wavelengths by determining cleavage and blastocyst development rates, cell numbers and DNA damage.

Method:

CBAF1 mice one cell presumptive zygotes were collected and cultured (5% O_2) until day 5. Embryos were exposed to light emitting diodes of Blue (616nm, 52sec), Green (525nm, 105sec), Yellow (590nm, 213sec) and Red (616nm, 83sec) wavelengths, delivering a total 1Joule/cm^2/day of energy from days 1-5. Cleavage and blastocyst rates were recorded. To determine inner cell mass and trophectoderm cell numbers, blastocysts were differentially stained. To measure the incidence of DNA strand breaks, histone modification antibody (γH2AX) was used. Area (in pixels) for γH2AX and DAPI positive cells was calculated using FIJI image analysis. Statistical significance ($p<0.05$) was analysed using GraphPad Prism.

Results:

Exposing embryos to the yellow wavelength had a significant impact by reducing cleavage ($p<0.01$) and blastocyst rates ($p<0.05$) when compared to control. No significant difference in cell numbers was noted across treatment groups when differentially stained, but DNA damage was evident across all treatments when stained for DNA strand breaks.

Conclusion:

Our data demonstrates that yellow light illumination at an equivalent energy level as other wavelengths, impedes embryo development, with reduced cleavage and blastocyst rates. Exposing embryos to all wavelengths of light in this study caused significant cell DNA damage, which may impact implantation potential of embryos.

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Mitochondrial distribution and trafficking is disrupted in oocytes from mitochondrial fission deficient and old mice

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Maternal ageing is associated with a dramatic decrease in fertility, which is primarily caused by an age-related decline in oocyte quality. Mitochondria are the most abundant organelle in the oocyte and play an important role in generating ATP during oocyte maturation. Importantly, during oocyte maturation, mitochondria undergo dramatic reorganisation that is dependent on motor protein dynein-mediated associations with microtubules, and surround the meiotic spindle. This study sought to determine whether the kinetics of mitochondrial re-localisation are disrupted in two different contexts of impaired mitochondrial function: i) oocytes that are deficient in fission due to null mutation in dynamin-related protein 1 (Drp1$^{-/-}$ mice) and ii) oocytes from aged females (1 year old mice). Both mouse models were on the Dendra background so that all oocyte mitochondria were endogenously tagged with green fluorescence. Oocytes were collected at the germinal vesicle stage and induced to undergo maturation in vitro and imaged continuously by confocal microscopy. Mitochondrial movements were monitored in real-time and re-localisation to the meiotic spindle was quantified. GV oocytes from both Drp1$^{-/-}$ mice and aged mice exhibited mitochondria that were clumped within the cytoplasm compared to homogeneously dispersed in young/wildtype mice. During maturation mitochondria in oocytes of young/ wildtype mice underwent rapid re-distribution and around 80% of mitochondria surrounded the meiotic spindle within 9h. Interestingly, oocyte mitochondria in Drp1$^{-/-}$ oocytes underwent spindle-associated aggregation with even greater efficiency than wildtype littermates. As these mitochondria are deficient in the fission machinery it suggests that enhanced mitochondrial connectivity results in more uniform relocalisation. In contrast, mitochondria in oocytes from old mice exhibited reduced trafficking to the developing meiotic spindle, with a greater proportion retained in the cytoplasm. This indicates mitochondrial clusters in old oocytes are either deficient in dynein-mediated trafficking or more fragmented than mitochondria in oocytes of young females.

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Oocyte-secreted factors measured in serum of women correlate with reproductive potential

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Growth differentiation factor-9 (GDF9) and bone morphogenetic protein-15 (BMP15) are oocyte-secreted factors essential for folliculogenesis. Despite known associations with reproductive pathologies, measurements of GDF9 and BMP15 as biomarkers of reproductive function have not been demonstrated. The aim of this study was to develop novel immunoassays for the detection of GDF9 and BMP15 in human peripheral blood and apply these to samples from women undergoing IVF. The immunoassays were developed in-house and validated for sensitivity, specificity and reproducibility and applied to clinical samples from women undergoing IVF. Serum samples were collected immediately before and on multiple days during FSH stimulation in antagonist treatment (100 bloods from 58 women). Serum GDF9 and BMP15 levels varied >10-fold between women, but, within an individual; were unchanged with GnRH antagonist controlled stimulation, and independent of FSH dose. GDF9 and BMP15 significantly correlated within individuals (n=58, r=0.39, p<0.01). Serum levels of GDF9, but not BMP15, positively correlated with the number of oocytes retrieved following a stimulation cycle in non-PCO(S) patients (n=39, r=0.38, p<0.05). Patients with ≥10 oocytes retrieved had 1.85-fold more GDF9 than those with <10 oocytes (p=0.05). This is the first report of the detection of serum GDF9 and BMP15 levels measured in human serum and their correlation with IVF outcome. This study demonstrates oocyte-secreted factors as potential serum biomarkers in reproductive medicine.

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Manipulating testicular androgen production to promote lifelong male health

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Androgen production by the testis is essential for fetal masculinisation and male fertility. More recently androgens have been shown to support lifelong male health, with low testosterone associated with a wide range of clinical co-morbidities such as cardiovascular disease, diabetes and obesity, and also age-related degeneration. Conversely androgen replacement therapy has also been associated with negative clinical outcomes in some cases, suggesting that alternative approaches to develop, maintain, and support a healthy androgen profile throughout life are worth exploring. To address this, we have utilised a series of transgenic mouse models to dissect out genetic, cellular and age-related influences on the androgen production machinery and in particular, the testicular Leydig cells. This has identified a functional paracrine network, working through multiple cell-types, to support the development and function of this important cell type throughout life, and identified novel pathways for manipulation to improve lifelong male health.

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Activin A target genes in fetal testis: clues to male infertility

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Activin A, the *Inhba* gene product, is produced by fetal testis interstitial cells and acts on Sertoli cells to regulate cord development[1]. In *Inhba*$^{-/-}$ testes, fetal Sertoli cell proliferation is reduced, yet some gonocytes proliferate longer than normal, yielding an imbalance between Sertoli cell and gonocyte numbers at birth[2]. The present study investigates how activin influences fetal testis development by examining gene expression in mouse fetal testes with altered activin bioactivity levels. Approaches were: (1) RNAseq to measure transcripts in isolated somatic cells (interstitial, endothelial and Sertoli cells; germ cells excluded) from wildtype and *Inhba*$^{-/-}$ testes at E13.5 and E15.5; (2) comparing these with pre-existing RNAseq data to identify transcripts enriched in interstitial or Sertoli cells[3]; (3) high-throughput qPCR to measure specific transcripts in fetal testes from *Inhba*$^{-/-}$(no activin A) and *Inha*$^{-/-}$ mice[4] (elevated activin bioactivity). Unaltered levels of steroidogenic enzymes (*Cyp11a1*, *Cyp17a1*, *Hsd3b1*) and other Leydig cell-specific transcripts (e.g. *Itih5*, *Itga8*) suggested fetal Leydig cell differentiation was unaffected by activin. However selected Sertoli cell transcripts were significantly altered between E13.5-15.5 by activin deficiency; qPCR confirmed these as activin-responsive genes in *Inhba*$^{-/-}$and *Inha*$^{-/-}$ testes. Transcripts positively regulated by activin included *Sel1l3* and *Vnn1*, and extracellular/secreted proteins *Masp1*, *Sfrp4* and *Kazald1*. Sertoli cell-enriched transcripts negatively regulated included *Cldn11*, *Tthy1* and *Erbb3*. Importantly, the Sertoli cell-enriched steroidogenic enzymes, *Hsd17b1* and *Hsd17b3*, were positively regulated by activin. Fetal Sertoli cells express these enzymes to produce testosterone[5] which influences gonocyte mitotic quiescence[6]. These outcomes suggest in conditions of activin insufficiency, androgen precursors production is normal in interstitial cells but inhibited within cords. Identification of these activin-responsive genes in fetal Sertoli cells may be central to understanding how disruptions to this signalling pathway lead to adult pathologies, such as hypospermatogenesis or testicular cancer.

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Sertoli cell proteins in testicular interstitial fluid; potential mediators of seminiferous tubule-interstitial cell communication

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Sperm are produced within the seminiferous tubules of the testis. Outside the tubules, the interstitial space contains blood vessels, steroid-producing Leydig cells and immune cells that modulate testicular function. These cells are bathed in testicular interstitial fluid (TIF), a rich source of proteins potentially involved in seminiferous tubule - interstitial cell communication. This study aimed to identify Sertoli cell-derived TIF proteins, because these cells directly modulate Leydig cell development and function via unknown mechanism(s)[1,2]. TIF was isolated from control adult mice and mice in which Sertoli cells had been acutely ablated[1,2] (n=12/group). TIF proteins were identified using a nano-flow HPLC coupled to an Impact II UHR-QqTOF mass spectrometer. Statistically different proteins (control vs Sertoli cell-ablation) were identified using a custom-designed pipeline[3]. The potential cellular origin of TIF proteins was assessed using an RNASeq dataset of testes from mice with germ cell +/- Sertoli cell ablation[4]. 44 proteins of likely Sertoli cell origin were identified based on mRNA expression and their significant reduction in TIF after Sertoli cell ablation. These proteins included well known Sertoli cell-specific proteins, e.g. CLU, CTSL, TUBB3 and VCL, as well as novel Sertoli cell proteins that were confirmed by immunohistochemistry. In silico data-mining showed most Sertoli cell proteins were unlikely to be secreted but 86% have been detected in exosomes, suggesting that Sertoli cells release exosomes basally to communicate with interstitial cells. Most mouse Sertoli cell proteins were also detected in TIF isolated from men with normal testis function, indicating that deposition of Sertoli cell TIF proteins is conserved in humans. This study has revealed multiple candidate proteins in TIF with potential roles in Sertoli-interstitial cell communication.

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Mechanistic insights into epididymosome-sperm interactions

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The mammalian epididymis is responsible for the provision of a highly specialized environment in which spermatozoa acquire functional maturity and are subsequently stored in preparation for ejaculation. Making important contributions to both processes are epididymosomes, small extracellular vesicles released from the epididymal soma via an apocrine secretory pathway. While considerable effort has been focused on defining the cargo transferred between epididymosomes and spermatozoa, comparatively less is known about the mechanistic basis of these interactions. To investigate this phenomenon, we have utilized an *in vitro* co-culture system to track the transfer of biotinylated protein cargo between mouse epididymosomes and recipient spermatozoa. Our data indicate that epididymosome-sperm interactions are initiated via tethering of the epididymosome to receptors restricted to the post-acrosomal domain of the sperm head. Thereafter, epididymosomes mediate the transfer of protein cargo to spermatozoa via a process that is dependent on dynamin, a family of mechanoenzymes that direct intercellular vesicle trafficking. Notably, upon co-culture of sperm with epididymosomes, dynamin 1 undergoes a pronounced relocation between the peri- and post-acrosomal domains of the sperm head. This repositioning of dynamin 1 is potentially mediated via its association with membrane rafts and ideally positions the enzyme to facilitate the uptake of epididymosome-borne proteins. Accordingly, disruption of membrane raft integrity or pharmacological inhibition of dynamin both potently suppress the transfer of biotinylated epididymosome proteins to spermatozoa. Together, these data provide new mechanistic insight into epididymosome-sperm interactions with potential implications extending to the manipulation of sperm maturation for the purpose of fertility regulation.

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Seminal fluid may alter cytokine and chemokine gene expression in human endometrium after coitus

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In rodents, seminal fluid establishes maternal immune tolerance for embryo implantation and optimal placentation by inducing inflammatory changes and priming the immune response to paternal antigens. Coital exposure to seminal fluid also induced marked changes in gene expression, cytokine and chemokine production and subsequent leukocyte recruitment within cervical tissues. This study was undertaken to determine whether seminal fluid also altered gene expression profiles in human endometrial tissues *in vivo,* and in Ishikawa endometrial epithelial cells lines *in vitro*. With informed consent, women (n=4) with a past history of tubal ligation and regular menstrual cycles were recruited via community notices to this randomised, cross over design, pilot study. Volunteers who used hormonal or barrier contraception, had irregular cycles or did not have a consenting sexual partner were excluded. Urinary ovulation prediction kits identified the luteinising hormone (LH) surge. At the first positive LH test, each participant was randomised to either have unprotected intercourse or abstain in the next 24 hours in 2 different menstrual cycles, 2 months apart. Twelve hours after timed intercourse or abstinence, endometrial biopsies were collected by pipelle and immediately stored in RNAlater before RNA extraction. Affymetrix microarray and qPCR analyses identified immune-regulatory and tissue remodelling genes that were consistently differentially expressed when women had had intercourse (n=3). CXCL8 (3.8-fold), MMP12 (3.6-fold), IL1RN (1.6-fold), CXCL5 (1.5-fold) and TNFSF10/TRAIL (1.7-fold) were upregulated, whereas CXCL14 (3.3-fold) and CSF1 (1.4-fold) were downregulated. These changes were not seen in the fourth woman who abstained in both treatment cycles. *In vitro*, whole seminal fluid, seminal plasma and washed sperm elicited similar gene expression profiles in Ishikawa cells. Our study provides the first evidence that coital seminal fluid exposure may alter gene expression in the human endometrium. Human endometrial responses to seminal fluid exposure potentially contribute to embryo implantation and subsequent fetal development.

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Characterization of seminal plasma anti-HIV-1 antibodies

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There is an urgent need for a HIV-1 vaccine. A better understanding of the immune parameters present during natural HIV-1 exposure might inform vaccine design. Semen - an overlooked factor in models of HIV-1 infection - not only acts as a vehicle for HIV-1 transmission, but might also initiate or attenuate anti-viral immune responses at the mucosal site of exposure. We have previously demonstrated seminal plasma (SP) to potently inhibit anti-viral NK cell and cytotoxic T-lymphocyte responses *in vitro*. In addition to inhibitory factors, SP from HIV-1-infected donors contain anti-HIV-1 antibodies. We now present data characterizing anti-HIV-1 antibodies within semen from paired samples obtained prior to and following initiation of antiretroviral therapy (ART).

Matched serum and SP samples were collected from 11 HIV-1-infected donors at pre- and during-ART time points. These samples were assessed by end-point titer ELISAs for anti-gp120 IgG. Both serum and SP samples collected after greater than six months of ART exhibited a non-significant decrease in levels of anti-gp120 antibodies, compared to pre-ART samples [median 2.0 $\log_{10}$ vs 2.5 $\log_{10}$, p=0.218, n=8]. Furthermore, we observed no significant differences in NK cell IFNγ production following stimulation with gp120-coated T cells in the presence of purified IgG from pre-ART or during-ART SP samples [2.85% (15.04–0.17) vs 4.57% (14.33–0.23), p=0.465, n=11]. Lastly, we noted that antibodies capable of mediating antibody-dependent cellular cytoxicity within serum and SP targeted similar gp120 epitopes. Pre-incubation of gp120-coated T cells with a FAb of an anti-HIV-1 antibody directed to a CD4-induced epitope within gp120 inhibited anti-HIV-1 antibody-dependent NK cell activation by sera and SP-derived IgG [median 70.0% inhibition, p=0.0039, n=9].

Our findings highlight the presence of functional anti-HIV-1- antibodies within SP that target similar epitopes on gp120 as sera antibodies. Future research should investigate the role these anti-HIV-1 seminal antibodies play during mucosal HIV-1 transmission.

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Living with the enemy: germ cell survival in the 21st century

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Many environmental contaminants are potentially toxic to eggs and sperm and there are increasing numbers of men and women of late reproductive age, who are having difficulty producing a sufficient number of good gametes to conceive. The evidence suggests that fertility is influenced by lifetime exposure, probably at very low levels to oxidative stress via reproductive toxicants.

In addition, despite antibiotic treatment, early life exposure to immune challenges suggest that increased proinflammatory signalling within the neonatal gonads may be responsible for the depletion of the primordial follicle pool and spermatogenic dysregulation. These findings also have implications for late female and male fecundity as major determinates of reproductive longevity.

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Chlamydia infection damages the ovary and depletes the ovarian reserve in mice

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Chlamydia trachomatis is the most commonly diagnosed sexually transmitted infection (STI) in Australia (1) and can cause severe and irreversible damage to the fallopian tubes, leading to ectopic pregnancies or complete infertility. Previous *Chlamydia* infections are also associated with elevated miscarriage rates, poor IVF outcomes and increased time to natural conception, suggesting that fertility may be compromised by mechanisms that extend beyond fallopian tube scarring. In this study, we used a well-characterised mouse model to investigate that hypothesis that *Chlamydia* infection, and the inflammatory response it induces, damages the ovary. Primordial and growing follicle numbers were found to be significantly reduced 35 days after a single infection compared to uninfected controls ($p<0.05$, n=4 mice/group). A second infection of *Chlamydia* caused a further decrease in the numbers of all follicle types ($p<0.05$, n=4-5 mice/group) and increased the proportion of follicles with apoptotic granulosa cells ($p< 0.05$, n=4-5 mice/group). Two infections was also associated with changes in the overall ovarian morphology and increased fibrosis in the ovary ($p<0.05$, n=5 mice/group). Notably, using immunohistochemistry, chlamydial bodies were detected in the ovarian stroma 35 days after a single or double infection, indicating persistence. Collectively, these observations provide evidence that *Chlamydia* penetrates the ovary and disrupts the ovarian reserve, ovarian function and the structural integrity of the ovary itself, suggesting that ovarian infection may underlie some cases of unexplained infertility and poor IVF outcome. The results obtained from this study will aid the development of improved therapeutic strategies, including novel vaccines, to reduce the damaging effects of *Chlamydia* infection.

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The consequences of multigenerational exposure to acrylamide on DNA damage in the male germ line

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Humans are chronically exposed to acrylamide in carbohydrate-rich foods cooked above 120°C. CYP2E1, an enzyme found within testes and epididymides, solely converts acrylamide to glycidamide. Alone CYP2E1 leaks reactive oxygen species leading to oxidative DNA damage, whereas in the presence of acrylamide the resulting glycidamide directly adducts to the DNA.

We have previously demonstrated chronic acrylamide administration to male mice, at a human relevant dose (1 µg/mL drinking water for 6 months (M)) increased DNA damage in mature spermatozoa and led to induction of the CYP2E1 protein within spermatocytes [1]. Notably, CYP2E1 induction also occurred in the testes of unexposed offspring of acrylamide-exposed fathers where it too was associated with an increase in oxidative DNA damage [1]. This raises concerns for increased susceptibility of offspring to acrylamide-induced DNA damage. To explore this possibility, we are currently exposing the male offspring of acrylamide-exposed fathers using a shorter treatment regimen (3M at 1 µg/mL) and extending the study to the F2 generation. We report here on the F0 and F1 generations. Our shorter exposure regimen increased DNA damage in spermatozoa of acrylamide-exposed F0 males (127% of control). Similarly, this shorter exposure increased DNA damage in spermatozoa of the F1 generation. As with the longer exposure, unexposed male offspring had increased DNA damage in their spermatozoa (115% of control, n=14-17 representing n=4 F0). Surprisingly, we found significantly higher DNA damage in spermatozoa of the unexposed F1 generation than that of their directly exposed (1 µg/mL for 3M) littermates. This suggests an increased level of CYP2E1 resulting from paternal acrylamide exposure preferentially generates increased DNA damage in the absence of the substrate.

This study demonstrates that a paternal acrylamide exposure regimen briefer than previously investigated has a detrimental effect on the offspring and their genetic potential with, or without, further exposure to acrylamide.

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The ovarian follicle reserve is preserved in inflammasome-deficient mice

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Ovarian ageing is defined by a progressive decline in oocyte/follicle number and quality, leading to loss of fertility and ovarian function, cycle irregularity and eventually menopause. The factors that contribute to the depletion of follicles throughout reproductive life are poorly characterised. However, with more women delaying childbearing beyond the age of 35 years, there is a growing need to elucidate the mechanisms underlying the reproductive ageing process. Several recent studies have shown that low level chronic inflammation, driven by the NLRP3 inflammasome, is associated with normal and pathological ageing in many organ systems. Notably, mice lacking key components of the NLRP3 inflammasome, ASC or NRLP3, live for longer and exhibit a delayed ageing phenotype compared to wild type (WT) mice. Therefore, in this study we investigated the hypothesis that inflammatory processes contribute to ovarian ageing by comparing follicle numbers in WT mice with age-matched *asc*$^{-/-}$ and *nlrp3*$^{-/-}$ mice (n=3-4/genotype). At 12 months of age, WT mice were nearing the end of their reproductive lifespan and had significantly depleted primordial follicle reserves, consistent with ovarian ageing. In striking contrast, the primordial follicle reserves remained extremely high in *asc*$^{-/-}$ mice (WT= 229±32 vs *asc*$^{-/-}$= 824±96, p<0.0001). Primordial follicle numbers were also significantly elevated in *nlrp3*$^{-/-}$ mice, though to a lesser extent (WT= 229±32 vs *nlrp3*$^{-/-}$= 399±41, p=0.0194). The number of primary and secondary follicles did not show any statistically significant differences. However, the number of antral follicles was also significantly higher in 12 months old inflammation-deficient mice compared with WT (WT= 14±3 vs *asc*$^{-/-}$= 36±6, p=0.017; vs *nlrp3*$^{-/-}$= 33±3, p=0.0044). These findings suggest that the inflammation, and the NRLP3 inflammasome in particular, play a critical role in follicle loss during the normal reproductive ageing and raise the possibility that ovarian ageing could be delayed by suppressing inflammatory processes in the ovary.

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Investigation of intratesticular inflammatory responses in humans and mice implicates activin A as a potential inducer of fibrosis in the testis

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Testicular inflammation is a cause of fertility disturbance in men with fibrosis being a common observation. Experimental autoimmune orchitis (EAO) in mice reflects this immunopathology (1). Yet the cells responsible for production of extracellular matrix components (ECMC) in testicular inflammation are unknown, but resident fibroblasts or peritubular cells (PTCs) are potential sources. Activins (Transforming growth factor-β superfamily members) regulate fibrosis in many tissues and control spermatogenesis and steroidogenesis in the normal testis. Activin A levels increase substantially during murine EAO, prompting the question of its involvment in the subsequent fibrosis.

Accordingly, we investigated the pro-fibrotic actions of activin A in isolated adult mouse PTCs and 3T3 fibroblasts. Activin A increased fibronectin mRNA levels, and production of collagen type I and fibronectin proteins in both PTC and 3T3 fibroblasts. Activin A also increased α-smooth muscle actin protein and mRNA expression in 3T3 fibroblasts, and collagen type IV mRNA levels in PTCs. Follistatin-288, an activin A antagonist, inhibited these effects. Furthermore, treatment with tumour necrosis factor (TNF) caused an increase of activin A production by isolated mouse Sertoli cells (SC) in vitro. Since TNF is elevated during the course of murine EAO (1), this indicates a role for activin production by SC in response to TNF in the outcome of testicular inflammation.

In line with our EAO model, where *Inhba* mRNA levels were correlated with collagen and fibronectin deposition, EAO damage score and severity of fibrosis (1), collagen and fibronectin, as well as activin A (*Inhba*) mRNA, were found to be increased in human testicular biopsies containing lymphocytic infiltrates and impaired spermatogenesis.

These data indicate that both resident fibroblasts and PTCs may contribute to testicular fibrosis under activin A control, subsequent to inflammation. Antagonists of activin action may be beneficial as therapeutics in limiting fertility impairment in patients with testicular inflammation.

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The effects of placental miRNAs predicted to target the placental renin-angiotensin system on trophoblast proliferation

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Placental development requires rapid trophoblast proliferation, which is activated by the renin angiotensin system (RAS). In the first trimester, when placental growth is maximal, the mRNA expression of placental prorenin (*REN*), angiotensinogen (*AGT*) and the type 1 angiotensin receptor (*AGTR1*) is highest. We have shown that expression of several miRNAs (miR-378, miR-181a-3p (targeting both *REN* and *ACE*), miR-663, miR-181a-5p (targeting *REN*), miR-892 (targeting *AGT*) and miR-483 (targeting *ACE, AGT* and *AGTR1*), are reduced in first trimester human placenta. We propose that they play a role in post-transcriptional regulation of the placental RAS. Shallow placentation may result from altered expression of these miRNAs, and therefore altered placental RAS expression, leading to pregnancy complications. In this study we examined the effects of these miRNAs on the expression of their predicted placental RAS targets, and on the ability of trophoblasts to proliferate.

The effects of miRNA mimics for the chosen miRNAs on RAS mRNA abundances were measured. In another series of experiments, HTR-8/SVneo cells were cultured in an xCELLigence real-time cell-analysis system for 24h to establish a baseline cell index (measured by electrical impendence). After 24h, varying concentrations of miRNA mimics (that mimic the activity of a

specific endogenous miRNAs) were added and their effect on trophoblast proliferation measured. The rate of cell proliferation was calculated as the rate of change in cell index (slope).

miRNA mimics for miR-378, miR-181a-3p, miR-663, miR-483 and miR-181a-5p significantly reduced the ability of trophoblast cells to proliferate. The miR-892 mimic had a biphasic effect on trophoblast proliferation, with low concentrations increasing proliferation and high concentrations decreasing proliferation. In cells treated with miR-181a-5p mimic, downregulation of *REN* mRNA was observed. Further experiments are ongoing.

Overall, this suggests that if these miRNAs are upregulated early in placentation they could repress RAS activity, decreasing trophoblast proliferation and reducing placental growth.

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Sperm contribute to the maternal neutrophil and T cell responses in early mouse pregnancy

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Seminal fluid interacts with epithelial cells lining the female reproductive tract to induce pro-inflammatory cytokines and initiate immune adaptations for pregnancy. The plasma fraction of seminal fluid plays a key role, however sperm may also contribute. To assess the impact of sperm, CBAF1 female mice were mated with intact or vasectomised Balb/c males.

Gene expression profiles of endometrial tissue were examined by microarray (n=4/group) and qPCR (n=16-20/group). Cytokine profiles from uterine luminal fluid were assayed by Luminex assays (n=12/group). Immune cells in uterus and uterine-draining lymph nodes were assessed using immunohistochemistry (n=6/group) and flow cytometry (n=10-14/group).

Microarray data showed that mating with intact males induced greater changes in gene expression than mating with vasectomised males, with 110 genes (78 up-regulated, 32 down-regulated) differentially regulated (>1.5-FC, FDR<0.01) in intact compared to vasectomised mating. Cytokines and miRNAs associated with neutrophil recruitment including G-CSF, IP-10, IL-6, MCP-1 and miR-223-3p were amongst those specifically regulated by sperm, and immunohistochemical analysis revealed greater increases in neutrophils in the endometrium of females mated to intact compared to vasectomised males ($p<0.05$). Amongst T cell populations in the lymph node, total CD4+ T cells (CD4+ cells), Treg cells (CD4+CD25+Foxp3+ cells) and thymic Treg cells (CD4+CD25+Foxp3+Nrp+ cells) were not altered, but fewer antigen-specific peripheral Treg cells (CD4+CD25+Foxp3+Nrp- cells) were induced in females mated with vasectomised males, compared with intact males ($p<0.05$).

This study provides evidence that sperm contribute to the immune-regulatory activity of seminal fluid, and particularly are involved in neutrophil recruitment. Although neutrophils play a fundamental role in innate immune responses, recent studies have highlighted a link between neutrophils and Treg cells, raising the possibility that sperm-mediated neutrophil recruitment may contribute to antigen-specific Treg cell expansion. Current studies aim to further understanding of the association between neutrophils and Treg cells in early pregnancy and identify sperm signalling factors.

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DNA repair mechanisms responsible for safeguarding oocyte quality and offspring health

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As oocytes are among the most long-lived cells in the body, it is essential that genomic integrity is maintained throughout reproductive life to ensure fertility and offspring health. Apoptotic elimination of DNA damaged oocytes is presumed essential to ensure that only high quality oocytes remain in the ovary and to prevent transmission of genetic defects to subsequent generations. However, primordial follicles from apoptosis-deficient mice (*TAp63*$^{-/-}$) survive irradiation-induced DNA damage, and females can produce live offspring. Furthermore, we identified both RAD51 and DNA-PKcs foci, key repair factors in the homologous repair and non-homologous end joining repair pathways respectively, in primordial follicle oocytes within 3 hours of irradiation. These observations suggest that despite their propensity for apoptosis, primordial oocytes have the capacity to undertake highly efficient DNA repair. Interestingly, super-ovulated irradiated *TAp63*$^{-/-}$ females produced significantly less mature oocytes compared to controls (Control 27.2±2.76 vs 0.45Gy 3.88±1.99, $p<0.0001$), while natural matings resulted in normal preimplantation embryo numbers (Control 6.4±1.97 vs 0.45Gy 5.67±1.33, $p=0.8$) and litter sizes (Control 4.833±0.75 vs 0.45Gy 5±0.63, $p=0.9$), without any reduction in number of litters over 6 months (Control 5±0.73 vs 0.45Gy 6±0.73, $p=0.4$). This indicates that DNA repair within damaged oocytes was sufficient to support embryonic development and sustain fertility. Interestingly, despite no difference in prepubertal weight, postpubertal male offspring from irradiated females were significantly lighter than controls, without any significant difference in body composition analysed by DEXA. In-depth histopathology health assessments of offspring from irradiated *TAp63*$^{-/-}$ mothers confirmed that all offspring were healthy, with no pathology to explain the difference in male weight. Whole-genome sequencing studies are underway in order to investigate offspring genetic integrity. This is the first study to examine DNA repair in primordial follicle oocytes in detail and represents important progress in our understanding of oocyte quality control and preservation of female fertility.

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miR-29c overexpression in endometrial cancer reduced proliferation and increased apoptosis and altered cell cycle and migration related genes

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Endometrial cancer is the most common gynaecological cancer and advanced disease has a very poor prognosis. There are also no biomarkers for endometrial cancer. Micro RNA (miRs) are known to be dysregulated in endometrial cancer. The expression of miR-29 family members (-a and –b) are downregulated in endometrial cancer. However, a role for miR-29c and its targets in endometrial cancer growth remain to be elucidated. The aim of this study was to investigate the functional effects of re-introducing miR-29c expression in endometrial cancer cell lines and identify which miR-29c targets may contribute to cancer progression.

Real-time PCR for miR-29c expression was performed on human endometrial tumour grades 1-3 and benign tissue as well as endometrial cancer cell lines Ishikawa, HEC1A and AN3CA. Cell lines were transfected with miR-29c mimic and negative control. xCELLigence real-time cell monitoring was performed to analyse proliferation and migration and Flow cytometry for apoptosis and cell cycle population percentages. miR-29c target genes in the 3 cell lines were analysed using real-time PCR.

miR-29c was down regulated in grade 1-3 endometrial cancer samples compared to benign endometrium ($p<0.0001$ respectively). miR-29c expression was reduced in Ishikawa ($p<0.0001$) and AN3CA ($p<0.01$) but not HEC1A cell lines compared to non-cancerous primary human endometrial epithelial cells. Overexpression of miR-29c reduced proliferation ($p<0.05$ HEC1A and $p<0.001$ AN3CA at 72hr), increased apoptosis ($p<0.001$ Ishikawa and $p<0.05$ AN3CA) and reduced miR-29c target genes (CAV1, CDC42, SIRT1, FBN1, HBP1, ITGB1, NUMB, MCL1, MCL1, SGK1, VEGFA and MDM2) across the three cell lines. Inhibition of miR-29c in HEC1A cells increased proliferation ($p<0.05$ at 72hr) and COL4A1 expression ($p<0.01$).

The re-introduction of miR-29c to endometrial cancer cell lines reduced proliferation, increased apoptosis and reduced miR-29c target genes in vitro. This suggests miR-29c may be a therapeutic target for endometrial cancer.

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Testosterone: The Biography of a Molecule

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Advances in our understanding of male infertility over the past 50 years –shores of need and reefs of greed

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Male infertility is a common condition affecting at least 1 in 20 of the male population. However, despite its prevalence, the aetiology of this condition remains poorly understood. Genetic factors play a minor role accounting for around 25% of cases of azoospermia and 10% of cases with idiopathic infertility. Aside from chromosomal disorders such as Klinefelter syndrome and rare single gene mutations, the most common genetic lesions responsible for male infertility are Y-chromosome deletions. Such minor causes aside, the causative factors underpinning a vast majority of male infertility remain unknown. In most infertile males sufficient numbers of spermatozoa are present to achieve fertilization, however the functional competence of these cells has, for some reason, become compromised. Such a dramatic loss of sperm function has been shown to involve a high incidence of oxidative stress affecting the ability of the spermatozoa to swim, interact with the zona pellucida, acrosome react and fuse with the vitelline membrane of the oocyte. In addition, oxidative stress compromises the integrity of DNA in the male germ line and thereby impacts the mutational load carried by children. This is a serious problem for the ART industry, particularly when ICSI is used as the preferred method of insemination. Oxidative DNA damage in spermatozoa targets areas of the genome involved in a variety of brain disorders (eg. autism, spontaneous schizophrenia and bipolar disease) the incidence which may become elevated in the offspring of patients treated for infertility associated with oxidative stress. The recent evidence indicating an increased risk of autism in ICSI children is a case in point. We must be careful that the uncontrolled use of such technology does not ultimately create a health burden for future generations to solve.

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Significant advances in male reproduction

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Male infertility was overlooked for decades as female factors were seen as paramount, male biology was relatively poorly understood and interventions limited. However, everything has changed in one lifetime; we now recognize male factor infertility as the sole, or part, cause for half of couples, and fatherhood is possible for many previously considered sterile. Australia has taken a leading role in these scientific, bioengineering, clinical practice and cultural advances.

In 1974, the Prince Henry's Reproductive Medicine Clinic provided the first integrated clinic involving endocrinologists, urologists, gynaecologists, nurses and counsellors. It had a specialised reproductive laboratory and a donor sperm service, and was run after-hours to suit working couples.

Knowledge of sperm ultrastructure and motility, sperm-egg interactions, reproductive hormone assay and regulation of hypothalamo-pituitary-testicular function and spermatogenesis, and life table analyses of male subfertility informed evaluation, the use of gonadotrophin therapy and clinical decision-making.

In the 1980s, ART allowed real time 'sperm function testing', revealing key determinants such as zona binding and acrosome reactivity. Conventional IVF was successful in milder male factor, even allowing the first pregnancy with surgically recovered epididymal sperm.

Success rates improved dramatically using ICSI, especially using testicular sperm retrieval in obstructive azoospermia (diminishing the role of surgery) and spermatogenic failure e.g. Klinefelter's syndrome (reducing need for donor sperm). Identification of genetic associations (aneuploidies, Y microdeletions, point mutations) along with embryo selection provided a degree of safety for offspring. Further genetic revelations are certain.

Overall data on the health of ART-conceived offspring is reassuring however; data specifically for ICSI/male infertility is extremely limited.

Culturally, male infertility is now widely discussed and consumers have access to excellent materials. Sadly, while all guidelines and RTAC evaluation criteria require evaluation of the male partner, this is not widely practiced nor are clinics audited. Even the national ART database (once NPSU now ANZARD) does not yet record male aetiologies or provide male factor specific outcomes.

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Targeted disruption of lipoxygenase enzymes to prevent oxidative stress-mediated pathologies in the male germline

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Infertility has become a critical health burden, with an estimated 80 million individuals experiencing the weight of this disease globally. At least 50% of infertile cases in Australia involve a male factor, making defective sperm function one of the largest, defined causes of human infertility. Unfortunately, due to a lack of mechanistic knowledge surrounding sperm function, there are few molecular tools available for the accurate diagnosis of male infertility and no successful preventative strategies. This is particularly concerning as there is now compelling evidence that poor sperm quality may be prescient of major systemic diseases such as cardiovascular disease, diabetes, and cancer. It is well established that germline oxidative stress is responsible for a large proportion of male infertility cases. Through comparative proteomic analyses targeted at understanding the induction of oxidative stress, we have recently reported on a novel enzyme responsible for its catalysis termed 15-arachidonate lipoxygenase (ALOX15). In somatic cells, the overproduction of damaging lipid peroxidation products such as 4-hydroxynonenal (4HNE) is primarily attributed to the action of lipoxygenase enzymes, which facilitate the oxygenation and degradation of polyunsaturated fatty acids. Our recent work has provided the first evidence for the mediation of oxidative stress by ALOX15 in the male germline and accordingly, cellular reactive oxygen species and lipid peroxidation events can be prevented through the selective inhibition of ALOX15 with 6,11-dihydro [1] benzothiopyrano [4,3-b] indole (PD146176). This, in turn, ameliorates changes to germ cell protein homeostasis that are commonly elicited by 4HNE and results in a recovery of human sperm-egg interaction under conditions of oxidative stress. Combined, our data establish ALOX15 as a key physiological target to prevent oxidative stress-mediated pathologies in both the developing sperm cells of the testis and in mature human spermatozoa.

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Highlights of placenta research past, present and future

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The placenta was known in Ancient times. We are likely familiar with the image of the Pharoah in ancient Egypt with his attendants carrying placentas with umbilical cords attached atop long stakes in a ceremonial procession. The placenta was thought to be the seat of the soul of the fetus and at least those of the wealthy were revered. Aristotle, in the third century BC, recognised the nutritive function of the placenta. The Japanese and Chinese also revered the placenta and umbilical cord and those of important people were often buried near shrines in their honour.

In the late 1960s and 1970s Robertson and Brosens established the placental bed biopsy and described the physiological changes in the spiral arterioles that occur in normal pregnancy and their failure to occur in preeclampsia. In the 1980s this was also shown for miscarriage, small for gestational age, preterm birth and stillbirth.

It also became clear that placental structural development was perturbed in women with pregnancy complications where utero-placental insufficiency is a feature. Then in the 1990s convincing evidence emerged that for most of the first trimester the placenta and embryo/fetus develop in a hypoxic environment as maternal blood flow into the placenta does not commence until about 10 weeks of gestation. It is thought that impaired remodelling of the spiral arterioles results in premature maternal blood flow into the intervillous space and oxidative damage that the early placenta is ill-equipped to counteract.

We now live in exciting times for placenta research. NIH has invested over $50million in the Human Placenta Project. Technological innovations in recent years are enabling unprecedented acquisition of data. Placental differentiation, growth and function are being explored using state of the art cellular, molecular and imaging modalities. Non-invasive screening of the placenta in real time is becoming a reality and will be increasingly used to identify and monitor pregnancies at risk.

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The Pathophysiology of Preeclampsia: 50 years of discovery but have we missed?

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3-5% of all pregnancies are affected by preeclampsia, which is associated with multiple adverse maternal and fetal outcomes, including preterm birth and fetal growth restriction. Preeclampsia isone of the leading causes of maternal mortality, particularly in developing countries, and is responsible for over 60,000 maternal deaths annually. Preeclampsia is a multi-system disorder affecting maternal blood vessels (causing high blood pressure and vascular injury), kidneys, liver, the haematological system, brain (causing seizures, or eclampsia) and the fetoplacental unit (resulting in growth restriction).A better understanding of the pathophysiology of the disease as well as early prediction of preeclampsia are essential to allow close monitoring of women at risk, and timely intervention to reduce the burden of preeclampsia.

It is now widely accepted that the pathophysiology of preeclampsia can be attributed to two main processes: inadequate placentation and subsequent maternal endothelial dysfunction. In women with preeclampsia, there is shallow implantation of the placenta resulting in inadequate invasion and remodelling of the maternal uterine spiral arteries. This compromises placental perfusion and leads to fluctuations in oxygen delivery that predispose to oxidative stress. What is seen to be the disease is in fact the maternal response to placental stress. Placental oxidative stress provokes the release of anti-angiogenic factors such as soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng). These factors enter the maternal blood stream causing widespread vascular dysfunction and multi-system maternal organ injury.

In this presentation I will give a brief overview of the key discoveries in the pathogenesis of preeclampsia across the last 50 years, before focusing on our most recent findings, identifying novel contributors of impaired placentation and novel soluble placental factors that promote endothelial dysfunction. After all of this research what questions remain and why have we not yet found a cure?

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Understanding placental regulation of gestational diabetes and programmed disease

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Placental adaptations to perturbations that occur during pregnancy strongly influence maternal and offspring health and disease. While it is clear that the placenta successfully adapts to *in utero* challenges in some individuals, in others, these adaptations are insufficient. A range of factors including fetal sex and maternal age impact how successful the placenta is at adapting to environmental influences. Largely attributed to poor placental function, Gestational diabetes mellitus (GDM) is rapidly becoming the most common complication of pregnancy. GDM is characterised by dysregulated placental hormone secretion which alters maternal glucose homeostasis. There are a large number of risk factors for developing GDM including maternal stress which can disrupt the balance of hormones secreted by the placenta that are involved with mediating insulin resistance in pregnancy. Stress during pregnancy is similarly known to increase maternal risk of a range of other pregnancy disorders and can cause fetal growth restriction, impaired development of key organs and cardiovascular and metabolic disorders in offspring. This highlights that key placental adaptations mediate both maternal disorders of pregnancy and programmed disease risk. This presentation will discuss the impact of maternal perturbations on placental processes that increase maternal risk of developing GDM and offspring risk of programming chronic disease.

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Therapeutic development for preeclampsia

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Preeclampsia is a serious complication of pregnancy. Responsible for over 70,000 maternal deaths worldwide and far greater rates of perinatal loss. More recently the field has gained better understanding of the key pathological steps in preeclampsia, including placental oxidative stress, major imbalances in angiogenic factors and systemic maternal endothelial dysfunction. *Despite this there has been minimal therapeutic advances over the past 50 years.* A therapeutic that is safe in pregnancy that can quench disease severity is urgently needed.

We have developed a preclinical screening approach utilising primary human cells/tissues and a mouse model of preeclampsia to develop therapeutic candidates for the prevention and treatment of preeclampsia, and importantly to translate these discoveries into clinical trials.

Through this approach we have identified several drugs (safe to be taken in pregnancy) that have the unique ability to reduce oxidative stress, induce cytoprotective antioxidant pathways, potently improve the angiogenic imbalance, reduce the aberrant pro-inflammatory cytokine secretion in the placenta. Furthermore, these agents are able to rescue endothelial dysfunction, enhance vasodilation and reduce hypertension in our mouse model of preeclampsia. We have already begun translating these findings into a Phase II prevention and treatment trials.

We have also embarked on the development of a placental targeted delivery approach. Using nanoparticles targeted to placenta to deliver gene silencing RNA directly to the placenta, we have demonstrated these targeted nanoparticles can silence the major toxic anti-angiogenic factor, soluble fms-like tyrosine kinase 1 (sFlt1), in the human placenta. Importantly these nanoparticles accumulate in the mouse placenta; their ability to rescue the preeclamptic phenotype in our mouse model of disease is currently being assessed.

These novel strategies have demonstrated exciting potential for the prevention and/or treatment of preeclampsia, they are focused toward clinical translation and offer hope for improved management of preeclampsia.

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Identifying new markers of placental insufficiency

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Stillbirth is a tragedy that ends 1:130 pregnancies in Australia. 50% of stillbirths are associated with fetal growth restriction (FGR) which arises from placental insufficiency. FGR is also a major determinant of perinatal morbidity, with low-birthweight infants experiencing poorer neurodevelopmental outcomes then infants of normal birthweight. Moreover, FGR is associated with adverse outcomes later in life; school-aged children who were growth restricted have higher rates of impaired cognition, memory, attention and gross motor proficiencies and its effects can persist lifelong - adults have a higher prevalence of major chronic diseases such as cardiovascular disease, stroke and diabetes. Surprisingly, current clinical practice only identifies 20-30% of pregnancies compromised by fetal growth restriction, thus new tools are desperately needed. Combining clinical and scientific expertise, our team have completed a prospective cohort collection of 2000 blood samples from women at both 28 and 36 weeks' gestation and have identified a panel of biomarkers present within the circulation of mothers preceding their delivery of a baby weighing <10th centile. Importantly, some biomarkers are deranged as early as 10-12 weeks before disease onset and correlate with fetal growth and neonatal body composition. We have subsequently used our biomarker findings to return back to the laboratory and use 'reverse translation' to provide a better understanding of how these deranged markers are likely to be contributing to the pathogenesis of placental insufficiency.

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Characterization of the granulosa-lutein cell transcriptome in women with Polycystic Ovary Syndrome

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Polycystic ovary syndrome (PCOS) is a common endocrinopathy that is associated with menstrual irregularities, anovulatory infertility and metabolic abnormalities. Although the aetiology and pathophysiology of PCOS remain unclear, granulosa cell dysfunction is a notable feature of the syndrome. In this study, we used a combination of RT-qPCR, RNA-Sequencing and pathway analysis to identify genes in granulosa cells that may be implicated in the pathogenesis of PCOS.

Granulosa-lutein cells were retrieved from women undergoing in-vitro fertilisation who had normal ovaries and regular cycles or polycystic ovaries and irregular cycles (anovPCOS) or polycystic ovaries with regular, ovulatory cycles (ovPCO). Our results show that the steroidogenic gene network is dysregulated in granulosa-lutein cells from women with either ovPCO or anovPCOS. Similarly, changes are also seen in androgen receptor expression and in the expression of some of its splice-variants. RNA-Sequencing identified 21,175 genes in granulosa-lutein cells with women with PCOS showing a distinct, global transcriptional profile of 450 differentially expressed genes. Subsequent pathway and network analyses highlighted a group of differentially expressed genes involved in cholesterol biosynthesis and metabolism that are highly enriched in women with PCOS. Finally, granulosa-lutein cells were cultured in the presence or absence of androgen, and the results suggest that changes in gene expression in women with PCOS may be, at least in part, a function of androgen action.

Granulosa cells play a major role in ovarian steroid synthesis including estrogen and progesterone. The significance of altered expression of genes involved in steroid and cholesterol metabolism remains to be fully elucidated, but these results lend further support to the notion of aberrant metabolic and endocrine function in granulosa cells of women with PCOS.

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New Insights into Mammalian Sexual Differentiation: From Frank Lillie's Freemartin to the Jost Paradigm

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Frank Lillie and Alfred Jost, the two pillars in the field of reproductive sciences, laid the foundations of modern knowledge on sexual differentiation. Lillie's description of Freemartin, a masculinized female calf born as a twin with a male calf, pioneered the hypothesis that shaped the concept of hormonal influence on sex determination: The masculinized reproductive tracts and gonads of the female twin is caused by diffusible factors or hormones that come from the male twin through the placenta. Alfred Jost further advanced the filed by establishing the paradigm of sexual differentiation, based on his discovery that testis-derived androgen in the male embryo is responsible for the maintenance of Wolffian duct, the precursor of male reproductive tract. In the female embryos, on the other hand, their reproductive systems arise by default as a result of a lack of androgens. In the past few years, my laboratory revisited Lillie's freemartin model and Jost' paradigm using modern mouse genetic approaches. In this presentation, I will discuss our efforts on identifying the freemartin factors and redefining the concept of the Jost paradigm.

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International Evidence Based Guideline for the Assessment and Management of PCOS

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Objective: To develop and translate rigorous, comprehensive evidence-based diagnosis, assessment and treatment guidelines, to improve the lives of women with polycystic ovary syndrome (PCOS) worldwide.

Participants: Extensive health professional and patient engagement informed guideline priority areas. International Society-nominated panels included consumers, paediatrics, endocrinology, gynaecology, primary care, reproductive endocrinology, psychiatry, psychology, dietetics, exercise physiology, public health, project management, evidence synthesis and translation experts.

Evidence: Best practice evidence-based guideline development involved extensive evidence synthesis and the Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) framework covered evidence quality, feasibility, acceptability, cost, implementation and ultimately recommendation strength.

Process: Governance included an international advisory board from six continents, a project board, five guideline development groups with 63 members, consumer and translation committees. The Australian Centre for Research Excellence in PCOS, funded by the National Health and Medical Research Council (NHMRC), partnered with European Society of Human Reproduction and Embryology and the American Society for Reproductive Medicine. Thirty seven organisations across 71 countries collaborated with 23 face to face international meetings over 15 months. Sixty prioritised clinical questions involved 40 systematic and 20 narrative reviews, generating 170 recommendations. Convened Committees from partner and collaborating organisations provided peer review and the guideline was approved by the NHMRC.

Conclusions: We endorse the Rotterdam PCOS diagnostic criteria in adults (two of clinical or biochemical hyperandrogenism, ovulatory dysfunction, or polycystic ovaries on ultrasound) excluding other causes. Where irregular menstrual cycles and hyperandrogenism are present, we highlight that ultrasound is not necessary in diagnosis. Within eight years of menarche, both hyperandrogenism and ovulatory dysfunction are required, with ultrasound not recommended. Ultrasound criteria are tightened with advancing technology. Anti-Müllerian hormone levels are not yet adequate for diagnosis. Once diagnosed, assessment and management includes reproductive, metabolic and psychological features. Education, self-empowerment, multidisciplinary care and lifestyle intervention for prevention or management of excess weight are important. Depressive and anxiety symptoms should be screened, assessed and managed with the need for awareness of other impacts on emotional wellbeing. Combined oral contraceptive pills are first-line pharmacological management for menstrual irregularity and hyperandrogenism, with no specific recommended preparations and general preference for lower dose preparations. Metformin is recommended in addition or alone, primarily for metabolic features. Letrozole is first-line pharmacological infertility therapy; with clomiphene and metformin having a role alone and in combination. Gonadotrophins and laparoscopic surgery are second line and in-vitro fertilisation third line in isolated PCOS. Overall evidence is low to moderate quality, requiring significant research expansion in this neglected, yet common condition. Guideline translation will be extensive including a multilingual patient mobile application and health professional training.

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Maternal-fetal dialogue during early pregnancy

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Miscarriage, preeclampsia and fetal growth restriction are pregnancy complications arising from poor implantation and placentation affecting up to 9% of all pregnancies. Remarkably there are currently no predictive biomarkers and limited pharmacological treatment options. Despite this, the critical processes which facilitate implantation, placentation and maternal tolerance towards the fetus in humans remain poorly understood and has contributed to the lack of treatment options. My research goal is to understand how maternal decidual and immune, and fetal cells interact to facilitate appropriate placentation and maternal tolerance to establish a healthy pregnancy.

The interplay between these cells in the decidua has not been extensively studied largely due to the lack of appropriate models. My team has developed unique in vitro and in vivo models to identify the key factors in maternal-fetal dialogue during early placentation. As trophoblast invading into the decidua represent the first contact between fetal cells and the maternal immune system, the decidua is a critical site for the maternal immune system to develop tolerance towards fetal antigens. My research has discovered a role for the decidua in the establishment of maternal tolerance and that invasive trophoblast actively secrete

factors which repress the production of pro-inflammatory cytokines by decidual and immune cells. Some key regulators identified using our unique models are also identified in maternal sera prior to the establishment of certain pregnancy disorders. My research paves the way for the development of much needed diagnostics and treatments for pregnancy disorders associated with abnormal implantation and placental development.

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How the maternal immune cells drive vascular adaptations to pregnancy

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Preeclampsia and fetal growth restriction remain important causes of maternal and perinatal morbidity and mortality. In these conditions, a deficiency in regulatory T (Treg) cells, a specialized subset of lymphocytes, has been observed. Treg cells prevent maternal immune rejection of the fetus, but also contribute to vascular homeostasis in non-pregnant rats. We hypothesized that a reduced Treg cell population would cause inflammation and uterine artery dysfunction during pregnancy. Using the Foxp3-DTR Treg cell depletion mouse model, we investigated how Treg cell depletion in early pregnancy affected maternal vascular function in pregnancy, as well as fetal growth. Treg cell depletion affected uterine artery function on day 10.5 post coitum, as measured by wire myography and ultrasound biomicroscopy. Pro-inflammatory cytokines were increased, as well as the rate of fetal resorption. Furthermore, there was greater nitric oxide regulation of maternal mean arterial pressure. In late gestation, fetuses were growth restricted, and uterine and umbilical artery function is perturbed, as measured by ultrasound biomicroscopy. We demonstrate an essential role for Treg cells in maternal vascular function in pregnancy. Given the severe implications of preeclampsia on the future health of the mother and her baby, investigation of therapeutic strategies targeting Treg cells offers a promising intervention.

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Towards the development of novel oncofertility strategies for female cancer patients

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Whilst chemotherapy can be life-saving for cancer patients it commonly results in infertility. *In vitro* fertilisation (IVF) offers patients the best chance of a successful pregnancy, however, IVF is not suitable for many patients such as girls. Although alternative treatments such as ovarian tissue cryopreservation exist, they have poor outcomes. Therefore, with improving cancer survival rates there is an urgent need for new medical options to protect the fertility of females before cancer treatment. I have established a 3-tiered research program whereby we are: 1)enhancing the efficiency of oocyte *in vitro* maturation (IVM); 2)improving transplanted ovarian tissue quality of and; 3)developing methods to protect the ovary *in situ* –the “holy grail” of oncofertility. Through our work, evidence has emerged that nicotinamide adenine dinucleotide (NAD^+) plays a critical role in maintaining female fertility and protecting the ovary against the damaging effects of chemotherapy. By manipulating NAD^+ metabolism during IVM, we are unravelling the contribution of NAD^+ to oocyte developmental competence. We have demonstrated that the NAD^+ precursor nicotinamide mononucleotide (NMN) as an adjuvant to IVM, increases oocyte quality following IVM and IVF. Using mouse-to-mouse ovarian tissue transplants we revealed positive effects of systemic NMN administration on follicle development within grafted tissue. Finally, to protect the ovary during chemotherapy, our results demonstrate a clear protective effect of the NAD^+ precursor NMN when administered systemically. However, we are cognisant that while NMN protects the ovary from chemotherapy it may also have the potentially undesirable effect of also protecting the cancer from chemotherapy. To circumvent this issue we are developing and assessing the efficacy of hydrogels to deliver NMN locally to the ovary. These findings illuminate the key role of NAD^+ actions in fertility and chemoprotection in the ovary, as well as providing novel avenues of basic research for translation into the clinic.

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The association between Metabolic Syndrome and time to pregnancy in a cohort of nulliparous women.

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Background and objective: Several lifestyle factors, such as smoking and obesity, have consistently been associated with infertility. The impact of metabolic syndrome (MetS), which is typically a consequence of adverse lifestyle factors, has not been assessed in relation to fertility. The study aimed to determine the association between MetS and infertility, and to examine associations between individual and number of MetS components and infertility.

Methods: Low-risk, nulliparous women recruited to the multi-centre, international, prospective SCOPE (Screening for Pregnancy Endpoints) cohort were assessed for a range of demographic, lifestyle and metabolic health variables at 15 ± 1 weeks' gestation.MetS was defined according to the International Diabetes Federation for adults. Infertility was defined as taking more than 12 months to conceive. Generalized linear models were conducted to estimate relative risks (RR) and 95% CIs associated with infertility, adjusted for a range of maternal and paternal factors.

Results: Of the 5500 women included, 12.3% (n=679) had MetS. Compared to women without MetS, women with MetS had a higher risk of infertility (adjusted RR 1.53; 1.25-1.88). Risk of infertility increased with reduced levels of high-density lipoprotein cholesterol (1.48; 1.12-1.95) and with raised levels of triglycerides (1.26; 1.07-1.49), but not with raised blood pressure, raised glucose or high waist circumference. Having more than one MetS component increased risk of infertility by up to 73% (0 metabolic components [reference] vs. 1 [0.95; 0.75-1.19], 2 [1.05; 0.82-1.34], 3 [1.48; 1.11-1.97], ≥4 [1.73; 1.13-2.64]).

Conclusions: MetS appears to be an independent risk factor for infertility. Future studies are needed to replicate our findings and to determine whether individual or a combination of components of MetS pose greater risk for reduced fertility. Assessment of metabolic profile and advice on improving metabolic health may be beneficial for women who experience difficulty in conceiving.

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Placental and fetal changes due to periconceptional alcohol exposure and the potential for mid-gestational choline supplementation to ameliorate these effects

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Alcohol consumption amongst women of reproductive age is widespread. Given that ~50% of pregnancies are unplanned, there is potential to expose the early embryo to alcohol before pregnancy detection. In rats, we have demonstrated that alcohol exposure during the periconceptional period (PCE) leads to fetal growth restriction and sex-specific changes in the placenta. Maternal choline supplementation can ameliorate specific neurological and behavioural abnormalities caused by alcohol exposure during pregnancy. We aimed to test whether mid-gestational choline supplementation could ameliorate the adverse effects of PCE on the late-gestation placenta and fetus.

Sprague-Dawley dams were given 12.5% ethanol (PCE) or 0% ethanol (control) liquid diet from 4 days prior to 4 days after conception. At embryonic day 10 (E10), dams were given choline enriched (2.5X-4X) or standard chow (8-10 litters/group). Late in gestation (E20), fetuses and placentas were collected and measured/weighed. All fetuses/placentas were sexed; only one of each sex/litter was used per analysis. Placentas were separated into labyrinth and junctional zones for molecular analysis or left whole and prepared for histology.

Placental efficiency was reduced by PCE but improved by choline supplementation in a sex-specific manner. Fetal growth restriction due to PCE was restored in both sexes by supplemental choline. Placental depth was increased by PCE but other placental dimensions were altered by choline, independent of PCE. Placental glycogen deposition was increased in the junctional zone in response to PCE, specifically in females, but there was no effect of choline. The glycogen cell marker, protocadherin 12 (*Pcdh12*), and glycogen synthase kinase 3 beta (*Gsk3B*), showed altered expression due to PCE and choline in a sex-specific manner.

Therefore, both PCE and choline supplementation resulted in sex-specific changes in the late-gestation placenta. Importantly, our results suggest that choline has the potential to improve placental efficiency and restore fetal growth following prenatal alcohol exposure.

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Relationship between endometrial stem/progenitor cells in the endometrial scratch biopsy and live birth outcomes following IVF – a pilot study

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Endometrial injury (scratch) via a biopsy in the cycle prior to embryo transfer (ET) increases live birth rates. However the mechanism is unknown and the "scratch" is controversial. Human endometrium contains small populations of N-cadherin$^+$ epithelial progenitor cells (eEPCs) located in the basalis and perivascular SUSD2$^+$ mesenchymal stem cells (eMSCs) in both basalis and functionalis. We hypothesised that endometrial injury activates quiescent endometrial stem/progenitor cells to proliferate and regenerate the damaged endometrium, producing a thicker more responsive endometrium 'ripe' for embryo implantation in the following cycle. Scratch biopsies were collected from 31 women aged 27-41 years who had failed ≥1 IVF-ET cycle and were scheduled to receive an embryo in the subsequent cycle. Endometrial tissue was weighed, dissociated to single cells using collagenase and DNAse and cells counted. Aliquots of 10^5 cells were assessed for N-cadherin$^+$ and SUSD2$^+$ cells

by single colour flow cytometry. Cell concentrations were calculated as number/mg tissue. Endometrial thickness was measured by ultrasound. Live birth/pregnancy outcomes were obtained from Monash IVF. 24 women underwent ET in the cycle following the scratch. Based on the distribution of tissue concentrations of N-cadherin^{+} and SUSD2^{+} cells, threshold values were >4500cells/mg for SUSD2^{hi} , >3000 for N-cadherin^{hi} and samples were categorised into 4 groups. Six of 9 samples with SUSD2^{hi}N-cadherin^{lo} concentrations resulted in live birth/pregnancy, 3/11 SUSD2^{lo}N-cadherin^{lo} samples resulted in live births and there were no pregnancies/live births for SUSD2^{lo}N-Cadherin^{hi} (n=3) or SUSD2^{hi}N-Cadherin^{hi} (n=1) samples. Age was similar between the groups, but endometrial thickness decreased from 11.8±1.5 to 7 mm across the 4 groups. This pilot data suggests that pregnancies/live births depend on the relative concentrations of eMSCs and eEPCs, with N-cadherin^{lo} indicating a thicker endometrium and N-cadherin^{hi} suggestive of thin basalis endometrium unable to support pregnancy. A fully powered study is required to confirm these findings.

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Faking its own death, why the syncytiotrophoblast appears to die in placental explant cultures.

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Background It is published that after 4-6 hours in explant culture, the human syncytiotrophoblast is unable to exclude viability stains like propidium iodide (PI). This is interpreted as the death of the syncytiotrophoblast, which is subsequently shed and replaced by a new syncytium. We confirm that the syncytiotrophoblast does not exclude non-viability stains after 24 hours but provide evidence that this is not due to the death of the syncytiotrophoblast.

Methods Replicate first trimester placental explants, obtained with written informed consent, were incubated for up to 24 hours. The explants were then; 1)stained with PI, or 2)mRNA was extracted and connexins and pannexins quantified by qRT-PCR, or 3)stained by immunohistochemistry with antibodies reactive with pannexin 1 or connexin 43.

Results At 0 or 4 hours, explants excluded PI but at 24 hours the syncytiotrophoblast nuclei stained strongly for PI. Quantitative RT-PCR indicated multiple connexins as well as, pannexins-2 and -3 were expressed at low levels in the explants. The levels of connexin-43 (p=0.004) and pannexin-1 (p=0.016) mRNA increased significantly between 0 and 24 hours of culture. Immunohistochemistry confirmed the increased expression of connexin-43 and pannexin-1 at 24 hours, and showed increased expression of pannexin-1 at four hours. Incubation with a pannexin-1 channel blocker, resulted in the exclusion of PI from the syncytiotrophoblast.

Discussion We show the uptake of PI by the syncytiotrophoblast of placental explants is mediated by pannexin-1 hemi-channels. Since the syncytiotrophoblast is a multinucleated cell, covering the entire placenta, we believe that the uptake of PI is not a marker of cell death but rather, a reflection of leakiness. In normal pregnancy there are extensive areas of damage to the syncytiotrophoblast which also presumably induce leakiness. The extent to which this leakiness disrupts the function of the syncytiotrophoblast is not yet clear.

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DNA repair and protection in the female germline

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The unique biology of the oocyte means that accepted paradigms for DNA repair and protection are not of direct relevance to the female gamete. Instead, preservation of the integrity of the maternal genome depends on endogenous protein stores and/or mRNA transcripts accumulated during oogenesis. To improve our understanding of these systems, the aim of this research project was to determine whether mature oocytes are able to utilize these resources to detect, respond and subsequently mount protective and/or reparative strategies to mitigate the impact of genotoxic insult. For this purpose, DNA double strand breaks (DSB) were elicited using etoposide (ETP); which led to a rapid increase in DSB (P = 0.0002). Utilizing this model, we documented 2 distinct responses, namely: (i) the MII oocyte engaged a reparative cascade known as the non-homologous end joining (NHEJ) DNA repair pathway and (ii) oocytes experience a developmental change in their vulnerability to ETP, with fertilisation leading to a rapid increase in the expression of permeability glycoprotein (PGP) which acts as a drug transporter to minimize the intracellular accumulation of ETP. In support of these responses, we were able to document a significant reduction in DSB lesions 4h post-ETP treatment. Notably, this repair was completely abrogated by pharmacological inhibition of key elements of the canonical NHEJ pathway (i.e. DNA-PKcs and DNA ligase IV), thus providing the first evidence implicating this reparative cascade in the protection of the maternal genome. Similarly, we were able to utilize dye exclusion assays, in the presence of a selective PGP pharmacological inhibitor (PSC833) to confirm this multidrug efflux transporter does provide a first line of defence to protect the zygote against genotoxic agents capable of inducing DSB DNA damage. Our collective data therefore encourages a reappraisal of the paradigm the oocyte/embryo is largely refractory to DSB DNA repair and protection.

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Chemotherapy-induced infertility is caused by direct damage to oocytes

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DNA-damaging cancer treatments cause female infertility and ovarian endocrine failure, due to depletion of the ovarian reserve. Attempts to address this have been hampered by limited understanding of the cellular mechanisms underlying follicle death after chemotherapy. We have previously reported that the pro-apoptotic BH3-only protein, PUMA, is the critical trigger of apoptosis in primordial follicles (PMFs) following cisplatin (Cis) or cyclophosphamide (Cy) treatment in mice. However, the specific targets of these drugs in the ovary, and the timing of follicular demise remain unclear. In this study we investigated the underlying processes leading to follicle death following these treatments. Adult wild-type mice were injected with saline (control), Cis 5 mg/kg, or Cy 300 mg/kg (N=5/group); ovaries were harvested after 8 hours, 24 hours, or 5 days. γH2AX immunofluorescence showed DNA double-stranded breaks (DSBs) in PMFs by 8 hours (saline-8h: 0% positive vs Cy-8h: 54±8% positive, $p<0.01$ vs Cis-8h: 38±8% positive, $p<0.01$). The proportion of γH2AX-positive follicles had reduced by 24 hours (saline-24h: 0% vs Cy-24h: 8%, NS vs Cis-24h: 16%, NS), and disappeared by 5 days (saline-5d: 0% vs Cy-5d: 8%, NS vs Cis-5d: 0%), indicating that PMF oocytes undergo either apoptosis or repair in this timeframe. Similar patterns of γH2AX positivity and disappearance were observed across all follicle stages. We then focused on the γH2AX-positive follicles to identify the cells targeted. In primordial, transitional, and primary follicles, only oocytes sustained DSBs, whereas in secondary and antral follicles, only somatic (granulosa ± theca) cells were affected. TUNEL staining further supported this conclusion. Given that 80% of oocytes are stored in PMFs in young adult mice, these data demonstrate that direct killing of PMF oocytes is the primary mechanism of ovarian reserve depletion caused by cisplatin and cyclophosphamide. Thus, future strategies to prevent chemotherapy-induced infertility must focus on preventing PMF oocyte death.

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Paternal miR-146a regulation of male reproductive parameters and its influence on the female immune response at conception in the mouse

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Seminal fluid interacts with female uterine epithelial cells to induce pro-inflammatory cytokine and chemokine expression, which in turn promotes immune tolerance and tissue remodelling for optimal pregnancy progression. Seminal fluid signalling factors, including transforming growth factor-beta (TGFß), have been identified but additional signalling molecules remain to be discovered. Our recent studies have suggested that microRNAs (miRNAs) may contribute to seminal fluid signalling capacity. One miRNA, miR-146a, regulates factors involved in spermatogonial differentiation and TGFß signalling pathway. This study aimed to investigate the physiological role of paternal miR-146a in seminal fluid signalling capacity using miR-146a-deficient mouse strain. Female endometrial responsiveness to seminal fluid was examined by qPCR in unmated estrus BALB/c females, or in BALB/c females 8 hours following mating with miR-146a$^{+/+}$ or miR-146a$^{-/-}$ males (n=7-11/group). Pregnancy outcomes were measured on day 17.5 post-coitum (pc) (n=28-30 pregnancies/group). Male reproductive parameters were determined after euthanasia at 6-7 months old (n=18-19). Contact with seminal fluid from miR-146a$^{-/-}$ males resulted in a significant decrease in endometrial expression of *Cxcl2* and *Il6* (49.8% decrease for both genes), compared to miR-146a$^{+/+}$-mated females. Day 17.5pc analysis showed that surprisingly, miR-146a$^{-/-}$-male-sired litters contained an increased number of viable pups (19.3% increase compared to miR-146a$^{+/+}$-male-sired litters). Fetal weight was not affected by paternal genotype, but placental weight was reduced (8.0% decrease compared to miR-146a$^{+/+}$-male-sired litters). Analysis of male reproductive tract parameters revealed that miR-146a$^{-/-}$ males had larger seminal vesicles (11.2% increase), but produced less motile (12.8% reduction) and less sperm with normal morphology (10.2% reduction). These findings suggest that paternal miR-146a influences the nature of seminal fluid signals in such a way as to impact the strength and quality of the female response, and this is linked to higher implantation rates. Further experiments are required to elucidate the specific mechanisms by which paternal miR-146a influences seminal fluid signalling capacity.

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Reversing decreased gamete quality and altered embryogenesis caused by advanced paternal age

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Men are seeking fatherhood later in life, yet aging is associated with decreased sperm quality. Men >40 years old undergoing autologous IVF cycles have lower pregnancy and live birth rates than young men. The increased use of ART to treat age-related infertility necessitates a better understanding of how male age impacts sperm quality and IVF outcomes, and new therapeutic strategies to improve success rates. Chaperone-inducing drugs improve oocyte quality and embryo development in obese mice, but the potential to improve sperm quality has not yet been investigated. The aim of this study was to determine whether treatment of sperm from aged male mice with a chaperone-inducing drug prior to IVF can improve gamete quality and embryo development.

Sperm from C57BL6J male mice that were either “old” (>1-year-old), or “young” (<8 months old) was collected and treated in vitro during capacitation. Sperm quality assessments included motility, zona-binding capacity and mitochondrial activity by flow cytometry. In parallel, sperm was used for IVF and embryo development was analyzed by timelapse imaging.

Old males had ~15% reduction in pregnancy rates compared to young males. Sperm from old males had reduced motility (N=9-12; P=0.03), lower mitochondrial membrane potential (N=6-11; P=0.04) and impaired zona-binding capacity (N=4-6; P=0.02) compared to young males. Each of these sperm quality parameters was improved by in vitro drug treatment. When sperm was used for IVF, morphokinetic analysis showed that embryos from old males had delayed time to first cleavage (N=21-27; P=0.01). Sperm from older males gave decreased 2-cell (N=7-12; P=0.04) and blastocyst rates (N=7-12; P=0.003). Drug treatment of 'old' sperm restored embryo development rates to those of sperm from young males. These results demonstrate that male age negatively impacts the earliest stages of embryo development but that treating sperm in vitro prior to IVF normalises sperm quality and improves embryogenesis.

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Animal sex determination by genes, chromosomes and the environment

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Humans and other mammals have an XX female: XY male system of sex determination, in which a gene *SRY* on the Y chromosome kick-starts testis-differentiation in the embryo. The testis makes male hormones, which induce male development of the fetus. Birds have a different system of ZZ male: ZW female, in which dosage of a gene *DMRT1* on the Z controls sex; the ZW pair is not homologous to the mammal XY. Other reptiles, frogs and fish have different sex chromosome systems and we now know of many distinct sex determining genes which act at different points of the conserved sex determining pathway. This astonishing variety of sex determining genes and chromosomes is the result of the rapid birth and death of sex chromosomes.

Many reptiles and some fish have no sex chromosomes. Sex is determined by environmental factors such as temperature (TSD), through epigenetic changes whose nature has been a longstanding mystery. We work with an Australian dragon lizard, which has a ZW system driven by yet another sex determining gene. However, when it's hot, all the eggs hatch as females. We have used this system to investigate how TSD works. We found that the transcriptome of ZZ females contains upregulated stress markers and unique transcripts of two epigenetic markers. This suggests that temperature acts, via the stress pathway, to activate epgenetic modifications involved in male determination. Have we discovered the mechanism of TSD at last?

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Heady Tales of Ewe

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In 1969, James R Goding et al published a paper describing what was arguably the first radio-immunoassay for luteinising hormone (LH) – a considerable advance on the ovarian ascorbic acid depletion test! This paper also reported that a single injection of estradiol caused a surge in LH secretion, synonymous with the preovulatory surge. This and other papers heralded a new era in reproductive biology, enabling easy measurement of hormones. Following shortly afterwards (in 1971), the structure of gonadotropin releasing hormone (GnRH) was published simultaneously by Guilleman and Schally. It was hypothesised that brain secretion of GnRH into the hypophysial portal blood system was responsible for the synthesis of the gonadotropins and secretion of the same, but it was some years before this was conclusively demonstrated. George Fink and colleagues were the first to measure GnRH surges in anesthetised rats, in 1977. As a young hopeful Australian Wool Corporation Fellow, I was fortunate to make the acquaintance of James T Cummins, a neurosurgeon. Jim and I devised a means of surgically accessing the portal blood system in the sheep and we were able to collect blood flowing out of the median eminence from sentient animals in real time. To our great satisfaction, we were able to measure GnRH in this portal blood and we then proceeded to describe the pattern of pulsatile GnRH secretion in conscious animals, including the estrogen-induced surge. The ensuing years allowed me to capitalise on this and other surgical procedures, to detail steroid feedback effects and the influence of season, stress and metabolic state on the secretion of GnRH and the gonadotropins. This paper will review highlights and landmarks of 40 years research on reproductive neuroendocrinology in the ewe.

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Fifty years of research. What a journey for the study of the ovary.

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SRB was founded by members who traditionally attended the scientific meetings of the ESA. Those early days saw the development of hormone RIAs for the gonadotrophins and ovarian steroid hormones and much of the text book knowledge of the reproductive cycle was discovered by SRB members. The local development of solid-phase assays advanced our technological capacity. Humans, sheep, marsupials and rats were generally the species of choice then. SRB offered more than endocrine research relating to the ovary. Developmental biology became important. In the follicle the two-cell hypothesis of oestrogen production and the differential gonadotrophin control was discovered. This was followed up with studies of the different luteal cells and later of immune cells in the ovary. IVF researchers were at the forefront of developments and

continued to attract a lot of press and ethical debates. They still do! With the advent of molecular biology the cloning of genes encoding inhibins and relaxins was largely conducted in Australia as were a number of other genes important in endocrinology. In situ hybridisation was pioneered in Australia. The study of steroid hormones morphed into the study of steroid hormone receptors and steroidogenic enzymes once they were cloned in the 1980's. Developmental biology continued. Phenomenal methods for manipulating the genetics of mice allowed lineage tracing of cells, tissue specific gene knockouts and over-expression and depletion of specific cells. The origins of granulosa cells went through different models resulting in a new concepts of how the ovary develops. Studies identified how the indifferent gonad makes decisions on becoming a testis or ovary. Some hypotheses in early 2000s were controversial, even until today, such as the formation of new follicles in adult ovaries and the existence of germline stem cells. PCOS was found to have metabolic consequences and even today new guidelines are being developed for the management of this condition, whose aetiology still remains a mystery. Some of the causes of premature ovarian failure have been identified but not all of them. The use of AMH for predicting ovarian reserve has focused the public's attention to reproductive health and scientists' attention on primordial follicles. Ovarian cancer is also increasingly studied with new hypotheses on their cellular origins. With current funding imperatives much future research is likely to be directed to translational health outcomes.

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The 3 Ps of Reproduction: Pheromones, Photons and Phood

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My first meeting of our Society was in 1978. As a PhD student, I had been wondering how the reproductive system responds to changes in the environment. Over the subsequent 40 years, I studied a variety of 'models', including ratites, canids, marsupials and ruminants. Here, I focus on the humble sheep – it serves as a model for human reproduction but has the added advantage of being important in industry. The sheep has helped us learn how the brain receives inputs about night length (photons), the odours of its flock mates (pheromones), the availability of food, and body status (eg, energy balance). The brain integrates all that information and enacts a strategy, fine-tuned over evolutionary timescales, to maximise reproductive success. My personal 4-decade scientific journey has been punctuated with unexpected discoveries that offered amazing new perspectives on reproductive biology. We used to think that brain cells cannot divide, but now we know that they do so in response to photons and pheromones. We used to accept a simple control system in which the brain produces a hormone that stimulates the gonads, but now we see brain-gonad communication as an intricate two-way exchange. We have discovered a plethora of control processes within the gonad – most astonishing, for me, being the 'non-coding RNAs' produced by DNA that interfere with the control of cells by genes. The implications? First, we are optimistic about the possibility of repairing brain tissue. Second, gone is the simple view that a gene produces RNA that produces a protein to control the cell, with non-coding RNAs offering a new suite of therapeutic possibilities. Third, photons, pheromones and food have presented 'clean, green and ethical' managementt of livestock – a complete about-turn in the thinking that dominated research on livestock reproduction when our Society first met in 1968.

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Oncofertility: a catalyst for significant advances in female reproduction

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In recent years, early diagnosis and improved treatments have led to a sharp rise in cancer survivorship. Unfortunately, a side-effect of many treatments is damage to the ovary and depletion of the ovarian follicular reserve, causing infertility and ovarian endocrine failure. This clinical need has driven the development of a new field of interdisciplinary science called oncofertility, which bridges reproduction, endocrinology and oncology. The goal of the basic scientists, medical researchers, oncologists and fertility specialists actively engaged in this area is to develop and implement effective treatments to enable cancer survivors to have children later in life, should they choose to. The fertility preservation options currently available include embryo, oocyte and ovarian tissue cryopreservation. These options harness knowledge and techniques that have been developed and optimized in the last 50 years. In addition, exciting new developments in the laboratory hold great promise for the future, including the bioprosthetic ovary, and the generation of fully mature oocytes from induced pluripotent stem cells, entirely *in vitro*. Such developments are made possible through discovery research aimed at understanding the biological and physical processes that regulate the growth and development of oocytes and follicles. Similarly, our recent research focusses on understanding the precise mechanisms by which oocytes and follicles die, both during normal life and following exposure to anticancer drugs. The intent is that the knowledge gained from these studies will facilitate the development of effective, non-invasive, targeted pharmacological treatments that protect the ovarian reserve from damage and depletion during cancer treatment. This is a desirable strategy because it would simultaneously ensure ovarian hormone production and fertility, offering a long-term solution. Collectively, these examples illustrate that oncofertility is both a beneficiary of, and catalyst for, significant advances in female reproduction.

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Altered Treg cell and antigen presenting cell populations are associated with LPS induced fetal loss in pregnant miR-223 deficient mice

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Maternal immune tolerance of the semi-allogeneic fetus requires CD4+Foxp3+ T-regulatory (Treg) cells, which suppress inflammation and anti-fetal immunity. In mice, expansion of the Treg cell pool is initiated by seminal fluid contact at coitus. Recent studies have demonstrated that microRNAs (miRNA) including miR-223 play a role in the regulation of immune responses. This study aimed to investigate the contribution of miR-223 to maternal immune environment, by evaluating Treg cell and antigen presenting cell activation and proliferation using flow cytometry in miR-223-/- or miR-223+/+ C57Bl/6 females at estrus or d3.5pc mated to Balb/c males (n=10-15/group). miR-223 deficiency resulted in a significant alteration in the Treg cell profile in early pregnancy in the PALN following mating where reductions were observed in the proportion of Treg cells amongst the CD4+ T cell pool (22%, $p<0.05$) and Treg number (28%, $p< 0.05$) compared to mated miR-223+/+ females. In the absence of miR-223, a reduction in macrophages as a proportion of total cells (58%, $p<0.05$) and in the number of activated macrophages (15%, $p<0.05$) was observed in the PALN on d3.5pc compared to mated miR-223+/+ mice. Additional miR-223-/- and miR-223+/+ mice (n=20=21/group) were administered low dose LPS on d9.5pc, to evaluate pregnancy outcomes. The absence of miR-223 led to altered outcomes in pregnancy following LPS inflammatory challenge, with an 10% reduction in fetal weight and a 19% reduction in the fetal:placental weight ratio in late gestation. LPS administration also significantly increased the resorption rate (8.78-fold, $p<0.05$) in miR-223-/- females compared to miR-223+/+ females.

Collectively, these data show that the absence of miR-223 alters the maternal immune profile in early pregnancy and this may increase susceptibility to inflammation-induced fetal loss later in gestation. These findings may be relevant to understanding how Treg-associated pregnancy pathologies such as preeclampsia arise in women where reduced miR-223 has been noted.

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miR29c overexpression in the infertile endometrium impairs endometrial epithelial adhesive capacity by downregulating COL4A1

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Successful implantation relies upon the firm adhesion of a good quality blastocyst to a receptive endometrium. Cross-talk between the blastocyst and the endometrium during the peri-implantation period is poorly understood. Microarray studies have identified microRNAs (miRs) to be differentially altered across the human menstrual cycle and dysregulated with infertility. The aim of this study was to identify miRs dysregulated in the pre-receptive phase infertile endometrium and define the effects on endometrial epithelial adhesion.

miR expression levels in endometrial tissue was determined by microarray and confirmed by qRT-PCR. In situ hybridisation was used to localise miRs in endometrium. Primary human endometrial epithelial cells (HEEC) were transfected with miR mimic (synthetic miR) and control and used in a functional trophoblast cell line (HTR8/SVneo) spheroid-HEEC co-culture adhesion assay to model blastocyst adhesion to the endometrium. miR predicted and confirmed targets were identified using bioinformatics and their expression levels determined in HEEC transfected with miR mimic via qRT-PCR. Protein production was assessed by immunohistochemistry.

miR-29c expression was elevated in pre-receptive phase infertile endometrium, compared to fertile endometrium ($p<0.05$). miR-29c localised to endometrial luminal and glandular epithelium. Transfection of HEEC with miR-29c mimic reduced HTR8/SVneo spheroid adhesion compared to HEEC compared to control ($p<0.05$). Collagen type IV alpha 1 (COL4A1) is an abundant basement membrane component and a predicted target of miR-29c. miR-29c overexpression in HEEC significantly reduced COL4A1 mRNA expression ($p<0.05$). COL4A1 is localised to the luminal epithelium basement membrane and vascular endothelial cells in human endometrium. Protein expression remained unchanged between fertile and infertile pre-receptive phase endometrium.

This is the first study to reveal miR-29c dysregulation in the infertile endometrium and a functional effect. Collectively, this data demonstrates that miR-29c overexpression significantly impaired HEEC adhesive capacity, suggesting that miR-29c dysregulation in the infertile endometrium may impair blastocyst adhesion and implantation, contributing to infertility.

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Low oxygen driven miRNA regulation of AT_1R mediated placental angiogenesis

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During the first trimester, placental development occurs in a low oxygen environment that is known to stimulate angiogenesis and proliferation via upregulation of vascular endothelial growth factor (VEGF). Expression of the placental renin-angiotensin system (RAS) is highest in early pregnancy when oxygen tension is low. Components of the RAS, including angiotensin II type 1 receptor (*AGTR1*), are significantly upregulated in low oxygen. This is also associated with increased VEGF expression in a first trimester extravillous trophoblast cell line (HTR-8/SVneo) which can be inhibited using losartan (AGTR1 antagonist). Changes in miRNAs known to target these RAS genes also occur. We postulated that low oxygen would increase the expression of *AGTR1* as well as *VEGF* in first trimester human chorionic villous explants and that this would be associated with decreased expression of miRNAs that target *AGTR1*.

Human first trimester chorionic villous explants (6-12 weeks gestational age) were cultured in 1%, 5% or 20% O_2 in DMEM-HG (supplemented with 10% FCS, 1 mg/ml antibiotic-antimycotic and 1% L-glutamine) for 48h (n=8). *AGTR1* and *VEGF* mRNA levels as well as expression of miRNAs predicted to target *AGTR1* were determined by qPCR.

Culture in low oxygen (1% O_2) significantly increased the expression of *AGTR1* mRNA compared with culture in 5% O_2 ($p=0.03$). *VEGF* mRNA expression was similarly enhanced by culture in 1% O_2 compared with culture in 20% O_2 ($p=0.01$). miR-483-3p, which when overexpressed in HTR-8/SVneo cells significantly reduces *AGTR1* expression ($p=0.03$), was significantly

reduced in villi cultured in 1% O_2 when compared with culture in 20% O_2 (p=0.028). Thus, this reduction in miR-483-3p may be responsible for the oxygen-induced upregulation of *AGTR1* and subsequent stimulation of angiogenic factors in first trimester chorionic villi.

This study highlights the potential role of miRNAs in promoting the low oxygen-induced AGTR1 expression in placental angiogenesis.

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Characterisation of the lipoxygenase – lipid peroxidation pathway in human spermatozoa

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Male fertility issues account for approximately 50% of all infertility cases in western society. With infertility affecting 1 in 6 couples in Australia, there is a pressing need to understand the underlying cause. Growing evidence suggests that the overproduction of reactive oxygen species (ROS) reduces the fertilisation potential of spermatozoa. The specific mechanism of this functional decline appears to be tied to a lipoxygenase-lipid peroxidation pathway. This occurs through the overproduction of ROS within the cell, liberating poly-unsaturated fatty acids in the plasma membrane, which are subsequently broken down by lipoxygenase proteins. We have recently confirmed that arachidonate 15-lipoxygenase (ALOX15) catalyses lipid peroxidation in human spermatozoa, yielding the cytotoxic aldehyde product 4-hydroxynonenal (4HNE). The involvement of ALOX15 within the lipid peroxidation pathway of human spermatozoa was assessed using the ALOX15 inhibitor, PD146176. PD146176 treatment, alongside an oxidative insult (H_2O_2), resulted in significant reductions to both cellular ROS ($p < 0.01$) and lipid peroxidation levels ($p < 0.01$) compared to our H_2O_2 treated controls. Excitingly, our functional data indicate that the inhibition of ALOX15 provides protection against oxidative stress with significant improvements in sperm motility ($p < 0.01$), acrosome reaction rates ($p < 0.05$) and the competence of these cells to interact with homologous human oocytes ($p < 0.05$). In addition to ALOX15, we have now established the presence of two additional lipoxygenase proteins, ALOX5 and ALOX12, in human spermatozoa that may contribute to cellular decline. To date, the use of a selective ALOX5 inhibitor (BW-B70C) and an ALOX5, 12 and 15 inhibitor (2-TEDC) have both shown significant reductions in lipid peroxidation levels compared to a treated control ($p < 0.05$ and $p < 0.005$ respectively). This work presents the possibility of targeting lipoxygenase proteins as a novel therapeutic strategy to lower oxidative stress in the male germline.

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Experimental polycystic ovary syndrome (PCOS) traits can be ameliorated by dietary intervention

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Polycystic ovary syndrome (PCOS) is a complex disorder characterized by reproductive, endocrine and metabolic abnormalities; however, its aetiology is unknown and current medical management relies solely on symptomatic treatment. Hyperandrogenism is a defining characteristic of PCOS, and diet is inherently associated since obesity is present in 40-80% of PCOS women. Yet, the optimal diet for PCOS treatment remains undefined. Therefore, to determine the impact of dietary macronutrient balance on the development of PCOS, we provided our experimental mouse model of dihydrotestosterone (DHT)-induced PCOS and control mice with *ad libitum* access to one of 10 diets varying in protein (P), carbohydrate (C) and fat (F) content. PCOS mice exhibit complete estrous acyclicity, but despite the presence of hyperandrogenism, cyclicity was restored in PCOS mice on a relatively balanced diet of P:C (ratio of 1:1.5; $p<0.05$), with fat having a negligible effect. Ovaries collected from PCOS mice with diet restored cyclicity exhibited corpora lutea, confirming the occurrence of ovulation. Interestingly, PCOS mice with restored cyclicity were not the leanest PCOS females observed, displaying a higher average body weight (24.4g +/- 0.2) compared to controls (21.7g +/- 0.2, $p<0.001$). In PCOS mice, obesity was minimised on a high P diet (P:C ratio of 2:1), with a reduction of adipocyte size correlating with a decrease in C intake ($p<0.05$). As a measure of energy expenditure, uncoupling protein 1 (UCP1) expressed in brown adipose tissue was 42% higher ($p<0.05$) in PCOS mice compared to controls, independent of diet. PCOS mice on diets with restored cyclicity had equivalent UCP1 levels to the peak UCP1 levels of control females. These findings demonstrate that PCOS traits can be ameliorated through dietary interventions to restore reproductive and metabolic abnormalities to control levels, although it appears that these hallmark PCOS traits are differentially affected by diet.

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Unravelling the underlying role of androgen- vs estrogen-mediated actions in development of the differing PCOS phenotypes

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Hyperandrogenism is a key defining characteristic of polycystic ovary syndrome (PCOS) and clinical and animal studies support an important role for androgen driven actions in the development of PCOS. Testosterone (T) and dihydrotestosterone (DHT) are the two bioactive forms of androgens which can act directly through androgen receptor (AR). T, unlike DHT, can also be converted to estradiol and act via the estrogen receptor (ER). Therefore, we aimed to determine if ER-mediated actions play a role in the development of PCOS, by assessing the ability of DHT vs T excess to induce PCOS traits in wild type (WT) and androgen receptor knockout (ARKO) mice. WT and ARKO prepubertal mice were implanted with a blank, T or DHT implant and examined after 12 weeks. In wild-type control mice, both T and DHT were able to induce the PCOS trait of anovulation as no corpora lutea (CL) were observed in their ovaries (CL number: WT+DHT: 0±0; WT+T: 0±0). In contrast, ARKO mice treated with DHT implants ovulated, however those treated with T exhibited ovulatory disruption (CL number: ARKO+DHT: 4±0.57; ARKO+T: 0.75 ±0.75, $P<0.05$). This finding implies that ER action in the absence of AR action can induce acyclicity, a key reproductive feature of PCOS. DHT, but not T, induced metabolic features of PCOS (e.g. body weight (WT+DHT: 27.1g±0.6; WT+T: 22.5g±0.67, $P<0.01$) in WT mice, however neither androgen had an effect in ARKO mice (Body weight, ARKO+DHT: 24.1g±0.7; ARKO+T: 23.5g±0.4). These findings demonstrate (1) the need for AR signalling for the metabolic traits of PCOS, (2) that ER-mediated actions may contribute to the reproductive features of PCOS and (3) suggest that lean PCOS patients lacking hyperandrogenism may have a distinct pathophysiology, prognosis and therapeutic approach from hyperandrogenic PCOS patients.

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Syncytialisation and the prorenin receptor ((P)RR)

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Background: Placenta from women with preeclampsia display insufficient syncytialisation and syncytial release of anti-angiogenic factors into the maternal circulation. Previous studies in our lab show that the prorenin receptor ((P)RR), a key component of tissue renin-angiotensin systems (RAS), is localised to the syncytiotrophoblast. Additionally, a soluble form of (P)RR (s(P)RR) exists and is detected in plasma and urine. Both placental (P)RR and plasma s(P)RR levels are elevated in preeclamptic women (Narita et. al 2016. Placenta). We hypothesise that (P)RR promotes syncytialisation and that s(P)RR is released from the syncytium.

Methods: Primary human trophoblasts and BeWo cells were incubated overnight then transfected with (P)RR siRNA, negative control siRNA or vehicle (lipofectamine/opti-MEM) for 48h. BeWo cells were also treated with 100mM forskolin (to induce syncytialisation) or vehicle (DMSO). Primary trophoblasts spontaneously syncytialise. hCG secretion and E-cadherin visualisation were used to assess syncytialisation.

Results: We are the first to show that s(P)RR is released from both BeWo and primary trophoblasts. Forskolin-induced syncytialisation of BeWo cells significantly increased s(P)RR secretion ($P<0.001$). There was no change in (P)RR mRNA expression with syncytialisation in either cell type.

Treatment with (P)RR siRNA significantly decreased (P)RR mRNA abundance and protein levels in BeWos (both $P<0.0001$), and (P)RR expression in primary trophoblasts ($P<0.0001$, $N=5$). (P)RR knockdown was associated with a significant decrease in forskolin-induced hCG secretion ($P<0.01$) and the percent of nuclei in the syncytium ($P=0.05$) in BeWos. Preliminary results in primary trophoblasts show no change in E-cadherin mRNA abundance upon (P)RR knock-down, although E-cadherin expression was significantly decreased with syncytialisation ($P<0.0001$).

Conclusion: In BeWo cells, (P)RR promotes syncytialisation however, preliminary studies in primary trophoblasts are yet to determine a role for (P)RR in syncytialisation. Both BeWo and primary trophoblasts secrete s(P)RR, highlighting that elevated levels of plasma s(P)RR in women with preeclampsia, could originate from the placenta.

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Polymer-based, biodegradable nanoparticles for the treatment of placental dysfunction

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Fetal growth restriction (FGR) is one of the leading causes of stillbirth and neonatal mortality. The majority of these cases are due to placental dysfunction and treatment options for FGR *in utero* are limited. We aimed to determine the suitability of a polymer-based, biodegradable nanoparticle in delivering DNA to human syncytiotrophoblasts using an *ex vivo* perfusion model and an *in vitro* culture model.

Nanoparticles (NP) were created by complexing Texas-Red fluorophore labelled polymer with plasmid (human *Insulin-like Growth Factor 1* (*hIGF1*) under the placenta specific promotor PLAC1). Term, human placenta cotyledons ($n=6$) were perfused for 3.5 hrs including approximately 1 hr with nanoparticle. Fluorescence (625 nm) was quantified in maternal and fetal perfusate using a fluorescent microplate-reader. For *in vitro* transgene expression, term, human cytotrophoblasts were isolated from

placenta tissue (*n*=4) and allowed to spontaneously syncytialise into syncytiotrophoblasts. Syncytiotrophoblasts were treated with nanoparticle for 48 hr and harvested to isolate RNA.

Fluorescence of the maternal perfusate significantly increased on addition of nanoparticle and declined by the conclusion of the experiment (mean minimum relative fluorescence units (RFU): baseline: -1.2±1.3 vs. NP addition: 322.4±62.1 vs. conclusion: 74.9±7.2; *P*<0.001, ANOVA). In contrast, negligible levels of Texas-Red were detected in the fetal perfusate (mean minimum RFU: baseline -0.7±0.6 vs. NP addition 1.5±1.5 vs. conclusion 3.7±2.0; *NS*). Histological analysis of placenta following perfusion showed Texas-Red localisation within the syncytiotrophoblasts of the placental villi. *In vitro*, treatment with NP significantly increased *hIGF1* expression after 48 hr compared to untreated and treatment with plasmid only (mean normalised gene expression: untreated 1.03±0.12 vs. plasmid-only 4.97±2.83 vs. NP 362.12±196.13; *P*<0.001, ANOVA).

We demonstrate successful NP-mediated delivery of nucleic acids to multiple models of human syncytiotrophoblast and increased expression of the transgene under a specific promoter representing a crucial advance in the development of treatment for placental dysfunction.

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Rcbtb2 is reduced in preeclamptic placentas and in placental tissue exposed to hypoxia

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INTRODUCTION: Placental dysfunction is a key contributor in the development of pre-eclampsia and fetal growth restriction. We have recently identified significantly reduced levels of the mRNA transcript encoding RCC1 and BTB domain containing protein 2 (*RCBTB2*) in the maternal circulation in pregnancies complicated by severe early onset fetal growth restriction (FGR) and pre-eclampsia (PE).

OBJECTIVE: The current study aimed to determine whether *RCBTB2* expression was altered in the human placenta from pregnancies complicated by PE and FGR, and with hypoxia.

METHODS: Placental tissue was collected from severe early onset PE (n=49), FGR (n=16) and gestation matched controls (n=47). Isolated primary cytotrophoblasts (n=5) and placental explant tissue (n=3) from normal term placentas were cultured under normoxia (8% oxygen) and hypoxia (1% oxygen) for 24 hours. *RCBTB2* expression was assessed by qPCR. Primary cytotrophoblasts were transfected with *RCBTB2* silencing RNA (siRNA) (n=3) to silence gene expression (under normoxia and hypoxia for 48 hours). Secretion of anti-angiogenic factor soluble fms-like tyrosine kinase 1 (sFLT-1) was assessed by ELISA and expression of cell growth and apoptosis associated genes (*BAX, BCL2, EGFR* and *IGF2)* by qPCR.

RESULTS: Comparison of RCBTB2 mRNA expression in dysfunctional placental tissue cohorts demonstrated significantly decreased expression in the PE placenta compared to healthy control placentas, but no significant change in the FGR placental tissue. *RCBTB2* mRNA expression was significantly reduced under hypoxic conditions in isolated human cytotrophoblast and placental explant tissue. Interestingly, silencing *RCBTB2* did not alter sFLT-1 secretion nor expression of the cell survival associated genes compared to controls.

CONCLUSION: *RCBTB2* is reduced in PE placenta and with hypoxic insult ex vivo. Loss of RCBTB2 does not alter sFLT-1 secretion, cell survival/growth or apoptosis genes. Loss of RCBTB2 may provide a new marker of placental insufficiency but whether it has a role in the pathogenesis of disease remains unknown.

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(P)RR remodels the trophoblast cytoskeleton and promotes trophoblast proliferation and migration

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The placental renin-angiotensin system, particularly prorenin and the (pro)renin receptor ((P)RR), are highly expressed in the early gestation placenta, when extravillous trophoblast migration and invasion is maximal. If trophoblast invasion and artery remodelling are inadequate, this can lead to shallow placentation and abnormal pregnancy outcomes. Our study aims to investigate the role of the (P)RR in early placental development.

A first trimester extravillous trophoblast cell line, HTR-8/SVneo, was transfected with siRNA targeting (P)RR mRNA. This was used either alone or in combination with an siRNA targeting the angiotensin II type 1 receptor (AT_1R). Rates of proliferation and migration after (P)RR and/or AT_1R knockdown were monitored in real-time using the xCELLigence Real-Time Cell Analysis system. In order to identify novel downstream pathways affected by knockdown of (P)RR, samples were prepared for proteomics analysis via liquid chromatography-mass spectrometry (LC-MS) using the TMT-10Plex Mass Tag labelling system.

The rate of trophoblast cell proliferation was significantly decreased when treated with (P)RR siRNA alone (P=0.03) or (P)RR siRNA + AT_1R siRNA used in combination (P<0.006). AT_1R siRNA alone had no effect on cell proliferation. Similarly, the rate of cell migration was significantly decreased in cells treated with (P)RR siRNA alone (P=0.0001) and (P)RR siRNA + AT_1R siRNA used in combination (P<0.007) but not with AT_1R siRNA alone. Analysis of mass spectrometry data showed that a number of phosphorylated cytoskeletal proteins were significantly upregulated in HTR-8/SVneo cells when (P)RR was knocked down compared to cells treated with negative control siRNA. These included metastasis suppressor 1 (*MTSS1*; P<0.03), kinectin (*KTN1*; P<0.03), and cytopsin (*SPECC1;*P<0.04).

These preliminary data suggest that proliferation and migration are regulated via (P)RR and not Ang II/AT_1R, and that (P)RR regulates pathways involved in cytoskeletal remodelling, which could explain the decrease in HTR-8/SVneo cell proliferation and migration after (P)RR knockdown.

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High levels of circulating HtrA4 may contribute to the development of preeclampsia by cleaving the main cell surface receptor of VEGFA to induce systemic endothelial dysfunction

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Objectives: Preeclampsia (PE), a serious disorder of human pregnancy is characterized by systemic endothelial dysfunction. PE can be categorized into subtypes of early-onset (EPE, ≤34 weeks of gestation) and late-onset (LPE, >34 weeks). EPE is often severe and poses a greater risk, but the only effective treatment is premature delivery. We have reported that high temperature requirement factor A4 (HtrA4) is a placenta-specific protease that is significantly up-regulated in EPE [1]. We have also shown that HtrA4 is secreted into the maternal circulation and serum levels of HtrA4 are significantly elevated in EPE [1]. We have further demonstrated that high levels of HtrA4 induce endothelial dysfunction [1, 2]. However, the mechanisms of HtrA4 action on endothelial cells are unknown. In this study, we investigated whether HtrA4 can cleave the main cell surface receptor of VEGFA, the kinase insert domain receptor (KDR), thereby inhibiting VEGFA action.

Methods: We first determined whether HtrA4 can directly cleave recombinant KDR *in vitro*. We then determined whether HtrA4 can cleave KDR from the surface of human umbilical vein endothelial cells (HUVECs) using western blot analysis and immunocytochemistry. Finally, we examined whether HtrA4 can inhibit VEGFA action in HUVECs and in mouse aorta rings.

Results: HtrA4 directly cleaved recombinant KDR *in vitro*. HtrA4 also reduced the amount of intact KDR in HUVECs. Treatment of HUVECs with high levels of HtrA4 inhibited the VEGFA-induced phosphorylation of Akt kinase, which is essential for VEGFA signalling. Functionally, HtrA4 prevented VEGFA-induced tube formation in HUVECs and inhibited VEGFA-induced angiogenesis in mouse aorta rings.

Conclusion: High levels of HtrA4 in the maternal circulation may cleave the main receptor of VEGFA on endothelial cells to induce wide-spread impairment of angiogenesis. Our study thus suggests that HtrA4 may be an important causal factor of EPE development and a potential target for treatment.

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Mitochondrial dysfunction and increased progesterone synthesis in syncytiotrophoblast mitochondria from gestational diabetes mellitus.

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The placenta is a transient organ consisting of underlying cytotrophoblasts (CT) which fuse to form the syncytiotrophoblast (ST). During this transformation, the associated mitochondria undergo substantial morphologic alterations. Mitochondria are dynamic organelles which maintain homeostatic balance of ATP, with ST mitochondria integral for the production of progesterone in the placenta and therefore maintenance of the pregnancy. As such, mitochondrial dysfunction may contribute to placental dysfunction, contributing to the pathophysiology of gestational disorders such as gestational diabetes mellitus (GDM). This study examined the bioenergetic and steroidogenic capacity of mitochondria from CT and ST in control and GDM placenta to investigate the role of mitochondria in the development and progression of GDM.

Villous placental tissue was collected from GDM (n=7) and matched control (n=7) pregnancies. Mitochondrial respiration was measure in an O2K oxygraph (Oroboros). ATP production was quantified by flourometric assay. Progesterone was analysed via ELISA.

In healthy control placentae, respiration and ATP production was higher and in CT mitochondria when compared to ST mitochondria (P=0.0202, P=0.0016). Whereas progesterone production was higher in mitochondria from ST compared to CT (P<0.0001). GDM mitochondria, respired lower in both CT and ST compared to control (P=0.0082). Similarly, mitochondrial ATP production was lower in GDM CT (P=0.0024), however greater in ST than control (P=0.0055). Progesterone was increased in GDM (P=0.0026) compared to controls, with ST mitochondria higher than ST controls (P=0.0118).

Results show decreased mitochondrial respiration in GDM, indicating a level of mitochondrial dysfunction in CT and ST. The excess production of ATP by GDM ST mitochondria appears to be for steroidogenesis, with a significant increase in progesterone observed in ST mitochondria, and GDM on the whole, when compared to controls. Progesterone has previously been shown to increase insulin resistance; our research suggests that ST dysfunction and overproduction of progesterone is associated with GDM.

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Defining a human embryo implantation 'adhesome'

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Background: Human embryo implantation is considered the 'black box' of reproduction. Knowledge of the basic biological processes underpinning initial stages of implantation is important to improve fertility outcomes, particularly in ART cycles where pregnancy rates remain ~30% despite production of high quality embryos. Elucidation of critical molecules involved in implantation could facilitate novel approaches/therapeutics to improve reproductive outcomes.

Aim: Develop a medium/high throughput model of human embryo implantation and interrogate it to provide a proteome of human embryo adhesion.

Methods: Human trophoblast stem cells ('trophectoderm') were formed into spheroids (TS), approximating size of human blastocyst, and co-cultured with endometrial epithelial cell-lines/ primary human endometrial epithelial cells (HEEC) to determine i) time course of adhesion; ii) adhesion with hormonal treatments; iii) adhesion to HEEC's from fertile versus infertile women. Hormonally-treated HEEC/TS co-cultures were subjected to proteomic analysis for proteins involved in embryo adhesion.

Results: The endometrial endometrial cell-lines exhibited differing degrees of adhesion for TS ranging from high (RL95-2 cells), moderate (ECC-1, Ishikawa) to low (Hec-1a). ECC-1 cells exhibited ~50% adhesion after 6-hours of co-culture; timing approximating human embryo adhesion. ECC-1 and HEEC's demonstrated increased adhesion in response to 'receptive' (estrogen/progesterone) and 'implantation' (estrogen/progesterone/hCG) versus 'proliferative' (estrogen) treatments. Importantly, TS adhered to 95% of HEEC's derived from fertile women but only 11% of those from infertile women, suggesting TS 'identify' endometrial infertility. Proteomic analysis identified 147 up-regulated and 145 down-regulated proteins associated with adhesion; the 'adhesome'. The most up-regulated proteins included PTGS2, RPL28, CNIH4, SERPINE1 and ATP5I, which have previously been implicated in endometrial receptivity/embryo adhesion, confirming the validity of our model.

Conclusion: The TS-HEEC model provides insight into embryo adhesion and a medium-throughput model for screening pro/anti-implantation factors. Our characterisation of an embryo implantation 'adhesome' provides a valuable resource for understanding and more effectively targeting this critical phase of pregnancy.

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A genetic mutation in the inhibin βB-subunit disrupts uterine function in mice

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Gonadal function is coordinated by pituitary hormones, including follicle stimulating hormone (FSH), which travel via the circulation to the ovaries and testes to drive folliculogenesis and spermatogenesis. In response to FSH stimulation, the gonads produce inhibin A and inhibin B, which act back at the pituitary to down-regulate FSH production. Inhibins (α/β dimers) block FSH release indirectly by antagonizing the actions of the related activins (β/β dimers) on the surface of pituitary cells. This circulatory hypothalamic-pituitary-gonadal (HPG) loop is integral to reproductive function, and consequently, imbalances in inhibin/activin can impact fertility. In a recent study, a homozygous genetic mutation (c.T1079C:p.M360T) arising from uniparental disomy of chromosome 2, was identified in the *Inhbb* gene (encodes the β-subunit of inhibin B and activin B) in a male patient suffering infertility (azoospermia). In the current study, we aimed to determine the impact of the *Inhbb*^{M360T}mutation on the function of activin B and inhibin B. Mechanistic analysis revealed that the M360T mutation resulted in increased *in vitro* production of both activin B and inhibin B, but had no effect on the bioactivity of these proteins. To ascertain if the M360T mutation was sufficient to disrupt fertility, we generated *Inhbb*$^{M360T/M360T}$mice. Although anything resembling azoospermia was not observed in male mice, we found that female *Inhbb*$^{M360T/M360T}$ mice had a significant reproductive phenotype. Indeed pregnant *Inhbb*$^{M360T/M360T}$ mice displayed abnormal labours (>8 hours) and increased litter sizes (up to 14 pups/litter). Serum hormone analyses support that the *Inhbb*^{M360T}mutation likely disrupts the HPG axis, and may result in a hormonal imbalance in pregnant *Inhbb*^{M360T}females. Our *Inhbb*^{M360T} mouse represents a unique model to study failure to progress in labour.

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The implantation reaction evolved as a mode of endometrial recognition of pregnancy in the first live bearing mammals

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In human pregnancy, recognition of a developing fetus within the uterus is essential to maintain uterine quiesence and support the embryo through an extended gestation. In most marsupials (with the exception of macropods), pregnancy is shorter than the estrus cycle, and for this reason it has been assumed that recognition of pregnancy is not necessary, and was a trait that evolved in the first eutherian (placental) mammals. To investigate whether there is uterine recognition of pregnancy in early live bearing mammals, we examined reproduction in the grey short tailed opossum (*Monodelphis domestica*) a marsupial with what is assumed to have the most pleiotropic mode of pregnancy. We examined the morphological and gene expression changes in

the uterus of females in the estrus cycle, and compared these to the observed changes during pregnancy. We found that while the morphology of the uterus undergoes substantial changes in pregnancy, these changes occur in a programmed fashion during estrus, and for the most part do not appear to be impacted by the presence of a fetus. However, transcriptionally we saw big differences between the uterus of pregnant and estrus animals. Gene ontology analysis shows us that the genes up-regulated due to the presence of a fetus are involved in macromolecular transport, inflammation, and metabolic activity. Our results suggest that while the uterus exhibits programmed changes in response to ovulation, the transcriptional landscape of pregnancy responds to the presence of a fetus, and upregulates suites of genes that may be essential for fetal support. These results are consistent with uterine recognition of pregnancy being an older feature dating back to the origin of live birth in mammals.

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Reduced SIRT1 levels are evident in 15 weeks maternal serum and in term placentas of pregnancies complicated by gestational diabetes mellitus

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Introduction: Gestational diabetes mellitus (GDM) is associated with significant maternal and perinatal complications. Although GDM affects 11% of all pregnancies on average, the incidence has reached epidemic proportions within Asian (>16%) and Indian (>20%) populations. Sirtuin 1 (SIRT1), a ''reverse thrifty gene'' that protects against metabolic diseases by directing the organism to limit energy consumption and expenditure, is a nutrient sensing protein deacetylase involved in glucose and insulin metabolism and nutrient transport. We aimed to characterize and determine the role of SIRT1 in human placenta and to investigate whether circulating concentrations can be used as an early marker for GDM.

Methods: Human term placentas were obtained from women whose pregnancies were normal or complicated by GDM and controlled by diet. SIRT1 activity was inhibited in primary syncytiotrophoblasts with the selective SIRT1 inhibitor Ex-527 *in vitro*. Gene and protein expressions were examined by real-time PCR and western blotting, respectively. Serum samples from GDM cases and controls who participated in the SCOPE study in Auckland, New Zealand were obtained at 15 and 20 weeks' gestation. SIRT1 levels in serum were analysed by ELISA.

Results: SIRT1 mRNA and protein expressions were down-regulated in term placentas from pregnancies complicated with GDM. Hyperglycaemia resulted in reduced SIRT1 protein expression in primary syncytiotrophoblasts *in vitro*. Whereas, SIRT1 inhibition resulted in up-regulation of fatty acid transporter proteins FATP2, FATP4 and GLUT4 after 24 hours and down-regulation of FATP6, amino acid transporters SNAT2 and ASCT1 gene expressions after 48 hours. SIRT1 levels were significantly lower at 15 weeks' in women who developed GDM compared to control women.

Conclusion: Our data implicate a role for SIRT1 in the pathogenesis of GDM and in the regulation of nutrient transporter proteins in human placenta. Manipulation of SIRT1 may constitute a novel future approach for the treatment of GDM.

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Maternal low protein diet programmes low ovarian reserve in offspring

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The ovarian reserve of primordial follicle oocytes is formed during *in utero* development and represents the entire supply of oocytes available to sustain female fertility. Maternal undernutrition during pregnancy and lactation diminishes offspring ovarian reserve in rats. In mice, maternal oocyte maturation is also susceptible to undernutrition, causing impaired offspring cardiovascular function. We aimed to determine whether programming of the ovarian reserve is impacted in offspring when maternal undernutrition extends from preconception oocyte development through to weaning. C57BL6/J mice were fed normal protein (20%) or low protein (8%) diet during preconception, pregnancy and lactation periods. Maternal ovaries were harvested at weaning and offspring ovaries collected at postnatal day (PN)21 and 24 weeks of age. Ovarian follicles were quantified (n=5/group). There was no impact of diet on maternal follicle numbers, however time to first litter was significantly delayed in protein restricted mice (n=11-12/group, $p<0.05$). In offspring, protein restriction significantly depleted primordial follicles by 37% at PN21 and 51% at 24 weeks ($p<0.05$). There were no effects of diet on other follicle classes. Histological analysis showed no differences in the proportion of proliferative follicles (pH3-positive), but increased atresia (cleaved caspase-3-positive, or TUNEL-positive), as well as altered patterns of mitochondrial (MTCO-1) and lysosomal (LAMP-1) distribution were detected in follicles of protein-restricted offspring at both ages ($p<0.05$). Our data show that maternal diet during the preconception period, *in utero* development and early life has significant impacts on follicle endowment and markers of follicle health later in life. In Australia, current National Health and Medical Research Council dietary guidelines are available for women during pregnancy. However, emerging evidence, including our findings, highlight the potential benefits for creating specific dietary guidelines and also improving clinical and public awareness of the importance of preconception diet for expectant mothers to develop healthy offspring.

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Characterisation of 5-methylcytosine and 5-hydroxymethylcytosine in human placenta cell types across gestation

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The placenta is one of the most important organs to human reproduction however, very little is understood about placental development at a molecular level, including how epigenetic mechanisms change throughout gestation. In addition, DNA methylation studies in this organ are complicated by the different cell types that make up the placenta. Placental dysfunction is often associated with pregnancy complications such as preeclampsia (PE), and aberrant DNA methylation in the placenta has been identified in pregnancy complications compared to uncomplicated samples. We used immunohistochemistry (IHC) and immunofluorescence (IF) to investigate the localisation of 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC), in placenta tissue across gestation. IHC analysis of tissue sections showed levels of 5-mC increased across gestation, as expected and previously shown. When specific trophoblast cell subtypes: cytotrophoblasts (CTB) and syncytiotrophoblasts (STB), were isolated and stained using IF, levels of both 5-mC and 5-hmC significantly increased in term CTBs compared to first/second trimester. Staining intensity of 5-hmC also increased in first trimester STBs compared to CTBs (P=0.0011). Finally, comparison of IHC staining in term tissue from PE and uncomplicated pregnancies revealed levels of 5-mC to be higher in placentas from PE pregnancies (P=0.028). Our analysis confirmed that 5-mC and 5-hmC staining intensity increased across gestation, and was different between CTB and STB. This provides a solid foundation for future research focused on single cell populations. Differences in DNA methylation profiles between different cell types of the placenta may be indicative of different functions and requires further study in order to elucidate what changes accompany placental pathologies.

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In vitro reactivation of the mink blastocyst from embryonic diapause

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Embryonic diapause is a period of developmental arrest in which the embryo is maintained in a dormant state for an extended period of time. Over 130 species of mammals undergo diapause but the molecular mechanisms that control it remain unknown. During diapause, the mink blastocyst maintains an almost complete arrest of the cell cycle and a greatly reduced metabolism, DNA and protein synthesis (1). Treating mink with prolactin during diapause results in precocious termination of diapause, whereas treatment with dopamine agonists prevents implantation. The first signs of reactivation of the mink blastocyst from diapause are increased DNA and protein synthesis on the third day after prolactin injection (d3 PRL), with the first expansion of the blastocyst occurring at day 4 PRL (1). However, prolactin cannot reactivate embryos in culture and it is unknown how prolactin acts at the uterine level to induce reactivation. Previous attempts to culture mink embryos *in vitro* have been relatively unsuccessful with no mono-culture system identified that is able to sustain growth (2-5). Here we show that culture of diapause mink blastocysts in feline optimised cat media 2 (FOCM2) maintained viability and growth for a minimum of 4 days with increases in cell number and significant increases in diameter. Furthermore, this is the first time that mink diapause blastocysts have been reported to reactivate spontaneously in culture. This suggests that in the mink, embryonic diapause is under uterine control as opposed to the absence of a critical factor that the blastocyst requires to reactivate.

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A living koala genome bank

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Wild koala populations in Queensland are under increasing threat of local extinction, associated with habitat fragmentation and disease. We are proposing a novel innovative approach to koala conservation in SE Queensland that makes use of proven breeding technologies, in combination with in-depth analyses of current wild and captive Queensland koala population genetics and chlamydia proofing vaccination therapy to deliver a “living koala genome bank”. The bank will provide practical

mechanisms by which koala researchers and managers will improve the genetic diversity of small fragmented populations, rescue genetic variation from animals that have to be euthanased or treated for disease in koala hospitals, and allow the propagation of additional disease-free koalas of high genetic merit for release back into the wild. This new paradigm takes advantage of redevelopment of Dreamworld's wildlife precinct that will include construction and maintenance of research facility in which animals are housed off-exhibit in a dedicated koala breeding centre; this centre will also be used to facilitate the practical management and preservation of wild koala genetic variation into existing captive koala populations throughout Queensland, with pilot studies concentrated in the Gold Coast region. Reproductive health of the genome bank will be assured through the translation of recently developed chlamydia vaccine technology by our QUT partners. Our "big vision", is for zoos, governments and universities to become joint custodians of wild koala genetics, to ensure not only the long-term conservation of koalas in the wild but also to ensure a sustainable tourism industry.

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The Role of the Progesterone Receptor in the Regulation of Endometrial Receptivity

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The uterus functions to support embryo implantation and fetal development until the offspring is able to survive at birth. The ability of the uterus to accomplish this function is dependent on the stimulation by the ovarian hormones estrogen and progesterone. These hormones act through their receptors the Estrogen Receptor, ESR1, and Progesterone Receptor, PGR. The latter receptor consists of two isoforms the PGRA and PGRB. These isoforms are encoded by the same gene through differential gene transcription. The PGRB as an additional transactivation domain at the amino terminus which is speculated renders it a more potent activator than PGRA. The mechanisms governing uterine receptivity regulated by PGR and its isoforms have been elucidated *in vivo* utilizing genetically engineered mouse models and *in vitro* utilizing cell lines and primary cell culture. Ablation of the PGR and specifically the PGRA renders the uterus incapable of supporting embryo attachment, and invasion. The uterine stromal cells in these mice are unable to differentiate to decidual cells and the hormone progesterone is usable to counter the mitogenic effects of estrogen. Utilizing transcriptomic approaches, it has been determined that PGR initiates the s a bidirectional crosstalk between the epithelium and stroma to prepare the uterus for pregnancy. The paracrine crosstalk is initiated by PGR stimulation of the expression of Indian Hedgehog IHH. Cistromic analysis identified the regulatory region flanking the *Ihh* gene which governs its expression. This region binds PGR and the transcription factors GATA2 and SOX17. Ablation of this enhancer utilizing CRISPR/Cas9 determined that this region is critical for the uterine expression of *Ihh*. Analysis of the mouse genome for regions that contain overlapping PGR, GAtA2 and SOX17 binding sites reveal a potential cassette of transcription factors that regulate uterine epithelial gene expression. These pathways are conserved in the human endometrium and have been associated with human endometrial disease.. Identification of the interactions of cis and trans regulation of genes will identify novel diagnostic and therapeutic targets for human reproductive diseases.

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Critical windows and experimental models programming developmental disease risk

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Professor Wlodek Mary is a global leader in developmental origins physiological research and will present her research findings using her successful experimental models that mimic human pregnancy complications. Her experimental model of growth restriction has growth profiles, organ deficits and phenotypes like those observed in babies born small who are susceptible to adult cardiorenal disease, diabetes and obesity. Generally, males, but not females, born small have a greater risk of developing such diseases despite females also presenting with organ deficits which might render them susceptible to additional lifestyle challenges including pregnancy, stress and obesity. How growth restricted offspring respond to lifestyle interventions such as nutrition and exercise can reprogram these diseases and deficits will be described. Experimental models of gestational diabetes and maternal stress with pregnancy onset have also been developed. How the key critical windows of adolescence, pregnancy and the postnatal lactational period program developmental disease risk will be discussed. Our experimental research approaches provide insight into critical windows and mechanisms programming and preventing disease risk with implications to improve human health and that of future unborn generations.

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To what extent is health predetermined? – How environmental exposures of gametes influence the developmental trajectory of embryos?

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Over recent years there has been increasing awareness that parental exposures to lifestyle and environmental toxins can increase the risk of non-communicable diseases such as obesity and metabolic syndrome in the next generation. Studies in rodent models have shown that this transgenerational transmission of disease occurs down both the maternal (oocyte) and paternal (sperm) line. The most studied example is obesity where oocytes from obese mothers have altered metabolic parameters which are associated with impaired embryo development and alterations in fetal growth trajectories. This appears likely to be mediated through metabolic sensor proteins that link metabolic dysfunction to altered epigenetics marks in both

DNA and histones. Similarly, obesity in males induces oxidative stress, which signals a cascade of perturbations including to DNA methylation as well as changes in microRNA signatures. This paradigm that the environment of the gametes and early embryo can influence long term development also has implications for clinical IVF where this occurs in the laboratory. Further elucidations of the pathways involved in this amplification of disease into the next generation will be essential for the development of effective interventions that can break this cycle.

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Epigenetic Regulation in the Germline: Setting a Foundation for the Next Generation.

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The development of a complex multicellular organism involves the production of a remarkable array of specialised cells from a single genomic sequence. This is facilitated by heritable epigenetic modifications that organise the DNA into active and repressed domains, allowing the regulation of specific gene expression programs that direct cell specialisation. The transmission of epigenetic signatures between cell generations ensures the maintenance of lineage specific expression profiles, and appropriate cell and tissue function throughout life. Cell type specific epigenomic information is not only important in somatic cell specialisation, but also regulates germline differentiation and the transmission of information that affects the development and physiology of offspring. This is of particular interest as it is proposed that the epigenome provides an interface between the environment and genome function. However, the mechanisms involved in epigenetic patterning of the germline and the epigenetic modifications that affect offspring development are poorly understood. While many studies have focussed on the impacts of various environmental challenges on epigenetic inheritance, our studies have concentrated on the role of Polycomb Repressive Complex 2 (PRC2) in the germline. PRC2 is a highly conserved epigenetic modifier essential for establishing the repressive histone modification H3K27me3 and regulating development through repression of target genes. Using genetic and pharmacological approaches, our work provides evidence that PRC2 regulates H3K27me3 in developing germ cells and alters epigenetic inheritance through the maternal and paternal germlines. Of particular interest in this context are systemic epigenomic drugs that directly target epigenetic modifying proteins in target tissues such as tumours. While these drugs offer improved therapeutic approaches, they also have the potential to alter the germline epigenome. Given growing evidence that epigenetic inheritance is affected by environmental influences such as diet, chemicals and drugs, further understanding of mechanisms regulating the germline epigenome is required to gauge potential for environmental impacts on the germline to alter disease inheritance and evolution.

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Prenatal alcohol exposure: intervening to prevent disease

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It is well established that a poor *in utero* environment may contribute to increased risk of developing adult disease. The period around the conception is now recognised as a window of particular susceptibility as women are often unaware they are pregnant and continue to engage in risky behaviours. WHO and NHMRC guidelines state “Not drinking is the safest option for pregnant women and women planning a pregnancy” but almost 50% of pregnancies in Australia are unplanned and up to 70% of women are drinking in the time leading up to conception. Our research program has developed a rodent model of periconceptional alcohol exposure and utilised this to explore the phenotypic outcomes and the underlying mechanisms involved.

Alcohol around conception resulted in fetal growth restriction and offspring with insulin resistance, increased fat deposition and impaired renal and cardiac function. Similar to other models of exposure to alcohol during periods of brain development, our model resulted in neuro-behavioural deficits including anxiety like behaviour and a depressive phenotype. Investigation of the early embryo suggested that the alcohol exposure caused sex-specific changes to the growth and differentiation of the pre-implantation embryo including changes to DNA methylation. This contributed to poor placental development and changes to the regulation of the hypothalamic-pituitary-adrenal axis including the isoforms of the glucocorticoid receptor. This work highlights that maternal alcohol consumption, even prior to blastocyst implantation and placental formation, can result in long lasting deficits in the offspring.

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Making Babies

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Australia is an international leader in human reproduction research and innovation, with many firsts in understanding fundamental processes of reproduction, and treatment options for infertility and subfertility. Infertility and subfertility affect a significant and increasing proportion of humanity and this is a ‘canary in the gold mine’ alerting us to environmental impacts on human health. WHO estimate 1 in 4 of women desiring pregnancy experience infertility / subfertility, and in men, sperm counts have dropped around 50% in major Western countries over the last four decades. IVF is an enormously successful technology, and 6 million IVF babies have now been born. However IVF remains expensive, invasive, and variably successful, and does not solve the underlying causes. There is a long way to go to deliver better options for couples and individuals who are unable to achieve and maintain pregnancy, especially in low resource settings. Current research priorities are to understand how environmental factors and genes interact to affect human fertility and reproductive senescence, and to define the significance of gamete health, peri-conception events and epigenetic processes in programming life course health and breaking the cycle of

intergenerational transmission of disease. Investing in human reproductive health will inform a better start to life for all children, build healthier communities and help governments tackle rising health care costs.

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Laser drilling for artificial shrinkage increases ROS levels in mouse blastocysts

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Laser drilling is used clinically for assisted hatching and for artificial shrinkage of the blastocoel cavity prior to vitrification. However, there is limited research on the safety of the heat emitted from the laser or its impact on embryo development and viability. Therefore, the aim of this study was to establish the effect of laser assisted hatching and artificial shrinkage on embryo development and ROS production.

Mouse zygotes were collected from super-ovulated pre-pubertal females (CBAF1, n=1182) and cultured in G1/G2 sequential media at 37°C in 6%CO_2:5%O_2:89%N_2 for 73h. Morula/blastocysts were lasered or artificial shrunk, by directly lasering the blastocyst, and cultured for a further 24h. Morphology, cell number, and ROS levels (MitoSOX Red) were assessed on day 5 (97h culture). Statistical analysis by univariate general linear modelling with LSD post-hoc test, performed in SPSS software.

There was no effect of laser drilling on blastocyst development on day 5 or cell number ($P>0.05$) between the groups, control (60.9±2.9, n=45), 5µm laser drilling (59.8±2.9, n=45), 5µm laser drilling + artificial shrinkage (58.0±2.9, n=45).

ROS levels (proportion of MitoSOX positive cells / total number of cells) were significantly elevated by 5µm laser drilling + artificial shrinkage (13.8%±1.3, n=45) compared to controls (9.4%±1.3, n=45) ($P<0.05$). Additionally, MitoSOX positive cells in artificial shrinkage treatment were localised to the site of laser pulse. Although there was an increase in ROS levels in the 5µm laser drilling treatment group (10.7%±1.3, n=45, $P<0.08$) this did not reach significance.

There appears to be no detrimental impact of laser drilling for assisted hatching on embryo development or ROS production. However, laser drilling for artificial shrinkage did increase ROS levels in the area of the laser pulse, suggesting cellular damage. Further research is underway to assess the impact of laser drilling on subsequent viability, particularly when the embryo is directly pulsed.

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Physiological oxygen facilitates the acquisition of a more embryonic stem cell-like metabolic response in human induced pluripotent stem cells

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Reprogramming to pluripotency involves not only profound epigenetic and transcriptional change but necessitates the restructuring of metabolism. Worryingly, resultant induced pluripotent stem cells (iPSC) retain a metabolic memory of their somatic origin[1]. While physiological oxygen, a known regulator of embryonic development and embryonic stem cell physiology, has been shown to increase the proportion of somatic cells reprogrammed to iPSC, whether this increased efficiency is accompanied by physiological alterations has not been examined. The aim of this study was, therefore, to determine whether oxygen concentration during reprogramming affected iPSC metabolic memory.

Neonatal human dermal fibroblasts (NHDF) were reprogrammed under either 20% or 5% oxygen in mTeSR1 medium. Resultant iPSC were maintained in their respective oxygen conditions, or challenged with the opposing oxygen concentration, and analysed for carbohydrate utilisation, mitochondrial metabolism, telomere length and transcriptional differences.

All iPSC lines retained aspects of somatic cell metabolic memory and failed to regulate carbohydrate metabolism, similar to their parental NHDF cells, in contrast to the metabolic response characteristic of embryonic stem cells[2]. Significantly, basal and maximal respiration, along with mitochondrial ATP production, were only modulated in iPSC reprogrammed under physiological oxygen. Consistent with an observed reduction in telomere length, RNA-seq revealed transcriptomic instability in iPSC reprogrammed under atmospheric oxygen.

These data reveal oxygen availability during reprogramming perturbs subsequent transcriptional and metabolic profiles of iPSC. Physiological oxygen enables the acquisition of a more embryonic stem cell-like physiology, although metabolic reprogramming remains incomplete. As metabolism links nutrient availability with epigenetic regulation, these perturbations may plausibly persist in iPSC-derived differentiated populations, and impact downstream application of iPSC for disease modelling, drug discovery and cell therapy.

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2. [2] Lees JG et al. (2015) Reproduction 150: 367 - 382.

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Expression of olfactory signalling proteins in spermatozoa

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The olfactory signalling cascade begins when an odorant binds to the olfactory receptor (OR) on the olfactory sensory neuron and activates the olfactory G-protein (G_{olf}) and Ca^{2+}-calmodulin-sensitive adenylyl cyclase (AC3). Olfactory Marker Protein (OMP) is an abundant, soluble acidic protein that is expressed in mature olfactory sensory neurons throughout vertebrates and immunologically co-localises with ORs. The expression of OMP in specific cell types of non-olfactory tissue suggests that it might reveal OR-mediated chemoreception in other functional systems.

We tested the hypothesis that chemoreceptors from the olfactory system could play a role in sperm chemotaxis if sperm were guided by factors secreted from the egg. Using light microscopic immunocytochemistry, we studied OMP, G_{olf}, and AC3 in spermatozoa of rats and humans. In rats, expression of these proteins was documented in epididymal spermatozoa. In humans, expression was investigated in ejaculated spermatozoa in three modes of activation (control, activated, and hyper-activated).

OMP expression was evident in compartment-specific locations of rat and human spermatozoa with some species-specific differences. OMP expression in the acrosomal cap of hyper-activated spermatozoa implies guidance of the sperm to the egg, whereas its presence in the equatorial segment suggests a role for the binding of the sperm to the egg after it undergoes its 90 degree rotation. The role of OMP in the connecting piece where the centriole is located and along the tail remains unknown. In epididymal spermatozoa, G_{olf} and AC3 were immunolocalised to the acrosome head and equatorial segment, the connecting piece, and along the tail.

OMP appears a reliable indicator of OR-mediated chemoreception and is heavily involved in reproductive biology. Much remains to be determined about the ligands that activate ORs but the resulting knowledge will have direct significance to understanding human fertility.

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Idebenone reduces mitochondrial ROS in mouse and human sperm with elevated baseline ROS

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Male obesity and ageing have been shown to impair male fertility. One common proposed mechanism is an increase in harmful ROS levels that can cause DNA damage in the sperm, resulting in reduced pregnancy rates and increases in miscarriage. ART treatments provide an opportunity to reduce sperm ROS during sperm washing and isolation in vitro. Idebenone is a synthetic analog of co-enzyme Q10 that is more readily dissolved in culture media and crosses the mitochondrial membrane, targeting mitochondrial ROS. The aim of this study was to determine if addition of Idebenone to the sperm preparation culture media could reduce ROS levels in human and mouse sperm.

Human sperm (n=6) from obese males or mouse sperm (n=5) with elevated ROS levels were incubated in GIVF medium (control) or GIVF medium supplemented with 5µM Idebenone for 2h. Mitochondrial ROS was measured by incubation with MitoSOX and assessed using flow cytometry of live sperm which were identified by exclusion of SYTOX Green. Fertilization in the mouse was performed by co-incubation of sperm with cumulus-enclosed oocytes for 4h and successful fertilization assessed by the presence of 2-cells the following morning. Statistical differences between treatments where assessed by generalised linear modelling using SPSS.

Incubation with Idebenone resulted in a significant decrease in sperm mitochondrial ROS levels, with a 16% decrease in the human (control 1392±15, Idebenone 1195±14, $P<0.01$) and 13% decrease in mouse (control 687±11, Idebenone 596±10). In the mouse, fertilization rates were increased when the sperm was incubated with 5µM Idebenone prior to insemination ($P<0.05$).

This study suggests that short term incubation with the antioxidant Idebenone in vitro can reduce sperm mitochondrial ROS, in samples that have elevated baseline ROS. In the mouse, this translated to improvements in fertilization rates. Further studies need to establish if this translates to similar improvements in the human.

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Analysis of the Role of PRDM14 in Lineage Specification in the Mouse Preimplantation Embryo

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The first round of embryo differentiation is accompanied by changes in the patterns of transcription in the resulting trophectoderm (TE) and inner cell mass (ICM). Two epigenetic modifiers show highly reciprocal patterns of gene expression: *Prdm14* (high in ICM, low in TE) and *Dnmt3b* (high in TE, low in ICM), leading to a hypothesis that PRDM14 acts as a negative regulator of DNMT3B [1]. Analysis of DNMT3B protein levels show it follows its gene expression pattern [2]. In this study, we examined: (1) PRDM14 across preimplantation development; and (2) the effect of PRDM14 knock-down with morpholino oligonucleotides on embryo development.

The pro-nuclei of zygotes showed a low level of PRDM14. Staining increased ~ 340-fold in 2-cell embryos. It was present throughout the cytoplasm but accumulated within the nuclei. From the 4-cell to blastocyst stages cytoplasmic staining decreased and antigen became predominantly localized to the nuclei. Staining was present in the nuclei of both the TE and

ICM, contrary to gene expression studies[1]. Treatment with anti-PRDM14 morpholino oligonucleotides for 72h resulted in > 80% loss of PRDM14. Despite this extensive knockdown, embryos still developed into the blastocysts without any notable phenotypical difference from controls.

Our results show that the reciprocal expression of *Prdm14* and *Dnmt3b* in blastocysts is not recapitulated at the protein level. The work does not provide evidence for PRDM14 being differentially allocated to the ICM, and by inference does not support it having a direct role in the differential expression of *Dnmt3b*. The failure of PRDM14 knockdown to affect blastocyst formation is inconsistent with reports of this epigenetic modifier having a primary role in committing cells to the ICM lineage.

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2 Li, Y., Seah, M. K. Y. & O'Neill, C. *Reproduction* **151**, 83-95 (2016).

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Human multipotent NT2/D1 cells model bipotential events in mammalian sex determination

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Sex determination is the process during embryogenesis when the bipotential gonads differentiate into either testes in males or ovaries in females. In males, the Y chromosome gene SRY turns up *SOX9* transcription, directing the somatic cells to differentiate into testicular Sertoli cells rather than ovarian granulosa cells. NT2/D1 cells are a human Sertoli-like cell line derived from a testicular seminoma. Despite being multipotent, they express important Sertoli cell markers at low levels that can be manipulated to recapitulate key male sex determination processes. For example, transfection of SRY turns up *SOX9* transcription. SRY and SOX9 play key roles in male sex development since human SRY and SOX9 mutations cause Disorders of Sex Development (DSD) in XX males (SOX9 duplications) and XY females (SOX9 mutations/deletions). However many causes of DSD remain unexplained genetically.

Using the NT2/D1 cells, we are unravelling the role of genes and their relevance to human sex determination and to DSD. We reasoned that the genes regulated by SOX9 are potential DSD genes. Previously we identified 119 candidate SOX9 target genes, including Nedd9, by integrating RNAseq analysis on mouse Sox9 knock-out gonads with Sox9 ChIPseq on wildtype mouse testes (Rahmoun et al., 2017, *NAR*). Using NT2/D1 cells, overexpression and knockdown of *SOX9* led to an increase or decrease in *NEDD9* mRNA and protein levels. This suggested that human *NEDD9* is a target gene of SOX9. As such, Nedd9 may have an important role to play in testis development. *Nedd9* knockout mouse testes show growth defects and a coelomic blood vessel defect. Nedd9 is a scaffolding protein from the Cas family, and using NT2 cells we are currently investigating its role in cellular processes including cell polarity, migration, adhesion, apoptosis and cell cycle control.

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The Roles of DNA Template Segregation in Cellular Differentiation in the Early Embryo

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Cellular differentiation results from the asymmetrical self-renewal of cells whereby one daughter cell retains the characteristics of the progenitor lineage while the other becomes committed to differentiation into a new lineage. Each daughter cell acquires a new epigenetic landscape, but the mechanism for this reprogramming is currently ill-defined.

Differentiation of some adult stem cells is associated with non-random DNA template segregation (NRTS) at mitosis. Analysis of NRTS is achieved by labelling the newly synthesized DNA strand in a given S-phase and tracking its (and its non-labelled template strand) allocation into daughter cells. In this study, we examined the fate of sister chromatids across each of the first four cell-cycles of the mouse embryo.

Embryos were incubated in KSOM + amino acids containing 1 µM bromodeoxyuridine (BrdU) for the duration of S-phase of a given cell cycle. They were then cultured through two cell-cycles and the allocation of template DNA between daughter cells assessed by immunolocalization of BrdU.

Labelling of blastomeres in the 1-cell, 2-cell and 8-cell embryo resulted in random allocation of template DNA in subsequent daughter cells. By contrast, labelling during the 4-cell S-phase resulted in non-random allocation of template DNA to the apolar daughter cells following the asymmetrical division of 8-cell embryos. Furthermore, tracking these cells through to blastocysts showed a significant accumulation of template DNA in the inner cell mass compared to the trophectoderm. Analysis of DNA methylation showed that there was a strong negative association between the location of template DNA and global immuno-detectable DNA methylation.

This study points for the first time to NRTS being involved in the asymmetric cell division leading to the first round of differentiation in the early embryo and is associated with global modification of the epigenetic landscape in each of the resultant cell lineages.

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Immunolocalisation of DNA Methylation and DNA methyltransferases In The Post-Implantation Mouse Embryo.

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Dynamic reprogramming of DNA methylation (5meC) is an essential feature of early embryonic development. It is a highly regulated process, with three conserved DNA methyltransferase enzymes responsible for maintenance (DNMT1) and de novo (DNMT3A and DNMT3B) methylation. The complex structure of chromatin within nuclei variably masks 5meC and DNMTs from antibody detection. Immunolocalization techniques that use both acid and tryptic digestion are required to unmask 5meC in the pre-implantation embryo. This method showed that there was a marked loss of immune-detectable global 5meC from the inner cell mass of the preimplantation embryo (1). In this study we used our validated immunolocalization approaches to assess the pattern of global immune-detectable 5meC and the DNMTs in each of the lineages that arise in the early post-implantation embryo.

Changes in 5meC and DNMT enzymes within serial cryo-sections of the post-implantation embryo (E5.5 & E6.5) were mapped. At both these embryonic stages, acid and tryptic digestion was required to retrieve the 5meC epitope while acid digestion was required for full antigenic retrieval of the DNMTs. E5.5 and E6.5 embryos showed similar global levels and patterns of nuclear 5meC staining in all cells of the placental cone, trophectoderm, the hypoblast and epiblast. DNMT1 showed a similar staining profile to 5meC. DNMT3A and DNMT3B were also localised to nuclei of all lineages in the embryo, however, both were strongly upregulated in the epiblast of the post-implantation embryo.

This study shows that the low levels of immuno-detectable 5meC in the inner cell mass of the preimplantation blastocyst was reversed in the post-implantation epiblast and this is associated with a marked upregulation of DNMT3A and DNMT3B within this lineage. All other lineages showed similar levels of global 5meC and DNMTs.

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TETs exhibit cell-specific differential expression in response to steroid hormones in human endometrium

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Background: The term DNA modification generally referred to the process where a methyl group is added to the cytosine to form 5-methylcytosine (5mC). However, recently, 5-hydroxymethylation (5hmc) has also been identified as a biologically critical epigenetic modification. The 5mC generated from DNA methylation down-regulates gene expression or leads to gene silencing whereas 5hmC, produced from DNA hydroxymethylation is known to perform alternative functions. Mediated by three Ten Eleven Translocation (TET) enzymes, the role of hydroxymethylation in the endometrium has not yet been explored. The aim of this study was to characterize TET1, 2 and 3 expression across the menstrual cycle and further, to determine the effect of steroid hormones on epithelial and stromal cells independently.

Methods: Endometrial tissue biopsies were collected from women of reproductive age with regular menstrual cycles. Endometrial epithelial (HES) and stromal (HESC) cell-lines were treated with either control, estradiol, progesterone or a combination of both. TET gene expression was determined using Real-time PCR.

Results: TET1 and TET3 mRNA expressions in the mid-secretory phase were significantly up-regulated compared to proliferative phase and the early secretory phase. TET1 and TET3 mRNA expressions were down-regulated in the late and early secretory phases, respectively. Preliminary data imply that estrogen up-regulated TET1 and TET3 mRNA in HESC cells post 48 hours of treatment. TET1, 2 and 3 mRNA expressions were down-regulated in HESC cells while in HES cells, TET3 mRNA was up-regulated in response to 48 hours of estrogen and progesterone treatment.

Conclusion: Our data imply that TET1, 2, 3 are dynamically expressed in the endometrium during the menstrual cycle. Furthermore, cell specific regulation of TET enzymes by the steroid hormones is also apparent. Further studies are underway to explore the relationship between DNA methylation and hydroxymethylation and their role in gene regulation in human endometrium.

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ESRP1 plays a role in gonadal cancer progression by regulation of alternative splicing

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Introduction

Alternative splicing (AS) plays critical roles in controlling normal developmental programs. ESRP1 (Epithelial Splicing Regulatory Protein) is important cell-type specific regulator that affects splicing of various genes. To date, a considerable number of cancer-related genes have been shown to undergo splicing alterations during ovarian and testicular cancer progression. In this study, we have compared ESRP1 expression in human ovarian and testicular, normal and tumor tissues. In addition, potential splicing targets of ESRP1 have been examined in a seminoma-derived cell line (TCam-2).

Methods

We examined expression of ESRP1 and its putative targets, (*FGFR2* and *CD44*), in human ovarian and testicular normal and tumor tissues using droplet digital PCR. In addition, RNA interference was used to knockdown *Esrp1* expression in TCam-2 followed by transcriptional and splicing analysis using RNA-Seq.

Results

Our results showed upregulation of Esrp1 in both ovarian and testicular cancer and a correlation with switching from mesenchymal to epithelial isoforms of CD44 and FGFR2 in ovarian but not testicular cancers. RNA sequencing of Esrp1 depleted cells resulted in the identification of 576 novel potential splicing targets for ESRP1 in this germ cell tumour cell line. Also, splicing changes in some of these targets genes have been confirmed using RT-PCR. IPA analyses demonstrated ESRP1 regulates alternative splicing of genes that are involved in directing critical pathways involved in cell migration and morphology that occur during EMT. Our data also showed that four mitochondrial complexes of oxidative phosphorylation are affected by differential gene expression after silencing of ESRP1.

Conclusion

Overall, these data suggest that ESRP1 is involved in gonadal cancer progression by regulation of AS of numerous genes that are related to EMT. Furthermore, differential gene expression after silencing of ESRP1 suggests that ESRP1 expression in testicular germ cells may alter ATP production and affect energy metabolism of these cells.

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ZFP708 mediates transgenerational epigenetic inheritance

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Parental epigenomes are drastically modified shortly after fertilization. These modifications include remodeling of the histone landscape and global changes in histone post-translational modifications and DNA methylation. The reprogramming is vital and carefully regulated rather than a radical purge of germ-cell inherited epigenomes. TRIM28 and its binding partners have been shown to regionally oppose global reprogramming to preserve vital methylation marks, yet the DNA binding factors guiding this complex to specific targets are largely unknown. Here we uncover a novel, maternally expressed KRAB Zinc finger protein (ZFP708), which interacts with TRIM28 to ensure target-specific inheritance of DNA methylation from germline to soma.

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Expression of the neutral amino acid transporter B^0AT1 (SLC6A19) in the mouse preimplantation embryo

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Amino acid transporters play important roles in different human diseases. An inherited mutation of amino acid transporter B^0AT1 (SLC6A19) in humans leads to poor absorption of amino acids from the gut, or poor retention of amino acids by the kidneys, leading to serious symptomatic diseases such as Hartnup disorder and iminoglycinuria (Seow *et al* 2004). Here we show that the B^0AT1 amino acid transporter protein is expressed in mouse preimplantation embryos and the positive action of L-proline on early embryo development may rely on uptake of L-proline into the embryo by this transporter. Proline is present in mouse oviductal fluid *in vivo* (Guerin *et al*., 1995) and is accumulated in mouse embryos when added to culture media *in vitro (*Day Lab, unpublished*)*. Some of the L-proline accumulation in zygotes can be attributed to expression of the transporter SIT1, which actively transports L-proline into the embryo after fertilisation until the 2-cell stage (Anas *et al.*, 2008). L-proline uptake also involves at least one other unknown transporter, that is betaine resistant (Anas *et al*., 2008).

Here we found that uptake of radiolabelled L-proline by 8-cell stage embryos was reduced by the addition of excess unlabelled L-proline, L-leucine and L-glycine but not betaine, suggesting that L-proline, L-leucine and L-gylcine are transported into the embryo by the same transporter at this stage. In 8-cell stage embryos from SLC6A19-/- mice, radiolabelled L-proline uptake was decreased, compared to embryos from wild-type mice, and not significantly reduced by addition of excess unlabelled L-leucine and L-glycine, suggesting that the B^0AT1 transporter may be partly responsible for L-proline uptake in the 8-cell stage embryo. Further analysis of other embryo stages will determine the role of B^0AT1 in L-proline uptake and whether uptake by this transporter is required for the positive effect of L-proline on early embryo development.

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Sexually dimorphic requirement for FGFR2 expression in testicular and ovarian development

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In mice, male sex determination in XY gonads critically depends on sufficient SOX9 expression in the supporting cell population, which drives differentiation of Sertoli cells at ~11.5 days post coitum (dpc). This largely relies on FGF9-FGFR2 mediated repression of pro-ovarian signalling pathways such as WNT4/RSPO1 and FOXL2, which inhibit SOX9. Since Sertoli cell identity in adult testes is reversible and retains potential for trans-differentiation into ovarian granulosa cells, we first asked whether FGF9-FGFR2 signalling is involved in Sertoli cell maintenance throughout testicular development. To investigate this, *Fgfr2* was deleted in the Sertoli cells of XY gonads after the stage of sex determination at 12.5 dpc using $Fgfr2^{fl/fl}$mice mated to the *Amh*-Cre deleter line. Surprisingly, we found that testicular development, as well as spermatogenesis was normal in XY $Fgfr2^{fl/fl}$*; Amh*-Cre mice up to 5 months of age. To support this, examination of FGFR2 localisation in XX and XY gonads at 11.5-13.5 dpc revealed that after 11.5 dpc (where robust somatic and germ cell expression is observed), FGFR2 expression in 12.5-13.5 dpc XY gonads is almost absent in somatic cells and largely restricted to germ cells. In contrast 12.5-13.5 dpc XX gonads showed strong FGFR2 expression in somatic cells but minimal expression in germ cells. To investigate the unexplored role of FGFR2 in the developing ovary we then examined XX $Fgfr2c^{-/-}$ gonads. Although differentiation of FOXL2-positive granulosa cells was normal at all stages examined, MVH-positive germ cell numbers were severely reduced after 11.5 dpc and were often not detected at 15.5 dpc. Together, these results suggest that FGFR2 expression in Sertoli cells is dispensable for testicular development after the stage of sex determination at 11.5-12.0 dpc, while FGFR2 expression is required in the somatic cell population of the ovary beyond 12.5 dpc for germ cell maintenance.

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Levels of oocyte-secreted BMP15 are altered by maternal age and polycystic ovaries in infertile patients

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Repeated mating progressively expands population size and suppressive capacity of regulatory T cells to build pregnancy tolerance in mice

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Female regulatory T (Treg) cells are activated by seminal fluid factors following coitus. These activated Treg cells exert immune-suppressive actions which are essential to prevent immune rejection of the fetus. Reduced abundance and/or impaired function of Treg cells are evident in women experiencing pregnancy pathologies including recurrent miscarriage and preeclampsia. It is well established that long term sexual co-habitation and seminal fluid exposure, in a partner-specific manner, reduces the risk of pathologies associated with immune dysregulation such as preeclampsia and in utero growth restriction. Thus we hypothesised that repeated priming with seminal fluid can progressively expand the Treg cell pool. To test this female C57Bl/6 (B6) mice were mated once, twice or four times to B6 or Balb/C males. To allow the impact of seminal fluid to be delineated, mated female mice where administered the progesterone receptor antagonist RU486 to prevent pregnancy progression, before re-mating at least 7 days later. Uterus and para-aortic lymph nodes (PALN) were recovered at day 3.5 post-coitum after the final mating and Treg populations were assessed by flow cytometry, immunohistochemistry and in vitro suppression assays. Treg cell numbers in the uterus and PALN increased with each mating event. This expansion was dependent upon exposure to male seminal fluid factors, including paternal MHC antigen, as expansion was greater in allogeneic matings. Higher proportions of Treg cells isolated from mice mated four times expressed the proliferation marker Ki67 and suppression marker CTLA4, and had a greater capacity to suppress T cell responses as assessed by in vitro suppression assays. These data provide evidence that repeated seminal fluid contact acts to increase the number and suppressive capacity of the maternal Treg cell pool during early pregnancy. This data provides a mechanistic explanation for the link between duration of sexual co-habitation and protection from immune-based pregnancy complications in women.

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Seminal vesicle growth is stimulated by anti-leptin treatment in immature male mice

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Leptin originally thought to be derived predominantly from adipose tissue is now known to be almost ubiquitously expressed in many tissues. The complete absence of leptin is not developmentally lethal and results in early onset obesity, stunted skeletal and brain growth, extreme insulin resistance, hyperphagia, a compromised immune system and infertility. In this study we examine the effects of passive immunization on seminal vesicle weight, and testosterone in prepubertal mice.

Immature Swiss random bred male mice, aged three weeks, were randomly allocated into four treatments groups (n=12), and were given three subcutaneous (sc) injections (100 µl) every 48 h of the following treatments (A) non-immune Ig (50 µg) as the control group; (B) anti-leptin antibody (JMCK#16, 50 µg); (C) eCG (40 IU) with non-immune Ig (50 µg); (D) anti-leptin antibody (50 µg) with eCG (40 IU). On the morning of day 6 of treatment, animals were euthanized, blood was collected and seminal vesicles dissected out and weighed. Testosterone concentration was measured in the plasma.

No difference in final body weight was observed, however seminal vesicle weight was significantly increased by the treatments ($P<0.001$) with lowest in the controls (31.7±5.6mg) followed by anti-leptin (47.0±7.1mg), eCG (57.0±8.7mg) and eCG plus anti-leptin (57.8±7.0mg). Plasma testosterone concentrations were significantly ($P<0.01$) elevated in the eCG group (3.00±0.10ng/ml), the anti-leptin treatment had the lowest testosterone (0.24±0.09ng/ml) although not significantly different from the control (0.82±0.42ng/ml) or eCG plus anti-leptin groups (1.20±0.55ng/ml). These results suggest that while leptin inhibits testosterone production it may also have a direct suppressive effect on seminal vesicle maturation in the immature male that is independent of the effects of androgens. This supports our earlier pharmacokinetic work showing the seminal vesicles are a target for leptin.

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To investigate sperm flagellar beating pattern in CRISP deficient mice

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In order for successful fertilization, the sperm has to travel a daunting distance to reach the site of fertilization and need to have the mechanical force to penetrate the oocyte. During the journey of sperm, the sperm flagellar waveform transitions from a symmetrical, undulatory flagellar movement to a high-amplitude, asymmetric whip-like motion called hyperactivation. Sperm which cannot achieve hyperactivated motility are unable to fertilize an egg. Hyperactivated motility and flagellar waveform is regulated by Ca^{2+} influx into the sperm tail via CatSper, a sperm-specific ion channel. The Cysteine-RIch Secretory Proteins (CRISPs) are expressed in the mammalian male reproductive tract and have been shown have ion channel regulatory activity. Data from our lab, and another lab, has suggested that CRISPs may regulate CatSper function, and thus fertility, raising the possibility that CRISPs are regulators of sperm motility. To test this hypothesis, we chose to characterize the sperm flagellar beating pattern in *Crisp* deficient mice using a novel high-speed high-resolution microscopy technique and a custom-made MATLAB program which utilizes shape mode analysis to study the complex dynamics and pattern of beating sperm flagella. The analysis revealed that sperm from *Crisp1*$^{-/-}$ and *Crisp1/4*$^{-/-}$ mice have irregular and disrupted flagellar beating patterns when compared to wild-type sperm. Whereas, *Crisp2*$^{-/-}$mice have a distinct motility pattern referred to as 'stiff mid-piece syndrome'. We find significantly lower beat frequency in sperm from *Crisp1*$^{-/-}$ and *Crisp1/4*$^{-/-}$ mice than their respective controls. Moreover,

the beat frequency from *Crisp1*$^{-/-}$ and *Crisp1/4*$^{-/-}$ mice does not increase upon capacitation. Our data suggests that CRISPs are key regulators of sperm motility and that deficiency could lead to disrupted sperm flagellar beating pattern and infertility.

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Mouse beta-defensin 22 contributes to male reproductive function and fertility

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Molecular agents in seminal plasma and on sperm have the capacity to interact with the uterine epithelium to prepare the female reproductive tract for pregnancy in mice. Recent findings suggest that sperm-associated molecules ligate TLR4 on uterine epithelial cells to assist in this process. Human DEFB126 is a ligand of TLR4 that is present on the sperm glycocalyx, and males homozygous for a *DEFB126* frameshift mutation have reduced fertility. The aim of this study was to identify and explore how the murine orthologue of DEFB126, Defb22 contributes to this interaction to affect pregnancy success.

Defb22$^{-/-}$ mice were generated using CRISPR-CAS9 technology at the University of Adelaide SAGE Facility. *Defb22*$^{+/+}$and *Defb22*$^{-/-}$mice were bred from a *Defb22*$^{+/-}$breeding colony. Males were housed as studs from 8 weeks of age. Fertility of males was assessed by mating *Defb22*$^{+/+}$or *Defb22*$^{-/-}$ males with Balb/c females. On d17.5pc, viable and resorbing fetuses were counted then fetal and placental tissues were weighed (n=8 dams/group). At 20-22 weeks of age, a full post-mortem analysis of organ and reproductive parameters was conducted on the males (n=16/group).

Paternal Defb22 deficiency resulted in reduced pregnancy rates, fewer total viable pups per litter, and reduced fetal weights and fetal:placental weight ratios. At post-mortem, *Defb22*$^{-/-}$ males had reduced epididymal sperm ($p<0.01$), a reduced proportion of progressive motile sperm ($p<0.001$), and increased proportions of immotile sperm ($p<0.01$) compared to wildtype males. There was no difference in the weight of male reproductive organs between groups.

This study provides evidence that Defb22 deficiency affects male reproductive potential by reducing the number and motility of sperm, which impacts fertility. Current studies aim to identify how sperm-associated Defb22 affects immune reprogramming in the female reproductive tract, and its role in driving female TLR4 activation to contribute to the female response to seminal fluid.

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The fundamental role of both classical and backdoor pathways in male reproductive function

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Male development, fertility, and lifelong health are all androgen-dependent. Perturbed androgen action at any stage of life significantly impacts quality of life (1), and low androgens are an independent risk factor for all-cause early death (2). Thus, understanding the fundamental regulation of androgen production and action is essential if we are to support healthy ageing throughout life.

Androgens are synthesized by Leydig cells within the interstitial compartment. Testosterone can act (i) directly within the testis and local environment, and or (ii) indirectly, once secreted in the bloodstream, via the conversion into dihydrotestosterone (DHT). This route, **the classical pathway**, initiates masculinization and later promotes male fertility and health. Recently, an alternative metabolic route to DHT conversion, **the backdoor pathway**, was identified bypassing the testosterone step (3-4). However, how these pathways intersect both inside and outside of the testis, and the relative importance of each pathway for specific androgen-responsive endpoints during sexual development and throughout later life has not been dissected yet. Using different mouse models: a HSD17B3 knockout that results in classical pathway disruption, a SRD5A1 knockout that impairs the backdoor pathway, and a model inhibiting both pathways, along with cell culture systems and in vivo gene therapy, we are dissecting the roles and interactions between the classical and backdoor androgen pathways.

Our preliminary data shows that our understanding of the androgen signaling system is far from being fully comprehended. This highlights the importance of deciphering the fundamental role of both classical and backdoor pathways in male reproductive function and wider androgen-related conditions affecting male health, if we are to address many of the age-related comorbidities associated with perturbed androgen signaling.

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The impact of environmental insult on the small RNA profile of spermatozoa

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Inheritance of epigenetic information, which occurs independent of an individual's DNA sequence, is now a well-accepted paradigm. Among the potential mechanisms for relaying epigenetic information between generations, considerable attention

has been focused on small non-protein-coding RNA (sRNA). Indeed, there is growing recognition that the profile of sperm sRNA is altered in response to diverse environmental insults with significant post-fertilisation consequences, including altered behavioural and metabolic phenotypes in the offspring. While these data firmly implicate the male gamete as an important vehicle for epigenetic inheritance, the precise timing and mechanism(s) responsible for effecting changes in the sperm sRNA profile have yet to be established. To begin to address these questions, we have employed next generation sequencing strategies to determine the impact of three physiologically relevant environmental stressors; (i) elevated ambient temperature, (ii) radiofrequency electromagnetic energy (RF-EME) and (iii) acute acrylamide exposure on the sRNA profile of mature sperm. Our data has revealed a subset of sRNAs that are significantly altered (i.e. experience an increased or decreased accumulation) in the spermatozoa of male mice exposed to these environmental insults. Interestingly, exposure to RF-EME led predominantly to decreased accumulation of a subset of micro RNAs (miRNAs), while conversely exposure to acrylamide and elevated ambient temperature primarily resulted in an increased accumulation of miRNAs. Our data also demonstrated conservation in miRNA alterations in response to stress, with seven miRNAs differentially expressed in more than one of the insults investigated. We now aim to determine the mechanism(s) by which these sperm sRNA candidates are altered, with a particular focus on the role of epididymal extracellular vesicles, as well as the impact these sRNA changes have on embryo development and offspring health.

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What is the role of the testis in regulating epididymal activin levels?

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Activins are homodimeric cytokines that regulate testicular and epididymal function. Activin A and B are encoded by the inhibin β-subunit genes, *Inhba* and *Inhbb*, respectively. Both activins are preferentially expressed in the caput epididymis. Activin action is opposed by inhibin in the testis, and by follistatin in the vas deferens, but the factors that control epididymal activin production are unknown. The potential role of the testis in this regulation was investigated.

Mice lacking the inhibin α-subunit (*Inha*$^{-/-}$), which cannot produce inhibin, have increased intratesticular activin A (34-fold) and activin B (38-fold), and increased testicular expression of *Inhba* (750-fold) and *Inhbb* (7-fold); however, activin A and B protein and mRNA levels were not different from wildtype in the epididymides of the *Inha*$^{-/-}$ mice. These data suggest that the testis is not a significant source of epididymal activins, and that inhibin is not involved in their regulation.

The efferent ducts connecting the testis to the caput epididymis in 10 week old C57/Bl6 mice were ligated (EDL) bilaterally, or unilaterally. Efferent ducts were manipulated, but not ligated, on the contralateral side as controls. Tissues were collected 7 days post-surgery. The seminiferous epithelium was disrupted in ligated testes, but control testes appeared normal. *Inhba* and *Inhbb* were significantly increased in the caput of bilateral EDL mice (2- and 6-fold, respectively), but not in the ligated or control caput of unilateral EDL mice. The gene encoding indoleamine-2,3-dioxygenase (*Ido1*), an immunoregulatory gene postulated to be regulated by activins in the epididymis, increased 60-fold in the bilateral EDL epididymis, but not in unilateral EDL mice.

These data suggest that caput epididymal expression of *Inhba, Inhbb* and *Ido1* are not acutely regulated by sperm or testicular lumicrine secretions. However, it appears that the testis influences the expression of these genes via a systemic, possibly endocrine or immunological, mechanism.

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Evolution of sperm shape and size in mice and rats

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In mammals, sperm morphology varies greatly in size and shape across species. Most mice and rats have a distinctive sperm morphology with the head having a highly decurved apical hook into which the nucleus, acrosome and perforatorium extend and long tail with most Australian old endemic rodents have two further processes that extend from the sperm head upper concave surface, the ventral processes. Nevertheless a few species of mice and rats have evolved highly divergent sperm morphologies that lack both an apical hook and ventral processes as well as having considerably shorter tails. What is the functional significance of the sperm head apical hook, and ventral processes where they occur, and why have a few species evolved such highly divergent sperm morphologies? Here the hypothesis is tested that sperm head shape and tail length are sexually selected traits that are determined by differences in intensity of intermale sperm competition.

The results show that in species where the sperm head has an apical hook, large relative testes mass (RTM) invariably occurs. By contrast where the sperm head lacks this extension, a greatly reduced perforatorium, often a large acrosome, and low RTM, are present suggesting depressed levels of intermale sperm competition and hence a monogamous mating system. It is suggested that, high levels of intermale sperm competition select for a *s*treamlined sperm head with an apical hook and large perforatorium to facilitate egg coat penetration at fertilisation, whereas depressed levels of intermale sperm competition result in a less streamlined sperm head with reduced perforatorium but greater acrosomal enzyme release to aid in sperm passage through the zona pellucida at this time. It is concluded that sperm morphology, at least in the murine rodents, is determined in large part by post-copulatory sexual selection, and hence breeding system, of the species concerned.

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Mitochondrial functions during oocyte development: beyond ATP

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Measurement of mitochondrial distribution and membrane potential in oocytes

Usama Alzubaidi, John Carroll

Measurement of mitochondrial distribution and membrane potential in oocytes

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Mitochondrial ATP production plays a critical role during oocyte maturation and early embryo development. ATP production is generated through oxidative phosphorylation, a process that is critically dependent on the mitochondrial membrane potential ($\Delta\Psi m$). In oocytes and embryos, it has been reported that $\Delta\Psi m$ varies according to stage of oocyte maturation and mitochondrial location within the cell. However, these measurements are complicated by the properties of fluorescent indicators, JC1 and tetramethylrhodamine, methyl ester, (TMRM), and the changing distribution and concentration of mitochondria in different sub-cellular compartments during meiosis and mitosis. Here we have set out to characterize and establish new reliable methods to measure $\Delta\Psi m$ that allows accurate comparisons across different cells and regions of an individual cell.

We confirm that the commonly used ratiometric $\Delta\Psi m$ indicator, JC-1, typically demonstrates a cortical elevation of $\Delta\Psi m$ that is not evident with the single wave-length potentiometric indicator, TMRM. We show that J-aggregates are subject to concentration and time-dependent distribution, thereby explaining apparent sub-cellular differences in $\Delta\Psi m$. By ratioing fluorescence images or values from TMRM with those from a mitochondria-specific, $\Delta\Psi m$-insensitive fluorescent probe (such as mito-GFP or MitoTracker-Green), we show that $\Delta\Psi m$ is relatively homogeneous across the oocyte and early embryo. Furthermore, the ratiometric approach allows comparison of $\Delta\Psi m$ across different cells independent of time and concentration of mitochondria.

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TGFβ-1 Regulation of Extracellular Matrix Genes in Cultured Primary Cells from the Bovine Theca Interna

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The theca interna contains fibroblast-like mesenchymal cells, endothelial cells and immune cells [Endocr Rev. 1985 Summer;6(3):371-99]. It also contains steroidogenic cells that are responsible for androgen production, and as such the theca interna is different to the other ovarian stromal compartments of the ovary. TGFβ-1 is an important growth factor that regulates ovarian stromal compartments [Reprod Sci. 2014 Jan;21(1):20-31] and inhibits ovarian steroidogenesis [Endocrinology. 1990 Dec;127(6):2804-11]. The aim of our study was to determine the effects of TGFβ-1 on the regulation of various extracellular matrix (ECM) genes in bovine thecal cells. Thecal cells from small antral bovine follicles (4-6 mm) were cultured for 4 days and then treated with TGFβ-1 or SB431542 (TGFβ signalling inhibitor) for 2 days. The cells were then harvested for gene expression analyses. TGFβ-1 stimulated the expression of various genes involved in the regulation of collagen fibrillogenesis such as *POSTN* ($P < 0.001$), *COL1A1* ($P < 0.05$), *TNC* ($P < 0.05$), *FN* ($P < 0.0001$), *DCN* ($P < 0.01$), *FBN1* ($P < 0.01$) and *FBN2* ($P < 0.001$), but decreased *OGN* ($P < 0.05$) and *GPC3* ($P < 0.05$) expression. The expression of *LTBP3* and *BMP1* was not affected by TGFβ-1. Since the theca interna is a highly vascularised tissue, we also examined the expression of several ECM genes associated with subendothelial basal laminae. TGFβ-1 had no effect on *COL4A1*, *COL4A4* and *COL21A1* expression, but significantly reduced *LAMA4* ($P < 0.0001$), *FBLN1* ($P < 0.01$) and *FBLN5* ($P < 0.05$) expression. SB431542 treatment decreased *TNC* ($P < 0.05$) and *COL4A4* ($P < 0.01$) expression, suggesting that endogenous TGFβ signalling was active in our cultures. Our observations suggest that TGFβ-1 could affect the matrix associated with the vasculature and the rigidity of the theca interna, and hence is involved in follicular dynamics.

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Defining the epigenetic origins of maternally inherited disease

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Germ cells undergo the most extensive epigenetic reprogramming of any *in vivo* cell type, ultimately resulting in the establishment of specialized epigenetic information in oocytes and sperm. Some of this information is transmitted via the oocyte and sperm to the next generation, and disruption of this inherited epigenetic information can lead to developmental defects and disease in offspring. EED is an essential component of Polycomb Repressive Complex 2 (PRC2), which establishes the epigenetic modification H3K27me3 in all animals, including humans. We recently demonstrated that PRC2 and H3K27me3 regulate epigenetic programming in mouse germ cells, which is important for early embryo development. We have now

developed a model for deleting *Eed* only from growing oocytes in mice, providing a unique opportunity to the study epigenetic inheritance in genetically identical offspring. Consistent with this, loss of EED function severely reduces H3K27me3 and results in a highly significant, epigenetically transmitted, overgrowth phenotype that persists in offspring into adult life. Similar overgrowth occurs in Weaver syndrome patients who arise from oocytes or sperm that carry *de novo* germline mutations in *EED*. These patients have skeletal defects and intellectual disability consistent with known functions of PRC2 in bone and brain development. Preliminary data using DEXA demonstrates an increase in bone mineral density in combination with changes to fat and muscle content in the offspring derived from *Eed* deficient oocytes. We have also observed a significant decrease in litter size which suggests that loss of *Eed* in oocytes may affect female fertility. To further dissect the mechanism(s) responsible for the offspring phenotype we plan to determine the role of EED in oocyte programming, preimplantation and on postnatal development in offspring, with focus on bone and brain development. This work will provide greater understanding of the relationship between germline epigenetic programming and the developmental origins of disease.

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The Role of Heparan Sulfate in Regulating Growth Differentiation Factor-9 (GDF-9) Signalling During Oocyte Maturation

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Oocyte-secreted growth differentiation factor-9 (GDF9) plays critical roles in oocyte development by directing differentiation of the cumulus cell lineage from the granulosa cells to provide essential metabolic support for the oocyte. How oocyte-secreted GDF9 effects are restricted to cumulus cells is unknown. Recent reports show that GDF9 effects on cumulus cells can be disrupted with exogenous heparin. Heparan sulfate proteoglycans (HSPGs) are cell-surface glycosaminoglycans with a similar structure to heparin. We hypothesised that endogenous HSPGs sequester GDF9 at cumulus cell surfaces thereby restricting signalling during oocyte maturation. To explore this, we determined the temporal expression of heparan-sulfate (HS) synthesising enzymes during maturation of cumulus oocyte complexes (COCs). We found that *Ext1* was significantly induced by 3- and 6.04-fold at 4- and 16-h of *in vivo* maturation (IVV), respectively, when compared to immature COCs ($P<0.05$). However, *Ext1* was dysregulated during *in vitro* maturation (IVM) with significantly less *Ext1* transcript at 4- and 16-h of maturation compared to IVV. Similarly, *Ext2* was significantly reduced in IVM at 8- and 16-h of maturation compared to IVV ($P<0.05$). This was supported by a significant increase in sulphated glycosaminoglycan (including HS) during IVV with no increase observed during IVM. Lastly, we investigated the role of HS in determining oocyte quality during IVM using heparin and heparin+exogenous-GDF9. Heparin treatment significantly dampened cumulus expansion, which was restored by co-treatment with exogenous GDF9, as previously shown [1]. Interestingly, heparin treatment during IVM impaired oocyte quality with a 1.3-fold reduction in blastocysts following fertilisation, which was restored by exogenous GDF9 co-treatment during IVM. Although not significant with the limited sample size, this data indicates that HS trapping of GDF9 during maturation may be required for oocyte quality. Collectively, the data suggests that HSs play an essential role in GDF9-signalling during oocyte maturation.

1. Watson et al Endocrinology 2012

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Connecting the fetal and genetic origins of PCOS

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Polycystic Ovary Syndrome (PCOS) is a multifactorial reproductive and metabolic endocrine disorder with increased ovarian stroma. It is believed that PCOS has genetic and fetal origins. Recent genotyping studies have discovered susceptibility loci containing candidate genes for PCOS [1]. However, little is known about expression patterns and interactions during fetal ovarian development. This study investigated the expression of these 19 candidate genes and additional (*AMH*, *AR*, *TGFB1I1*) genes throughout gestation. Ovaries of bovine fetuses across gestation (n=27) and adults (n=5) were collected for gene expression analyses. Except *DENND1A.V2* and *SUMO1P1*, all genes were expressed in fetal and adult ovaries. During ovarian development, the expression of *GATA4, HMGA2, TOX3, DENND1A1.V1* and *FBN3* was initially high and decreased at about the end of the first trimester, whilst *FSHR, AMH, INSR, AR* and *TGFB1I1* increased from around the second trimester. Those genes were strongly correlated with gestational age. *LHCGR* expression was high in the first trimester, decreased to its lowest by 130 days of gestation, and then sharply increased until the end of gestation. The expression of the remaining genes was not correlated with gestational age. In the adult, most genes were expressed significantly lower compared to fetal developmental stages ($p<0.05$), except *LHCGR, FSHB* and *ERBB4*. *FBN3*, *HMGA2* and *TOX3* expression decreased significantly during follicle formation in the fetal ovary and were lowest in the adult ovary ($p<0.05$), whereas *AMH* and *FSHR* were expressed highly in the adult compared to the fetal ovary ($p<0.05$). Collectively, *GATA4, HMGA2, TOX3, LHCGR* (before 150 days) and *FBN3* were highly expressed and positively correlated with each other during early development when stroma first develops, while *FSHR, AMH, INSR, AR* and *TGFB1I1* were expressed during folliculogenesis and negatively correlated with the early expressed genes. Dysregulation of these genes during gestation might cause the PCOS phenotype later in life.

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Determining the impacts of epigenetic modifying drugs on the female germline

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Epigenetic modifications, including DNA methylation and histone modifications, regulate gene expression to facilitate the differentiation and maintenance of distinct cell lineages in multicellular organisms. Activity of the enzymes that mediate epigenetic modifications can be influenced by environmental factors such as drugs or diet. Polycomb Repressive Complex 2 (PRC2) is an essential epigenetic modifier that catalyses histone 3 lysine 27 trimethylation (H3K27me3) at developmental genes in many tissues, including the germline. Germ cells give rise to mature sperm and oocytes that transmit genetic and epigenetic information to offspring. Integrity of this information is critical as alterations in the germline epigenome can affect offspring development and health. Common dysregulation of epigenetic modifications in cancers has driven the development of drugs that inhibit epigenetic enzymes. For example, Tazemetostat inhibits EZH2, the catalytic component of PRC2, and is currently in phase I/II trials for treatment of tumours, including in patients of reproductive age. While epigenetic drugs have great therapeutic potential, they act systemically and may detrimentally affect the germline epigenome. Using genetic models that lack PRC2 function in the oocyte, we are examining how H3K27me3 establishment is regulated in the growing oocyte and determining how Tazemetostat depletion of H3K27me3 affects oocyte maturation, and offspring growth and development. We demonstrate that oocytes are enriched with H3K27me3 during their growth and that genetic deletion of PRC2 activity alters offspring growth and development. Consistent with this, *de novo* germline mutations result in Weaver or Cohen-Gibson Syndromes in humans, characterised by overgrowth, skeletal abnormalities and learning deficits. Moreover, treatment of adult female mice with Tazemetostat severely depleted H3K27me3 in mature oocytes, indicating that this drug will detrimentally affect offspring. This work aims to determine the impacts of epigenomic drugs on the germline epigenome and offspring in mice, and ultimately improve clinical guidelines for these drugs.

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Visualisation of oocyte mitochondria and transzonal projections using 3d quantification methods

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Morphological markers associated with oocyte metabolism such as the motile mitochondria population and the filamentous transzonal projections (TZPs), that connect the oocyte to its attendant cumulus cells, have been traditionally studied by 2D imaging methods that miss most of the detail within the 3-dimensional oocyte.

We have developed a 3D quantification procedure using fluorescent labelling of live cultured oocytes with both Tetramethylrhodamine (TMRM) and Mitotracker Green to map active and total mitochondrial populations respectively, or with the Sir-Actin stain to visualise the TZPs. High resolution 3D modelling/visualisation of Z-stack confocal images using Imaris software allowed each fluorescent point to be tracked and analysed in a 3D space and quantitative comparison of details of fluorescent signal position and intensity. This novel method for visualising oocyte metabolic morphology was tested with a simple oocyte stress model. GV and MII stage cumulus oocyte complexes were subjected to either control culture conditions or 50uM H_2O_2 for 30min followed by fluorescent staining. As expected H_2O_2 treatment reduced the volumetric ratio of active compared to inactive mitochondria of 0.638 and 0.338 between control and H_2O_2 treated oocytes respectively. We analysed the spatial arrangement of 'active' mitochondria in relation to the meiotic spindle and each other. On average 4000 individual TMRM fluorescent points per oocyte were clustered predominantly between 10-15um from the meiotic spindle and 1.29um from the nearest fluorescent spot. For TZPs, the mean control oocyte contained 1445±647 filaments, on average 3.57um long whereas H_2O_2 treated oocyte had 725±396 filaments, averaging 3.06um long, though these differences were non-significant. In conclusion the short H_2O_2 treatment had a limited effect however this proof of concept experiment demonstrates that 3D quantification techniques can allow a new level of investigation into subcellular features providing a tool for greater understanding of conditions that impact oocyte morphology and competence.

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The roles of TRAK2 and Miro in microtubule-mediated mitochondria dynamics in mammalian oocytes

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Oocytes have individual mitochondria that undergo a dramatic reorganization as the oocyte progresses through meiosis from the Germinal Vesicle (GV) stage to metaphase II(MII). From a relatively homogeneous cytoplasmic distribution at the GV stage, mitochondria aggregate around the developing meiotic spindle, and then migrate to the cortex with the spindle. After polar body extrusion, as oocytes progress to MII, mitochondria are dramatically and rapidly redistributed throughout the cytoplasm.

Mitochondria trafficking in mammalian cells generally occurs along microtubule tracks via motor proteins, dynein, and kinesin. Recent studies in neurons have led to the discovery of mitochondrial adaptor proteins, TRAK1/2 and Miro. We hypothesize that the dynamics of mitochondrial localization during meiosis is controlled by TRAK and Miro. By using mutant variants of these proteins, microinjection, live-cell imaging and quantitative microscopy approaches, we study the role of Trak2 and Miro in mitochondrial trafficking in mouse oocytes. Immunofluorescence reveals TRAK2 and Miro are localized to mitochondria at all stages of oocyte maturation. Over-expression of TRAK2 caused a dramatic increase in mitochondrial aggregation at all stages and very few mitochondria remained distant to the GV or spindle associated cluster of mitochondria. Over-expression of fluorescently tagged TRAK2 deletion mutants lacking the kinesin-binding domain (TRAK2KDM) had a negligible effect on the GV stage but led to an increased accumulation around the spindle, presumably due to reduced kinesin-mediated trafficking to microtubule located +ends in the oocyte cytoplasm. These results suggest that microtubule dynamics in oocytes are regulated in a cell cycle specific manner and involve the adaptor proteins TRAK2 and Miro.

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Loss of PUMA results in elevated primordial follicle reserves in adult mice and delays ovarian ageing

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More than two thirds of the germ cells produced during female embryonic development undergo apoptosis shortly after they are generated. This leaves a reduced number of oocytes within the ovary at birth, and, because new germ cells cannot be made after this point, this limits female fertility and reproductive lifespan. Despite the critical role of apoptosis in controlling the number of available oocytes, the proteins involved have not been fully characterised. Our previous study (Myers et al. Reproduction 2014; 148:211-219) showed that targeted disruption of the Bbc3 gene encoding PUMA (p53 upregulated modulator of apoptosis), a pro-apoptotic protein belonging to the BH3-only subgroup of the BCL-2 family, resulted in an increased number of primordial follicles in the mouse ovary on postnatal day (PN) 10. In this study, we further investigated the role of PUMA in maintaining the primordial follicle pool during puberty and adulthood. Stereological analyses of ovaries showed that *Puma*$^{-/-}$ mice had significantly more primordial follicles than WT control animals during the transition through puberty (PN20, 30, 40), and females reached sexual maturity with more than twice the number of follicles in their ovarian reserve (PN50:WT 2850±202 vs *Puma*$^{-/-}$ 6651±307, P<0.0001). Follicle numbers remained elevated during adulthood (PN60, 100 and 200) in *Puma*$^{-/-}$ mice compared to controls. Furthermore, *Puma*$^{-/-}$ mice had dramatically increased follicle numbers at PN300, an age when WT mice are nearing the end of their reproductive lifespan because their follicle reserves are significantly depleted (WT 485±198 vs *Puma*$^{-/-}$ 2257±787, P<0.05). Interestingly, however, preliminary studies suggest that elevated follicle numbers do not confer prolonged fertility in *Puma*$^{-/-}$ females, possibly due to other reproductive deficits. These findings reveal an important role for PUMA in determining the number of primordial follicles established in the initial ovarian reserve and then subsequently maintained in the ovary throughout reproductive life.

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The potential role of haemoglobin in sequestering menadione-induced ROS during *in vitro* maturation of mouse cumulus-oocyte complexes

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Reactive oxygen species (ROS) are known to have detrimental effects across many stages of early development, including oocyte maturation and embryo implantation, with downstream health impacts such as pre-eclampsia and birth defects. This is especially pertinent when gametes and embryos undergo artificial reproductive technologies (ART), as the *in vitro* environment may lack the endogenous *in vivo* mechanisms to quench ROS. Various strategies have attempted to quench ROS during *in vitro* culture through the use of antioxidants, including L-carnitine and glutathione. We have recently shown that haemoglobin (Hb) is present at high levels in oocytes and embryos developed *in vivo*, but absent from those derived from *in vitro* culture. Hb is a gas-binding protein commonly found in red blood cells and acts to decrease oxidative stress in other cell types, namely hepatocytes, brain and retina cells, where it binds ROS such as hydrogen peroxide and other superoxides. This raises the possibility that Hb acts as an antioxidant in the oocyte and embryo and that this antioxidant system is lacking during *in vitro* culture. Whether Hb acts as an effective antioxidant during the *in vitro* maturation (IVM) of cumulus-oocyte complexes (COCs) has not been determined. We first established a model of increased ROS during IVM through the addition of menadione (100uM), as demonstrated by increased CellROX Deep Red fluorescence compared to control. High concentrations of menadione (400 mM) during IVM was detrimental to oocyte quality with a significant 83% and 60% reduction in fertilisation and blastocyst rate respectively. In the presence of high levels of menadione during IVM, the addition of Hb could not restore the negative impact on oocyte quality. Experiments are now being pursued to evaluate if Hb has an antioxidant mechanism in COCs under a range of menadione concentrations.

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Oxidative damage is exacerbated by dysregulation of proteasomal activity in naturally aged oocytes

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An increase in oxidative protein damage is a leading contributor to age-associated decline in oocyte quality. Through the removal of such damaged and / or dysfunctional proteins, the proteasome plays an essential role in maintaining the fidelity of oocyte meiosis. Our study reveals a decrease in proteasome activity in the naturally aged germinal vesicle (GV) mouse oocyte that is positively correlated with increased protein modification by the lipid aldehyde, 4-hydroxynonenal (4-HNE). This highly reactive lipid aldehyde, generated as a by-product of lipid peroxidation cascades, can covalently adduct and dysregulate the function of key oocyte proteins including α-tubulin, a structural component of the oocyte's meiotic spindle; thus, contributing to a reduction in the integrity of oocyte meiosis. Further, we have shown that inhibition of proteasome activity (MG132; 50 μM) in oxidatively stressed GV oocytes obtained from young animals leads to an attendant increase in the accumulation of 4-HNE-modified proteins, including α-tubulin. Among alternative protein targets for 4-HNE adduction, we identified several subunits of the proteasome as being particularly vulnerable. Accordingly, proteasomal activity was also significantly attenuated in a dose-dependent manner in young oocytes exposed to an acute oxidative insult (H_2O_2 or 4-HNE). Notably, the inclusion of the antioxidant, penicillamine, to limit propagation of oxidative stress cascades led to a complete recovery of proteasome activity and enhanced clearance of 4-HNE adducted α-tubulin during a 6 h post-H_2O_2 treatment recovery period. This strategy also proved effective in reducing the incidence of oxidative stress-induced aneuploidy following *in vitro* oocyte maturation. Taken together, our data implicates proteasome dysfunction as an important factor in the accumulation of oxidatively induced damage in the female germline.

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Nicotinamide enhances the developmental competence of porcine oocytes matured in vitro

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Sirtuin activity has previously been implicated in cellular aging and oocyte quality. Nicotinamide (NAM) treatments have been shown to elevate the level of nicotinamide adenine dinucleotide (NAD+), a key cellular metabolite that modulates Sirtuin activity in the cell. Resveratrol is a well-recognised activator of Sirtuin proteins, and both NAM and resveratrol treatments have been shown to improve oocyte quality in mice[1,2]. The aim of this study was to examine the effects of NAM and resveratrol on the in vitro maturation (IVM) of porcine oocytes. Oocytes were matured over 44 h in a defined IVM system and subjected to treatment with NAM (5 mM) and resveratrol (2 μM) alone and in combination. Mature oocytes were chemically activated with ionomycin and the presumptive zygotes were cultured for 7 d. Cleavage and development to the blastocyst stage were assessed and total blastocyst cell numbers were determined. Nuclear maturation rates did not differ among the groups ($P > 0.05$). The rate of cleavage for oocytes matured in the presence of NAM alone was greater than that for untreated oocytes (84.7 ± 4.0% vs 42.8 ± 11.0%; $P < 0.05$). Also, treatment with NAM alone significantly increased the mean blastocyst cell number compared with the other treatment groups (88.4 ± 9.0 cells vs 51.3 ± 5.5 to 65.6 ± 5.6 cells; $P < 0.05$). In contrast, treatment with resveratrol, either alone or in combination with NAM, did not improve embryo development compared with the untreated control group ($P > 0.05$). These results indicate that the positive effects of NAM on oocyte maturation were diminished in the presence of resveratrol. Further studies are needed to determine whether this treatment interaction involves the modulation of Sirtuin activity or other biochemical processes that influence oocyte quality.

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2. 2. Liu M-J, Sun A-G, Zhao S-G, Liu H, Ma S-Y, Li M, Huai Y-X, Zhao H, Liu H-B (2018) Resveratrol improves in vitro maturation of oocytes in aged mice and humans. Fertil. Steril. 109(5):900-907.

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Dairy cows with high genetic merit for fertility produce better quality oocytes than those with low genetic merit for fertility

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We hypothesised that the superior reproductive performance in cows with high genetic merit for fertility is, in part, explained by a better follicular environment resulting in high quality oocytes. To test this, trans-vaginal ovum-pickup was used to collect preovulatory cumulus-oocyte complexes and follicular fluid from spring-calving primiparous Holstein-Friesian dairy cows with either low (n = 23) or high (n = 26) genetic merit for fertility. Only cows in their second or third oestrus were sampled, (62 ± 17 days postpartum). Multiplex quantitative PCR was used to measure the expression of gene markers of oocyte quality in the cumulus cell masses and oocytes. High performance liquid chromatography was used to measure the concentration of amino acids, steroids, and metabolites in the follicular fluid and plasma. Compared with low fertility cows, oocytes from high fertility cows had higher expression of gene markers for good oocyte quality (*HAS2, VCAN, PDE8A*, $P < 0.05$). The follicular environment of these cows was also markedly different, with lower concentrations of serine, proline, methionine, and isoleucine than cows with low genetic fertility ($P < 0.05$). High fertility cows also tended to have lower ($P = 0.053$) follicular fluid concentrations of non-esterified fatty acids than cows with low genetic fertility. Plasma concentrations of asparagine, alanine, proline, tyrosine, methionine, and phenylalanine were lower in high fertility cows than low fertility cows. High non-esterified fatty

acids concentrations are reportedly harmful to the developing oocyte, suggesting the follicular fluid composition of low fertility cows may be detrimental to oocyte quality. These differences in gene expression and follicular fluid constituents are consistent with cows with high genetic merit for fertility producing higher quality oocytes than cows with low genetic merit for fertility and this may be related to an altered follicular environment.

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A modified form of human GDF9 with cumulin-like Smad-2/3 activity

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Human growth differentiation factor-9 (GDF9) is expressed almost exclusively by oocytes, but is secreted in a latent form. Recent studies have indicated that human GDF9 is activated by forming a heterodimer (called cumulin) with the closely related molecule bone morphogenetic protein-15 (BMP15). Cumulin added to *in vitro* maturation (IVM) media of immature porcine or human oocytes markedly increases subsequent embryo yield, identifying this molecule as an attractive therapy for the treatment of female infertility. However, the heterodimeric nature of cumulin makes mass production difficult and prone to batch-to-batch variability in potency. To address this, we sought to generate an engineered form of GDF9 with 'cumulin-like' ability to activate the Smad-2/3 transcription pathway. BMP15 residues (Arg363, Gly366, His369), predicted to activate GDF9 within the cumulin heterodimer, were introduced into the GDF9 homodimer. Collectively, the introduced mutations were non-disruptive for GDF9 assembly and secretion. Excitingly, the modified GDF9 form was capable of inducing Smad-2/3 activity in human granulosa COV434 cells, with greater potency than cumulin. Replacement experiments indicated that all three residues are required for maximal activation of the Smad-2/3 pathway. Ongoing studies will determine if, like cumulin, our modified GDF9 can improve oocyte quality and blastocyst production.

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Incubation of cumulus-oocytes-complexes (COCs) from unstimulated mice with C-type natriuretic peptide (CNP) prior to *in vitro*maturation (IVM) did not improve outcomes compared to IVM alone

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Nuclear and cytoplasmic synchrony is important during oocyte maturation within antral follicles. CNP binds with its receptor, supporting high cyclic-adenosine-monophosphate (cAMP) levels thus holding the oocyte in meiotic arrest. COCs are removed *in vitro*, leading to a decrease of cAMP causing spontaneous nuclear maturation and an asynchrony with the oocytes' cytoplasmic maturation. Adding CNP to pre-IVM media has the capacity to maintain cAMP levels in the oocytes, emulating *in vivo* conditions and improving IVM outcomes.

COCs (N=908) were matured either directly in IVM media for 18h or incubated with 25, 50,100,150 and 200nM CNP for 4 or 24h prior to IVM. After IVM, the COCs were denuded and assessed for germinal vesicle breakdown (GVBD) and some were inseminated to develop embryos.

The 25, 50, 100, 150 and 200nM CNP maintained meiotic arrest after 4h (75%, 87.5%, 89.7%, 97.3%, 100%) and 24h pre-incubation (37.8%, 55.3%, 54%, 87.8%). Maturation rates did not differ ($P<0.05$) between groups except that 24h pre-incubation with 25nM CNP gave significantly ($P>0.05$) lower rates (53.1%, $P<0.05$) than conventional IVM (75.3%). Two-cell per MII in the conventional IVM was 54.8% while at 4h was 43.3%, 55.9%, 43.7%, 37%, 37.8%, respectively; and 24h was 23.5%, 46.7%, 56.7%, 52.6% and 58.8%, respectively. Blastocyst rate per two-cell in the conventional IVM was 67.5% while at 4h was 76.9%, 52.6%, 71.4%, 70% and 71.4%; and at 24h was 50%, 50%, 38.1%, 40% and 50%. The two-cell and blastocyst rates did not differ between groups ($P<0.05$).

These results did not align with previous studies, but subtle variations in protocols, even within the same species, would suggest caution is required before clinical translation.

This study suggested that when conventional IVM works well, small differences in cytoplasmic and nuclear synchrony may not have a significant impact overall, using unstimulated animals.

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Does exposure to ionising radiation damage mitochondria within oocytes?

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Anticancer treatments can damage oocytes and deplete the ovarian reserve, leading to premature ovarian failure and infertility. To devise new strategies to improve the long-term fertility and health of women post-treatment, it is necessary to understand the mechanisms by which treatments inflict this damage. Recent studies have largely focused on the ability of radiation therapy

to damage oocyte nuclear DNA. However, the treatment may also damage intracellular organelles, such as mitochondria. We are investigating the hypothesis that radiation causes damage to mitochondria in oocytes, thereby contributing to depletion of the ovarian reserve and loss of oocyte quality. In this study, neonatal C57Bl6 female mice were untreated (controls) or subjected to whole body γ-irradiation (0.1Gy), and small (diameter≈20μm) and growing (diameter≈50μm) oocytes were collected 3 and 6 hours later. Staining with TMRM revealed a decrease in mitochondrial membrane potential in both small and growing oocytes after γ-irradiation, indicating that mitochondria were damaged. However, MitoTracker staining indicated that mitochondrial localization and abundance was not immediately affected by irradiation. Interestingly, mtDNA copy number was increased in growing oocytes after γ-irradiation compared to controls ($p<0.05$), which may be a compensatory mechanism to ensure the maintenance of mitochondrial function. When mice were superovulated more than 40 days after γ-irradiation, there was a significant reduction in the number of oocytes harvested compared to controls (0.1Gy 4±1/Control 18±1, n=16/6 mice, $p<0.0001$), though mitochondrial localization and membrane potential was similar between groups. As these irradiated mice are able to produce healthy pups, these preliminary/initial results indicate that oocytes that survive γ-irradiation and develop through to ovulation contain healthy mitochondria. Overall, if these studies indicate that anticancer treatment-induced mitochondrial damage contributes to depletion of the ovarian reserve or loss of oocyte quality, then protection of mitochondria may represent a novel strategy for alleviating anticancer treatments-mediated insult to the ovary.

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The dynamics of mitochondrial organisation during oocyte maturation

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Publish consent withheld

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Cyclin A2 is necessary for the oocyte to embryo transition in mice

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In mitosis, cyclin A2 is destroyed in prometaphase and is necessary for stable progression through mitosis. However, in female meiosis cyclin A2 persists during metaphase of meiosis II where it regulates microtubule stability, allows normal MII spindle formation, and prevents merotelic attachments and lagging chromosomes on MII exit. There remains little known about the role and regulation of cyclin A2 during the transition from meiosis to mitosis in mammals. We developed a conditional knockout mouse model by crossing cyclin A2 floxed mice with *Zp3 Cre* mice, and investigated the functions of cyclin A2 in the transition from egg to embryo. Our studies with *cyclin A2*$^{-/-}$oocytes showed delays and abnormalities in proncleus formation, changes in activity of Cdk1 and levels of p-Plk1. After parthenogenetic activation there was an increase in abnormalities during early development and a reduced rate of progression to the blastocyst stage. These abnormalities underlie an overall reduction in fecundity of *cyclin A2*$^{-/-}$ mice. These results demonstrate a role of cyclin A2 in the oocyte-embryo transition.

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The effect of absent kisspeptin signalling on locomotor activity in mice.

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Kisspeptin regulates reproduction by activating gonadotrophin-releasing hormone (GnRH) neurons through its receptor, Kiss1r. Previous evidence shows that the neuropeptide kisspeptin and Kiss1r have an important, previously-uncharacterised role in metabolic status, energy balance, and glucose homeostasis. Specifically, infertile kisspeptin receptor (Kiss1r) knockout (KO) mice have reduced energy expenditure. This important discovery advances our understanding of the interconnections between fertility, obesity and energy balance with important etiologic and therapeutic implications. For this study, we used Kiss1rKO and wild-type (WT) male mice to investigate the relationship between absent kisspeptin signalling and locomotor behaviour by allowing mice free access to running wheels. We examined the real-time characteristics of wheel running activity in mice over a 3-week period and its flow on effects on body weight. We used Lafayette Mouse Activity Wheel Chambers (23.5 cm × 35.3 cm; Model 80820; Lafayette Instrument, IN, USA) equipped with a 12.7 cm diameter exercise wheel with a 5.72 cm wide running surface (Model 80820RW, Lafayette). Each chamber was equipped to constantly record distance travelled (0.40 m/revolution) and speed (m/min). The Activity Wheel Monitoring (AWM) Software (Model 86065) was used to record all data sets. Our data show an 82% reduction in total distance travelled per 24 h in KO mice compared to WTs (WT, 4804 ± 632 m; KO, 871 ± 270 m; $P<0.01$). Moreover, the circadian pattern of wheel running activity (dark phase activity) clearly present in WT mice was severely diminished in KO mice. These experiments are ongoing but suggest that absent of kisspeptin signalling may act as a regulator of voluntary activity and patterns of locomotion behaviour, potentially involving circadian rhythm.

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Kisspeptin (Kp) and RFamide-related peptide 3 (RFRP-3) neurons in the hypothalamus of dairy cattle; expression of oestrogen receptor alpha (ERα), and inputs to gonadotrophin releasing hormone (GnRH) neurons.

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Fertility of high producing dairy cattle is very low, most likely related to the extreme metabolic demands associated with high milk production. To address this issue, a better understanding of the brain control of reproduction in dairy cattle is needed. In cattle, Kp treatment stimulates gonadotrophin secretion, while RFRP-3 inhibits it. Conversely, RFRP-3 mRNA levels are stimulated by reduced feed intake. We have used immunohistochemistry to map the distribution of Kp and RFRP-3 neurons in the hypothalamus of dairy cattle, determined their co-expression with ERα, and whether they make apparent contact with GnRH neurons. The heads of male and female calves (n=3 each), steers, heifers and lactating cows (n=2/group) were perfusion fixed (4% paraformaldehyde) and the brains dissected out and frozen. Dual label immunohistochemistry for Kp or RFRP-3 with ERα or GnRH was performed on 40µm cryostat sections, using fluorescently labelled secondary antibodies. Kp neurons were located primarily in the arcuate nucleus, with some cells in the preoptic area, although few were observed in the calves. Most Kp neurons in the adult animals of both sexes, but few in the calves, expressed ERα. We were unable to determine whether Kp neurons contact GnRH neurons. RFRP-3 neurons were localised in the dorsomedial hypothalamus and paraventricular nucleus, with fewer cells observed in the cows than the heifers & steers. ERα was not expressed in this region. Fewer than 20% of GnRH neurons received close contact from RFRP-3 neurons, with a few RFRP-3 neurons receiving apparent contact from GnRH fibres. These data show that Kp and RFRP-3 neurons in dairy cattle are neuroanatomically like other known species, but further studies are required to clarify their physiological role.

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The effect of dietary protein content on reproductive physiology in mice

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It is known that the amount of protein in the diet can affect reproductive performance in many species. However, the mechanism of action is not entirely clear. In this study we compared the effect of feeding mice a low (8%) or high (35%) protein mouse chow to mice fed standard (22%) mouse chow on the ovary, uterus, and pregnancy outcomes. Energy and fat content were identical in all three diets. Adult virgin female mice (20 per group) were fed one of the three diets for 6 weeks and then 10 mice per group were euthanized on the day of oestrus as determined by vaginal lavage. The ovaries and uterus were collected and weighed. The remaining mice were then joined with a male mouse (previously fed on standard mouse chow) for two weeks and examined twice daily for vaginal plugs to determine conception day and then allowed to complete the pregnancy.

The mean ovarian weights at oestrus from mice fed the normal (43.8±4.2mg) and high (47.3±4.9mg) protein diets were not significantly different but significantly ($P<0.02$) heavier than those on low (29.2±4.1mg) protein diet. The oestrus uterine weight from the low protein diet (89.4±23.1mg) was significantly lower than those from the normal (169.0±24.3mg) and high (137.2±10.6mg) protein diet mice. However, the pregnancy rates were lower in both the low protein (70%) and high protein (80%) than the normal diet (90%).

In conclusion low protein diets result in low ovarian weights at oestrus which may reflect reduced follicular development and subsequent corpus luteum number and consequently lower steroid production reflected in the lower uterine weight. Although high protein did not affect either the ovarian and uterine weights the pregnancy success rate was similar to that of low protein diets. We are currently examining the endocrine and histological differences between the groups.

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Characterisation of the glucocorticoid receptor isoform expression profile in the rat placenta and fetal liver in pregnancies exposed to periconceptional alcohol

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Prenatal alcohol exposure alters the maternal hypothalamic-pituitary-adrenal-axis, elevating concentrations of glucocorticoids. Glucocorticoids exert their effects via binding to the glucocorticoid receptor (GR), for which there are a number of isoforms that regulate specific downstream signaling pathways. This study aimed to characterise the GR isoform profile in the placenta and fetal liver in the rat following periconceptional alcohol exposure (PCE).

Sprague-Dawley dams were given 12.5% ethanol (PCE) or 0% ethanol (control) liquid diet from 4 days prior to 4 days after conception (6-9 litters/group). At embryonic day 10 (E10), dams were given standard chow. Late gestation (E20) fetuses and placentas were collected and measured/weighed. All fetuses/placentas were sexed. Placentas were separated into labyrinth and junctional zones for molecular analysis and fetal livers collected (only one of each sex/litter used for each analysis). Tissues were snap-frozen for analysis of GR isoforms by western blot and densitometry. Cytoplasmic and nuclear protein fractions were isolated at extraction and were analysed separately.

Multiple GR isoforms were identified within the fetal liver (12 isoforms, including GRα, GR-P, GRα-D1-D3) while a different profile of isoforms was identified in the placenta (13 isoforms, including GRα-A, and GRα-C). We also identified previously

uncharacterised GR-positive bands in both tissues. Isoform profiles varied by cellular compartment and, in the placenta, by zone. PCE induced changes in the GR isoform profile in each tissue in a sex-specific manner, with females showing alterations in the placental labyrinth and males showing alterations in cytoplasmic liver fractions. Specific isoforms were also significantly correlated with fetal, liver and placental growth.

These results have identified, for the first time, GR isoforms in the rat placenta and fetal liver, and suggest that PCE alters the GR isoform profiles in these tissues. This data provides a possible mechanism for glucocorticoid-mediated programming of offspring metabolic disease following prenatal alcohol exposure.

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Cardiovascular risk factors in offspring of preeclamptic pregnancies – systematic review and meta-analysis

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Background: Preeclampsia is a pregnancy complication that affects 2-5% of pregnancies and is a leading case of maternal and infant morbidity and mortality. Women who develop preeclampsia are at approximately twice the risk of developing cardiovascular disease (CVD) in later life. Emerging evidence also demonstrates that children born of preeclamptic pregnancies may also be at increased risk of CVD in adulthood. We aimed to examine evidence for increased CVD risk factors in children exposed to preeclampsia *in utero*.

Methods: We performed a systematic review and meta-analysis on studies reporting cardiovascular risk factors among offspring of preeclamptic pregnancies compared to offspring of non preeclamptic pregnancies. The following electronic databases were searched: PubMed, CINAHL, the Cochrane Library and EMBASE with an end of search date of December 01, 2017. Information was extracted on established CVD risk factors including blood pressure, lipid profile, blood glucose, fasting insulin, body mass index (BMI) and endothelial/microvascular function. The review protocol is registered in PROSPERO (CRD42017074322)

Results: Thirty six studies provided cumulated data on 53,029 individuals. *In utero* exposure to preeclampsia was associated with 5.17 mmHg (95% CI: 1.60 to 8.73) higher systolic and 4.06 mmHg (95% CI: 0.67 to 7.44) higher diastolic blood pressure and 0.36 kg/m^2 (95% CI: 0.04 to 0.68) higher BMI, during childhood or young adulthood. Blood glucose, lipids and insulin levels were not significantly different between the two groups.

Conclusion: Offspring of preeclamptic pregnancies demonstrate risk factors for CVD during childhood and young adult life. Early screening of children born after preeclamptic pregnancies may identify those that require interventions or preventive strategies to reduce the risk of CVD in later life.

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Cardiovascular risk factors in those born preterm – systematic review and meta-analysis

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Background: Spontaneous preterm birth complicates approximately 5-8% of all pregnancies and is a leading cause of infant morbidity and mortality. Emerging research demonstrates that children born preterm may be at increased risk of cardiovascular disease (CVD) in adult life. We aimed to examine evidence for increased CVD risk factors among children and young adults born preterm.

Methods: We performed a systematic review and meta-analysis on studies reporting cardiovascular risk factors among those born preterm (< 37 weeks gestation) compared to those born at term (≥ 37 weeks gestation). The following electronic databases were searched: PubMed, CINAHL, the Cochrane Library and EMBASE with an end of search date of May 01, 2018. Information was extracted on established CVD risk factors including blood pressure, lipid profile, blood glucose, fasting insulin, body mass index (BMI) and endothelial/microvascular function. The review protocol is registered in PROSPERO (CRD42018095005).

Results: Thirty nine studies provided cumulated data on 892,024 individuals. Those born preterm had 3.45 mmHg (95% CI: 2.31 to 4.58) higher systolic and 1.49 mmHg (95% CI: 0.79 to 2.19) higher diastolic blood pressure and 0.26 mmol/l (95% CI: 0.01 to 0.50) higher total cholesterol compared to those born at term.

Conclusion: Risk factors for CVD are evident during childhood and early adulthood among those born preterm. Early screening of children born preterm may identify those at risk who may benefit from interventions targeted at improving lifestyle factors to reduce the risk for CVD in adult life.

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Maternal corticosterone exposure in pregnant mice alters the expression of placental oxidative stress, antioxidant enzymes and mitochondrial content in a sex specific manner.

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Many of our most debilitating chronic diseases are caused in part by events that occur before birth. Maternal health is essential for maintaining a healthy pregnancy and optimal birth outcomes. The placenta is an essential organ for pregnancy as it forms the interface between the mother and baby. It is required for nutrient delivery, oxygen transfer and waste removal from the foetus. Maternal stress during pregnancy can impair placental development and foetal growth, which can contribute to the development of chronic disease within offspring in adulthood. Maternal stress increases concentrations of stress hormones, known as glucocorticoids (GCs). GCs hinder placental development, although the mechanisms remain unknown. This study aimed to investigate the role of oxidative stress and antioxidants in glucocorticoid induced placental dysfunction.

Pregnant C57bl6 mice were exposed to corticosterone for 60 hours from embryonic day 12.5 (E12.5). Placentas were collected at E14.5, RNA extracted and reverse transcribed and gene expression of Thioredoxin Reductase 1 and 2 (*TXNRD1* and *TXNRD2*), Glutathione Peroxidase 1 and 3 (*GPX1* and *GPX3*) and Superoxide Dismutase 1 and 2 (*SOD1* and *SOD2*) was analysed by QPCR. Protein was extracted and used for protein carbonyl assays.

Maternal corticosterone exposure significantly increased *TXNRD1* gene expression in female placentae. Interestingly, males from the corticosterone treated placentas had significantly decreased *TXNRD2* gene expression. Maternal corticosterone did not affect *GPX1*, *GPX3*, *SOD1* and *SOD2* within the placenta in both sexes. Corticosterone exposure significantly increased protein carbonyl levels in female placentae. Mitochondrial content was significantly reduced in female placentas affected by maternal corticosterone.

The sex specific changes in the expression of mitochondrial content, antioxidant enzymes and protein carbonyl levels demonstrated in the current study may infer different levels of protection from glucocorticoids in males and females. These outcomes may underlie the increased disease outcomes in male offspring compared to females.

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Combination sulfasalazine and metformin are additive in reducing placental secretion of antiangiogenic factor sFlt

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Introduction

Preeclampsia is associated with increased placental secretion of antiangiogenic factors sFlt-1 and sENG and a reduction in angiogenic proteins placental growth factor secretion (PlGF) and vascular endothelial growth factor (VEGF). We have identified two medications, safe in pregnancy, that mitigate key aspects of preeclampsia *in vitro*. We have demonstrated metformin and sulfasalazine independently reduce placental sFlt-1 secretion and vessel dysfunction.

In this study we investigate the possibility of combining these medications to reduce the dose required to mitigate key aspects of preeclampsia; sFlt-1 and sENG secretion and PlGF and VEGF expression, using primary human tissues.

Methods

Metformin and sulfasalazine, at low doses individually and in combination, were administered to primary trophoblasts and placental explants and sFlt-1 secretion assessed. At the mRNA level there are two sFlt-1 splice variants; the primate and placental specific isoform sFlt-1 e15a and the predominately vascular isoform sFlt-1 i13. Both were measured following combination treatment. In addition the expression of angiogenic factors PlGF and VEGFα were assessed.

Results

Combining low-dose metformin and sulfasalazine additively decreased sFlt-1 secretion from primary trophoblast compared to either drug alone. Similarly, an additive decrease in sFlt-1 e15a and i13 was identified following combination treatment. In placental explants, combination treatment also additively decreased sFlt-1 secretion.

Low dose sulfasalazine potently upregulated PlGF expression in primary trophoblasts and this was maintained with the addition of metformin. Metformin and sulfasalazine individually increased VEGFα, and this effect was additive when the two drugs were combined.

Conclusion

Combination metformin and sulfasalazine additively reduced sFlt-1 secretion and the expression of its splice variants, sFlt-1 e15a and i13. Furthermore, in combination metformin and sulfasalazine upregulate angiogenic factor VEGFα. Therefore we conclude that they might be promising combination therapeutics that could reduce the burden of this devastating disease.

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Advanced maternal age affects rat placental nutrient transport in a sex-specific manner

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INTRODUCTION: The age at which women deliver their first child has increased steadily in recent years. Advanced maternal age (≥35 years) is associated with increased pregnancy complications (e.g., gestational diabetes mellitus and preeclampsia), in turn leading to maternal and perinatal morbidity and mortality. In a rat model of advanced maternal age, we have previously demonstrated maternal hypertension, fetal growth restriction and increased placental weight. We hypothesised that advanced maternal age affects placental function.

METHODS: Female Sprague Dawley rats aged 9-10 months (equivalent to a ~35 year old woman), and 4 months (young controls) were mated. On gestational day 20, fetal weights were recorded, and placentas were fixed for morphological assessment using stereology, or snap frozen for qPCR analysis of nutrient transporters and growth-regulatory genes.

RESULTS: Aged dams had a compromised capacity to carry viable pregnancies (young=90%, aged=50%), 40% smaller litters, and fetal weights were reduced by 10% compared to young dams. Preliminary data where fetal weight was assessed according to sex suggests that females from aged dams tended to be lighter. Placental weight was increased by 15% in aged dams, although calculated placental efficiency was reduced. There was no difference in the proportion of the placental zones, however the calculated volume of labyrinth and junctional zones were enlarged by 15% and 51%, respectively. Placentas from female fetuses from aged dams had increased expression of the glucose transport gene *Slc2a3* ($p<0.01$) and amino acid transporter *Slc38a4* ($p=0.07$). Nutrient transporter genes were unchanged in placentas from male fetuses from aged dams, but expression of angiogenic *Vegf* and the tumour suppressor *p53*, were reduced ($p<0.05$), compared to young dams.

CONCLUSION: Genes regulating placental nutrient transport, angiogenesis and apoptosis were altered in a sex-specific manner in aged dams. This provides insight into potential mechanisms responsible for poor pregnancy outcomes in advanced maternal age women.

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Periconceptional alcohol exposure alters female kidney function and circadian regulation of renal gene expression

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Introduction: Alcohol exposure throughout gestation has been shown alter kidney development and function. Although many women decrease consumption of alcohol upon pregnancy recognition, exposure during the periconceptional period (PC) is common. Many genes that regulate renal function are expressed in a circadian manner. This study aimed to investigate the effect of periconceptional alcohol exposure (PC:EtOH) on renal function in offspring and if the renal circadian clock was altered.

Methods: Female Sprague Dawley rats received a control liquid diet or 12.5%v/v ethanol diet (PC:EtOH) for four days before and after mating. In cohort 1, at 19 months (m) male and female offspring underwent 24hour metabolic cage studies to examine renal function and plasma aldosterone was measured by ELISA. Kidneys from a second cohort of female offspring were collected at 5m at six Zeitgeiber times (ZT). Renal mRNA expression of clock circadian regulator (*Clock*), period circadian regulator 1 (*Per1*), nuclear receptor subfamily 3 group C member 2 (*MR*) and epithelium sodium channel alpha subunit (*αEnac*) were analysed using cosinor analysis.

Results: At 19m, PC:EtOH exposure increased urine flow and water consumption, with post-hoc analysis showing the increase in female offspring only ($PTrt<0.05$, $P<0.05$). PC:EtOH decreased plasma aldosterone levels ($PTrt<0.05$). At 5m, following Cosinor analysis, PC:EtOH exposure shifted the acrophase (time of peak expression) of *Clock*, *Per1*, *MR* and *αEnac* ($P<0.05$). PC:EtOH decreased mRNA expression of *MR* and *αEnac* at ZT2.5 ($P<0.05$) and *αEnac* and *Clock* at ZT6.5 ($P<0.05$).

Conclusion: PC:EtOH causes diuresis in 19m female offspring associated with reduced plasma aldosterone and altered circadian expression of genes involved in renal function at 5m. Aldosterone is known to affect mRNA expression of *MR* as well as *Per1*, which regulates *αEnac* in the kidney. Together, this study demonstrates altered renal function in female PC:EtOH could be directly influenced by the renal circadian clock.

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Maternal Endothelium and Risk of Cardiovascular Diseases

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Background:
In Pakistan, It has been strongly suggested that women with hypertensive disorders of pregnancy are at risk of developing cardiovascular disorders.

Objective:
Our objective was to ascertain the persistence of endothelial activation in hypertensive pregnancies compared to women with normal pregnancies.

Methadology:
Case control study design was chosen in two matched group. Endothelial activation was determined by the evaluation of adhesion molecules namely P selectin, E-selectin, Intercellular adhesion molecules-1(ICAM-1) and vascular cellular adhesion

molecules-1 (VCAM-1).

Results:
In the first study, adhesion molecules were measured in 40 women with hypertensive pregnancies and in a matched control group with an uncomplicated pregnancy one month and three months after delivery. In the second study, adhesion molecules were measured in 40 patients with a history of HELLP syndrome several years after pregnancy and in 40 matched controls. Shortly after the delivery, increased levels of soluble adhesion molecules were found in women with hypertensive complications. However women with uncomplicated pregnancy did not have any increase level of soluble adhesion molecules. Significant differences were still present, several years after delivery comparing levels of adhesion molecules in women with a history of HELLP syndrome with those found in control patients.

Conclusion:
An abnormal activation of endothelium was seen in hypertensive pregnancies. It has also been concluded that is abnormal activation of endothelium remains increased even after delivery which pre disposes the patient towards cardiovascular disorder. The risk of cardiovascular complications including ischemic heart diseases, chronic hypertension and stroke is more commonly seen in women experiencing HELLP syndrome

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The Effects of Maternal Melatonin Supplementation in Mid to Late Gestating Singleton and Twin-bearing Merino Ewes on Growth Parameters at Birth

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Supplementing the maternal environment with a neuroprotectant, melatonin, may improve neonatal survival through increased placenta efficiency, umbilical cord blood flow, and higher birth weight. While melatonin has mainly been investigated as a means of improving the development and survival in human babies, there is also potential that this neuroprotectant may help to reduce the high levels of neonatal mortality in Merino sheep, which can be as high as 40% for twins. From day 80 of gestation, ewes were supplemented with melatonin via a subcutaneous 18 mg Regulin® implant (MEL-IMP *n* = 13) or 2 mg gel capsule fed orally daily (MEL-FED *n* = 14). Thirteen ewes received no supplementation (CTL). Ewes were intensively monitored during parturition and weight of lambs was measured at 4, 24, 72 hours and 7 days following birth. Morphology was measured at 4 hours then again at 7 days following birth. Preliminary analysis showed twin lambs (*n* = 45) were consistently lighter compared to singleton lambs (*n* = 15) at all time points (4h P=0.001; 24h: P=0.001; 72h: P=0.001, 7 days: P=0.001), irrespective of melatonin treatment. Similarly, morphological measures; crown-rump length (P=0.013), crown width (P=0.001), forelimb length (P=0.003), thoracic (P=0.076) and abdominal circumference (P=0.019), tended to be greater in singleton lambs compared to twin lambs. When analysing the morphology of just twins lambs and lamb weight was accounted for within treatment, both MEL-IMP and MEL-FED lambs were larger, with longer crown-rump (P=0.001) and forelimb length (P=0.006) as well as larger crown width (P=0.001) compared to CTL twin lambs. While these early results are promising, further research is being conducted which will include analysing the effects of pre-natal melatonin on lamb behaviour, such as time to stand and suckle, as indicators of lamb survival during the first few days of life.

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A murine model of IUGR induced by embryo transfer

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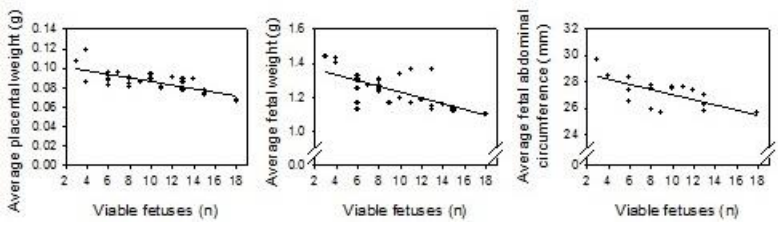
Intrauterine growth restriction (IUGR) and low birth weight (LBW) affect 5-15% of babies and increase their risk of perinatal death and poor health in later life (1, 2). Rates of LBW increase in multiple pregnancies; 5% singletons, 54% twins and 98% higher-order multiples were LBW for 2015 Australian pregnancies (1), likely due to restricted implantation and competition for nutrients. Spontaneous variation in litter size is confounded by maternal factors. We therefore aimed to utilise embryo transfer to produce murine IUGR due to large litter size. CBAF1 embryos were collected at gestation day 0.5 (GD0.5) and 6, 8, 10 or 12 embryos transferred into each uterine horn of pseudopregnant CD1 mice (n=32, 26 completed). At GD18.5, umbilical (3-4 fetuses/litter) and uterine blood flows were analysed by Doppler ultrasound prior to post-mortem. Fetal morphometry has been measured in 16 litters to date. Litter size (viable pups, R^2=0.334, P=0.002) increased linearly as more embryos were transferred. At GD17.5, litter average placental weight (R^2=0.456, P<0.001), and fetal weight (R^2=0.404, P<0.001), abdominal circumference (R^2=0.452, P=0.004) and head width (R^2=0.255, P=0.046), but not crown-rump length (R^2=0.133, P=0.166) correlated negatively with litter size (Figure). Fetuses from larger litters had lower weight:length ratios (R^2=0.332, P=0.020) and tended to have increased head width:abdominal circumference ratios (R^2=0.214, P=0.071). In preliminary analysis (data from 7 litters), litter average umbilical and uterine arterial resistance and pulsatility indices were not correlated with litter size or litter average fetal weight.



Increased litter size in embryo transfer-generated pregnancies induces an IUGR phenotype, including lighter placentas and fetuses, which are thinner with evidence of head sparing. Analysis of Doppler blood flow parameters for individual fetuses is currently in progress. We will now utilise this approach in combination with murine genetic models to identify mechanisms and test new intervention strategies to prevent IUGR due to chronic restriction.

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Effects of conceptus sex and genetics on maternal circulating thyroid hormone and insulin-like growth factor concentrations

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Pregnancy is characterized by significant changes in maternal metabolism that are essential for adequate fetal growth and subsequently lactation. These changes are driven by conceptus demand and maternal supply that is under hormonal control. Here we use a bovine model with purebred and reciprocal cross *Bos taurus taurus* (Bt, Angus) and *Bos taurus indicus* (Bi, Brahman) concepti in primiparous dams to demonstrate significant effects of conceptus sex and genetics on maternal circulating thyroid hormone and insulin-like growth factor concentrations. We analysed total and free thyroxine and triiodothyronine, reverse triiodothyronine and insulin-like growth factors 1 and 2 as well as total IGFBPs at midgestation (Day153). In Bt and Bi dams total thyroxine concentrations were significantly higher ($P<0.05$ and $P<0.01$) when carrying a male conceptus while triiodothyronine was not affected by conceptus sex ($P>0.10$). However, free thyroxine was higher in Bt dams carrying females ($P<0.05$), while Bi dams showed a tendency for the opposite effect ($P<0.10$). In Bt dams, insulin-like growth factors 1 and 2 were not affected by conceptus sex ($P>0.10$), but in Bi dams IGF1 tended to be higher ($P<0.10$) with a male conceptus. Furthermore, we found significant effects of conceptus genetics (BtxBi versus BixBi) on total thyroxine ($P<0.001$) and a tendency for effects on reverse triiodothyronine ($P<0.10$) in Bi dams only. Apart from the likely impact on dam and conceptus growth, our data suggest that conceptus sex and genetics may affect hormones that are crucial for mammary gland development and lactation. The current data therefore provide a physiological basis for reported conceptus sex effects on lactation in mammals (Hinde et al. 2014, PLoS One 9(2):e86169) while the impact of dam genetic background may help explain conflicting results (Græsbøll et al. 2015, PLoS One 10(4):e0124051).

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Dealing with the stress of preeclampsia through placental mitochondrial adaptations

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Preeclampsia is a devastating pregnancy disorder that can quickly and unpredictably become dangerous for both mother and fetus. Severity varies widely, and while severe preeclampsia often requires preterm delivery, women with mild preeclampsia may reach term with minor interventions. The mechanisms that mediate disease severity are poorly understood, but may include adaptive processes by the placenta. We aimed to establish whether placental response to preeclampsia was different in pregnancies that reached term *versus* those that delivered preterm, and explore potential adaptive mechanisms.

This study included placentae collected from 16 patients with preeclampsia (term delivery n=10, preterm delivery n=6) and 16 control pregnancies (term delivery n=10, preterm delivery n=6). Markers of oxidative stress, antioxidant, mitochondrial, and apoptosis function were analysed.

Hydrogen peroxide production (p=0.003, 1.15 fold) and antioxidant activity (p=0.016, 1.13 fold) were increased in term preeclamptic placentae, whereas preterm preeclamptic placentae had reduced function of the antioxidant superoxide dismutase (activity p=0.029, 0.79 fold). Mitochondrial fusion (L-OPA1/S-OPA1 p=0.0007, 1.62 fold; MFN1 p=0.011, 1.78 fold)

and anti-apoptosis (BCL2 p=0.016, 1.53 fold) proteins were increased in term preeclamptic placentae. Mitochondrial respiration (mitochondrial complex I, p=0.008, 1.19 fold) and mitochondrial content (p=0.009, 1.46 fold) were increased in term preeclamptic placentae, but mitochondria had a lower respiratory reserve capacity (p=0.001, 0.46 fold).

Placentae from preeclamptic pregnancies that reached term showed multiple mitochondrial-related adaptions that were not present in preterm preeclamptic placentae. Increased antioxidant activity, and expression of markers of mitochondrial fusion and apoptotic suppression, may relate to salvaging damaged mitochondria. Increased mitochondrial content may be the reason for increased respiration, allowing ongoing tissue function even with reduced respiratory efficiency in term preeclamptic pregnancies. Reactive oxygen species signalling in term preeclamptic placentae may be at a level to trigger compensatory antioxidant and mitochondrial responses, allowing tissue level maintenance of function when there is organelle level dysfunction.

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Validation studies of a fluorescent method to measure placental glucose transport in mice

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Placental nutrient transport assessment is essential in experimental models to provide a functional measure of fetal nutrient supply and uptake and their responses to interventions. In small animals including mouse, radiolabelled tracers have been used to measure passive and active transport of solutes across the placenta, which is reduced in IUGR (1,2). Use of fluorescent rather than radioactive labels for solutes has benefits for safety and waste disposal. In pilot studies, we demonstrated transfer of fluorescently-labelled 2-deoxyglucose (IRDye800CW 2-DG, LI-COR Biosciences, Lincoln, NE) across the placenta into fetal circulation in anaesthetised late pregnant mice. When injected into conscious mice, transfer from placenta to fetus stabilised by 30 minutes after injection and similar levels were maintained for 120 minutes. Our next aim was to test whether dye transport differed between littermates with the lowest (low PE) and highest (high PE) placental efficiency for their litter, defined as fetal:placental weight ratio. Fluorescently-labelled 2-DG was injected into the tail vein of pregnant CBAF1 mice 2 or 24 h before tissue collection at 17.5 or 18.5 d gestation. Fluorescent signals were measured (800 nm channel, Odyssey Imaging System, LI-COR) in homogenised tissues of low PE and high PE littermates. Fetal uptake of fluorescently-labelled 2-DG across the placenta was calculated as total fetal fluorescence per gram of placenta. Littermates were compared using paired t-test. Fetal 2-DG uptake was higher in high PE than low PE littermates of dams injected either 2 h (P=0.032, n=3 litters) or 24 h (P=0.020, n=6 litters) before tissue collection. This method is therefore able to differentiate littermates that differ in placental function in the late pregnant mouse. We are currently performing additional studies investigating the relationship between fetal 2-DG uptake and Doppler indices of fetal blood supply, and effects of IUGR on fetal 2-DG uptake in late gestation.

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Elemental metabolomics in the characterisation of placental mitochondria in control and gestational diabetes mellitus

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Elements are central to many cellular functions and enzymatic activities within living systems. Elemental metabolomics quantifies elements in biological samples, giving novel insight into how cellular processes are altered within disease states. The placenta is a highly metabolically active organ with numerous enzymatic functions that require elements. The aim of this project is to establish an elemental profile of placental mitochondria from cytotrophoblast (CT) and syncytiotrophoblast (ST), of healthy and gestational diabetes mellitus (GDM) pregnancies. We aim to translate these elemental profiles to investigate functional enzymatic changes.

CT and ST mitochondria were isolated from placental tissue collected from GDM (n=13) and matched control (n=10) pregnancies. Inductively coupled plasma mass spectrometry was utilised to create proteomic profiles of 65 elements. Superoxide dismutase (SOD) activity was measured by activity assay.

CT and ST mitochondria had distinct profiles within healthy pregnancies (16 different elements; $p<0.05$). In GDM, CT and ST mitochondria profiles were altered, with six additional elements (including Mn), distinguishing CT and ST mitochondria. 11 of the elements distinct between mitochondrial populations in healthy placentae were no longer different in GDM samples ($p<0.05$). Five elements, Ti, Cu, As, Zr, and Au, were distinct between CT and ST mitochondria in both healthy and GDM pregnancies ($p<0.05$). SOD activity was increased in GDM (p=0.01).

This research provides the foundation for the use of elemental metabolomics in the creation of translatable elemental profiles in placental tissue. Furthermore, this methodology allowed for differentiation between control and GDM samples in both CT and ST mitochondria. Differences observed in Mn and Cu through elemental profiling predicted a functional increase in SOD activity

in GDM. This study shows the translatable potential of elemental metabolomics in characterising and predicting functional alterations between mitochondrial populations in gestational disorders.

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Antenatal dexamethasone treatment in mice exerts reciprocal effects upon fetal and placental weight at E14.5 and E17.5

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Pregnant women at risk of preterm delivery are administered potent synthetic glucocorticoids (betamethasone, dexamethasone) to reduce neonatal mortality should preterm delivery ensue. This precedes the natural rise in endogenous glucocorticoids shortly prior to parturition. However, studies in animals and humans suggest that glucocorticoid exposure may adversely affect fetal and placental outcomes, dependent on timing. We hypothesise that in mice, dexamethasone will differentially affect fetal and placental growth and development if administered prior to the rise in endogenous fetal glucocorticoid synthesis (initiates at embryonic day [E]14.5) *versus* exposure at peak endogenous glucocorticoid concentration (E16.5-E17.5).

Female C57BL/6 mice were time-mated (day of plug designated E0.5) and received an IP injection of vehicle (Veh) or dexamethasone (Dex; 500µg/kg) at E13.5 or E16.5 (n=4-11/group). A control group (Con) received no injection, controlling for stress of procedure. *In vivo* ultrasound scanning was conducted 24h post-injection at E14.5 or E17.5, respectively, to measure umbilical arterial blood flow (a key regulator of fetal and placental growth) and fetal heart function. Dams were sacrificed immediately after the scan. Fetal and placental weights were recorded, and tissues collected. Data were analysed by ANOVA with post-hoc Tukey's test. Mean ± SEM; $^{\#}$p<0.05 *vs* Veh; *p<0.05, ***p<0.001 *vs* Con.

24h treatment with dexamethasone significantly reduced fetal weight at E14.5 (Dex: 0.22±0.01g$^{\#/***}$, Veh: 0.25±0.01g, Con: 0.29±0.02g, n=4-11); placental weight did not differ. At E17.5, fetal weight was unaffected by dexamethasone treatment but placental weight was significantly reduced (Dex: 0.077±0.001g*, Veh: 0.083±0.003g, Con: 0.083±0.001g, n=9-11).

Dexamethasone injection differentially affected fetal and placental growth at E14.5 and E17.5 in a reciprocal manner. Analysis of ultrasound data is ongoing to determine alterations in placental hemodynamic function following dexamethasone treatment. Future experiments will analyse placental morphology and molecular pathways to develop a comprehensive understanding of how these parameters translate to function of dexamethasone exposed placentas.

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The effect of oestrous synchronisation and breed on anti-Mullerian hormone (AMH) concentrations in Merino and Suffolk ewes

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Anti-Müllerian hormone (AMH) is a transforming growth factor family glycoprotein expressed exclusively by the granulosa cells of growing antral follicles. AMH plays an inhibitory role during initial primordial follicle growth recruitment and regulates the continued growth of large pre-antral and small antral follicles. AMH is an endocrine marker of ovarian reserve in humans, a reliable marker of the response of cows to super-ovulatory stimulation protocols, and can be used to identify which ewes should be used as donors for laparoscopic ovum pick up. This study had two primary aims; one, to determine if there is an optimum time to measure AMH relative to synchronisation of the oestrous cycle, and, two, to determine whether AMH levels differ between breeds of sheep. Suffolk (n = 19) and Merino (n = 19) ewes were used, and oestrus cyclicity was synchronised using an intravaginal progesterone insert, which was removed after 12 days. Blood samples were collected into lithium heparin coated tubes on the day of the insertion of the intravaginal progesterone insert (day 0), and again on the day of its removal (day 12). Samples were centrifuged at 1500 rpm for 15 minutes and AMH levels measured in the plasma using an ovine AMH ELISA kit (ANSH labs, Texas, USA). Data were analysed using an ANOVA (unbalanced design; Genstat 15th Edition SP2). Mean AMH levels were similar on Day 0 and Day 12 (2.61 ± 0.25 and 2.64 ± 0.25 pg/ml, respectively; P = 0.315) and for Merino and Suffolk ewes (2.55 ± 0.25 and 2.69 ± 0.25 pg/ml; P = 0.315). The current data indicates no differences in AMH levels between non-synchronised and synchronised ewes, or between breeds, suggesting it may be a useful tool with which to select donor ewes, regardless of breed, prior to commencing oestrous synchronisation.

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Linoleic acid alters inflammatory responses and increases reactive oxygen species generation in human trophoblast like cells

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Linoleic acid (LA) is an essential omega-6 fatty acid (FA) which is important in the normal functioning of the body. The consumption of LA is increasing worldwide, with elevated LA exhibiting pro-inflammatory and pro-oxidative properties. Adequate concentrations of LA are critical for fetal development and must be obtained from the maternal diet, however excess maternal LA may have detrimental effect on offspring health. FAs are transported from mother to fetus through the placenta via fatty acid transporters; however, the specific fatty acid transport proteins (FATPs) and fatty acid binding proteins (FABPs) responsible for this transport are unknown. We hypothesised that exposure of trophoblasts to high LA may change expression of specific FATPs and FABPs, which may affect inflammatory responses and mitochondrial activity. In our present study, 300 and 500 µM of LA increased *FATP1* and *FATP4* mRNA expressions in Swan71 trophoblasts. 500 µM of LA elevated *FABP5*, while 100, 300 and 500 µM of LA decreased *FABP3* expression. There was no changes in mRNA expression of *IL-6*, however, 300 and 500 µM of LA decreased IL-6 secretion in cell supernatant. 500 µM of LA increased mRNA expression of *IL-8*, while 300 µM of LA decreased its protein expression. Further, 100, 300 and 500 µM of LA increased prostaglandin E2 and leukotrienes B4 secretion. Moreover, 300 and 500 µM of LA increased hydrogen peroxide production suggesting elevated reactive oxygen species (ROS) generation. In conclusion, high LA altered the expression of FA transport related proteins, inflammatory responses and increased ROS generation in human trophoblasts. While these trophoblast adaptations to LA may act to limit transport of LA to the fetus, these results indicate that they may also induce placental dysfunction. Thus, these findings suggest the importance of consuming adequate amount of LA during pregnancy and warrant further experimental animal and human studies.

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New insights into the regulation of uterine contractions in human labour

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The Effects of Maternal Melatonin Supplementation on Blood Glucose and Thermoregulation in Neonatal Lambs

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Melatonin has previously been investigated as a means to mitigate the adverse effects of premature birth or growth restriction in human infants. Supplementing Merino ewes with melatonin during mid to late gestation may also help to reduce the high level of lamb mortality. We aimed to determine the effects of supplementing the maternal environment with melatonin on lamb vitality and survival at birth. From day 80 of gestation, ewes were supplemented with melatonin via a subcutaneous 18 mg Regulin® implant (MEL-IMP *n* = 13) or 2 mg gel capsule fed orally daily (MEL-FED *n* = 14). Thirteen ewes received no supplementation (CTL). Ewes were intensively monitored during parturition and rectal temperature and blood glucose measured in lambs (*n* = 65) at 4 and 24 hours following birth. Survival was measured daily and is continuing to weaning (in August 2018). Preliminary analysis showed there was no difference in the duration of parturition (P=0.334); however, more CLT twin-bearing ewes needed assistance during lambing (CLT=3, MEL-IMP=0; MEL-FED=0. P=0.039), and human intervention for five twin lambs (CTL=4; MEL-IMP=1; MEL-FED=0) was required to ensure survival past eight hours. Rectal temperature was highest in CTL singleton lambs at 4 hours (P=0.050); however, temperature dropped significantly by 24 hours in both singleton and twin CTL lambs (P=0.013). Both MEL-FED (P=0.002) and MEL-IMP (P=0.083) lambs maintained or tended to have increased rectal temperature at 24 hours. While blood glucose was significantly higher in singleton compared to twin lambs at both 4 and 24 hours (4h: P=0.001; 24h: P=0.006), MEL-FED singleton lambs had consistently higher levels of blood glucose compared to other treatments (P=0.003). The latter may be indicative of the lamb's ability to stand and suckle, and subsequently, a measure of higher colostrum intake during the first day of life.

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Establishing an *in-vivo* magnetic resonance imaging (MRI) protocol for measurement of placental vascular function in rats

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Placental vascular physiology and function are critical for appropriate fetal growth and health outcomes in later life. Understanding how placental vasculature aligns with placental function, particularly *in-vivo*, may reveal underlying mechanisms that can inform interventions to improve long-term fetal development outcomes. Classically, these research questions are addressed using invasive and terminal techniques such as histology and gene expression. However, *in-vivo* study of the placenta can permit repeated measures to serially investigate function and track development. Magnetic resonance imaging (MRI) is a noninvasive tool which can be used to study both placental morphology and function, as well as monitor development *in-vivo*. This study aimed to establish in our laboratory an efficient and effective protocol of *in-vivo* MRI scans to assess placental function in rats. There are technical challenges in using MRI to assess placental function of rats *in-vivo*, e.g., motion, variable feto-placental position, image contrast, etc., which must be considered. Pregnant Wistar rats were scanned serially at embryonic day (E) 15, 18, and 21 using 9.4 T MRI. Our established *in-vivo* MRI protocol includes: (i) T2 TurboRARE for fetal and placental anatomical imaging; (ii) an oxygen-challenge paradigm (air vs O_2) obtaining 3D T1 and T2* relaxation maps for investigating tissue perfusion and blood oxygenation; (iii) bidirectional arterial spin labelling for feto-placental ordering; and (iv) dynamic contrast enhanced MRI for dynamic evaluation of placental perfusion (E21 only). After the E21 scan session, dams were euthanized for tissue collection and the number and ordering of feto-placental units confirmed. Our preliminary data is congruent with the literature indicating that the relaxation mapping is sensitive to the oxygen-challenge paradigm. Research is on-going to validate the approach to study a maternal stressor model and how the MRI outcomes align with placental vascular structure.

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Context is all - Characterisation of progesterone receptor-chromatin binding properties in mouse reproductive tissues and its implication of tissue-specific gene expression

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Progesterone receptor (PGR), the nuclear receptor transcription factor activated by progesterone, regulates vastly different tissue-specific processes in each organ of the reproductive tract, enabling temporal coordination of ovulation, fertilisation and implantation. Ligand activated PGR interacts with chromatin through specific sequence motifs in order to regulate gene expression; however, the mechanism through which it regulates different gene sets in different tissues is still poorly understood. In this study, we provide the first genome-wide description of PGR action in peri-ovulatory mouse granulosa cells using chromatin immunoprecipitation followed by massively parallel DNA sequencing (ChIP-seq). We found more than 15000 PGR binding sites in granulosa cells, the majority of which were enriched in proximal promoter regions and within close proximity to H3K27ac-bound chromatin. Motif analysis indicated that while the PGR response element was highly targeted, PGR also interacted with other transcription factor binding motifs. Using a gene expression microarray on wild type and *Pgr*-null mice we identified 61 significant PGR-dependent genes in granulosa cells, 82% of which possessed PGR binding in ChIP-seq. We also performed a systematic comparison of PGR action in the reproductive tract. We compared PGR-regulated genes between granulosa, uterus and oviduct, showing a high level of tissue specificity in PGR target gene profiles with no PGR-regulated genes found in common between all three tissues. We performed a parallel analysis of our new granulosa cell ChIP-seq with uterus ChIP-seq data, illustrating distinct cistromes and PGR-gene interaction properties between the two tissue types. These findings indicate that PGR relies on interaction with unique co-modulators in each tissue in order to achieve tissue specificity. Our study offers further understanding of the finely-tuned context-specific roles of PGR across the reproductive tract with implications on contraceptive and cancer therapeutics, while also revealing potential targets for the management of anovulatory infertility.

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Changes in proteomic profile of testicular interstitial fluid after short-term heat treatment in the adult rat

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Spermatogenesis involves interactions between the seminiferous tubules, which contain developing germ cells and somatic Sertoli cells, and the interstitial space which contains steroidogenic Leydig cells, macrophages and blood vessels. Because little is known about soluble factors that potentially participate in intercellular communication between the various cell types, our aim was to investigate changes in the proteome of testicular interstitial fluid (TIF) that surrounds the seminiferous tubules following selective germ cell damage induced by heat treatment. Adult (70 days old) Sprague-Dawley rats were anaesthetised and their scrota immersed in a water bath at 43°C for 15 minutes. This treatment results in the loss of pachytene spermatocytes (PSC) and round spermatids (rST) from the epithelium with maximum damage 2-6 weeks later, but spermatogenesis recovers

by 10-12 weeks[1]. TIF was collected from treated and age-matched untreated control rats (n=4/group) at 1, 2, 4, 8, 12, and 14 weeks following heat treatment. After removal of abundant proteins, TIF proteins were identified by LC-MS/MS with statistically different candidates selected by ANOVA. The potential cellular origin of TIF proteins was assessed using datasets from a microarray of isolated germ and Sertoli cells[2] and an RNASeq of mouse testes with germ- +/- Sertoli cell ablation[3]. Overall, 1729 unique proteins were identified in TIF, with 31 proteins significantly ($p<0.05$) suppressed 2-22 fold at the 8 week maximal damage timepoint. These proteins included potential markers of Sertoli cells (n=3), PSC and rST (n=7), spermatogonial (n=1) and interstitial cells (n=1). This group included several germ cell heat shock-related proteins, along with ubiquitin-conjugating and calcium regulating proteins. Interestingly, this strategy also revealed various Sertoli and spermatogonial proteins altered in the 1 week group, likely indicative of an early response to heat by the epithelium. This proteomic approach opens a new direction to study intercellular communication in the testis.

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Exploring changes to protein homeostasis in the male germ line in response to oxidative stress

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Oxidative stress has been implicated in an extensive range of age-related pathologies including neurodegenerative disease, and is a well-known cause of infertility. The negative impacts of oxidative stress in the male germline are primarily underpinned by the peroxidation of fatty acids, resulting in the production of highly reactive lipid aldehydes, such as 4-hydroxynonenal (4HNE). In many cell types, the portion of the cell proteome that is targeted for 4HNE-modification often experiences severe protein misfolding that, in turn, leads to a disruption of protein homeostasis (proteostasis). This study was designed to explore a relationship between oxidative stress and protein aggregation in the male germline with a key focus on determining the protective mechanisms employed by germ cells to prevent protein damage.

Through the development of several robust strategies for the detection of protein aggregates, this study has revealed a causative role for oxidative stress in the induction of protein aggregation in both pachytene spermatocytes and round spermatids. Specifically, the exogenous application of 4HNE to these cells resulted in a significant increase in aggregation ($P<0.005$) detectable with the amyloid-specific fluorophores Proteostat and Thioflavin T, and the conformer specific antibodies anti-A11 and anti-OC. In this study, nucleocytoplasmic transport machinery was examined as a potential mechanism for the subcellular compartmentalisation of aggregating proteins. The inhibition of transport proteins karyopherin beta 1/alpha 2 (KPNB1/A2) and exportin 1 (XPO1), resulted in a significant increase in cellular protein aggregates ($P < 0.005$). Similarly, the inhibition of the molecular chaperone Heat Shock Protein 90 (HSP90) also resulted in a significant increase in protein aggregation ($P<0.005$). These results shed light on two mechanisms that may assist in the management of misfolded proteins in the male germline. These important leads provide new targets to enhance the innate defences of germ cells against oxidative stress and better maintain germline proteostasis.

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The role of zinc transporters in male fertility

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Male infertility is an under-appreciated, yet highly relevant medical issue, affecting approximately 7% of men, in which most cases the aetiology is unknown. However, it is estimated that at least 50% of cases are contributed to by genetic abnormalities. With the advent of Assisted Reproductive Technologies, such as *in-vitro* fertilization, genetic mutations causing infertility are more likely to be passed on to future generations. Zinc is essential for the functioning of the male reproductive system, with studies showing a correlation between seminal plasma zinc and male infertility. However, this has not been investigated from a genetic standpoint.

In order to explore the relevance of Zinc metabolism to male fertility, we assembled a panel of *Drosophila melanogaster* lines containing genetic modifications in Zrt-, Irt-like Protein (ZIP) and Zinc Transporter (ZnT) genes, involved in zinc transport across cellular membranes. These fly lines were used to target ZIP and ZNT over expression and knockdown to either the somatic or the germline cell compartments of the developing male gonad, and male fertility was tested.

Preliminary data has shown that in the germline, manipulation of *ZnT86D* and *ZIP71B* caused partial or complete infertility. In the gonadal somatic cells, over-expression or knockdown of *ZnT49B*, *fear-of-intimacy*, *catsup* and *ZIP89B* also demonstrated sub-fertility or sterility. We are currently exploring the effect of these transgenes on the morphology of the larval gonad and adult testis.

This research demonstrates that multiple Zinc transporter genes are required for male fertility and that they function in both germline and somatic cells of the testis and further indicates the importance of Zinc and its transport in male fertility. Future work will aim to mutate the mouse orthologues of the genes of interest, in order to determine whether these infertility-causing mutations are conserved in a mammalian model.

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An orthodox samurai: KATNAL2 interacts with novel targets to co-ordinate manchette dynamics and sperm tail development

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Recently, we showed that the putative microtubule-severing protein, KATNAL2, is essential for spermatid differentiation. In mice, *Katnal2* loss results in spermatid head-shaping defects due to excessive constriction and elongation of the manchette microtubules, accompanied by a lack of manchette movement and delays in its disassembly. An absence of axoneme generation and, in the few sperm flagella that did form, mitochondrial sheath abnormalities were also observed.

Here, we show, unlike its paralogues, KATNAL2 does not sever α/β tubulin microtubules. Instead, our data suggests KATNAL2 targets multiple microtubule-associated proteins. We identify δ (TUBD1) and ε (TUBE1) tubulin as components of the manchette and as strong candidates for being the mysterious manchette microtubule-microtubule and microtubule-nuclear links that modulate manchette constriction and movement. Importantly, through co-immunoprecipitation and proximity ligation assays, we show TUBD1 and TUBE1 bind KATNAL2 and that these complexes localize to the manchette. To investigate the manchette elongation phenotype, we also characterised the expression of the microtubule nucleator, γ-tubulin, in spermatids. We show, γ-tubulin complexes localise to the caudal tips of the manchette microtubules, and in *Katnal2* knockout mice the removal of these γ-tubulin foci is delayed, suggesting KATNAL2 is required for the release of manchette microtubules from the microtubule organising centre. Finally, through a forward yeast two-hybrid screen using *Katnal2* as bait against a mouse testis prey library, we identified CPEB2 and CPEB4, two RNA-binding proteins, as KATNAL2 binding partners. Our characterisation of these proteins in spermatogenesis supports a model wherein CPEB2 and CPEB4, in partnership with KATNAL2, facilitate a manchette microtubule-based mRNA transport pathway to deliver components to the site of spermatid flagella assembly. Collectively, our data reveals TUBD1, TUBE1, CPEB2, and CPEB4 as new components of the spermiogenesis machinery, and supports a model wherein KATNAL2 modulates the association of the manchette microtubules with diverse targets to facilitate spermatid remodelling.

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Essential post-transcriptional regulators of germline maintenance and spermatogenesis

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Utilising RNA interference in *Drosophila melanogaster* to pre-screen for novel genes required for male fertility

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Male infertility is a common disease with implications beyond an inability to conceive a child, affecting approximately 7% of men in the Western world. Most male infertility is idiopathic in origin, meaning many men go without an accurate diagnosis and thus precise treatments cannot be provided. Further, infertile men as a cohort die younger than their fertile counterparts, suggesting that conserved mechanisms required for male fertility play roles in other tissues. Despite its unknown aetiology, it is estimated that genetic causes are responsible for up to 40-50% of all male infertility cases. We therefore set out to investigate the effect of mutations in single genes that lead to male infertility. Ongoing genetic screens performed on infertile versus fertile men have identified (and are continuing to identify) pathogenic mutants in potential male fertility genes. An initial whole exome screen on azoospermic infertile males has highlighted 10 genes that have been assessed for their role in male fertility, by means of a *Drosophila* RNA interference system screen. For each candidate gene, RNA interference has been performed to target expression in the germline (nanos-enhancer) or somatic (traffic jam-enhancer) lineages of the testis, independently, utilising 1-4 available RNAi lines commercially available. While many of these genes were proven to be dispensable for male fertility, one heat shock protein gene (HSPA4L) has shown a pivotal role in early germ cell development, with knockdown resulting in male sterility and abnormal testis histology. Such effects were absent when HSPA4L was ablated in the somatic lineage of the testis. This sterility in germ cell derived knockdown of expression was validated in two independent fly lines targeting HSPA4L, highlighting a severe reduction in testis size. Further study is focusing on understanding this loss of fertility, hypothesised to be due to a maturation arrest during spermatogenesis.

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Using focused ion beam scanning electron microscopy (FIB-SEM) tomography to define the three dimensional architecture of spermatogenesis

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FIB-SEM tomography is a new powerful tool for visualization of various biological structures which provides three-dimensional information with nanometer scale resolution [1]. Sample preparation for FIB-SEM is almost the same with the cells and tissue preparation for conventional TEM, except the slicing.

Spermatogenesis is the process by which spermatogenic stem cells undergo division and differentiation to produce spermatozoa. Microtubules having a crucial role in this process [2]. FIB-SEM tomography provides unparalleled information about the spatial distribution of microtubules in the cell, and thus, a greater understanding of intracellular processes. The possibility of reconstruction of microtubules spatial distribution has been shown for different cells involved in spermatogenesis: Sertoli cells, all phases of germ cell development and mature spermatozoa.

In comparison with conventional SEM, FIB-SEM tomography provides not only exterior morphology of the cell but also detailed visualization of internal structures. Detailed 3D reconstruction (resolution up to 5 x 5 nm/pixel and section thickness of 20 nm) was performed for mouse sperm. FEI Helios DualBeam plasma FIB with Auto Slice & View software was used (operating 2 kV for backscattering electron imaging and 30 kV for oxygen FIB milling).

Ion beam interaction with embedding medium is of crucial importance in the FIB-SEM technique. Three embedding mediums were studied: Epon, LR White and Lowicryl HM20. The best results have been achieved with Epon resin.

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Germ cell development in cryptorchid testes during first 18 month of life

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Introduction

Neonatal testicular germ cells/gonocytes, transform into stem cells for spermatogenesis during 'minipuberty' (3-6 months old), drive change in the timing of ochidopexy. This study aimed to examine the timing of gonocyte transformation of cryptorchid testes ≤18 months of age with unilateral and bilateral UDT, and in patients with complete androgen insensitivity syndrome (CAIS) and partial androgen insensitivity syndrome (PAIS).

Material and methods

Testicular biopsies (42) from 35 patients with unilateral and bilateral UDT, and PAIS/CAIS aged 10 days-18 months were used for immunohistochemistry with antibodies (Oct4, Ki67, Sox9 and C-Kit) followed by confocal imaging, cell accounting and statistical analysis.

Results

Both Number of Sertoli cells/tubule and germ cell (GC)/tubule decreased with age, and % empty tubules (no GC) increased with age but with no significant differences between patient groups. $Oct4^+$ germ cells/tubule decreased with age and disappeared after 12 month of age. There were $Oct4^+$ GC in unilateral and CAIS UDT ≤12 month old, but no Oct4+ GCs were observed when the age reached 9 months old in bilateral UDT, except one 9 month old patient with bilateral UDT) that showed persisting gonocytes in a testicular tubule with $Oct4^+/Ki67^+/C\text{-}kit^+$ germ cells clustered at the centre of the tubule. There were a few GCs and Sertoli cells proliferating ($Ki67^+$) during the first year and very few afterwards. Most proliferating $Oct4^+$ germ cells ($Oct4^+/Ki67^+$) were located off the tubular basement membrane.

Conclusion

Our study showed that expression of Oct4, the gonocyte marker, gradually decreased after minipuberty and transformation into spermatogonia. Germ cells and Sertoli cells do undergo mitosis during the first 12 months although not abundantly. We propose that Oct4+ gonocytes transformation into spermatogonia via proliferation and migration to the basement membrane may be delayed in UDT.

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Gene expression profiling of epigenetic chromatin modification enzymes, chromatin remodelling factors and ubiquitination enzymes in *Dcaf17* knockout mouse testis

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The DDB1– and CUL4–associated factor 17 (DCAF17) is a member of DCAF family proteins that act as substrate receptors for Cullin-4 (CUL4) based E3 ubiquitin ligase complexes (CRLs) and regulate selective ubiquitylation of proteins. In Human, mutations in DCAF17 encoding gene cause a rare autosomal recessive genetic disorder known as Woodhouse-Sakati Syndrome (WSS), which is characterized by hypogonadism, partial alopecia, diabetes mellitus, mental retardation, and deafness. Recently, it has been shown that loss of DCAF17 in mice caused male infertility due to defective spermatogenesis. The *Dcaf17*$^{-/-}$ mice produced low number of sperm with abnormal shape and significantly low motility. Testis of *Dcaf17* KO

mouse displayed defects in nuclear compaction, acrosome biogenesis, and manchette assembly in spermatids and mature sperm. CRLs play critical roles in regulation of normal spermatogenesis. However, the molecular function of DCAF17 in normal sperm biogenesis is not known. Using quantitative real-time PCR and Western blot techniques, we show abnormal expression pattern of several epigenetic chromatin modification enzymes, chromatin remodelling factors and ubiquitylation enzymes in the *Dcaf17* KO mouse testis. The aberrant expression of the different chromatin modification enzymes, chromatin remodelling factors and ubiquitylation enzymes due to the loss of DCAF17 suggests that DCAF17 may play a critical role in chromatin compaction, an important aspect of sperm development. These data provide a rich resource for further elucidating the cellular/molecular mechanisms of DCAF17 in chromatin compaction that may contribute to the onset of male infertility.

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Investigating activin A effects on fetal male germ cell fate

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Germline development in the fetal testis underpins adult male fertility, and its disruption is considered permissive for testicular germ cell tumour emergence in young men. Signalling by TGFβ superfamily members, including activin A, affects germ cell proliferation and differentiation. Fetuses may be exposed to elevated activin A during pre-eclampsia, maternal infections and from maternal medications and the effects on male fertility are unknown. This study uses the human TCam-2 seminoma cell line, which is characteristic of fetal male germ cells, termed gonocytes, as well as mouse and human gonads to identify activin actions on the fetal male germline. TCam-2 cells were cultured with activin A (1.25 to 50 ng/mL) for 48 hours (n=3), or for 6, 24 and 48 hours with 5 ng/mL (n=5) in serum-free conditions, and transcripts were measured by qRT-PCR. Because TCam-2 cells produce an activin inhibitor, TDGF1 (the Nodal co-receptor), *TDGF1* was reduced using siRNA prior to activin exposure (24 hours, n=2). Downstream targets were significantly upregulated (*KIT, NODAL*; gonocytes proliferation markers) and downregulated (*DNMT3L*, *NANOS2*; differentiation markers). This was similar in both the normal and knockdown conditions, indicating these genes are direct activin targets and that activin promotes a less differentiated state in TCam-2 cells. TGFβ1 and TGFβ2 exposure had minimal effect. Preliminary data from human first trimester cultured gonads indicates *KIT* is downregulated by activin A (5 ng/mL, 72 hours), consistent with previous outcomes using cultured human testis cancer (seminoma) fragments (1). Additionally, embryonic day 13.5 mouse testes cultured in serum-containing media with 10 μM SB431542 (activin/nodal/TGFβ inhibitor) or vehicle control for 48 hours (n=5) resulted in significantly higher *Kit* and significantly lower *Dnmt3l* levels. These approaches demonstrate that the somatic cell environment regulates gonocyte responsiveness to activin A, and highlight the importance of this ligand on their development.

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Understanding poor chromatin compaction within human spermatozoa

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The main role of the spermatozoa can be summarized as the delivery of the paternal genome to the oocyte and central to this is the compaction and protection of its DNA. Poor chromatin compaction is a major issue associated with both natural and artificial pregnancies.

The accepted theory behind poor chromatin compaction is an alteration in the Histone/Protamine1/Protamine2 ratios. Thus, Balhorn has shown that poor nuclear condensation is associated with decreased levels of protamine 2[1]. However, other investigations into the levels of these proteins in poor quality sperm have reached alternate conclusions regarding these relationships[2]. As such, there has been little consensus to solely support this idea.

To further understand chromatin condensation, we isolated spermatozoa with both good and poor chromatin compaction. The sperm nucleus was purified and protein composition compared using quantitative proteomics. In total, 342 proteins were identified, and 20 were found to be more abundant ($p<0.05$, min 1.5-fold change) in the poor quality population. In contrast no proteins were found to be lower within a poorly compacted nucleus. Immunoblotting confirmed higher levels of two of these proteins (Topoisomerase 2A and an ODF2 isoform) in poor quality populations. Of particular interest, the level of detected histones (H4, H3.3, H1T, H2A/B variants) and protamine 2 remained unchanged. This suggests that poor chromatin compaction is highly correlated with retained/excess nucleoplasm. There may be two explanations for this. Firstly, it is possible that chromatin compaction is unrelated to the Histone/Protamine ratio, but rather excess nucleoplasm may hinder condensation. Alternatively, it may be that the mechanism responsible for poor chromatin compaction is highly related to nucleoplasm removal.

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The role of TOB1 in spermatogenesis: conservation of function in *D.melanogaster* and mice

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Introduction:

TOB1 as a member of the BTG/TOB family and recruits factors such as the CCR4-Not complex to regulate mRNA transcription, translation, and turnover. TOB1 also can interact with CCR4-CAF1 and the cytoplasmic poly (A)-binding protein to stimulate mRNA decay and blocking translation of target gene. Existing literature on the *Tob1* gene demonstrates its expression in many adult tissues. Analyses of TOB1 expression in the mouse reproductive tissues indicated TOB1 presence in oocyte and round spermatids and its association with DCP2 suggested that TOB1 is involved with mRNP granules. However, it is still unclear what role TOB1 plays during gametogenesis. In this study to investigate the role of TOB1 in gametogenesis, the effects of genetic deletion of *Tob1* on mouse and *Drosophila* spermatogenesis was studied.

Methods:

Sections of testes from *Tob1*$^{-/-}$and *Tob1*$^{+/+}$mice were examined by routine histology and immunofluorescence to quantify changes in structure, organization, and markers of spermatogenesis. The expression levels of *Tob* in male flies was reduced utilizing Tob RNAi. Resulting phenotypes were characterized by immunofluorescence.

Results:

Lack of TOB1 resulted in fragmented acrosomes in the mouse testis implying a role for TOB1 in acrosome biogenesis. Co-localization of TOB1 with PNA-lectin and ACRBP markers confirmed the loss of *Tob1* affected acrosomes in round spermatids. *Drosophila Tob* expression was observed in the individualization complex, that develops from the acrosome precursor in spermatids. Knockdown experiments in *Drosophila* demonstrated a requirement of *Tob* in the individualisation of sperm.

Conclusion:

TOB1 is involved in the regulation of acrosome biogenesis in mice, and spermatogenesis in *Drosophila*. The similarity of the phenotypes in TOB-deficient models of fly and mouse suggest some level of evolutionary conservation for *TOB* genes in spermatogenesis. The data presented in this study are consistent with TOB1 playing an important role in gametogenesis.

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The role of the prorenin receptor in male fertility

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The prorenin receptor (PRR) is best known for its role in the renin-angiotensin-system (RAS) to regulate blood pressure and salt homeostasis. However, more recently, PRR has been shown to be a multi-functional protein, which is involved in a number of downstream pathways including MAPK signalling, protein sorting and folding and receptor-mediated endocytosis and recycling through its interaction with the vacuolar H^+-ATPase (V-ATPase), as well as canonical and non-canonical WNT signalling. Therefore, we hypothesised that PRR plays a role in gonadal development and function. To test this hypothesis, we deleted *Prr* specifically in gonadal somatic cells using the *Nr5a1*-Cre mouse. While these mice appear to develop normally and are born at the expected Mendelian ratio, both males and females are infertile. Here, we present our analysis to date of the testicular phenotype of the conditional *Prr*-null mice.

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Prednisolone treatment in early pregnancy alters pregnancy outcomes in mice

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In recent years, the immune suppressant corticosteroid drug prednisolone has been increasingly utilised in women undergoing IVF treatment. Prednisolone is administered in the peri-implantation phase and first trimester in the hope of improving embryo implantation rate, by lowering the risk of immunological rejections. The use of prednisolone in early pregnancy is not clinically proven except in women with specific autoimmune disorders, so the validity of its wider use is under debate. This study sought to investigate the effects of prednisolone treatment on Treg cells and pregnancy outcome in mice. CBAF1 female mice mated with Balb/c males were treated with 20 µg prednisolone or vehicle, i.p on gestational day (GD) 0.5, 1.5 and 2.5 and assessed on GD 3.5. Additional females were assessed for fetal and placental development on GD 18.5 or allowed to give birth. On GD 3.5, a decline in T helper cells ($p<0.05$) and a trend towards reduced Treg cell ($p=0.085$) was observed in prednisolone-treated compared to the control group. Amongst the Treg cells, thymus-derived Treg cell numbers showed a trend towards reduction after prednisolone treatment ($p=0.063$). In the blood, expression of the marker CTLA4, an indicator suppressive capacity, was lower in peripheral blood Treg cells from prednisolone treated mice ($p<0.05$). Prednisolone-treated mice were found to have increased pregnancy viability and increased litter size with reduced fetal and placental weight. Prednisolone-treated females deliver later than vehicle treated females, with higher rates of postnatal loss. This study demonstrates that treatment with prednisolone at a dose and time frame comparable to that utilised in women causes decreased Treg cell abundance and reduced suppressive capacity of Treg cells during early pregnancy in mice. This suggests that prednisolone may impair an essential process of the maternal immune adaptation for pregnancy, and result in poor maternal quality control on embryo implantation.

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The role of Wnt signalling in endometrial regeneration

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The regenerative properties of the endometrium have been attributed to epithelial and stromal stem/progenitor cells. Wnt signalling is a developmental and stem cell pathway that is important for normal uterine development. In the intestine, Wnt signalling maintains a niche containing epithelial stem cells expressing the Wnt target gene Lgr5. Lgr5 is a stem cell marker in other organs including the hair follicle and stomach.

We investigated whether Wnt signalling and Lgr5 expression also defines a niche containing stem/progenitor cells in the endometrium. An Lgr5-GFP reporter mouse was used to determine whether Lgr5 expressing cells were present in the endometrium of adult mice undergoing an estrous cycle. Immunofluorescence for the Wnt target gene Axin-2 was used to detect Wnt activation in mouse and human endometrium.

Lgr5-GFP was not expressed in the adult mouse endometrium, but was readily detected at sites of known Lgr5 expression such as the intestinal crypt and ovarian surface epithelium. The Wnt target gene Axin-2 was widely expressed in mouse and human endometrial epithelium, without any restricted localisation that might be indicative of a stem cell niche. In the human endometrium, Axin-2 was prominent in the epithelium of differentiated functionalis endometrium. Axin-2 was not highly expressed in the basal N-cadherin positive glands that are enriched for cells possessing the stem cell property of clonogenic growth.

We conclude that endometrial Wnt activity is linked to epithelial differentiation rather than stem/progenitor cell function. Endometrial epithelial stem/progenitor cell populations are likely to be maintained by mechanisms distinct from the Wnt/Lgr5 system that operates in the intestine and other organs.

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COBL: The regulator of microvillar length in uterine epithelial cells

Samson Dowland, Chad Moore, Laura Lindsay, Christopher Murphy

During early pregnancy the luminal uterine epithelial cells (UECs) are extensively remodelled, where the microvilli are lost from the apical plasma membrane to enable interaction with the blastocyst. Microvilli are also dramatically altered in the rat model of ovarian hyperstimulated (OH) pregnancy. At the time of implantation in OH pregnancy microvilli are more numerous and longer than all stages of normal pregnancy, which is suggested to inhibit blastocyst implantation.

These dynamic changes in microvilli are unique to UECs, however the underlying molecular mechanisms driving the growth and retraction of these microvilli are currently unknown.

The present study has investigated the actin nucleation factor “Cordon-Bleu” (COBL) in UECs. COBL has recently been discovered to control the growth of microvilli in enterocytes. In these cells COBL is recruited to the base of microvilli, where it regulates the length of microvilli through its actin nucleation and severing abilities.

At the time of fertilisation in normal and OH pregnancy, UECs possess regular microvilli supported by an actin terminal web. COBL is concentrated at the base of these microvilli, associated with the terminal web. At the time of implantation during normal pregnancy, correlated light and electron microscopy (CLEM) demonstrates that UECs lose microvilli and the terminal web becomes disorganised. COBL remains localised to the region of the terminal web, where it may sever actin microfilaments to reduce the length of microvilli.

However, during OH pregnancy COBL is lost from UECs and CLEM indicates the unusually large, branching microvilli are not supported by an actin terminal web.

This study demonstrates for the first time that COBL is localised to the base of microvilli in UECs. During normal pregnancy, COBL may regulate the length of microvilli. However, the abnormal microvilli present during OH pregnancy do not appear to be associated with COBL or an actin terminal web.

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Endometrial miRNA profile varies with maternal age in a novel bovine model

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Increasing maternal age is associated with decreased endometrial receptivity, and thus diminished fertility in both humans and domestic species. Endometrial microRNAs (miRNAs) play an important role in establishing an optimal uterine environment, and thus the successful establishment of a pregnancy. The aim of this study was to determine the variability of endometrial miRNA with maternal age, using a novel bovine model that removes potential confounders of nuclear genetics, environment and diet. Endometrium samples of five young (4 years old, equivalent to women in their mid-twenties) and five old (11 years old, equivalent to women >37 years old) cloned cows with an identical genetic background, and managed as one herd, were collected on day 7 of a natural cycle, and total RNA was extracted. Through Next-Generation Sequencing, 786 differentially expressed transcripts were identified between the young and old group. Of those, 46 were significantly different after FDR correction ($p<0.05$) including increased levels of mir99A, mir140, mir125a, mir125b and mir145 in the old group. Further studies are required to confirm the functional pathways effected as well as the expression of regulated genes, and the role these may play in endometrial receptivity.

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Regulatory T cells protect against fetal loss in pregnancies compromised by peri-implantation disruption of progesterone signalling

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Early pregnancy is characterised by adaptations in the maternal immune system to allow development of genetically disparate embryos. Immune tolerance is mediated by a subset of anti-inflammatory, homeostatic T cells known as regulatory (Treg) cells, and is influenced by maternal hormones, through mechanisms that are not well understood. We previously demonstrated a key role for P4 in regulating Treg cell abundance and phenotype in early pregnancy, using a mouse model of peri-implantation disruption in P4 signalling with the P4-receptor antagonist, RU486. Administration of low-dose RU486 (1 mg/kg) on day (d) 1.5 and 3.5 post-coitus (pc) to allogeneically mated C57Bl6 females caused a decreased Treg cell pool in mid-pregnancy, followed by a reduced pregnancy rate in late pregnancy (d18.5 pc) along with increased fetal resorptions. Here, we investigated the effect of transferring donor Treg cells to RU486-treated females. $CD4^+CD25^+$ Treg cells (or vehicle control) isolated from pregnant females (d11.5- d14.5 pc) were transferred intravenously on d3.5 pc to mated recipients treated with RU486. Strikingly, the reduced pregnancy rate was rescued in RU486-treated females administered Treg cells. Furthermore, Treg cell transfer to RU486-treated females restored fetal viability to levels comparable to control-treated females. In another group naïve/effector T cells ($CD4^+CD25^-$) were administered, and although pregnancy rate was improved, the number of viable pups per litter was reduced and fetal resorption was elevated compared to control-treated and Treg cell-treated females. This data shows that Treg cells can rescue pregnancy outcome in females where Treg cells are insufficient due to peri-implantation P4 signalling disruption. This demonstrates that P4 is a key determinant of Treg cells in pregnancy and that Treg cells are a key effector mechanism for P4 actions. Luteal phase and early pregnancy P4 is thus essential to prime Treg cells to drive a robust anti-inflammatory immune response necessary for optimal pregnancy success.

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Macrophage depletion in the post-implantation phase elicits implantation failure through effects on the ovary and uterus in mice

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Pregnancy is a unique example of immune tolerance where the mother's immune system is able to tolerate and support the growth and development of the semi-allogeneic fetus. This process is mediated by a range of tolerance-promoting immune cells that suppress inflammation to which can impair placental and fetal development. Macrophages are involved in generating tolerance, as well as maternal tissue remodelling and vascular adaptations required to establish pregnancy. We hypothesised that macrophages are crucial for embryo implantation and placental development. The experiments utilised the CD11b-DTR murine macrophage depletion model wherein CD11b-DTR mice express the monkey diphtheria toxin receptor (DTR) via the CD11b promoter causing transient depletion of $CD11b^+$ cells upon diphtheria toxin (DT) treatment. CD11b-DTR or wild-type FVB females were mated to BALB/c stud males and administered DT on day 5.5 pc. Pregnancy outcomes were then assessed on days 6.5 and 7.5 pc. Pregnancy rate was not altered 24 h post macrophage depletion, however by 48 h viable implantation sites were reduced by 60%. To assess the cause of pregnancy failure, uteri and ovaries were collected and stained to assess morphometric changes. The ovaries from mice treated with DT were significantly impacted with haemorrhagic infiltration in the corpora lutea. There was no change to serum progesterone on day 6.5 but a significant decrease was evident on day 7.5. Furthermore, there were anomalies in the structure of the decidua and reduced vascularisation around implantation sites. In conclusion, macrophage depletion during embryo implantation affects pregnancy rate, corpus luteum structure and potentially the decidual response and neovascularisation around implantation sites. These results add to earlier work demonstrating a key role for macrophages in development of the corpora lutea in the pre-implantation phase, and indicate a crucial role for macrophages in sustaining progression of early pregnancy through effects in both the ovary and uterus.

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First trimester trophoblast cells induce regulatory properties in B cells

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A successful outcome to pregnancy is critically dependent upon the initiation of maternal immune tolerance upon contact with foreign paternal antigens at insemination and during implantation, a process that requires optimal expansion of regulatory T cells and tolerogenic dendritic cells. Recently IL-10-producing regulatory B cells (Bregs) have also been implicated as players in maternal tolerance. Clinically, significantly higher numbers of circulating Bregs are found in women who experienced normal pregnancies compared to those who had suffered spontaneous abortions. In abortion-prone mouse models, pregnancy can be rescued with early administration of Bregs. Furthermore, our mouse studies have shown a significant increase in uterine Breg frequency during early implantation. Therefore we hypothesized that during implantation, the conceptus influences the maternal B cell profile to acquire regulatory properties. To test this, the human trophoblast cell line SWAN-71 was co-cultured with human peripheral blood B cells. Results show that trophoblast cells enriched memory and plasmablast-like B cells, and increased the frequency of IL-10-producing cells within the transitional B cell subpopulation. These 'educated' B cells also upregulated IL-10 and TGFβ mRNA expression, all hallmarks of regulatory B cells. To confirm a regulatory function, we show that these B cells modulate the capacity of stromal cells to recruit $CD8^+$ T cells under inflammatory conditions by inhibiting IP-10

production and inducing TGFβ release. Soluble factors from educated B cells also altered the $CD4^+/CD8^+$ T-cell ratio as well as the corresponding cytokine profile. Lastly, using trophoblast spheroids as an *in vitro* implantation model, we demonstrate that the addition of conditioned media from educated B cells protect the spheroid from damage and facilitate migration and invasion, despite the presence of deleterious T-cell cytokines. Thus, we posit that first trimester trophoblast cells can educate B cells to acquire regulatory properties which contribute in securing successful implantation during pregnancy.

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Defining and interrogating new protein markers of human endometrial receptivity: the epithelial 'receptome'

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Background: Endometrial receptivity is critical for successful reproduction. The endometrium must be receptive and the blastocyst hatched and appropriately developed to allow adhesion and subsequent implantation. Assisted reproductive technologies have optimised the production of quality embryos. However, success rates have not changed significantly; ANZARD data indicates no change in the live birth rate from 2010 – 2015 (18.1%). These data indicate a need to gain greater understanding of, and the factors involved in endometrial receptivity to provide targets for improving reproductive outcomes.

Aim: Determine and validate a proteomic signature of human endometrial epithelial receptivity and assess the functional role of these proteins.

Methods: Hormonally primed (estrogen/progesterone) primary human endometrial epithelial cells (HEEC) were co-cultured with spheroids of human trophoblast stem cells (TS) to mimic blastocyst adhesion to the endometrium. HEEC monolayers were designated "adhesive" or "non-adhesive" based on assessment of TS adhesion after 6-hours co-culture. Matched hormonally primed HEEC monolayer-only cultures were designated 'receptive' or 'non-receptive' based on TS adhesion and subjected to proteomic comparison to provide a signature of receptivity, the epithelial "receptome". GO analysis determined functional processes regulated by these proteins. Immunohistochemistry validated proteins in 'receptive' (mid-secretory phase) versus 'non-receptive' (proliferative phase) human endometrium.

Results: 136 proteins were upregulated and 132 downregulated in the human epithelial receptome. Highly upregulated proteins include CDA, ACOT1, MAGT1, STMN1, PC4, and KYNU. CDA and STMN1 have previously been identified in the receptive endometrium, confirming the validity of this model. Cellular protein complex disassembly, translation, neutrophil degranulation and associated immune processes were enriched in the upregulated receptome proteins. CDA and ACOT1 have been validated as localized and elevated in the receptive human endometrium.

Conclusion: A proteomic signature of human endometrial epithelial receptivity has been determined and validated in the human endometrium.

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Rab13 is involved in the redistribution of desmosomes in uterine epithelial cells at the time of receptivity in the rat

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The luminal uterine epithelial cells (UECs) are the first point of contact with the implanting blastocyst and are only receptive for a narrow period of time. Specific changes in UECs are required for receptivity, termed the Plasma Membrane Transformation. These include deepening of the lateral tight junctions, a loss of adherens junctions and a redistribution of desmosomes to the apical part of the lateral plasma membrane (pm). While these morphological changes are important for uterine receptivity, mechanisms of regulation are currently unknown.

Rab13, a member of the Rab (Ras-related in the brain) family of GTPases, has a critical role in endosomal trafficking. While individual members of the Rab family have specific sub-cellular localizations, Rab13 is associated with endosomal trafficking to the lateral pm and is involved with modulation of tight junctions in several cell types. The aim of this study is to investigate the role of Rab13 in UECs during early pregnancy and to determine if Rab13 is involved in modification of the lateral junctional complex at the time of receptivity.

Confocal microscopy localised Rab13 along the entire lateral pm at the time of fertilization, which was shifted to the apical part of the lateral pm at the time of receptivity. Rab13 was consistently found below ZO-1 staining, an integral tight junction protein. There was no colocalisation between Rab13 and E-cadherin, a component of adherens junctions. Our modified immunofluorescence protocol demonstrated colocalisation between Rab 13 and desmoglein, a component of desmosomes at the time of fertilization and receptivity.

This is the first study to localize a member of the Rab family of GTPases, Rab 13, in the uterus. Rab13 was localized to the lateral pm where it colocalised with Dsg. We speculate that Rab13 is involved in the redistribution of desmosomes, which is an essential component of uterine receptivity.

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Maternal ovarian hormones drive changes in the localisation and abundance of keratin 18 and keratin 19 intermediate filaments in uterine epithelial cells

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Blastocyst implantation is a finely tuned process under hormonal control which requires significant remodelling of the luminal uterine epithelial cells (UECs). This includes a reorganisation of the cytoskeleton and is collectively referred to as the plasma membrane transformation. The keratin cytoskeleton is increasingly being identified as having a role in maintenance of cell polarity, apoptosis signalling and vesicular trafficking. We hypothesised that individual keratins would undergo specific reorganisation in UECs under the influence of ovarian hormones.

Ovariectomised rats were treated with estrogen or progesterone, either separately or in combination. Immunofluorescence and Western blotting revealed the unique localisation and changes in abundance of K18 and 19 filaments in each condition.

Immunofluorescence and Western blotting studies on uteri from ovariectomised rats indicated that the simple keratins are influenced differently by estrogen and progesterone. Keratin 18 expression is increased at the time of fertilisation in normal pregnancy as well as when ovariectomised rats are treated with estrogen and progesterone in combination. K19 expression was most abundant in the estrogen treated rats and notably reduced in all other conditions. Interestingly, K19 abundance is reduced at the time of receptivity in normal pregnancy.

This study has identified that simple epithelial keratins of uterine epithelial cells are under hormonal control, and that specific keratins are influenced by different hormones. An increase in K18 abundance associated with the hormonal conditions required for uterine receptivity is correlated with that seen at the time of implantation in normal pregnancy. Due to its known interaction and potential modulation of the actin and microtubule cytoskeleton, the K18 network may be an important component of the cellular transformation required for uterine receptivity. These results support the hypothesis that there are specific functions associated with individual keratins in the uterine epithelial cells and that they are under ovarian hormonal control.

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Furin cleaves the soluble prorenin receptor (s(P)RR) in BeWo choriocarcinoma cells but is it actually involved in syncytialisation?

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Background: The soluble prorenin receptor (s(P)RR) is a newly discovered protein in the renin-angiotensin system pathway that is elevated in the plasma of women with preeclampsia (Narita et. al. 2016. Placenta). The pro-protein convertase, furin, is known to cleave the (P)RR to produce s(P)RR in human glomerular epithelial cells. We hypothesise that this also occurs in the placenta. Furin has previously been shown to promote BeWo choriocarcinoma and primary human trophoblast syncytialisation (Wang et. al. 2013. Cell Death and Disease). However, we have evidence to suggest the contrary.

Methods: BeWo cells were incubated overnight before transfection with furin siRNA, negative control siRNA or vehicle (lipofectamine/opti-MEM) or treatment withDEC-RVKR-CMK (furin inhibitor) or vehicle (DMSO). BeWo cells were also treated with 100mM forskolin (to induce syncytialisation) or vehicle (DMSO) for 48h. Spontaneously syncytialising primary human trophoblasts were transfected as above and incubated for 72h. hCG secretion and E-cadherin visualisation were used to assess syncytialisation.

Results: *FURIN*mRNA expression and 'active' furin protein levels were significantly increased with forskolin treatment (both $P<0.01$) in BeWos. In primary trophoblasts, syncytialisation significantly decreased *FURIN*mRNA expression ($P<0.0001$, $N=5$). Furin siRNA significantly decreased *FURIN*mRNA expression, soluble and pro-furin protein levels (all $P<0.0001$) but had no effect on active furin protein levels. Neither furin knockdown or inhibition had any effect on the percent of nuclei in a syncytium or on the secretion of hCG from BeWo cells. Additionally, furin siRNA had no effect on E-cadherin mRNA expression in primary trophoblasts and DEC-RVKR-CMK had no effect on E-cadherin expression in BeWos. Both furin siRNA and DEC-RVKR-CMK significantly decreased s(P)RR secretion from BeWo cells (both $P<0.0001$).

Conclusion: Contrary to previous reports, furin knockdown/inhibition had no effect on syncytialisation of BeWo cells and preliminary results in primary trophoblasts suggest similar results. Furin cleaves the s(P)RR in BeWo cells.

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A role for α -parvin and β -parvin in implantation and decidualisation during early pregnancy in the rat

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During early pregnancy, the uterine luminal epithelial cells (UECs) and the endometrial stromal cells (ESCs) exhibit morphological changes to promote successful blastocyst implantation and decidualisation. The present study investigated cytoskeletal-associated proteins, α-parvin and β-parvin, during uterine receptivity in the rat. α-parvin and β-parvin are both involved in cell adhesion and cytoskeletal changes through binding with proteins such as actin and integrin-linked kinase (ILK). α-parvin was found present in UECs at fertilisation, decreasing by the time of implantation. β-parvin acted in opposition; significantly increasing in both UECs and ESCs at the time of implantation, suggesting a role in the process of decidualisation. Additionally, the presence of α-parvin phosphorylated at the serine-8 residue, a post-translational modification associated with cell morphology changes, was found in the nuclear region of both UECs and ESCs during implantation and decidualisation. The

current study confirmed that the presence of both β-parvin and phosphorylated α-parvin in ESCs was dependent on decidualisation occurring. This study demonstrated that the alternating balance and localisation of α-parvin and β-parvin is dependent on uterine receptivity, suggesting a co-dependent role in the cytoskeletal re-organisation crucial to the changing conditions necessary for implantation and decidualisation.

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MicroRNA biogenesis machinery is dysregulated in the endometrium of infertile women suggesting a role in receptivity and infertility

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Implantation failure is a major contributor of female infertility and a major cause of implantation failure is abnormal endometrial receptivity. Recent studies identified that there is a widespread alteration of small non-coding RNA, microRNAs (miRs) in infertile endometrium. We aimed to determine the gene expression and spatial and temporal immunolocalisation of the miR biogenesis molecules, Argonaut 1 and 2, Drosha and Dicer in endometrium.

Human endometrium was collected from women with normal fertility and primary unexplained infertility across the menstrual cycle (proliferative, early-secretory (pre-receptive), mid-(receptive) and late-secretory tissue; N=6/group). RNA was isolated from tissue and primary endometrial stromal (HESC)(N=5/group) and epithelial cells(HEEC)(N=5/group) and the mRNA expression determined by real-time RT-PCR. Protein production was determined by immunohistochemistry (N=6/group).

Drosha, Argonaut 1 and 2 mRNA were significantly reduced in endometrial tissue of women with infertility compared to fertile controls in pre-receptive and receptive phases respectively ($P<0.01$), while Dicer mRNA was not altered. By contrast, Drosha, Dicer, Argonaut 1 and 2 mRNA expression were not altered in primary HEEC and HESC isolated from infertile women compared to fertile controls respectively.

Immunohistochemistry for all 4 biogenesis machinery revealed that the staining intensity was maximal in the luminal (surface) and glandular epithelium and to a lesser extent to the stroma and vascular cells in the endometrium. Drosha immunostaining intensity was reduced in the luminal epithelium and in the pre-receptive phase in infertile endometrium compared to fertile ($P<0.01$), while in the glandular epithelium and stroma Drosha was reduced in the glandular epithelium in the receptive phase in the infertile endometrium ($P<0.01$).

This is the first study to reveal specific miR biogenesis machinery were dysregualted in the endometrium during receptivity and has identified a mechanism by which miR expression and their targets may be dysregualted in infertility and may therefore contribute to implantation failure and infertility.

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Exposure to bisphenol A and 4-tert-octylphenol result in embryo implantation failure in mouse

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In human, miscarriage due to blastocyst implantation failure is up to 2/3 of all cases. Calcium (Ca^{2+}) has been showed to involve in many cellular signal transduction pathways as well as regulation of cell adhesion, which is necessary for the process of embryo implantation. Previous studies have been reported that EDs can regulate the expression of genes associated with calcium transport in during pregnancy period such as TRPV5, TRPV6, PMCA, and NCX1. Additionally, exposure to EDs during early gestation results in disrupt intrauterine implantation, uterine receptive, leading to implantation failure. In this study, E2, BPA, OP and/or ICI 182,780 were inject by subcutaneous from gestation day 1 to gestation day 3 post coitus. The expression levels of calcium transport genes were assessed in maternal uterus and implantation site. The number of implantation sites were significantly decreased in OP group and no implantation site was observed in EDs+ICI groups. There was different in the expression of calcium transient transport channel between maternal uterus and implantation site. The levels of *TRPV6* and *TRPV5* genes were significantly increased by EDs and/or ICI treatment in uterus. The *NCX1* and *PMCA1* mRNA levels were significantly decreased in OP and BPA groups, but no significantly different in protein level in uterus. In contrary, *NCX1* and *PMCA1* mRNA levels were significantly decreased by OP- and BPA- treated in implantation site. Both mRNA and protein levels of MUC1 markedly higher in all groups, except BPA-group when compared to the VE in uterus. The *LIF* and *HOXA-10* mRNA were significantly decreased by E2; BPA+ICI; OP and/or ICI in both uterus and implantation site. The expression of estrogen receptor (*ERα*) and progesterone receptor (*PR*) were significantly lower in all groups. Taken together, these results suggest that E2, BPA and OP impairs embryo implantation through altered expression of calcium transport genes.

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High-throughput screen identifies new non-hormonal contraceptive

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The oral contraceptive pill has had enormous social and economic benefit by preventing unwanted pregnancies. Yet, unplanned pregnancy remains one of the most troubling global reproductive health issues. Global yearly estimates suggest that roughly 85 million pregnancies are unplanned, leading to millions of unsafe abortions and over 500,000 maternal deaths worldwide [1]. A major reason for this are the serious side-effects of hormone contraceptives, including cardiovascular and breast cancer risks, which lead to their non-use or discontinued use. There is an acute need for new safer contraceptives that can overcome the systemic side-effects of hormone therapy and offer wider contraceptive choices to women.

We have recently developed an *in vitro,* high-throughput screening model of ovulation, using a well-established cumulus-oocyte-complex (COC) adhesion assay. Using this approach to screen drug libraries, we identified a new class of drugs that potently and dose-dependently inhibit COC adhesion. *In vivo* testing in a mouse ovulation assay demonstrated highly significantly reduced ovulation with one candidate compound (11 vs. 26 oocytes/ovary;p5.8*10^-6) compared to controls. Notably, while there was no difference in the growing follicle counts, there was a clear increase in large pre-ovulatory follicles that failed to ovulate in the drug treated group. Gene expression analysis revealed that LH-Receptor expression and downstream signalling remained intact. Importantly, immunohistochemical analysis showed no difference in proliferative or apoptotic follicle counts between groups, suggesting minimal drug toxicity. Further, mechanistic investigations showed that drug treatment severely inhibited COC expansion and oocyte meiotic maturation when treated during *in vitro* maturation. Overall, this study is the first to 1) develop a unique high-throughput model for screening drugs for contraceptive indication; 2) identify and validate a new class of drugs with potent *in vitro* and *in vivo* potential; and 3) demonstrate a critical role for this target in oocyte-cumulus signalling during folliculogenesis and ovulation.

1. 1. Sedgh G, Singh S, Hussain R, Intended and Unintended Pregnancies Worldwide in 2012 and Recent Trends, New York: Guttmacher Institute, 2014

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Oxygen and acetyl-CoA modulate blastocyst H3K9 and H3K27 acetylation

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Preimplantation embryo development is accompanied by the restructuring of the epigenetic landscape to ensure appropriate gene activation for ongoing development. Nutrient availability modulates the levels of acetyl-CoA, a necessary cofactor for acetylation, linking metabolism with the regulation of the epigenome. Importantly, in vitro culture has recently been shown to significantly impact histone 3 lysine 9 acetylation (H3K9ac) levels. Plausibly, the detrimental effects of atmospheric oxygen on embryo development and metabolism are mediated in part via altered acetylation. Therefore, the aim of this study was to elucidate the impact of modulating oxygen, and acetyl-CoA directly using the pyruvate dehydrogenase kinase inhibitor dichloroacetic acid (DCA), on blastocyst formation, total cell numbers and acetylation.

Two PN embryos were collected from superovulated female mice and cultured in G1/G2 media, supplemented with or without 2 mM DCA under 5 or 20% oxygen. Day 4 blastocyst rates were calculated, with resultant embryos immunostained for H3K9ac and H3K27ac and counterstained with DAPI to quantify acetylation levels and total cell number. Blastocyst formation was not altered by oxygen or DCA treatment, however 20% oxygen significantly reduced total blastocyst cell number, relative to 5% cultured embryos ($p<0.0001$). Cell number was further reduced with DCA treatment under 20% oxygen ($p= 0.0044$). Significantly, both 20% oxygen and DCA treatment, alone or in combination, increased blastocyst H3K27ac ($p<0.0001$), while 20% oxygen alone and in combination with DCA also increased H3K9ac ($p<0.0001$).

These data reveal that 20% oxygen culture, and modulation of acetyl-CoA levels, significantly alters blastocyst H3K9ac and H3K27ac, highlighting how metabolite availability can markedly alter acetylation dynamics. Plausibly, increased H3K9 and H3K27 acetylation could alter fetal and placental development, and is currently being investigated.

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The amount of L-proline in mouse preimplantation embyos

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The addition of L-proline to embryo culture medium improves development of preimplantation embryos *in vitro*. Throughout preimplantation development, embryos are capable of L-proline uptake, although the rate of uptake is greatest during the zygote, 2-cell, and blastocyst stages. Unlike other amino acids, the concentration of L-proline in embryos has not been studied. We hypothesized that embryos developed *in vitro* in the absence of L-proline would contain less L-proline than those developed in the presence of L-proline. Thus, the aim of this this project was to use liquid chromatography and mass spectrometry to quantify L-proline in embryos that were developed *in vivo* and *in vitro* in the absence and presence of L-proline. It was found that *in vivo* developed 2-cell embryos contained significantly more L-proline than in vivo developed oocytes, zygotes, and 4-cell embryos. In addition, oocytes, zygotes, and 2-cell embryos were found to contain more L-proline when cultured with 400 µM L-proline, compared to those that were developed *in vivo* or cultured in the absence of L-proline. Comparatively, 4-cell embryos that had been cultured in the absence and in the presence of 400 µM L-proline contained the same amount of L-proline, which was significantly greater than that of *in vivo* developed 4-cell embryos, suggesting that L-proline may be produced endogenously. The elevated amount of L-proline in 2-cell and 4-cell stage embryos corresponds with the time at which L-proline improves development during *in vitro* embryo culture. Further knowledge of the regulation of the proline metabolic cycle and uptake in embryos is required to understand the mechanisms underlying the changing amounts of L-proline described in this study.

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Understanding female fertility and taking control of reproductive health: preliminary data from a fertility knowledge survey

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The age-related decline in oocyte quality and quantity remains a major limitation in the treatment of female infertility. This is particularly important when considered in the context of the trend for women to have children later in life (22% of Australian mothers over ≥ 35 years of age). Delays in childbearing have been concomitant with a rise in the number of couples seeking Assisted Reproductive Technologies (ART). Additionally, recent findings suggest the general fertility knowledge of Australian women at reproductive age is lacking. The current study aims to examine this gap in the fertility knowledge of Australian women, investigating the sources on which women base their understanding of reproductive health. To explore these aspects, we have conducted a survey with questions targeted towards understanding how women receive information about reproductive health. In the survey, seven general fertility knowledge questions were incorporated – including age of fertility decline, cyclic fertility, impacts of lifestyle factors and ART success. At the termination of the survey, 643 participants qualified for analysis. In assessing these responses, participants averaged only three correct answers ($P<0.001$). Among participants, 43% reported to use smartphone apps for reproductive health functions, with the top functions listed as menstrual tracking (91% of users), natural contraception (42%), and pregnancy (18%). This study has found that application may be linked to increased fertility knowledge, as app users were found to perform better than non-users, particularly when asked about the most fertile time in a menstrual cycle ($P<0.01$). These data also indicated that those who relied on school education as a source were more likely to score lower in fertility knowledge questions ($P<0.05$). This study highlights the need for development of applications with professional involvement. These findings will assist in the design of educational interventions appropriate to improve reproductive health and fertility knowledge across Australia.

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Cumulus cell membrane integrity is rapidly lost at the time of cattle oocyte activation during time-lapse microscope imaging

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During fertilisation the oocyte undergoes a dramatic increase in metabolic activity as it transitions into an embryo in a process called oocyte activation. Our pilot study shows a loss of Fluo4AM-labelled calcium fluorescence in the cumulus cells during time-lapse imaging of oocyte activation, but it is unknown how the cumulus cells are changing. This study hypothesises that this fluorescence loss is due to the cumulus cells losing their membrane integrity.

Abattoir-derived ovaries were aspirated to collect COCs, which were matured for 22 hours and fertilised with frozen-thawed spermatozoa including a no sperm control. Approximately 3 hours later, COCs were stained with 5μM Fluo4AM to label calcium, washed and put into individual 2μl drops of 1X DeadRed stock solution in a glass-bottomed confocal dish to assess cumulus cell membrane integrity. The COCs were imaged every 5 minutes for 6 hours before being returned to culture to assess development at Day 8. FIJI image analysis software was used to measure the fluorescence intensity changes in the cumulus vestment.

The mean fluorescence intensity of DeadRed staining in the cumulus cells significantly increases (Paired t-test, $p<0.001$) following a significant decrease in Fluo4AM fluorescence (Paired t-test, $p<0.0001$) at the time of oocyte activation in cattle. This loss was co-ordinated in sperm exposed COCs compared to random loss in the no sperm control but there was no relationship to embryo development. A 30% DMSO concentration with DeadRed showed the oocyte fluoresce red with no cumulus cell change, indicating the loss of membrane integrity is not due to oocyte death. However, the DeadRed fluorescence increase is only seen when both Fluo4AM and laser exposure are present.

It is concluded the change in cumulus cells at the time of oocyte activation is a result of membrane integrity loss, but this effect is induced by the imaging methodology.

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microRNA-223 mediated regulation of macrophages impacts lesion development in a mouse model of endometriosis

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Endometriosis, the ectopic growth of endometrial lesions, affects 10% of reproductive-aged women. Aberrant innate immune responses resulting in incomplete clearance of refluxed menstrual tissue in the pelvis have been implicated. The persistence or clearance of lesions appears to be governed by macrophages that exhibit either a pro-inflammatory (M1-like) or remodelling (M2-like) phenotype. microRNA-223 (miR-223) promotes M2-like macrophage activity and is elevated in ectopic endometrial tissue. We hypothesised that a miR-223 deficiency promotes M1-mediated inflammation, facilitating clearance of ectopic endometrium and that these cellular events would be reflected in a high-throughput RNA sequence analysis.

A menstrual model of endometriosis was established in miR-223 deficient mice by inoculating decidualised donor endometrial tissue subcutaneously into recipients. Lesions from miR-223-/- and miR-223+/+ mice were compared at days 7 (D7) and 14 (D14) –post inoculation (n=10-12/group). The Illumina Next-Seq500 platform was used in a RNA-seq analysis comparing un-inoculated donor endometrium, D7 and D14 lesions (n= 4/group). Differentially expressed gene (DEG) analyses ($-0.5 < logFC < 0.5$; FDR 0.1) were performed using R Studio and Qiagen IPA Software.

We found that miR-223-/- mice had significantly larger lesions, an early influx of F4/80+ macrophages, and cystic-like lesions by D14 compared to miR-233+/+ mice. RNA-seq comparison of D7 and D14 lesions identified 23 and 39 DEGs respectively; with DEGs associated with networks involved in phagosome maturation, iNOS and IL-4 signalling, indicating elevated M1 activity in miR-223-/-mice. Interestingly, when miR-223-/- and miR-233+/+ donor endometrium were compared, 646 DEG were identified with *CSF1* and *CXCL12* upregulated and *FGFR1*, *CEBPB*, and *VEGFA* downregulated in miR-223-/- endometrium. This suggests that signalling factors from donor endometrium may influence macrophage activity and subsequent lesion development in recipients.

This data demonstrates that miR-223 regulates macrophage activity, promoting endometriotic lesion development, and survival. Antagonism of miR-223 activity holds potential for future targeted therapeutics.

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Can hyperspectral microscopy detect metabolic variance in the cumulus oocyte complex and predict oocyte quality?

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Despite its wide-spread use, the success rate of assisted reproductive technologies (ART) is less than 20%. The ability of an embryo to result in a live birth is dependent of the quality of the gametes, in particular the oocyte. Thus, the development of a non-invasive tool to segregate oocytes with better developmental potential would likely improve ART success rate. During development and maturation, the oocyte and its surrounding cumulus cells have a high metabolic rate for the production of ATP: necessary for oocyte developmental competence. Autofluorescence generated by endogenous fluorophores as a result of cellular metabolism especially NAD(P)H and FAD, can be used to predict metabolic state of cells. Hyperspectral imaging captures hundreds to thousands of spectra instead of single spectrum using multiple emission wavelengths, including near-infrared, making it an alternative to metabolomics through non-invasively assessing intracellular fluorophores. This provides the capability to measure the metabolic heterogeneity between and within cells. Hyperspectral imaging was recently shown to be capable of discriminating between cancer cells of varying survival rate. In order to show that hyperspectral imaging of cumulus oocyte complexes (COCs) can be a predictive of oocyte quality, we used well-characterised metabolic inhibitors during in vitro maturation (IVM) that are known to negatively impact oocyte quality. COCs underwent IVM in the presence or absence of 2-Deoxy-D-Glucose to block glycolysis; Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrozone as a potent uncoupler of oxidative phosphorylation or etomoxir an inhibitor of β-oxidation. Following IVM (14h), metabolic heterogeneity of COCs was assessed by 40-channel hyperspectral microscopy. Preliminary results show that hyperspectral imaging of COCs in the presence of all metabolic inhibitors revealed drastic shifts in spectral profile compared to control. Advanced autofluorescence microscopy such as hyperspectral imaging may be a useful tool in assessing oocyte quality prior to fertilisation in order to improve the success rate of ARTs.

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Lactate availability affects preimplantation mouse embryo acetylation and viability

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Metaboloepigenetics, the interrelationship between metabolic function and epigenetic regulation, is increasingly recognised as a plausible mechanism underlying the developmental origins of adult health and disease. Significantly, suboptimal culture conditions during preimplantation embryo development impair embryonic metabolism and epigenetic regulation, influencing embryo viability and post-transfer developmental outcomes. Lactate availability has been shown to alter preimplantation mouse embryo metabolism accompanied by a reduced NAD^+:NADH ratio. As NAD^+ availability modulates the activity of histone deacetylases, lactate availability plausibly influences the preimplantation embryo epigenome. Therefore, this study aimed to determine whether altered lactate availability modified H3K9 and H3K27 acetylation (ac) levels. Pronucleate mouse embryos from superovulated females were cultured in G1/G2 in which the lactate:pyruvate (L:P) ratio was reduced (L:P=0) or increased (L:P=60) from control levels (L:P=30). Day 3 morula NADH autofluorescence, and day 3 morula and day 5 blastocyst H3K9ac and H3K27ac immunofluorescence were quantified, along with blastocyst total cell number A reduction in the L:P ratio did not alter total cell number, however it significantly increased endogenous NAD^+ levels in morulae ($P<0.01$), decreased H3K9ac in both morulae ($P<0.01$) and blastocysts ($P<0.01$), and reduced H3K27ac in morulae ($P<0.01$). Conversely, increasing the L:P ratio had the opposing effect, inducing a significant decrease in endogenous NAD^+ availability ($P<0.05$) and increasing H3K9ac levels at the morula stage ($P<0.01$). Increasing the L:P ratio however decreased H3K27ac levels in blastocysts ($P<0.05$). These data reveal that altered lactate availability profoundly affects endogenous NAD^+ levels, accompanied by significant restructuring of the embryo acetylation landscape. These findings highlight the ability of culture media composition to affect metaboloepigenetic regulation within the preimplantation embryo.

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Making Meat! Challenges and solutions.

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Animal sourced protein will remain a major protein source for the majority of the world's population. Indeed, the growth predictions for animal sourced protein demand by emerging economies is significant. The United Nations Environment Programme (UNEP) predicts the demand for livestock products will escalate over the next 30 years, to meet the needs of a global population of 9B by 2050[1]. By 2050, emerging economies are predicted to consume 326 million metric tons (mmt) of meat and 585 mmt of milk, being far greater than for developed economies (126 mmt of meat and 296 mmt of milk, respectively).

Furthermore, livestock breeding is faced with rapidly changing environmental conditions brought on by climate change and salination. Urban expansion is reducing available farming land and societal issues around animal welfare and minimising communicable diseases and greenhouse gas emissions are changing the way that livestock are managed.

Never before has there been a need for adoption of technologies that enable rapid genetic improvement to counter these challenges. Adaptive changes in livestock phenotypes will be met with application of genetic selection based on genotyping, now available at an affordable cost and accuracy, in conjunction with advanced reproductive technologies that capture both female and male genetics, through nucleus breeding herds focussed on particular desirable traits. Generation of new phenotypes not currently available can be met by gene editing technologies, such as CRISPR and TALENS, but societal and therefore commercial acceptability of such animals is a continuing challenge.

Finally, production of "meat" in a 'petri-dish' has been demonstrated. It offers an alternative approach with little to no environmental or welfare issues, but is likely to remain a niche product even if made affordable in the future, and has yet to be tested for societal acceptance.

[1]Nellerman et al. *United Nations Environment Programme*, GRID-Arendal, www.grida.no

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Saving the Environment

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Australia is listed as one of the world's few megadiverse countries, containing a highly diverse array of flora and fauna not found anywhere else on the planet. Unfortunately, Australia also has one of the world's worst extinction records. Many of our native species have suffered from the impacts of introduced species and habitat loss, but they are also subject to invisible threats that can affect their reproductive capacity. For example, exposure to endocrine disrupting chemicals can dramatically affect reproductive development and fertility. An understanding of reproductive biology can help mediate each of these threats. Manipulating reproduction provides opportunities to help save and preserve our threatened native fauna, it can help to control the spread of invasive pest species and help to identify environmental hazards such that policies can be put in place to protect our fauna. Reproductive strategies have helped to save the mountain pigmy possum from the brink of extinction through the introduction of virile males from a nearby population. Control of reproduction has been suggested as a method to help control the cane toad and more recently gene drive techniques, used successfully in insect species, are being suggested as a viable method to control invasive rodent (mouse and rabbit) populations in Australia. These studies highlight the importance of research in reproduction, which clearly has a pivotal role to play in saving the Australia environment.