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ABSTRACTS

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Homeobox gene *DLX3* regulates forskolin induced trophoblast differentiation

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Trophoblast cells carry out important functions required for the development of the normal placenta. Disruption of these functions is associated with significant pregnancy disorders such as fetal growth restriction and pre-eclampsia. Transcription factors regulate trophoblast functions. We are interested in one such class of transcription factors known as homeobox genes. The homeobox gene *Distal-less 3* (*DLX3*) plays a vital role in the development of the mouse placenta (Morasso, Grinberg et al. 1999) and increased levels of *DLX3* have been found in placentae affected by human fetal growth restriction (Murthi and Chui, unpublished data). However, the function of *DLX3* in the human placenta is not well established. Here, we investigated whether *DLX3* regulates trophoblast differentiation using a plasmid construct to over-express *DLX3* in the human trophoblast cell line, BeWo. Real-time PCR showed a significant increase in *DLX3* mRNA (3.1±0.1 vs. 1.0±0.2 control, $p<0.05$, n=3), as well as the mRNA of two known markers of differentiation 3-beta-hydroxysteroid dehydrogenase (3β-HSD) (8.1±1.8 vs. 1.2±0.1 control, $p<0.05$, n=3) and β-human chorionic gonadotropin (β-hCG) (54.9±0.9 vs. 49.2±1.6 control, $p<0.05$, n=3). Furthermore, forskolin mediated trophoblast differentiation was verified in BeWo cells. Following forskolin induction, real-time PCR showed a significant increase in the expression of *DLX3* mRNA (12.9±1.2 vs. 3.8±0.9 control, $p<0.05$, n=4), as well as a significant increase in the mRNA expression of the differentiation markers previously tested, 3β-HSD (28.3±2.4 vs. 1.0±0.08 control, $p<0.05$, n=4) and β-hCG (2.3±1.9 vs. 30.9±0.08 control, $p<0.001$, n=3). The expression of an additional differentiation marker, syncytin, was also significantly increased (4.0±1.9 vs. 1.0±0.08 control, $p<0.05$, n=4). Thus, we have shown that *DLX3* is a regulator of human trophoblast cell differentiation, and that forskolin acts through *DLX3* to induce trophoblast differentiation.

(1) Morasso, M. I., A. Grinberg, et al. (1999).

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Differential dietary regulation of placental and muscle myostatin in a transgenerational rat model of maternal under-nutrition

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Myostatin, a negative regulator of muscle growth, affects glucose uptake independent of insulin and also regulates placental glucose uptake *in vitro* in the human placenta. Maternal under-nutrition during early gestation has been associated with fetal programming for increased risk of metabolic disorders in later life. The aim of this project was to investigate changes in myostatin expression in placental and muscle samples in an established rat model of fetal programming. Pregnant rats were fed either a standard diet ad libitum (AD) or 30% of ad libitum (UN) throughout gestation. Female F1 offspring were in turn subjected to AD or UN conditions throughout pregnancy, giving three groups of F2 offspring: AD-AD, AD-UN and UN-AD. The F2 rats were then fed either a chow or a high fat (HF, 65% kcals fat) diet. Muscle and placental samples were collected at various time-points for analysis of myostatin and pro-myostatin expression. Myostatin mRNA expression was found to be significantly higher in placentae compared to skeletal muscle samples, and in UN-AD muscle compared to AD-AD muscle. At the protein level, expression of both myostatin dimer and precursor varied with time and across groups: particularly, placental myostatin dimer expression was significantly higher in UNAD females compared to ADUN and ADAD groups; muscle myostatin precursor expression was reduced in AD-UN and UN-AD females compared to the AD-AD group at embryonic day20; while at day140, there was a trend towards an increase in muscle myostatin dimer expression in UN-AD HF fed compared to UN-AD CHOW animals.Our data demonstrate that in response to maternal diet myostatin is differentially regulated in placentae compared to skeletal muscle, with both myostatin dimer and myostatin precursor levels affected. Modulation of placental myostatin activity through changes in relative expression of myostatin dimer and myostatin precursor in response to maternal diet may contribute to aberrant nutritional uptake by the fetus.

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Partial progesterone withdrawal during late gestation increases placental expression of 11β-HSD1 in the rat

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Fetal glucocorticoid excess programs adverse outcomes in adult offspring, including hypertension, obesity and insulin resistance. Access of maternal glucocorticoids to the fetus is regulated by the placental glucocorticoid barrier which consists of the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme and P-glycoprotein (Abcb1). Both proteins act to reduce fetal and placental exposure to active, circulating glucocorticoids. In addition, placental expression of 11β-HSD1 is thought to limit the effectiveness of the barrier by local reactivation of inert glucocorticoids. The present study measured expression of placental 11β-HSD1 and 11β-HSD2 in normal rat pregnancy and after either partial progesterone withdrawal or treatment with dexamethasone, both of which reduce fetal growth. Placentas were collected and dissected into their morphologically- and functionally-distinct zones (junctional and labyrinth) on days 16 and 22 of normal pregnancy (term=23 days) and after either dexamethasone treatment (0.75 μg/ml in drinking water from day 13) or ovariectomy (day 16) plus full estrogen and partial progesterone replacement (to approximately one third of day 22 levels). Junctional and labyrinth zone expression of 11β-HSD1 and 11β-HSD2 mRNA were determined by qRT-PCR. Labyrinthine expression of 11β-HSD1 increased markedly between days 16 and 22 and there was a concomitant decrease in labyrinthine 11β-HSD2 expression. Dexamethasone administration had no effect on the expression of either 11β-HSD isoform in either placental zone. Partial progesterone withdrawal increased 11β-HSD1 expression in both placental zones (1.9 and 3.1-fold in LZ and JZ respectively, $P<0.05$), but had no effect on 11β-HSD2 levels. In conclusion, these data confirm the pattern of placental 11β-HSD isoform expression in late rat pregnancy and suggest that that labyrinth zone 11β-HSD1 is normally suppressed by progesterone. Thus, the normal pre-partum decline in circulating progesterone may provide a key stimulus for the marked rise in labyrinth zone 11β-HSD1 that occurs between days 16 and 22 of pregnancy.

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Proteomic analysis of the effluent from perfused placental cotyledons identifies proteins associated with pre-eclampsia

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Pre-eclampsia (PE) is proposed to involve maternal systemic endothelial cell dysfunction which may be caused by excessive shedding of syncytiotrophoblast microparticles (STBM) from the placenta into the maternal circulation. We have examined STBM harvested by perfusion of placental cotyledons from PE and gestation-matched control (GMC) pregnancies. Following proteomic analysis, the proteins Calreticulin, Chloride intracellular channel 3 (CLIC3), Valosin-containing protein (VCP), Protein disulfide-isomerase A3 (ERp57) and Protein kinase C inhibitor protein 1 (14-3-3) were identified as having increased expression in PE samples. This result suggests that there may be increased rate of release of these proteins from the placenta into the maternal circulation. The aims of this work are to measure the expression of these proteins in placental extracts and maternal plasma, and to compare expression between PE and GMC using Western blots and/or ELISA with antibodies specific to each protein. Placental expression was confirmed for all five proteins, however, CLIC3 was the only protein significantly increased in the placenta in PE (17.858μg+1.62, n=27, t-test $p<0.05$) compared to GMC (10.478μg+0.76, n=25). Western blot determined there was a significant increase in calreticulin expression in plasma from PE women compared to GMC (as shown by Gu *et al.* 2008). Likewise, VCP was also increased in PE plasma (1810+280 density/0.25μl, n=10) compared to GMC (884+260, n=9). In contrast, the protein 14-3-3 was decreased in PE plasma (4 out of 27 PE plasma samples tested expressed 14-3-3) compared to GMC (10 out of 27 control plasma samples expressed 14-3-3). One of the Western blots determined that there was a significant decrease in 14-3-3 expression in plasma from PE women (199.24+186 density/2.5μl, n=10, un-paired t-test $p<0.05$) compared to GMC (2174.91±723.29, n=11). Neither ERp57 nor CLIC3 was detected in maternal plasma. Those proteins found to be significantly different in PE compared to GMC may be involved in the pathophysiology of PE. These results demonstrate that proteomic analysis of the effluent from perfused placental cotyledons is a reliable screening method to successfully identify proteins that have altered expression with PE.

(1) Gu et al. Mole. Human Repro. April 16 2008

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Homeobox gene *TGIF* is increased in human idiopathic fetal growth restriction

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Fetal Growth Restriction (FGR) is a clinically significant pregnancy disorder in which the fetus fails to achieve its full growth potential in utero. Recently, we identified a novel homeobox gene *TGIF,* in the placenta using microarray expression profiling (1). Targeted mutation of *tgif* in mouse results in placental dysfunction (2). In this study, we have investigated *TGIF* expression levels in idiopathic FGR. FGR-affected placental samples were collected based on strict clinical criteria to ensure inclusion of cases at the severe end of the spectrum of the disease. *TGIF* mRNA expression was analysed in placentae obtained from pregnancies complicated by idiopathic FGR and gestation-matched control pregnancies (n=25 each). Real-time PCR showed a significant increase in *TGIF* mRNA levels in FGR-affected placentae and gestation-matched controls [1.29±0.06 FGR versus 0.78±0.04 Control, $p<0.001$]. Western blotting using a TGIF polyclonal antibody revealed significantly increased levels of TGIF protein in term FGR-affected placentae compared with term controls [3970±1101 (n=10) versus 2323±644 (n=10), $p< 0.05$]. The spatial distribution of TGIF protein by immunohistochemistry revealed immunoreactive TGIF protein in residual cytotrophoblast cells, syncytiotrophoblast cells, microvascular endothelial cells and in stromal cells. We conclude that increased expression of homeobox gene TGIF may be a contributing factor to the developmental abnormalities seen in the FGR-affected placentae.

(1) Murthi P, Hiden U, Rajaraman G, Kalionis B. Placenta May 29, [Epub ahead of print].

(2) Bartholin L, Melhuish TA, et al. Dev Biol. 2008 May 2. [Epub ahead of print].

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Differential regulation of activin and inhibin production by interleukin 1 (IL1), transforming growth factor β1 (TGFβ1) and protein kinase C (PKC) in the Sertoli cell and granulosa cell

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Activin and inhibin are gonadal regulatory proteins comprising an α-subunit and either a β_A-subunit or β_B-subunit (inhibin A or B), or two β_A-subunits (activin A). Synthesis of the α-subunit, and the inhibins, is regulated by FSH via cAMP/protein kinase A. Regulation of the β-subunits in the gonads is less well-defined, but the IL1/MAP kinase, TGFβ /Smad and PKC pathways have been implicated. Sertoli cells and granulosa cells were isolated from 18-22 day-old Sprague-Dawley rats under standard conditions and cultured with IL1, TGFβ1 and the PKC agonists, gonadotrophin releasing hormone (GnRH) or phorbol myristate acetate (PMA). Activin A, inhibin A and inhibin B were measured in culture medium (at 48h) by ELISA. Subunit mRNA expression was measured in cell extracts (at 4h and 8h) using quantitative RT-PCR. IL1 stimulated β_A-subunit and activin A production and inhibited α-subunit and β_B-subunit expression and inhibin B production in Sertoli cells, but had no effect in granulosa cells. TGFβ1 stimulated activin A in both cell types, as well as the inhibins in granulosa cells. Surprisingly, TGFβ1 had no effect on Sertoli cell α-subunit or β_A-subunit mRNA expression, but did cause a slight reduction of β_B-subunit expression. GnRH increased activin A and inhibin A, but not inhibin B, production by granulosa cells and had no effect on Sertoli cells, which lack the GnRH receptor. However, direct activation of PKC by PMA stimulated β_A-subunit mRNA expression and activin A production and decreased β_B-subunit and inhibin B production by Sertoli cells, with marginal effects on inhibin A. These results indicate that activation of the TGFβ or PKC signalling pathways preferentially stimulates β_A-subunit expression and/or translation, leading to increased activin A secretion by Sertoli cells and both activin A and inhibin A secretion by granulosa cells. The ability of IL1 to stimulate activin A is confined to the Sertoli cell.

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Effect of TGFB1 on mammary gland development is dependent on cellular source of gene expression

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The cytokine TGFB1 is implicated in development of the mammary gland through regulation of epithelial cell proliferation and differentiation during puberty and pregnancy. We have compared mammary gland morphogenesis in virgin *Tgfb1*+/+, *Tgfb1*+/- and *Tgfb1*-/- mice, and transplanted *Tgfb1*+/+ and *Tgfb1*-/- epithelium to determine the impact of TGFB1 deficiency on development. When mammary gland tissue was evaluated respective to timing of puberty, invasion through the mammary fat pad of the ductal epithelium progressed similarly irrespective of genotype, albeit fewer terminal end buds were observed in mammary glands from *Tgfb1*-/- mice. The terminal end buds appeared morphologically normal, and a comparable amount of epithelial proliferation was evident. However, when transplanted into wildtype recipients, *Tgfb1*-/- epithelium showed accelerated invasion compared with *Tgfb1*+/+ epithelium. This suggests that the normal rate of ductal extension in *Tgfb1*-/- null mutant mice is the net result of impaired endocrine or paracrine support acting to limit the consequences of unrestrained epithelial growth. By adulthood, mammary glands in cycling virgin *Tgfb1*-/- mice were morphologically similar to those in *Tgfb1*+/+ and *Tgfb1*+/- animals, a normal branching pattern was observed, and the tissue differentiated into early alveolar structures in the diestrus phase of the ovarian cycle. Transplanted mammary gland epithelium showed a similar extent of ductal branching and evidence of secretory differentiation of luminal cells in pregnancy. These results reveal two opposing actions of TGFB1 during pubertal mammary gland morphogenesis, dependent on the cellular source of gene expression. When expressed in the epithelium, TGFB1 inhibits epithelial ductal growth, when expressed systemically, TGFB1 promotes epithelial ductal growth.

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Absence of GH-R exon 3 in marsupials and monotremes argues for a eutherian specific origin and fetal specific purpose of this domain

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Growth hormone receptor (GH-R) plays a critical role in the control of growth and metabolism in all vertebrates. GH-R consists of 9 coding exons (2-10) in all eutherian mammals, while the chicken only has 8 coding exons, and does not have an orthologous region to eutherian exon 3. To further understand the evolutionary origins of exon 3 of the GH-R we have cloned the full-length GH-R sequence in a marsupial, the tammar wallaby to determine whether exon 3 was present or absent in marsupial liver cDNA. There was no evidence for the presence of an exon 3 containing mRNA in sequence of tammar pouch young and adult livers. We next examined the genomes of the platypus (a monotreme mammal) and the grey short-tailed opossum (another marsupial). Like the tammar, the GH-R gene of neither species contained an exon 3. GH receptor can obviously function in the absence of this exon, raising speculation about the function of this domain, if any, in eutherians. A comparison of exon 3 protein sequences within 16 species of eutherian mammals showed that there was approximately 75% homology in the domain despite only 3 residues being identical (Leu12, Gln13 and Pro17). Interestingly, we detected greater evolutionary divergence in exon 3 sequences from species that have variants of GH or prolactin (PRL) in their placentas. These data show that exon 3 was inserted into the GH-R after the divergence of marsupial and eutherian lineages at least 130 million years ago.

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The absence of betaglycan affects Sox9 m RNA expression at the time of sex determination in a mouse model

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Betaglycan is a co-receptor that binds both TGF-beta and inhibin, and thereby acts as a modulator of the activities of multiple members of the TGF-beta superfamily. We have previously shown that the murine betaglycan gene is expressed in somatic cells within the interstitium of the fetal testis from 12.5 dpc-16.5 dpc. Betaglycan protein was predominantly localized to the interstitial cells surrounding the developing seminiferous cords which stained positive for Cyp11a (p450 Scc), a Leydig cell marker. In order to determine the impact of this receptor on fetal Leydig cell biology, RNA was extracted from two independently collected sets of betaglycan knockout and wildtype male and female gonads at 12.5 *dpc* and 13.5 *dpc* (n = 4 gonad pairs/set), and quantitative real time PCR was performed to determine changes in the expression levels of key genes involved in fetal Leydig cell differentiation and function. This analysis revealed that the levels of mRNA expression of *SF1*, *Cyp11a* and *Cyp17a1* were down-regulated between 12.5-13.5 *dpc* in the betaglycan knockout embryos compared to wildtype embryos immediately after the time of sex determination. Interestingly, the expression level of the key Sertoli cell marker SRY-(sex determining region Y)-box 9 (*Sox9*) was transiently decreased at 12.5 *dpc* by 50% in the knockout testis in comparison with that of the wildtype testis. No significant change was found one day later at 13.5 *dpc*. Our data show that betaglycan is predominantly expressed in the fetal Leydig cells of the murine testis and that the presence of this receptor is required for normal fetal Leydig cell differentiation. Furthermore, the transient down-regulation of Sox9 expression in null testis suggests that Sertoli cell differentiation may also be affected in betaglycan knockout mice, and that this defect may precede the defect in Leydig cell development. Supported by: the NHMRC Australia (RegKeys 338516; 241000).

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Fibroblast growth factor 9 (FGF9) in the developing marsupial

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The testicular differentiation pathway in mammals is controlled by a complex array of factors acting in multiple signaling pathways. An important group of factors that regulates this process are the fibroblast growth factors (FGFs). In particular, fibroblast growth factor 9 (*FGF9*) in mice is critical for normal sexual differentiation. *Fgf9* is thought to be important in tipping the balance of development in the bipotential gonad down the testis differentiation pathway. In the developing mouse testis, *Fgf9* is antagonistic to the expression of the ovarian differentiation factor *Wnt4*. In order to examine the conserved role of *FGF9* in mammalian testis formation, we cloned *FGF9* in a marsupial, the tammar wallaby. Tammar *FGF9* nucleotide and protein sequences are highly homologous to the human and chicken orthologues. *FGF9* is expressed during the period of testicular differentiation and our data shows that *FGF9* is upregulated during testicular differentiation in an opposite but complementary manner to that of *WNT4* expression. FGF9 protein was widely distributed in the bipotential gonads but become restricted only to the testis as seminiferous cords formed and was markedly down regulated in the ovary as differentiation proceeded. This strongly suggests that *FGF9* has a highly conserved and crucial role in male sexual differentiation similar to the mouse, and supports the suggestion that *FGF9* is involved with the control of *WNT4* expression in a balancing act to direct the developmental outcome of the gonad.

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Proteomic identification of caldesmon as one of the physiological substrates of proprotein convertase 6 during decidualization

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We have previously demonstrated that proprotein convertase 5/6 (PC6), a member of the proprotein convertase (PC) family, is a critical endometrial factor for implantation. PC6 is up-regulated in the endometrium specifically at implantation in association with epithelial differentiation (in human and monkey) and stromal cell decidualization (in the mouse, human and monkey). Knockdown of endometrial PC6 during early pregnancy in mice *in vivo* led to complete failure of implantation, while blocking of PC6 production in human endometrial stromal cells *in vitro* inhibited decidualization. PCs convert a range of precursor proteins of important functions into their bioactive forms; they are thus regarded as critical "master switch" molecules. We hypothesize that PC6 exerts its roles in the endometrium by regulating proteins of diverse functions essential for implantation. In this study, we utilized proteomic technology and aimed to identify proteins that are specifically cleaved by PC6 in human endometrial stromal cells (HESC) during decidualization. HESC were decidualized with cyclic AMP, the cell lysates were treated with and without recombinant human PC6-A (rPC6-A), and the 2D Differential in Gel Electrophoresis (2D DiGE) protein profiles were compared between the two treatments. We identified several proteins which were differentially cleaved following the addition of rPC6-A. Mass spectrometric analysis confirmed that the most abundant of these were caldesmon, tropomyosin-2, tropomyosin-4, hypoxia Inducible factor-1 and chloride intracellular channel-1. These proteins showed spot shifts in hPC6-A treated HESC lysates consistent with hPC6-A cleavage. Western blot analysis confirmed the specific cleavage of caldesmon by PC6 in HESCs, and immunohistochemical analysis showed co-localisation of caldesmon and PC6 in decidual cells in human endometrial tissue. Given that caldesmon is a structural protein previously found to be involved in actin filament reorganisation, our results strongly suggest that PC6 is a mediator of structural remodelling of stromal cells during decidualization in the endometrium.

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Specific targeting of uterine proprotein convertase 6 (PC6) facilitates the development of dual function contraception

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Proprotein convertase 6 (PC6), is a key player during embryo implantation in humans and mice. We have previously shown that PC6 is essential for decidualization in the mouse and knockdown of endometrial PC6 leads to implantation failure. The PC family of proteases, including PC6, are necessary for transmission of human immunodeficiency virus (HIV). It has been postulated that inhibition of PC activity could prevent HIV infection. We hypothesize that PC6 is a prospective target for the development of a dual role contraceptive for women to avoid pregnancy and protect from HIV infection. The aim of this study is to evaluate if a PC6 inhibitor that is capable of preventing HIV transmission can block implantation in mice. We used a generic PC peptide substrate to assess the potency of the inhibitor to block PC6 activity *in vitro*. The substrate releases a fluorochrome when cleaved by PC6; no fluorescent signals were observed in samples when inhibitor concentrations were 10μM or higher. We then gauged inhibitor uptake by the uterus over 24 hours in mice by two delivery routes; intrauterine injection (IU) and vaginal delivery (VD) with a neutral gel. Uptake was tracked with a FITC-conjugated inhibitor at 50μM (IU) and 500μM (VD). Strong fluorescent signals were seen at 2, 4, 6 and 24 hours at sites of endometrial PC6 activity in the IU and VD groups. Administration of a 50μM dosage (20μl) to the uterine lume*n* (IU) caused a significant reduction ($p=0.002$) in the number of implantation sites compared to controls (saline only) when treated between 2000-2100 on E3.75. The inhibitor's ability to block uterine PC6 activity and implantation via VD was assessed and to date outcomes have suggested that correct timing is crucial to prevent implantation and decidualization. These outcomes show the potential of the inhibitor to block implantation in mice.

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Chemokines: key players at the maternal-fetal interface

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Establishment of pregnancy requires extensive communication at the maternal-fetal interface and involves a plethora of locally acting molecules, including the chemokines. Chemokines are multifunctional molecules initially described for roles in leukocyte trafficking, but since found to participate in many other processes such as differentiation and directed migration. Previously we have shown that the chemokines, CX3CL1 and CCL14, are abundant in human endometrial vasculature, leukocytes, epithelial and decidual cells at the time of implantation and that their receptors, CX3CR1 and CCR1, are present on invading human trophoblast. CX3CL1 and CCL14 directly promote human trophoblast migration. We hypothesized that these endometrial chemokines promote trophoblast migration by regulating adhesion molecules and extracellular matrix (ECM) components on the trophoblast, similar to mechanisms used in leukocyte trafficking. Trophoblast cells (AC1M-88) used previously, showed a marked increase in adhesion to fibronectin following treatment with CX3CL1 and CCL14. Alterations in trophoblast adhesion associated and ECM genes following chemokine stimulation were examined using pathway specific oligo-arrays and quantitative real-time RT-PCR. Over 30 transcripts were affected by CX3CL1 treatment and 15 were regulated by CCL14 treatment. Real-time RT-PCR confirmed significant changes in the mRNA transcripts of alpha-catenin (CTNNA1), extracellular matrix protein-1 (ECM1), osteopontin (SPP1), integrin α6 (ITGA6), matrix metalloproteinase-12 (MMP12) and integrin β5 (ITGB5) following chemokine treatment. Several of these molecules have previously been implicated in implantation. Immunohistochemistry confirmed the presence of integrin alpha6, SPP1 and ECM1 protein in first trimester human implantation sites. The temporal and spatial expression of chemokines, their receptors and adhesion related molecules at the maternal-fetal interface emphasizes an important role in the controlled directional migration of trophoblast through the maternal decidua. For the first time this study demonstrates direct effects of CX3CL1 and CCL14 on trophoblast adhesion and ECM molecules suggesting mechanisms by which trophoblast cells migrate during early pregnancy.

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Altered placental gene expression following disruption of mitochondrial metabolism in mouse embryos

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Metabolic changes in the pre-implantation embryo are known to alter blastocyst viability. We used a mouse embryo model, inhibiting the malate-aspartate shuttle (MAS) which is required for energy metabolism in the early embryo, to examine the consequences on subsequent fetal and placental development, and placental gene expression.

Mouse zygotes were obtained from C57BL6xCBA females after gonadotrophin stimulation. Following culture in G1.2 for 48h, 8-cell embryos were cultured to the blastocyst stage in either G2.2 control media (C), G2.2 media without pyruvate (-P), or -P with 0.5mM aminooxyacetate, an inhibitor of MAS (-P+AOA). Blastocysts were transferred to day 4 pseudopregnant Swiss mice, and fetuses and placentas were harvested on day 18 of pregnancy. RNA was extracted from placentas for real time PCR expression analysis by ddCt relative to 18S. Expression of mitochondrial transcription factors (*mTERF, mTFAM, Nrf-1, Nrf-2*), glucose transporters (*Glut1 and Glut3*) and amino acid transporters (*Slc38a2 and Slc38a4*) were analysed.

Following transfer, fetal development per implantation was significantly reduced when embryos were cultured in –P+AOA conditions (25.9%) relative to both C and –P conditions (57.5% and 68.8% respectively). Although placental weight did not differ between treatment groups, fetal weight was significantly reduced for –P and –P+AOA groups ($p<0.05$), suggestive of altered placental function thus gene expression was examined. There were no differences in gene expression between placentas from C and –P conditions for any of the genes analysed. When compared to C placentas, -P+AOA conditions reduced the expression of *Glut 1* ($p=0.055$) and *Glut 3* ($p=0.043$). The –P+AOA placentas also tended to have significantly reduced expression of *Glut 3* ($p=0.06$) compared to placentas from –P conditions, but all other genes were expressed similarly.

This data suggests that decreased glucose transport may be the cause of reduced fetal growth as a consequence of metabolic perturbations in the embryo.

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Placental expression of uncoupling protein-2 is reduced by glucocorticoid treatment in late pregnancy: implications for placental oxidative stress

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Placental oxidative stress plays a key role in the pathophysiology of placenta-related disorders in humans, most notably in preeclampsia (PE) and intrauterine growth restriction (IUGR). Protection from oxidative stress is provided by antioxidant enzymes including superoxide dismutase-1 and 2 (SOD-1 and -2) and catalase (CAT), which convert reactive oxygen species (ROS) to inert products. It has also been proposed that uncoupling protein-2 (UCP2) may limit oxidative stress by reducing ROS production, but little is known of UCP2 expression in placenta. Here we measured placental UCP2, SOD-1, SOD-2 and CAT mRNA expression (by qRT-PCR) in normal gestation and after glucocorticoid-induced IUGR. The latter was included because glucocorticoids can increase oxidative stress in other tissues, and placental glucocorticoid exposure is elevated in both PE and IUGR. Placentas were collected on days 16 and 22 of normal pregnancy (term = day 23) and on day 22 after dexamethasone treatment (0.75 mg/ml in drinking water from day 13). The two morphologically-distinct regions of the placenta, the junctional (JZ) and labyrinth (LZ) zones, were analysed separately because effectively all growth occurs in the LZ over this period. Expression of UCP2 in LZ exceeded that in JZ ($P<0.001$) and increased in both zones between days 16 and 22 (LZ: 2.0-fold; JZ: 3.2-fold). Dexamethasone treatment reduced UCP2 in LZ (44%; $P<0.05$) but not in JZ. SOD1 and SOD2 increased with gestational age in LZ ($P<0.01$) and JZ ($P<0.05$), but neither were affected by dexamethasone. CAT expression was higher (2.4-fold, $P<0.001$) in LZ compared with JZ but did not change with gestational age or dexamethasone. In summary, these data suggest that endogenous protection against oxidative stress increases in the rat placenta during late pregnancy. Moreover, this protection may be compromised by reduced placental UCP2 expression in a model of glucocorticoid-induced IUGR.

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Activin A regulates trophoblast cell adhesion: implications for uterine receptivity and embryo implantation

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Embryo implantation involves blastocyst attachment to the endometrial luminal epithelium, followed by trophoblast invasion. This process involves a coordinated crosstalk between the implanting blastocyst and the endometrium. Adhesion molecules play an instrumental role during implantation and are regulated by a variety of factors including cytokines and growth factors. Activin A, a TGF-β superfamily member, has been detected in uterine washings,[1] and its subunit, β A, is produced by endometrial glands during the secretory phase of the menstrual cycle.[2] In endometriosis, a disease that associated with sub-fertility, β A immunostaining is increased in endometrial glands,[3] suggesting higher levels of activin A secreted into the uterine lumen could contribute to sub-fertility observed in endometriosis. Therefore we hypothesised that activin A secretion into the uterine cavity affects the adhesive properties of the cells present at the maternal-fetal interface. The aims of the study were to measure and compare activin A secretion in uterine washings from women with and without endometriosis and to demonstrate whether activin A regulates adhesion to extracellular matrix (ECM) components. Uterine washings (5ml of sterile saline) were collected from women with and without endometriosis during the secretory phase. Activin A was measured by ELISA. HTR8 (human trophoblast cell-line) cells were treated with rhActivin A (50 ng/ml) and assessed for binding to fibronectin, laminin, vitronectin, collagen I and IV. Activin A (>10pg/ml) was detectable in uterine washings from women with and without endometriosis and levels were elevated in endometriosis patients. Untreated HTR8 cells adhered maximally to fibronectin, collagen I and collagen IV with low binding to vitronectin and laminin. Following activin treatment, HTR8 cell binding to fibronectin, collagen I and IV was significantly decreased ($n=3$, $p<0.05$). These results suggest that activin A regulates the adhesive properties of the blastocyst during implantation. This study also implies that abnormalities in local activin A levels during endometrial receptivity may contribute to sub-fertility in women.

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Inflammatory stimulation of human decidual cells by periodontopathic bacteria

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Periodontal disease is associated with increased risk of preterm birth, although a mechanistic connection has not yet been confirmed. We hypothesised that circulating endotoxins (e.g. lipopolysaccharide, LPS) from periodontopathic organisms might possess the ability to exert highly potent immunostimulatory effects in extraplacental membranes, thereby triggering inflammatory activation sufficient to precipitate preterm labour and birth in the absence of overt intrauterine infection. We therefore tested the stimulatory effects of LPS prepared from three periodontopathic bacteria [*porphyromonas gingivalis (P.g), Aggregatibacter actinomycetemcomitants (A.a), Fusobacterium nucleatum (F.n)*] in comparison with 'standard' LPS from *E.Coli* (O55:B5). Human decidual cells were isolated by collagenase/dispase digestion with Percoll purification from term decidual membranes delivered before the onset of labour by Caesarean section. Cells were stimulated overnight with 0.02, 0.2 and 2 mg/L LPS or equivalent doses of whole cell non-viable bacteria. As an index of inflammatory stimulation, cytokine (TNF-alpha) production was measured by ELISA and normalised to cellular protein.

The different LPS preparations all stimulated decidual cytokine production, with ranked potencies as follows: Ec > Aa > Fn >> Pg. Maximal stimulation of TNF-alpha production achieved was 15-, 4.5-, 23- and 7-fold above control by Ec, Aa, Fn and Pg, respectively. Overall, the LPS preparations were more potent stimulators than whole cell bacteria, achieving greater levels of stimulation at lower doses. However, whole cell Aa was notable for its inflammatory effects, generating a >60-fold increase in TNF-alpha levels relative to control at the highest dose tested (2 mg/L).

These data highlight wide variability in the ability of periodontopathic bacteria to stimulate an inflammatory response in the human decidua, both in terms of their potency and efficacy. The potential significance of bacterial molecular patterns other than LPS as potential triggers of inflammation-driven preterm labour warrants further investigation.

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Homeobox gene *HLX* is expressed in choriodecidua mesenchymal stem cells and regulates their ability to migrate

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Mesenchymal stem cells (MSCs) can be prepared from the placenta (PMSC) and the choriodecidua component of the fetal membranes (CDMSC). PMSCs and CDMSCs share basic stem cell properties with adult MSCs but differ in their gene expression profiles and ultrastructure, showing features of more primitive and metabolically quiescent stem cells (1). Homeobox gene transcription factors are critical markers for identifying stem cells and they regulate important stem cell functions. Our lab showed the homeobox gene *HLX* is expressed in the placenta and the choriodecidua component of the fetal membranes, and is a regulator of proliferation in placental cells (2). In this study, our aim was to determine whether *HLX* was expressed in CDMSCs and to use short interfering RNAs (siRNAs) to specifically inactivate *HLX* and determine the effect on CDMSC function.

cDNA was prepared from CDMSCs and RT-PCR using *HLX*-specific primers generated the expected band size of 485bp following agarose gel electrophoresis (n=3). At the protein level, HLX expression was detected in the nuclei of CDMSCs using immunocytochemistry. The expected HLX protein product was detected at approximately 50kDa using Western blotting (n=3). Conditions were optimised for the use of short interfering RNAs (siRNA) to decrease *HLX* expression in CDMSCs with 5nM giving the most efficient downregulation. Two independent siRNAs were tested (*HLX*si3-4) and of these, *HLX*si4 resulted in significantly decreased *HLX* mRNA levels in CDMSCs as shown by real-time PCR (0.66±0.08, p=0.03, n=3). Functional assays to measure stem cell migration were carried out in quadriplicates on two samples. 10,000 cells were placed on one side of a filter and the number of cells that migrated to the other side of the filter was stained and densitometric analysis was carried out using Axiovision image analysis software. These results suggest that the *HLX*si4-mediated decrease in *HLX* expression resulted in reduced CDMSC migration ($2.6x10^3$±401 vs $1.3x10^3$±225 densitometric units, p=0.02). Therefore, *HLX* may play a role in stem cell migration.

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Parkin co-regulated gene (Pacrg) is an axonemal protein involved in sperm tail and ependymal cell function and is a candidate primary ciliary dyskinesia gene

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A leading cause of male infertility is genetic variation in genes required for sperm formation or function. Considerable evidence suggests *PACRG* is involved in spermiogeneis. The loss of Pacrg function causes infertility in mice (Lorenzetti et al. 2004) and we have shown an association between variability in the 5' untranslated region of *PACRG* and human male infertility (Wilson et al, in preparation). Evidence from studies in *C.reinhardtii* and *T.brucei* indicate Pacrg is crucial for axonome formation and microtubule stability. To assess this possibility in mammals, we generated and characterised *Pacrg* knockout (Quaking viable, Qkv), wildtype and *Pacrg* transgenic mice (Qkv-Tg). Using confocal and immunoelectron microscopy we showed that Pacrg was localised to the axonemal microtubule doublets of sperm, tracheal and ependymal cilia. The absence of *Pacrg* was associated with compromised sperm flagella formation and MRI analyses revealed the occurrence of hydrocephalus. Specifically, Qkv mice showed an inward expansion of the lateral ventricles, resulting in a significant reduction in distance between ventricles (1.0± 0.6mm, mean ± SD, n=5) and a ~250% increase in ventricle area (70±13 arbitrary units, mean ± SD, n=5) compared to wildtype littermates (1.38± 0.09mm; area 26±12, n=3). Transgenic expression of *Pacrg* was necessary and sufficient to correct the hydrocephalus (1.45±0.05mm; area 26±9, n=2) and infertility phenotypes (evidenced by daily sperm counts and litter sizes). In conclusion, we have shown Pacrg is a novel axoneme associated protein in a subset of motile cilia/flagella and loss of *Pacrg* function results in spermiogenic defects and hydrocephalus in mice. Further, we have shown that variations in the human *PACRG* promoter are a risk factor in human male infertility. Collectively these data suggest *PACRG* is a candidate gene in the human syndrome of primary ciliary dyskinesia.

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Homeobox gene *HLX* is a regulator of HGF/c-met mediated trophoblast migration

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Homeobox gene transcription factors play critical roles in normal placental development and are expressed in specialised trophoblast cells. Abnormal trophoblast cell function is associated with pregnancy disorders including fetal growth restriction. Our studies show homeobox gene *HLX* expression in trophoblast cells (1) and that *HLX* is significantly decreased in fetal growth restriction (2). *HLX* gene inactivation in cultured trophoblast cells shows that *HLX* is a regulator of cytokine-dependent trophoblast proliferation (3). Hepatocyte growth factor (HGF) activates trophoblast cell migration in a paracrine fashion and its receptor, c-met, is expressed in trophoblast cells. This study investigates the regulation of HGF/c-met mediated trophoblast migration by *HLX*, in two human trophoblast cell lines SGHPL-4 and HTR-8/SVNeo. HGF stimulation significantly increased *HLX* mRNA expression (e.g. 43.2 ± 2.5, HGF vs. 18.4 ± 1.7 control, densitometric units, $p<0.001$, n=3). siRNA-mediated inactivation of *HLX* resulted in significantly decreased trophoblast migration (e.g. 32 ± 4, siRNA vs. 127 ± 12 control, migrated cells, $p<0.05$, n=4). When *HLX* was inactivated in the presence of HGF stimulation, migration remained significantly decreased (e.g. 112 ± 15, siRNA + HGF vs. 368 ± 44 HGF, migrated cells, $p<0.05$, n=4). In order to determine if HGF is acting via the c-met receptor, the Met tyrosine kinase inhibitor, SU11274, was employed to inhibit c-met activity. c-met inhibition resulted in significantly reduced *HLX* mRNA expression (e.g. 2.1 ± 0.32, SU11274 vs. 12.3 ± 1.4 control, densitometric units, $p<0.05$, n=3). *HLX* expression remained significantly reduced with HGF stimulation and SU11274 mediated c-met inhibition (e.g. 8.02 ± 1.3, SU11274 vs. 38.3 ± 5.4 HGF, densitometric units, $p<0.05$, n=3). This is the first study to show that homeobox gene *HLX* is a downstream effector gene of HGF, that *HLX* regulates trophoblast migration and that HGF, via its receptor c-met, acts through *HLX* to control cell migration.

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Localisation of relaxin receptors (Rxfp1) in the uterine artery and the effects of blocking circulating relaxin on passive mechanical wall properties in the uterine artery of late pregnant rats

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During pregnancy, the uteroplacental circulation undergoes dramatic alterations to allow for the large increase in blood flow to the feto-placental unit. These alterations are achieved through a number of mechanisms including structural changes in the uterine artery wall and endothelium-dependent vasodilation. Small renal arteries of relaxin-deficient mice and rats have enhanced myogenic reactivity and decreased passive compliance, and are relatively vasoconstricted (Novak *et al*, 2001, 2006). To date, no study has identified relaxin receptors (Rxfp1) in arteries or investigated the effects of relaxin deficiency in pregnancy on uterine artery function. The aims of this current study were to: 1) localise Rxfp1 in the uterine arteries, 2) measure myogenic reactivity in small uterine arteries after relaxin treatment, and 3) test the hypothesis that blocking circulating relaxin in late pregnancy will increase uterine artery wall stiffness. We demonstrated that *Rxfp1* is expressed in the uterine arteries of pregnant mice and rats. Bright field immunohistochemistry and immunofluorescence using antibodies specific for rat Rxfp1, α-smooth muscle actin and CD31 localised Rxfp1 protein predominantly to the vascular smooth muscle in the uterine artery of pregnant rats. Administration of recombinant human H2 relaxin (4 ug/h) for 6 hours or 5 days in intact and ovariectomised rats reduced myogenic reactivity of small uterine arteries *in vitro*. Pregnant rats were treated with a monoclonal antibody against circulating relaxin (MCA1) or control (MCAF) for 3 days (Days 17-19) and uterine arteries were mounted on a pressure myograph to assess passive mechanical wall properties. Neutralising circulating relaxin in late pregnancy resulted in a significant increase in uterine artery wall stiffness. These data demonstrate that relaxin acts on the vascular smooth muscle cells in the uterine artery and may be involved in the pregnancy-specific vascular remodelling of uterine arteries to increase vasodilation and blood flow to the uterus and placenta.

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A new role for activin in endometrial restoration after menses

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10% of Australian women suffer from abnormal uterine bleeding (AUB). To stop endometrial bleeding after menstruation, the endometrium must repair adequately. We propose that endometrial restoration after menstruation has characteristics of wound healing and that inadequate endometrial repair may result in AUB . *In vivo* studies support a contribution of activins to skin wound healing: in mice over-expressing activins' natural inhibitor, follistatin, wound healing is significantly delayed (1).

We hypothesised that activin would enhance endometrial repair and examined its contribution using an in vitro wound healing model and our well-characterised in vivo mouse model of endometrial breakdown and repair (2). For the *in vitro* model, confluent human endometrial epithelial cells (ECC-1 cell line) were wounded and treated with carrier protein (control, 0.1% BSA), activin A (50ng/ml) or EGF (positive control: 50ng/ml). Wound areas were quantitated daily for 6 days. For the *in vivo* study, serum follistatin levels were measured by ELISA in follistatin over-expressing mice (FS) (2) and wild-type (WT) littermates. Mice were induced to undergo endometrial breakdown and repair (mimicking menstruation in women). Activin βA was immunolocalised during endometrial repair, and extent of repair assessed using our morphological scoring system (2).

ECC-1 wound repair was significantly ($p<0.05$) enhanced by activin A treatment vs control from days 2-6 of culture. In WT mice, activin βA localised to areas of endometrial repair. Serum follistatin was significantly elevated in FS mice vs controls (33.3±3.8 v 7.07±1.8 ng/ml, $p<0.01$). In FS mice ($n= 8$) only 50% of uterine sections showed complete repair after endometrial breakdown, significantly less than those from WT animals ($n=15$, $p<0.05$) where 85% of sections demonstrated complete repair. These results demonstrate for the first time that activin A functions to promote endometrial restoration following menses and that this can be delayed under physiological conditions: such studies indicate potential treatments for AUB.

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Interleukin-11 inhibits human trophoblast invasion via STAT-3 and not MAPK, indicating a likely role in the decidual restraint of trophoblast invasion during placentation

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Successful pregnancy depends on the precise regulation of extravillous trophoblast (EVT) invasion into the uterine decidua, primarily by decidua-derived factors. In humans, during early pregnancy, interleukin (IL)-11 is maximally expressed in the decidua[1], with its receptor, IL-11-receptor alpha (Rα) also identified on invasive EVT *in vivo*[2]. While a role for IL-11 in EVT migration has been established[2], whether it also plays a role in regulating EVT invasion is unknown. We investigated whether IL-11 influences human EVT invasion and the signalling pathways and underlying mechanisms involved using the HTR-8/SVneo immortalized EVT cell-line and primary EVT as models for EVT. The effect of IL-11 on tyrosine phosphorylation (p) of signal transducer and activator of transcription (STAT)-3 was determined by Western Blot. EVT invasion was assessed using *in vit*ro Matrigel invasion assays. To elucidate the mechanisms by which IL-11 may influence EVT invasion, matrix metalloproteinase (MMP) and urokinase plasminogen activator (uPA) activity were assessed by gelatin and plasminogen zymography / uPA activity assay respectively. Tissue inhibitor of MMPs (TIMPs)-1 and -2, plasminogen activator inhibitor (PAI)-1 and -2 and uPA receptor (uPAR) were assessed by ELISA whereas TIMP-3 was assessed by Western Blot. EVT adhesive properties and integrin expression were assessed by *in vitro* adhesion assays. IL-11 (100 ng/ml) significantly inhibited invasion of EVT cells by 40-60% ($p<0.001$). This effect was abolished by inhibitors of STAT-3 but not of mitogen-activated protein kinase pathways. IL-11 (100 ng/ml) had no effect on MMP-2 and -9, TIMP 1-3, uPA, uPAR, PAI-1 and -2 in EVT conditioned media and / or cell lysates. IL-11 (100 ng/ml) also did not regulate EVT cell adhesion or integrin expression. These data demonstrate that IL-11 inhibits human EVT invasion via STAT-3 indicating an important role for IL-11 in the decidual restraint of EVT invasion during normal pregnancy.

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Disruption of bi-directional oocyte-cumulus paracrine signalling during oocyte in vitro maturation reduces subsequent mouse fetal survival

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During folliculogenesis, oocyte to cumulus cell (CC) bi-directional communication is essential for normal development of the oocyte. We recently showed that addition of recombinant oocyte paracrine factor growth differentiation factor 9 (GDF9) during mouse oocyte *in vitro* maturation (IVM) increased fetal viability. GDF 9 signals through SMAD 2/3. Hence the effects of disrupting SMAD2/3 signalling and its interaction with FSH/EGF during IVM on oocyte development and subsequent fetal outcomes were investigated.

Cumulus-oocyte complexes (COCs) from antral follicles (N=400-500) of eCG treated pre-pubertal (C57BL/6xCBA F1 hybrid) mice were cultured for 18h in Waymouth's medium+5% serum, with or without 50mIU/ml FSH and 10ng/ml EGF, SMAD2/3 inhibitor SB-431542 (4μM), or its 0.04% DMSO control. Meiotic maturation was assessed by first polar body (PB1) extrusion immediately after culture. COCs were fertilised and cultured to the blastocyst stage in G1.2/G2.2 media at 37 ° C in 6%CO_2:5%O_2:89%N_2. Blastocysts were either transferred to pseudo-pregnant Swiss females or differentially stained. Pregnancy outcome was assessed on Day 18 of pregnancy.

Inhibition of SMAD 2/3 signalling did not alter meiotic maturation. No differences were observed in the percentage of blastocysts or hatching blastocysts from cleaved embryos with SMAD2/3 inhibition or the absence of FSH/EGF. However, IVM with SB-431542 or without FSH/EGF significantly decreased ($P<0.001$) blastocyst inner cell mass percentages (26% vs 35% control;18% vs 28% control respectively). Fetal survival (fetuses per embryo transferred) of oocytes matured with SB-431542 was significantly decreased (30% vs. 50% controls; $P<0.05$) although implantation rates and subsequent fetal weights were unaffected.

These findings demonstrate the importance of oocyte-CC communication throughout IVM. Inhibition of oocyte signalling through SMAD2/3 resulted in reduced blastocyst quality and fetal survival; outcomes similar to that of oocytes matured without FSH/EGF. Oocyte-cumulus cell bi-directional communication is thus an important feature of oocyte viability and has a substantial impact on subsequent fetal outcomes.

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Characterization of a diverse secretome generated by the mouse preimplantation embryo

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This study investigated the suitability of surface-enhanced laser desorption and ionization time-of-flight (SELDI-TOF) and electro-spray ionization (ESI) mass spectrometry for the analysis of the proteins released by the mouse preimplantation embryo in vitro. SELDI-TOF analysis with CM10 or IMAC30 (but not Q10) chips detected a protein peak at m/z 8570 released by both C57BL6 and hybrid embryos. No other peaks unique to the embryo were identified with this method. ESI mass spectrometry of tryptic digests of embryo conditioned media identified a total of 20 proteins released during development from the zygote to blastocysts stage. Four proteins were expressed in at least 7 out of 8 cultures tested, one of these (lactate dehydrogenase B) was in all cultures. A further five proteins were in at least half of the cultures and 11 more putative proteins were detected in at least one culture. The pattern of protein secretion was not obviously different for C57BL6 or hyrid embryos. The expression of two of these proteins is essential for preimplantation embryo development (NLR family, pyrin domain containing 5 protein and peptidyl arginine deiminase, type VI). A further four proteins detected have roles in redox regulation of cells, and three others are capable of inducing post-translational modification of proteins. This study shows the feasibility of ESI mass spectrometry and the limitations of SELD-TOF mass spectrometry for identifying the proteins sceretd by the preimplantation embryo in vitro. This analysis identifies a range of targets that now require detailed functional analysis to assess whether their release by the embryo is an important property of the early embryo, or an artifact of in vitro culture.

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Characterisation of a putative mouse sperm-zona pellucida receptor complex

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Mammalian spermatozoa acquire the ability to fertilise an oocyte as they ascend the female reproductive tract. This process is characterised by a complex cascade of biophysical and biochemical changes collectively known as capacitation. The attainment of a capacitated state is accompanied by a dramatic reorganisation of the surface architecture which renders the spermatozoa competent to recognise the heterogenetic matrix of the zona pellucida surrounding the oocyte and initiate fertilisation. Emerging evidence from our laboratory indicates that this process is facilitated by molecular chaperone-mediated assembly of a multimeric receptor complex on the sperm surface. However, to date the presence and composition of such a complex has yet to be described. Through the novel application of blue native polyacrylamide gel electrophoresis (BN-PAGE), we have provided the first evidence that capacitated mouse spermatozoa express high molecular weight, multimeric protein complexes on their surface. Interestingly, at least two of these complexes contain heat shock protein 1 (HSPD1), a molecular chaperone that has previously been implicated in sperm-zona pellucida interaction. Furthermore, we were able to demonstrate that one of these complexes also possessed an affinity for solubilised zona pellucida as determined by Far-Western blotting. 2D BN-PAGE was employed to further delineate the individual constituents of this high molecular weight complex, with a number of other chaperonin proteins not previously reported in functional sperm indentified. Collectively, these results support the notion the sperm-zona pellucida interaction are mediated by a multimeric receptor complex. Our current work is focused on the identification of the key zona adhesion molecules that comprise this complex.

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Eggs with a surprise: the "sperm-specific" protein SPRASA is also expressed in the oocyte

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SPRASA is a newly identified protein which i*n silico* analysis suggests is not expressed in other tissues. Antibodies reactive with SPRASA have been identified in some infertile men and an antiserum reactive with recombinant SPRASA prevented human sperm binding to hamster oocytes *in vitro*, indicating an important role in sperm/oocyte recognition. The aim of this study was to investigate the spatial and temporal expression of SPRASA in reproductive and other tissues.

Brain, thymus, heart, spleen, kidney, liver and the reproductive organs from duplicate female and male Balb/C mice were collected at several postnatal timepoints. RNA was extracted, reversed transcribed and analysed by quantitative real time PCR for SPRASA expression. Abattoir-derived, *in vitro* matured, bovine oocytes were examined for SPRASA expression by fluorescent immunochemistry. To examine SPRASA binding sites on oocytes, matured bovine oocytes were exposed to biotinylated recombinant human SPRASA or biotinylated alpha-lactalbumin (control), then visualised by confocal microscopy using DTAF-conjugated streptavidin.

We found SPRASA mRNA was expressed in the reproductive organs of both females and male mice from postnatal day 10. Fluorescent immunochemistry indicated SPRASA was expressed on the oolemmal membrane and in the few cumulus cells remaining attached to zona-intact oocytes. Control preimmune serum did not stain the oocytes or cumulus cells.

Recombinant human SPRASA bound to the oolemmal membrane of both zona intact and zona free bovine oocytes.

To date the expression of SPRASA has only been reported in the testes/sperm with an additional single EST identified in brain. Our quantitative real-time PCR analysis demonstrated SPRASA is also expressed in the female reproductive organs. This was confirmed by our immunoassays which show oocytes and possibly cumulus cells express SRPASA while the oolemmal membrane has the ability to bind (sperm-derived) SPRASA. That SPRASA expression is restricted sperm and oocytes confirms the likely function of this protein in reproduction.

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Proteasomal activity during mouse preimplantation development

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Function of the 26S proteasome, a proteolytic organelle directed at proteins targeted for turnover by polyubiquitination, in preimplantation embryos is unclear. But it is well known to play a role in regulating meiosis. This paper reports the distribution of the proteasome and assessment of its functional importance in preimplantation development.

Embryos from superovulated mice were either paraformaldehyde fixed for immunolabelling with a rabbit polyclonal antibody against the 20S proteasome core or cultured in KSOM medium with and without reversible (MG132) or irreversible (beta-lactone) proteasomal inhibitors. Morphology, cell number, apoptosis and proteolysis were measured. Although diffuse throughout embryonic cytoplasm, there were distinct proteasomal concentrations in pronuclei, nuclei and cortical cytoplasm. When beta-lactone was used to block blastocyst proteasomal proteolysis, about 25% of protein degradation was found to be proteasome-specific. Treatment of 2-cell embryos for more than 3 h with MG132 blocked blastocyst formation completely, even after washout, whilst both inhibitors reduced cell proliferation over the ensuing 48 h. Two hours exposure to MG132 tripled the proportion of apoptotic cells in expanded blastocysts 96 h post hCG.

The nuclear concentration of proteasomes suggests a particular role in nuclear protein degradation possibly including the timed destruction of cell-cycle regulators and anti-apoptotic factors. This is supported by the loss-of-function studies which show that cell proliferation as well as morphogenesis require proteasomal activity at the late 2-cell stage and that without it apoptosis is dramatically increased. The mechanisms involved in the activation of apoptosis as a result of proteasomal inhibition in the early embryo are unknown but may include JNK signalling although this is controversial. More intriguing however is the identity of the proteasomal targets in the 2-cell embryo that must be degraded to permit continued morphogenesis.

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Identification of transforming growth factor beta2 (TGF-β2) and its receptors TGF-βRI and TGF-βRII in the possum (*Trichosurus vulpecula*) prostate: evidence of seasonal changes

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Benign Prostatic Hyperplasia is an enlargement of the prostate affecting the ageing male population. The common Brushtail possum (Trichosurus vulpecula) has been identified as a possible model to study factors regulating prostate growth because its prostate grows and regresses seasonally. Transforming growth factor Beta 2 (TGF-β2) is present in human prostatic tissue. In vitro, TGF-β inhibits epithelial cell, but stimulates stromal cell proliferation (Mori et al., 1990). TGF-β2 binds to TGF-β receptor II (TGF-βRII), which then recruits the type 1 receptor (TGF-βRI) (Saez et al., 1998) The aim of this study was to identify any seasonal changes in expression of TGF-β2 and its receptors in the possum prostate. Six wild-caught possums were sacrificed in each of the months of January, March, May, July, September and November. The prostates were divided into a cranial and caudal region and immunohistochemistry and Western Blot analysis performed. In each animal the glandular and periurethral areas of the caudal and cranial prostates were examined separately. Immunohistochemistry identified the presence of TGF-β2 in both the stromal and epithelial cells of the glandular and periurethral areas of the cranial and caudal regions. In the cranial tissue, more immuno-positive stromal cells than epithelial cells were present, whereas in the caudal tissue immuno-reactivity was predominantly localised to the epithelial cells. Analysis of the Western blots suggested that TGF-β2 expression was lowest immediately before and during the breeding season (March, May). Both TGF-βRI and TGF-βRII were identified in all regions of the prostate. Furthermore, immunohistochemistry revealed that the receptors were co-localised in the epithelial and stromal cells in all areas. TGF-β2 and its receptors are present in the possum prostate. TGF-β2 localisation varies between the caudal and cranial regions and as predicted from in vitro experiments TGF-β2 expression decreases during prostate growth.

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Megalin, RAP and Nkx3.1 expression in the developing reproductive tract of a marsupial, the tammar wallaby

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Androgens induce the differentiation of the urogenital sinus (UGS) to form a prostate. An early marker of this response is upregulation of the transcription factor Nkx3.1 in the urogenital epithelium in the precursors of prostatic buds. In tammars, prostate differentiation begins about 3 weeks after birth and after the time the testis starts to secrete androgens, and 2 weeks after androgen stimulated Wolffian duct differentiation. The reason for this delay in prostate differentiation is unexplained. Androgen receptors are present in the UGS, and the potent androgen, androstanediol, induces prostatic development in females. Whilst androgens may diffuse into cells by across the cell membrane, there is increasing evidence that steroids are also internalized actively via the cell-surface transport molecule Megalin. We are exploring the possibility that the delay may be related to the establishment of a Megalin-mediated pathway. Megalin is a cell surface receptor expressed on epithelia and mediates the endocytosis of a wide range of ligands, including SHBG-bound sex steroids. Megalin action is regulated by Receptor Associated Protein (RAP), which acts as an antagonist to Megalin action.

This study cloned partial sequences of *Megalin*, *RAP* and *Nkx3.1* and examined their expression in the developing urogenital sinus of the tammar wallaby using RT-PCR. The cellular distribution of Megalin protein in the developing UGS was examined using immunohistochemistry. *Megalin, RAP* and *Nkx3.1* in the tammar were all highly conserved with eutherian orthologues. Megalin and Nkx3.1 transcripts were detected in the liver, kidney, ovary, testis and developing urogenital sinus of male and female tammars. In the developing UGS of the tammar, there was strong staining for Megalin protein in the urogenital epithelium with some diffuse staining in the surrounding mesenchyme. Together, these results suggest that Megalin could be a key gene in the mediation of androgen action in prostatic development in the tammar wallaby.

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Sertoli cells de-differentiate in men after chronic gonadotrophin suppression

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Recent studies suggest that adult Sertoli cells are not part of a homogenous and terminally differentiated population in phenotypes infertility[1-4]. The aims of this study were to compare the differentiation status of Sertoli cells from normal men undergoing gonadotrophin suppression by hormonal-based contraception (androgen plus progestin), as well as in pre-malignant and malignant testicular cancer. Confocal microscopy was performed to assess the expression of markers of cell proliferation (PCNA) and differentiation (androgen receptor) in Sertoli cells in all tissues. As additional markers of differentiation, Sertoli cell tight junction (claudin-11, JAM-A) and associated proteins (ZO-1) were assessed in men with testicular cancer. Samples from five different men were assessed in each group.

In normal men, Sertoli cells exhibited a differentiated phenotype (i.e. PCNA negative, androgen receptor positive). However, after gonadotrophin suppression, 1.7 ± 0.6% of Sertoli cells exhibited intense PCNA reactivity and a reduction in androgen receptor immunoreactivity, demonstrating an undifferentiated phenotype. PCNA-positive Sertoli cells were never observed in pre-malignancy, and were only rarely observed in malignant testicular cancer, indicating a potential change in differentiation. Tight junction protein localisation was disrupted in pre-malignant cancer, with a reduction in JAM-A reactivity in Sertoli cells from pre-malignancy and strong JAM-A reactivity in malignant cancer; suggesting a potential role for JAM-A expression in the progression of testicular cancer. We conclude that Sertoli cells are not a homogenous and terminally differentiated population in men and their differentiation is modifiable by hormones and in the disease state.

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Developmentally regulated activin A signal transduction by Sertoli cells is required for normal mouse testis development

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Activin A, a TGF-beta superfamily ligand, is critical for normal mouse testis development and quantitatively normal sperm production. Testicular activin production changes during development, being substantially higher in the immature testis relative to the adult [1, 2] . Activin influences the Sertoli cell, the nurse cell to developing sperm, enhancing proliferation during its immature phase, but not following terminal differentiation [3] . In the *Inha*$^{-/-}$ mouse, chronic excessive activin production results in Sertoli cell-derived tumours [4] whereas reduced activin bioactivity, in the *Inhba*$^{BK/BK}$ mouse, delays fertility [5] . Activin signals are transduced by the phosphorylation and nuclear accumulation of the transcription factors SMAD2 and SMAD3. By comparing activin signal transduction in immature versus terminally differentiated Sertoli cells, using quantitative confocal microscopy and Western blot analysis of total and phosphorylated SMAD2 and SMAD3, we discovered that mouse Sertoli cells exhibit developmentally regulated activin responses. Activin induces nuclear accumulation of SMAD3, but not SMAD2, in immature cells, although both proteins are phosphorylated. In contrast, terminally differentiated cells exhibit nuclear accumulation of both SMAD2 and SMAD3. We observed that this shift coincides with decreased SMAD3 production at puberty and changes in FSH-induced *Smad* transcription, which favours *Smad3* in immature cells but promotes *Smad2* synthesis in terminally differentiated cells. Furthermore, whereas removal of SMAD3 from the *Inha*$^{-/-}$ mouse rescues the tumour phenotype [6] , we demonstrated that insufficient SMAD3 production impairs testis growth . We hypothesized that this developmentally regulated SMAD utilization drives specific transcriptional outcomes. Using microarray and quantitative PCR, we identified novel activin target genes displaying developmental stage-specific expression patterns coinciding with differential SMAD usage, including *Gja1* and *Serpina5* which are required for male fertility. These mRNAs are also modulated *in vivo*, increased 1.5-2 fold in *Inha*$^{-/-}$ testes and decreased by half in *Inhba*$^{BK/BK}$ testes, confirming that normal testis development requires carefully regulated activin production and responsiveness.

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A role for activin/inhibin in mouse gonocyte relocation and proliferation

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Gonocytes in the testis resume proliferation after birth and relocate to contact the basement membrane of the seminiferous cords where they become spermatogonia. A previous *in vitro* study indicated that activin can increase gonocyte numbers on day 3 post partum (dpp) rat testis, while the activin antagonist, follistatin, together with FSH , increased the number of spermatogonia (Meehan *et al.,* 2000). The aim of this study was to understand how FSH, activin and inhibin, a potent activin antagonist, interact to influence gonocyte proliferation and relocation in the newborn mouse testis using in vivo and in vitro approaches.

Two mouse models were analysed, the inhibin alpha knock out (*inh a -/-*) mouse and the *InhbaBK* mouse. The *Inh a -/-* mouse lacks inhibin, and thus activin acts unopposed by its most potent antagonist (Matzuk *et al.,* 1992). The *InhbaBK* mouse has the *Inhbb* allele inserted into the *Inhba* locus, thus directing the expression of the less bioactive activin βB, in the spatiotemporal pattern of activin βA (Brown *et al.,* 2000). In addition, an *in vitro* model was developed in which 1dpp wild type testis fragments were cultured in hanging drops for 24 hours with the addition of combinations of activin, inhibin and FSH. Gonocyte proliferation in *inh a -/-* was assessed using proliferating cell nuclear antigen (PCNA). A significant increase in germ cell proliferation and relocation to the basement membrane was measured in 0dpp *inh a -/-* , while no difference was observed at 4dpp. The opposite was observed in *InhbaBK* mice, with reduced gonocyte migration in mutant animals at 0dpp. In vitro, inhibin seemed to inhibit proliferation and reduce the percentage of relocated gonocytes while FSH showed a tendency for the opposite effect on gonocyte migration. These findings show that inhibin levels affect germ cell development during early postnatal development in mouse testis influencing both cell maturation and proliferation.

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Hedgehog signalling components in adult rat testis spermatogonial cells

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In normal tissues, Hedgehog-induced progenitor cell proliferation is transient and tightly regulated, preventing continuous regeneration. However, activation of constitutive Hedgehog signalling results in unregulated self-renewal of progenitor cells in association with several human cancers. Although the contribution of Hedgehog signalling to cancers is widely accepted, its impact on spermatogonial stem cells and impact on male fertility are unknown.

In this study, we aimed to clarify the possible role of Hh signalling on normal spermatogenesis in the adult rat and in adult testicular stem cells in the irradiated model {1}. Adult male rats were obtained from Monash University Central Animal Service and killed by cervical dislocation before tissue removal and fixation in Bouins for routine histochemical procedures. For studies on irradiated testes, adult LBNF1 male rats (hybrids between Lewis and Brown–Norway) were purchased from Harlan Sprague–Dawley, Inc. (Indianapolis, IN, USA). Testes were irradiated with 6 Gy to deplete all maturing germ cell types. At 15 weeks after irradiation the animals were injected simultaneously with 1.5mg each of Cetrorelix pamoate and Cetrorelix acetate. Testes were collected 1, 2 or 4 weeks after injection. *In situ* hybridization combined with immunohistochemistry was performed using DIG-labeled cRNA probes to identify the cells in which Hedgehog signalling components are made {2}.

Signals for mRNAs encoding t he transmembrane receptors Ptc2 and Smo are most intensely detected in spermatogonia and spermatocytes and are much less intense in the round spermatids. The mRNA for the cytoplamic regulator, Fused, is restricted to the earliest germ cell types, whereas expression of the negative cytoplasmic regulator, SuFu, only begins in the round spermatids and persists in elongating spermatids. Gli1 and Gli3 are expressed from spermatogonia through to round spermatids, whereas Gli2 is restricted to spermatogonia and spermatocytes. This pattern mimics what was reported for mouse {2}. Examination of the irradiated rat testis model revealed that Hedgehog signalling machinery is produced by resting spermatogonial stem cells but is turned off when they differentiate in response to hormones. This matches the emerging understanding of Hedgehog signals in cancer stem cells and provides the first demonstration that Hedgehog signaling may influence stem cells in the adult testis.

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Activin C antagonizes activin A *in vitro* and over-expression leads to prostate pathologies *in vivo*

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Activin A is a well characterised inhibitor of proliferation in most epithelial cells. The actions of activin A on cell growth are mediated through Smad-dependent pathways. Activin A is potent at low levels, therefore its synthesis and bioactivity must be tightly regulated. Follistatin binding or inhibin subunit heterodimerisation block access to the activin receptor and/or receptor activation. We postulate that another mechanism of regulating activin A bioactivity is through the activin-β_C subunit. In order to test our hypothesis produced recombinant activin C and mice over-expressing activin-β_C. Recombinant activin C abrogated activin A-induced growth inhibition *in vitro* and the mechanism of action was down-regulation of activin A-induced Smad signalling molecules. In the prostate over-expression of activin-β_C increased epithelial cell proliferation while there was no significant difference in apoptotic epithelial cells. This imbalance between proliferation and apoptosis led to a significant increase in ventral prostate weight, prostatic hypertrophy and epithelial cell hyperplasia. A significant decrease in nuclear localisation of Smad-2 was associated with activin-β_C over-expression in the prostate which implies antagonism of activin signaling also occurs *in vivo*. This is the first study to provide evidence that activin-β_C is an antagonist of activin A *in vitro and in vivo* and implicates a role for the activin-β_C subunit in maintenance of tissue homeostasis in the prostate.

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Effects of long term recombinant rat follicle-stimulating hormone replacement on the restoration of spermatogenesis after chronic suppression of gonadotrophins in adult rats

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Follicle stimulating hormone (FSH) in short term rat studies supports spermatogenesis at multiple levels, notably spermatogonial development. The role of FSH in supporting full spermatogenesis in rats is still in question as long term studies have not been possible due the development of neutralising antibodies to heterologous FSH preparations. This study sought to assess the effects of a homologous recombinant rat FSH (rr-FSH) preparation on the long term restoration of spermatogenesis. Adult rats were GnRH-immunised (GnRH-im) for 12 weeks then, administered an anti-androgen; flutamide (flut), alone or together with rr-FSH (8µg/rat/daily) for 56 days (1 spermatogenic cycle). Germ and Sertoli cell numbers were quantified using an optical disector stereological method. Testis weight, serum FSH and inhibin B and Sertoli cell nuclear volume were significantly reduced to 15%, 13%, 25% and 57% of controls respectively, following GnRH-im+flut treatment. GnRH-im+flut treatment reduced A/I spermatogonial, type B spermatogonial+preleptotene, leptotene+zygotene and early pachytene spermatocyte numbers to 28%, 68%, 50% and 19 % ($P < 0.001$) of controls respectively, with later germ cells rarely observed. After FSH treatment, no significant affect on testis weight, serum FSH and inhibin B or Sertoli cell number were observed. However, rr-FSH treatment significantly increased numbers of A/I spermatogonia, leptotene+zygotene and early pachytene spermatocytes from 28=> 42%, 50=>69% and 19=>27% of controls, respectively, while no differences were observed in later germ cell types. rr-FSH also increased ($p<0.05$) the volume of Sertoli cell nuclei from 57=> 66% of control. In conclusion, FSH is unable to support full rat spermatogenesis; however, FSH can partially support germ cells notably spermatogonia through to early pachytene spermatocytes, despite the absence of androgenic support.

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AR-mediated androgen actions are essential for normal mouse uterine growth and development but not implantation and embryo development

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Recently the androgen receptor (AR) has been shown definitively to play a role in female reproduction. We generated a homozygous $AR^{-/-}$ female mouse using Cre/LoxP recombination for an in-frame excision of exon 3, encoding the second zinc finger essential for DNA-binding, while allowing production of an exon 3 deleted mutant AR protein which is nonfunctional as a nuclear transcription factor. $AR^{-/-}$ females were sub-fertile due primarily to ovulatory dysfunction (*1*) . However, the mechanism(s) of the observed sub-fertility remains to be fully defined. To evaluate the role of AR in uterine function we carried out a morphological and function analysis of the $AR^{-/-}$ uterus. Uterine weights did not differ, however, $AR^{-/-}$ females exhibited a significant increase in uterine horn length ($P<0.01$), and a significant reduction in uterine diameter ($P<0.01$) , total uterine area ($P<0.01$), endometrial area ($P<0.05$) and myometrial area ($P<0.01$), indicating a role for genomic AR-mediated actions in physiological uterine growth and development. Furthermore, during late pregnancy $AR^{-/-}$ females had significantly fewer implantation sites ($P<0.01$), fetuses present in utero ($P<0.05$) and a lower serum progesterone concentration ($P<0.01$). In spite of these findings, $AR^{-/-}$ females had normal gestational length, parturition and pup weights, as well as similar pre- and post implantation losses compared with $AR^{+/+}$ females. Therefore, although AR is not essential for normal uterine reproductive function, disrupting genomic AR signalling in the uterus leads to dysfunctional uterine development which may have important long-term functional consequences for hormone dependent uterine disorders such as endometrial hyperplasia and cancer.

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Endometrial vessel morphology is altered following progestin treatment in a mouse xenograft model

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Human endometrium undergoes cyclic changes under the influence of oestrogen and progesterone. When progestins are used for contraception, the endometrium regresses and breakthrough bleeding often occurs. The aim of this study was to investigate the short term effects of progestin on human endometrium in a mouse xenograft model. Uterine tissue was placed subcutaneously into NOD/SCID mice (n=12). Mice were given estradial valerate every fourth day for two weeks. Mice then received an implant containing medroxyprogesterone acetate (MPA) or an empty implant. After two weeks, mice were dissected and the xenografts formalin fixed and serially sectioned (5μm) for immunohistochemical analysis. Sections were double immunostained for alpha-smooth muscle actin and either FVIII (blood vessels) or D2-40 (lymphatic vessels). The endometrium from the progestin treated group contained decidual-like stroma cells and glandular epithelium with morphology ranging from squamous to columnar. The endometrium from the control group also contained fibroblast-like stromal cells and glandular epithelium with tall columnar epithelium. The endometrial blood vessel density was significantly reduced in the progestin-treated group (156.3 ± 13.4 vessel profiles/mm^2) compared to the control group (273.5 ± 41.5 vessel profiles/mm^2) (P=0.02); there was no significant difference in lymphatic vessel density (progestin: 43.5 ± 5.9 vs control: 35.6 ± 9.6 vessel profiles/mm^2). Blood vessel area was significantly increased in the progestin-treated group ($3.7x10^{-4} \pm 1.7x10^{-5}$ mm^2) compared to controls ($1.8x10^{-4} \pm 1.2x10^{-5}$ mm^2) (P=0.0001) and the lymphatic v essel area was also significantly increased in the progestin-treated group ($8.8x10^{-4} \pm 7.8x10^{-5}$ mm^2) compared to controls ($2.9x10^{-4} \pm 5.7x10^{-5}$ mm^2) (P=0.0001). This work has provided a model for the study of human endometrial vasculature, illustrating a significant increase in blood and lymphatic vessel size during progestin treatment. The increase in blood vessel size was associated with a significant reduction in blood vessel density in progestin treated samples.

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Expression of PAF-R and p53 in the endometrium during entry into and reactivation from diapause in the tammar wallaby

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Embryonic diapause is widespread amongst mammals, but is especially common in the kangaroos and wallabies. In the tammar, *Macropus eugenii*, the sequence of endocrine events leading to embryonic diapause and reactivation are well defined and the blastocyst can remain in diapause for up to 11 months without cell division or apoptosis occurring (Renfree and Shaw 2000). The ovarian hormones exert their effects on the blastocyst by alterations in the endometrial secretions, but the molecular cross-talk between the endometrium and blastocyst is unknown. One possible regulator of diapause is the phospholipid PAF, an embryotrophin that acts as a trophic/survival factor for the early embryo (O'Neill 2005) partly by inactivating the expression of p53, a cell cycle inhibitor, via the PI3-K pathway. PAF is released from the tammar endometrium around the time of reactivation from diapause (Kojima et al., 1993). This study examined the expression of PAF-R and p53 in the tammar endometrium at entry into, and reactivation from, diapause. PAF-R and p53 were highly conserved with orthologues in human and mouse. *PAF-R* and *p53* expression was assessed by RT-PCR and both genes were expressed in the endometrium at all stages examined. Quantitative PCR (QPCR) studies performed for PAF-R in the endometrium show that levels of PAF-R vary depending on the stage examined and appear to be increasing at entry into diapause and decreasing at exit from diapause. Immunohistochemical (IHC) studies are in progress to determine the cellular location of PAF-R in the endometrium and confirm the QPCR results. QPCR and IHC studies are in progress to determine if there is any change in levels of expression or cellular location of p53 between the stages examined and how this relates to PAF-R availability. These results suggest that the control of diapause in the tammar involves interactions between multiple factors.

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Reduced endometrial angiogenesis during early pregnancy in relaxin-deficient mice

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Relaxin is a peptide hormone with important roles in the reproductive tract, including the growth and remodelling of endometrial vasculature. It has been shown to stimulate VEGF secretion from human endometrial stromal cells *in vitro* and increases endometrial vascularization in ovariectomized steroid-primed primates *in vivo*. We have used mouse models to show that oestrogen and progesterone stimulate angiogenesis (new blood vessel growth) within the endometrium. Endometrial angiogenesis also occurs in the early stages of mouse pregnancy, which coincides with an increase in circulating progesterone. To date, no studies have investigated the effects of relaxin on endometrial angiogenesis in early pregnancy. Our aim was to test the hypothesis that endometrial angiogenesis would be reduced in relaxin-deficient mice ($Rln^{-/-}$) in comparison to their wildtype ($Rln^{+/+}$) counterparts. Uterine tissues were collected from $Rln^{-/-}$ and $Rln^{+/+}$ mice on days 1 to 4 of pregnancy, before implantation. All mice were treated with BrdU prior to dissection to allow the number of blood vessel profiles containing proliferating endothelial cells (PVPs) to be quantified by double CD31/BrdU immunohistochemistry. Consistent with published studies, PVPs were first observed on days 3 and 4 of pregnancy. However, the percentage of PVPs was reduced in $Rln^{-/-}$ mice compared to $Rln^{+/+}$ mice (Day 3: median = 4.4% versus 19.6%, Day 4: 9.6% versus 22.2%). We subsequently identified relaxin and relaxin receptors in the mouse endometrium in early pregnancy. Our data suggest that locally synthesized relaxin acts in synergy with progesterone to initiate endometrial angiogenesis in early pregnancy.

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Relaxin and INSL3 receptors and their signaling in primary human myometrial cells

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In model species such as the pig or rat, the ovarian peptide hormone relaxin is able to induce uterine quiescence, suppressing natural or oxytocin-induced contractility via a cAMP-dependent mechanism. This is not observed in humans. The present study investigated the ability of relaxin to activate adenylate cyclase in primary human myometrial smooth muscle cells from non-pregnant tissue obtained at hysterectomy. We show that relaxin is indeed able to stimulate the generation of cAMP, and it does this in a manner which is dependent upon the mediation of a tyrosine phosphorylation activity as has been shown by us earlier in the endometrium and THP-1 cells, though likely not involving PI3-kinase. Furthermore, we identified transcripts for the relaxin receptor RXFP1 (formerly LGR7) as full-length variants, though a minor splice variant missing exon 2 was also present in low amounts. Interestingly human myometrial cells also express transcripts encoding the full-length receptor, RXFP2 (formerly LGR8), for the closely related peptide hormone, INSL3. This receptor can also respond to relaxin at high concentration. However, this receptor does not appear to function by contributing to the cAMP production in human myometrial cells, nor does INSL3 act as a functional agonist or antagonist of relaxin action. In conclusion, therefore, the well-known inability of relaxin to inhibit contractility in human myometrial cells appears to be due to uncoupling events downstream of cAMP generation. *Research supported by NH&MRC project grant349502.*

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Reproductive performance of Australia Cashmere goats supplemented with lupin grain

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The productivity of Cashmere goats depends on their reproductive performance, which, in turn, depends on their level of nutrition. Ovulation rate and pregnancy in sheep are both affected by nutrition, but little is known about the response of female goats (does) to supplementary feeding. The lupin group (n = 40) received 250g lupin per head per day in addition to pasture whereas the control group (n = 40) received no nutritional supplement. Both groups were synchronised for 17 days with intravaginal progestagen pessaries. The supplement was fed for 21 days, commencing 7 days before the bucks were introduced and intravaginal pessaries were removed (Day -2). Does were expected to ovulate 2 days later on Day 0 and the bucks were removed on Day 3. Blood was sampled for progesterone every 3 days from buck removal (Day 3) until Day 18. Ovulation rate was assessed by trans-rectal ultrasonography on Day 13 and pregnancy was diagnosed by trans-abdominal ultrasonography on Day 61 of the experiment. Does supplemented with lupins had a numerically higher ovulation rate than does fed only on pasture, but this difference was not significant (1.76 ± 3.21 versus 1.52 ± 3.79; $p > 0.05$). Similarly, there was no difference in the numbers of does conceiving to the first service between the lupin and control group (89% versus 94%; $p > 0.05$). Progesterone concentrations on Day 12 were higher in does supplemented with lupins than does fed only pasture (6.29 ± 0.27 ng/ml versus 5.41 ± 0.27 ng/ml; lupin and control group; $p < 0.05$). In conclusion, lupin supplementation induced a numerical increase in ovulation rate but this difference failed to reach significance. Does supplemented with lupins had higher concentrations of progesterone during early pregnancy, which is the opposite effect to that previously reported in sheep.

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Interleukin-6 is an essential regulator of parturition and perinatal viability in mice

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IL-6 is an immune-regulatory cytokine which has functional overlap with LIF and IL-11 due to shared use of the gp130 receptor. IL-6 is synthesised abundantly in the uterus throughout pregnancy and at the time of parturition. Previously we have shown that mice with a null mutation in the *Il6* gene (*Il6*-/-) have elevated rates of fetal resorption (45% versus 14% in WT controls) and a high incidence of postnatal death. Additionally, timing of birth is delayed approximately 24h in *Il6*-/- mice (1), in association with altered uterine expression of several parturition-associated genes including PGHS-2, PTGFR and CX-43, and delayed progesterone decline (2). To investigate the effect of exogenous IL-6 replacement on perinatal parameters, *Il6*-/- and WT females were mated with males of the same genotype and on day 11.5 pc were surgically implanted subcutaneously with Alzet micro-osmotic pumps (7 day) containing 1 µg rhIL-6 (R&D Systems), or PBS+0.1% BSA carrier alone (n=12 females per group) . The mean time of parturition was advanced after rhIL-6 replacement in *Il6*-/- females from day 20.3 pc to day 19.6 pc ($p<0.05$), but was unchanged in WT mice (day 19.5). As expected, the number of viable pups delivered by *Il6*-/- mice was less than in WT mice, but was unchanged in either group by IL-6 replacement. However rhIL6 replacement in *Il6*-/- mice substantially increased the proportion of neonates that survived to weaning (from 60% to 98%, $p<0.05$), with no effect on postnatal survival in WT mice (76% versus 88%). Together these data provide further evidence supporting a central role for IL-6 in the events of parturition and postnatal survival, and indicate that exogenous IL-6 replacement in IL-6 deficient mice can improve perinatal outcomes.

(1) Robertson et al. ASRB 31 Abstract 97, 2000

(2) Robertson et al. SGI Abstract 729, 2008

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Effects of relaxin treatment on hyaluronic synthase expression in the cervix of pregnant relaxin-deficient (*Rln*$^{-/-}$) mice

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The cervical extracellular matrix (ECM) changes extensively in a process called cervical ripening in late pregnancy. This occurs through a complex series of processes leading to collagen fibre degradation and dispersal. We tested the hypothesis that relaxin increases cervical water content by stimulating hyaluronic synthase (HAS) enzymes that regulate hyaluronic acid (HA) synthesis. HA is thought to recruit water molecules into interstitial spaces between collagen fibrils, causing them to disperse. In the first study, cervices were collected from *Rln*$^{+/+}$ and *Rln*$^{-/-}$ mice on days 14.5, 16.5, 18.5 and 19 gestation, and day 1 postpartum (PP) to compare *has* expression between the genotypes by qPCR analysis. In the second study, *Rln*$^{-/-}$ mice were implanted with Alzet osmotic minipumps on day 12.5 gestation to infuse either recombinant H2 human relaxin (200 µg/ml; BAS Medical Inc) or 0.9% saline at a continuous flow rate of 0.5 µl/h. Cervices were collected after 4 or 6 days of infusion. *has1* and *has2* gene expression increased significantly at term and decreased immediately postpartum in the *Rln*$^{+/+}$ mice. Although *has1* and *has2* were expressed in *Rln*$^{-/-}$ mice, there was no increase in expression on day 18.5 gestation and cervix wet weight did not increase compared with *Rln*$^{+/+}$ mice. Relaxin infusion for 4 or 6 days in pregnant *Rln*$^{-/-}$ mice significantly increased cervical *has2* but decreased *has1* expression compared with saline-controls. Additionally, relaxin treatment caused a 6-fold increase in cervix wet weight, a 5% increase in water content and a significant decrease in relaxin receptor (*Rxfp1*) expression compared with saline-controls. These data suggest that relaxin promotes cervical hydration through an *has2*-mediated increase in HA, and may facilitate cervical ripening by causing collagen fibril dispersal in the ECM.

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Aristaless-related homeobox gene is involved in early development and spermatogenesis in mammals

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The aristaless homeobox gene, *ARX*, belongs to a large family of homeodomain transcription factors with essential roles in forebrain, pancreas, muscle tissues and testes development in human and mouse. Mutation of *ARX* in humans results in mental retardation with or without ambiguous genitalia. We used comparative analyses to examine the evolutionary conservation of the mammalian *ARX* gene. We characterized *ARX* in a marsupial, the tammar wallaby, to determine if this gene is highly conserved in the homeodomain, aristaless domain, octapeptide motif and polyalanine tracts of all mammals. We further investigated the mRNA distribution in the developing head of tammar with *in situ* hybridization, and found that it is expressed in forebrain and olfactory bulb as expected. Besides these regions, very strong expression was detected in the epithelium of the tongue and nasal pits. In the gonads, there is very strong staining in the interstitial cells and some of the germ cells in the developing ovary; strong staining was also seen in the cytoplasm of Sertoli cells and some of the germ cells, weak staining was also detected in the interstitium of the testis, possibly within the vessel endothelial cells and interstitial fibroblast-like cells. In addition, we investigated mRNA distribution in adult testes based on a very strong signal observed with Northern blotting. Interestingly, mRNA expression was restricted to the round spermatids, and was not seen before or after this stage. In order to confirm this new role for ARX in the adult testis, we further investigated mRNA distribution of *Arx* in adult mouse testis, and found the same expression pattern, which implies a conserved function for ARX in spermatogenesis and may explain why humans with *ARX* mutations are infertile. This is the first report that *ARX* gene is involved in spermatogenesis in addition to its conserved roles in early mammalian development.

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Developmental switches in male sex determination and spermiogenesis; importin-chromatin remodeling factor interaction

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Spermatogenesis, the complex process of generating haploid sperm capable of fertilizing the female gamete, requires precisely scheduled transport into the nucleus of transcription factors and chromatin remodeling factors to implement changes in nuclear gene expression, as well as genome compaction during sperm formation (spermiogenesis). This transport is mediated by members of the importin (IMP) superfamily, which display distinct expression patterns in the rodent testis, consistent with the idea that they may carry specific cargo(es) at discrete stages of testis development. A key cargo during foetal testis development is the sex determining chromatin remodeling factor SRY, whose role in the nucleus in modulating the expression of male-specific genes such as SOX9 is critically dependent on the efficiency of its nuclear import by the specific transporter IMPbeta1; specific mutations in SRY that reduce binding by IMPbeta1 result in XY female Swyer syndrome individuals. A second cargo of significance is Cdyl (Chromodomain Y chromosome-like), involved in the histone H4 hyperacetylation which precedes the replacement of histones with protamines during spermiogenesis. We recently identified IMPalpha2, together with IMPbeta1, as Cdyl's specific nuclear transporter. Using site-directed mutagenesis to perturb Cdyl recognition by IMPalpha2, IMP/Cdyl cotransfection approaches and quantitative confocal laser miscroscopic analysis, we established that the efficiency of Cdyl nuclear import is critical to its function in facilitating histone H4 acetylation, supporting the idea that one of the specific roles of IMPalpha2 is to localize Cdyl in the nucleus of elongating spermatids. Our findings are consistent with precisely scheduled, efficient IMP-mediated nuclear import of key chromatin remodeling factors being critical to testis development, reflecting an emerging paradigm for developmental processes in general.

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BMP signalling in the induction of germline precursors from mouse embryonic stem cells *in vitro*

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BMP signalling is critical for germline lineage establishment during mouse embryogenesis. To assess its importance in the induction of germline precursors *in vitro*, a mouse embryonic stem (ES) cell line harbouring an Oct4 promoter-EGFP reporter construct was used to derive embryoid body (EB) aggregates cultured in the absence or presence of combinations of BMP2, BMP4 and BMP8b for 3 to 10 days. At both day 5 and 10 of culture, clearly defined clusters of Oct4-EGFP were observed in EBs cultured with BMPs, while these clusters were minimal to absent in untreated EBs. Quantitative mRNA analysis of day 3 to day 10 EBs revealed a significant elevation in the expression of genes associated with primordial germ cell specification in EBs grown in the presence of BMPs. Moreover, a transient elevation of early germ cell markers *Blimp1, Fragilis* and *Stella* was detected in day 3-4 EBs cultured with BMPs, followed their decline by day 5. In contrast, levels of the pluripotency markers, *Oct3/4* and *Nanog*, and the later germ cell markers, *Dazl* and *Vasa,* progressively increased from day 3 to day 5. Levels of TGFβ superfamily signalling components *ALK2*, *Smad1* and *Smad5* remained relatively constant during this period. Wholemount immunofluorescence of day 5 Oct4-EGFP EBs exposed to BMP4 demonstrated co-localization of primordial germ cell markers Oct3/4, Stella, and the cell surface antigen SSEA-1 with EGFP+ clusters. These results demonstrate that signalling by BMP2 and 4 enhances germ cell marker expression within EBs and identifies the day 3 to 5 timeframe in EB differentiation as a critical window when putative germ cells are first specified *in vitro*.

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Oxidative stress and DNA damage in human spermatozoa

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Unusually high levels of DNA damage in the male germ line are, unfortunately, characteristic of our species. A great deal of circumstantial evidence has linked DNA damage in human spermatozoa with adverse reproductive outcomes including reduced fertility and high rates of miscarriage. Although oxidative stress is thought to make a significant contribution to DNA damage in the male germ line, the mechanisms responsible for creating this stress have not yet been elucidated. We have undertaken a detailed analysis of the ability of estrogens, electromagnetic radiation and xenobiotics including metal ions to trigger reactive oxygen species (ROS) production and/or DNA damage in human spermatozoa *in vitro*. This investigation was completed using a range of techniques validated for use in these highly specialized cells. DNA integrity was assessed using the Comet and TUNEL assays, oxidative DNA adducts were detected by an anti-8-oxo-dG assay and cross-linking adducts were characterised by mass spectrometry. Intracellular redox activity was monitored using dihydroethidium as the probe. Of the factors evaluated, catechol estrogens, certain transition metal ions, radio frequency electromagnetic radiation and heat were all capable of stimulating ROS production in human spermatozoa. The oxidative stress created by exposure to such factors lead to the induction of significant DNA damage. Generally, redox inert compounds including non-catechol estrogens and xenobiotics such as phthalate esters did not lead to ROS production or measurable DNA damage. Mass spectrometry also indicated that catechol estrogens were capable of forming dimers that can cross-link the densely packed DNA strands in sperm chromatin. These findings raise fundamental questions about the importance of xenobiotics, environmental factors as well endogenous compounds in creating oxidative stress and DNA damage in the male germ line.

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Regulated expression of KIT protein in juvenile and adult germ cells of the rodent testis

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KIT receptor is an established marker of differentiating spermatogonia and its activation is required to trigger spermatogonial maturation. *KIT* mRNA, however, can be detected in undifferentiated spermatogonia in the absence of protein expression, as previously established by us in the irradiated adult rat testes [1] . This differential regulation of mRNA and protein is presumably modulated by either local hormone action or by cues from the adult testicular microenvironment. Endogenous regulatory factors known to stimulate *KIT* synthesis in juvenile male germ cells *in vitro* are bone morphogenetic protein 4 (BMP4) and retinoic acid (RA), while factors known to suppress *KIT* at the onset of spermatogenesis have not yet been identified. Activin A, implicated in KIT down regulation in a murine erythroleukemia cell line [2] , is produced within the juvenile mammalian testis and influences activities of spermatogonia and Sertoli cells. We hypothesized that activin acts to repress *KIT* expression in spermatogonia and therefore modulate spermatogonial behavior. Evidence for this was first derived from Sertoli and germ cell co-cultures of day 8 wild type mouse testes in which exogenous activin addition caused a dose-dependent reduction of *KIT* mRNA. Whole testes mRNA analyses of two activin transgenic mouse models, the newborn *Inhba*$^{-/-}$ (lacking activin A) and postnatal *Inhba*$^{BK/BK}$ (decreased bioactive activin), revealed a significant elevation in *KIT* expression relative to wild type littermates. In the postnatal day 7 *Inhba*$^{BK/BK}$ testes, an elevated proportion of differentiated spermatogonia, increased cell surface KIT protein levels, enhanced mRNA levels of a known downstream target of KIT signaling pathway, *cyclind3* and a meiotic marker, *Sycp3*, were observed. These data provide the first comprehensive evidence for activin modulation of KIT expression at spermatogenesis onset, in germ cells of the juvenile testis. This finding is of fundamental importance to other KIT-dependent processes.

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(2) Hino, M., et al., Down-modulation of c-kit mRNA and protein expression by erythroid differentiation factor/activin A. FEBS Lett, 1995. 374(1): p. 69-71.

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Importin α2-recognised testis cargoes; relevance to spermatogenesis

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Spermatogenesis, the morphogenetic process of generating haploid sperm capable of fertilizing the female gamete, requires transport into the nucleus of transcription and chromatin remodeling factors to implement genome compaction. This transport is mediated by members of the importin (IMP) superfamily, which exhibit distinct expression patterns during male germ cell development, consistent with the idea that each IMP carries specific cargo(es) at discrete stages of spermatogenesis. To identify IMP cargoes in the testis to help identify potential developmental switches critical to the spermatogenic process, we performed a yeast two-hybrid screen using full length IMPα2 as bait and an adult mouse testis library. Binding partners identified included coilin, Hop2, Chrp (cysteine and histidine rich protein), TAF9 (TATA Binding Protein Associated Factor 9) and Cdyl (Chromodomain Y chromosome-like). These proteins may be important in cell cycle regulation, homologous chromosome pairing and recombination, transcriptional regulation, splicing, mRNA storage or histone-protamine exchange during spermatogenesis. IMPα2 interaction with these cargoes was verified by binding assays, coimmunoprecipation and cotransfection approaches, while immunohistochemical staining of rodent testis sections indicated their co-expression with IMPα2 in specific testicular cell types. A key cargo in this context is the chromatin remodeling component, Cdyl where we could show that interaction with IMPα2 (and not IMPα4 or IMPα6 which are known to be expressed in the same testicular cell types as IMPα2) is necessary for its efficient nuclear import and function in facilitating histone H4 acetylation, the prerequisite step for incorporation of protamines during the final stages of spermatogenesis. A similar approach is currently in progress to demonstrate the importance of IMPα2 for the efficient nuclear transport of the other cargoes identified for their function(s) during spermatogenesis. IMPα2-mediated nuclear localisation of specific cargoes would thus seem to represent a critical developmental switch during spermatogenesis.

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Production of donor-derived live lambs following testis germ cell transplantation

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Testes germ cell transplantation in livestock has the potential for amplification of transgenic genotypes and for use as an alternative to artificial insemination. This study investigated a workable protocol for testis germ cell transplantation in sheep between animals of the same breed and different breeds. Testes of two groups of recipients at the stage of pre-pubertal (transition from gonocytes to spermatogonia, n=2) or peri-pubertal (spermatogenesis initiated, n=2) were treated with a single dose of 9 gray (Gy) or 15 Gy with a 6MV photon beam irradiation, respectively.

In the first experiment, using pre-pubertal irradiated animals, testis germ cell transplantation between the same breed was performed at 16 weeks post irradiation. The left testes of recipient rams were injected with donor cells labelled with fluorescent dye PHK26, while the right testes were given unlabeled cells. The left testes of recipients were removed by castration after 2 weeks following transplantation to evaluate the location of the transferred cells, while the right testes were kept in place for long-term assessment of sperm output. In cryosections of the left testes, PKH26 positive cells were found both on the basement membrane as single cells or in the interstitial area. In the second experiment, animals irradiated at the peri-pubertal stage, received donor cells at 5 weeks post irradiation and animals were kept intact for semen production.

For a period of two years after transplantation, semen samples were collected routinely from two groups of rams and analyzed using microsatellite markers. Two recipients (50%) demonstrated the presence of donor DNA in their ejaculates. In order to investigate the fertility of the donor-origin sperm in recipient ejaculates, 99 ewes were artificially inseminated with semen from two positive rams. Four lambs (8%) have been identified as being sired by donor-derived sperm produced in the recipient ram that received a Merino to Merino transplantation, while no donor-derived offspring was obtained from the recipient with Border Leicester to Merino transplantation. This study represents the first report of the production of live progeny following testis germ cell transplantation in sheep.

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Regulated nuclear import of TATA binding protein associated factor 9 (TAF9) in spermatogenesis

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Spermatogenesis, the differentiation process resulting in the production of haploid germ cells able to fertilise an oocyte, is driven by by nuclear transcription factors, and changes in nuclear morphology and function itself. Signal-dependent transport into and out of the nucleus is mediated by members of the importin (IMP) superfamily of transporters, which we have previously shown to change dynamically in expression profile during spermatogenesis. One IMP family member, IMPα2, is expressed during mid to late phase spermatogenesis, implying that it may transport specific cargoes important for these stages, with a potential cargo of significance identified in a yeast 2-hybrid screen being TAF9 (TATA-Binding Protein Associated Factor 9). TAF9 is an integral part of transcription initiation for many genes, such as those with a Downstream Promoter Element in the core promoter and those with activators such as SOX18, p53, HSF1, NF-IL6 or NF-κB . In addition it participates in histone acetylation complexes which have been previously described to be important for the completion of spermatogenesis. Our preliminary data confirm the interaction of IMPα2 and TAF9 using a cotransfection approach. We have also shown that the expression of these proteins in the testis is correlated using both publically available Affymetrix data, and immunohistochemistry staining. TAF9, like IMPα2, is expressed in the nucleus of elongating spermatids of the adult rodent testis. Our data are thus consistent with the idea that specific nuclear import of TAF9 by IMPα2 may be a critical step in the later stages of spermatogenesis.

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Toxic effects of hyperglycaemia arise from induced O-linked glycosylation in early mouse embryos

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Glucose flux through the hexosamine biosynthetic pathway (HBP) which is essential for preimplantation development (1) produces uridine 5'-diphospho-N-acetylglucosamine, a donor substrate for multiple glycosylation reactions including O-linked glycosylation. This novel signaling arm of the HBP, known as the hexosamine signaling pathway (HSP) operates via reversible addition of an O-linked β-N-acetylglucosamine (O-GlcNAc) unit to serine and threonine residues of proteins including transcription factors, cytoskeletal components, metabolic enzymes and cellular signaling components. O-linked glycosylation is functionally reciprocal to phosphorylation at the same residues, altering the activity and/or stability of targeted proteins, thus providing a mechanism for modulating cellular physiology in response to glucose availability. The enzymes regulating this O-GlcNAcylation are the β-linked-O-GlcNAc transferase (OGT) and an O-GlcNAc-selective β-N-acetylglucosaminidase (O-GlcNAcase). We hypothesized that the toxicity of hyperglycemia on early embryos arises from increased flux through HBP and increased O-GlcNAcylation of key proteins. Mouse zygotes (18h post hCG) were cultured under conditions of modified flux through the HSP including hypoglycemia, hyperglycemia or supplemented with glucosamine which feeds exclusively into the HBP to increase downstream O-GlcNAcylation. BADGP was used to inhibit OGT and O-GlcNAcylation. Blastocyst formation, cell proliferation and apoptosis were assessed.

Treatments that perturb levels of intracellular protein O-GlcNAcylation inhibited embryo development. Whilst some flux through HBP is required to activate embryonic differentiation (1), excess flux arising from a hyperglycemic environment or glucosamine supplementation reduced cell proliferation and blastocyst formation, confirming the criticality of this novel post-translational signaling pathway. Inhibition of OGT using 2 mM BADGP blocked the negative impact of hyperglycemia on blastocyst formation, cell number and apoptosis supporting our hypothesis that O-GlcNAcylation is a key mechanism used by the embryo to sense and respond to perturbations of glucose in its environment.

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Evidences for a novel cAMP-phosphodiesterase expressed in the bovine ovarian follicle

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3'5'-Cyclic adenosine monophosphate (cAMP) is an important second messenger in the mammalian ovarian follicle implicated in gonadotrophin signalling as well as oocyte meiotic arrest. Cyclic AMP-degrading phosphodiesterases (PDE) modulate cAMP levels in the ovarian follicle, but the specific PDE subtypes responsible for this degradation in the different cellular compartments within the bovine follicle remain unknown. The current dogma, established principally in rodent, presents PDE3A as the "oocyte PDE", while PDE4D is the "granulosa/cumulus PDE". Our PDE activity measurements suggested that a PDE3 (cilostamide-sensitive, 10μM) was representing 79% of the total cAMP-PDE activity in the bovine oocyte, in agreement with the dogma. However, our results suggested that PDE4 (rolipram-sensitive, 10μM) is representing only 19% of the cAMP-PDE activity in the cumulus cells, while 65% of the activity was due to PDE8 (IBMX-insensitive, 500μM), a result in direct opposition with the accepted PDE distribution in the ovarian follicle. Mural granulosa cells were displaying equal amounts of PDE4 (31%) and PDE8 (30%) cAMP-PDE activities. Interestingly, cAMP-PDE activities were not varying during the first 9 hours of IVM in the bovine cumulus-oocyte complexes (COC), as seen in rat. COCs treated with an adenylyl cyclase stimulator (forkolin 100μM) in combinaison with the only known inhibitor for the PDE8 family, dipyridamole, are showing a dose-dependant increase of cAMP levels and a significant delay nuclear maturation, whereas a potent PDE4 inhibitor, rolipram (up to 100μM), was ineffective. This study provides the first insight into subtype-specific PDE cAMP degrading activities in the bovine ovarian follicle, especially around oocyte nuclear maturation. It demonstrates dramatic differential PDE subtype compartmentalisation between ovarian somatic cells and the germ cell, including the important contribution of a new PDE family member in the ovarian follicle, PDE8. PDE8 could be a novel pharmacological target to improve bovine oocyte IVM conditions and to increase developmental competence.

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Hormonal manipulation on the phenotype of ArKO female mice

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Gonadotrophins and steroid hormones are vital in controlling the cyclical pattern of ovarian follicular development essential for fertility. Previous studies have shown that ArKO (aromatase knockout) female mice are infertile due to the absence of estrogen, elevated levels of circulating gonadotrophins and testosterone and folliculogenic disruption. Therefore, the aim of this study was to determine the effects of E_2 (estradiol-17β) replacement, Acyline (GnRH antagonist) and Flutamide (anti-androgen) treatment on ArKO female mice.

WT and ArKO female mice (C57B6/J129; 16 weeks old; n = 6-8/grp) were assigned into three main groups: group 1 - received either E_2 (0.05 mg) pellet or placebo, group 2 - received either a single s.c. injection of acyline (1.5 mg/kg/week) or placebo and group 3 – received either flutamide (25 mg) pellet or placebo for 3 weeks. Mice were subjected to daily vaginal smears. The ovaries and uterine horns were collected and weighed. One ovary and the uterine horns were fixed in formalin for histological assessment, while the other ovary was snap frozen in Ultraspec solution for RNA isolation and gene expression studies. Serum was collected for hormone measurements.

All female ArKO mice exhibited an abnormal cycle that alternated between diestrus and early estrus . E_2 replacement restored the estrus cycle in ArKO female mice but acyline and flutamide treatment did not. Histologically, hemorrhagic cystic follicles were present in all placebo, acyline and flutamide treated ArKO ovaries, however, E_2 replacement improved the ovarian and uterine phenotypes. E_2 replacement and acyline treatment also led to a decrease in serum gonadotropin levels in ArKO mice.

In summary, E_2 replacement could reverse the abnormal reproductive phenotype of the ArKO female mice. This study suggests that the reproductive phenotype of the ArKO female mouse is due to the direct effect of estrogen and not due to the elevated circulating levels of gonadotrophins and testosterone.

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Ovarian lymphatic vascular development is hormonally regulated and Adamts1-dependent

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The lymphatic system is important for return of extra-vascular fluid to the blood circulation, conductance of hormones and immune cell trafficking. Delicate hormonal control of fluid conductance during reproductive cycles is exemplified by the ovarian hyperstimulation syndrome, a dangerous condition of hypovolemia caused by fluid accumulation in the abdomen and reproductive tissues, in response to hormonal hyperstimulation. This study is the first to investigate the relationship between ovarian lymphatic development and follicle growth. Quantitative morphometric analysis of vessel size and number in mouse ovary revealed, for the first time, that the ovarian lymphatic vasculature develops postnatally and in synchrony with the induction of ovarian *CYP19a1* (*Aromatase*); the time when secondary follicles become FSH-responsive and estrogenic. Mechanistically, we found that the FSH-analogue eCG mediates induction of lymphatic vascular endothelial growth factor *Vegfd* and the receptor *Vegfr3* (*Flt4*) in granulosa cells. Importantly, stimulation with eCG also enhanced ovarian lymphatic vessel number and size. However, formation of ovarian lymphatics also required the matrix-remodelling protease Adamts1, since ovaries from *Adamts1*$^{-/-}$ mice failed to undergo normal lymphatic vascular development. Treatment of *Adamts1* null mice with eCG significantly increased the number and size of ovarian lymphatic vessels, however, the vessels were still smaller and fewer in number than wildtypes. These combined results indicate that the ovarian lymphatic system develops in response to hormonal signals, which promote folliculogenesis, through induction of lymphangiogenic factors in granulosa cells; as well as involving Adamts1-dependent mechanisms. This study is the first demonstration of the novel principle of hormonal regulation of lymphangiogenesis in any tissue and suggests a requirement for functional lymphatics during normal folliculogenesis. In addition our results inform the elucidation of the tightly regulated processes that control fluid dynamics and immune cell surveillance within reproductive tissues.

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Activation of a calcium-activated chloride channel by paf is required for normal preimplantation mouse embryo development *in vitro*

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Platelet activating factor (paf) is an autocrine survival factor for preimplantation embryo. Binding of paf to its receptor activates PI_3kinase, causing an IP_3-dependent release of Ca^{2+} from intracellular stores as well as activation of Ca^{2+} influx via a dihydropyridine-sensitive Ca^{2+} channel. These actions result in the generation of a defined intracellular calcium ($[Ca^{2+}]_i$) transient in the 2-cell embryo[1]. By using combined whole-cell patch-clamp and real-time $[Ca^{2+}]_i$ analyses, we have shown that paf also induces a concomitant hyperpolarisation of the membrane potential in 2-cell embryos, accompanied by an increased net outward ion current. Both the membrane hyperpolarisation and outward current were dependent upon the occurrence of the paf-induced $[Ca^{2+}]_i$ transient[2]. The aim of this study was to investigate the characteristics of the paf-induced outward current in 2-cell embryos and to assess whether it has a role in normal mouse preimplantation development. We show that: (1) removal of extracellular anions or treatment with niflumic acid (NFA, 100 μM, a Ca^{2+}-activated Cl^- channel blocker) prevented activation of the outward current by paf but had no effect on the paf-induced $[Ca^{2+}]_i$ transient; and (2) The culture of embryos with NFA (100 μM) from the 1-cell to late 2-cell stage significantly reduced their development to the blastocyst stage ($P<0.001$), but treatment with NFA from the late 2-cell stage had no effect on development. The results show that paf induces an increase in $[Ca^{2+}]_i$ which in turn activates a Ca^{2+}-activated Cl^- channel. The activity of this NFA-sensitive channel during the zygote to 2-cell stage is required for normal embryo development.

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Differential expression of H2A and H3 variant histones in mouse preimplantation embryos and R1 ES cells

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Histone variants replace canonical histones in nucleosomes to serve numerous biological processes. This integration alters DNA properties to ultimately regulate gene expression. Previous mouse studies have indicated that some variants (H2AZ and H3.3) are essential for survival, but here we document and correlate histone expression patterns with key developmental events. Using quantitative reverse-transcribed PCR (qRT-PCR) we investigated the expression of 7 genes coding for H2A variants and 4 genes coding for H3 variants in mouse preimplantation embryos and in pluripotent R1 ES cells. mRNA was extracted from pools of 3 embryos flushed from superovulated mice. Embryos were collected at five stages, zygotes, 2-cell embryos, morulae, blastocysts and hatching blastocysts (20 h, 44 h, 68 h, 92 h and 116 h post hCG respectively). The expression of H2A variant genes typically peaked within blastocysts. *H2AZ* and *H2AX* expression was 80 – 95% higher in blastocysts than other stages. Conversely, genes coding for H3 variants showed elevated expression in zygotes, where *H3.3* expression was 85 – 95% higher and *CENPA* was approximately 75% higher than in later preimplantation stages. The expression profiles of histone remodelers *SWI/SNF* and *CAF-1* correlated with the variants they are known to remodel (H2A and H3 variants respectively), suggesting an increased integration of those variants into nucleosomes. We also compared blastocyst and embryonic stem cell (ES cell) expression patterns. R1 ES cells express all histone variants, including *H2A.Bbd*, *H3.1* and *H3.2* which were not expressed in preimplantation embryos. Further, expression levels of every histone variant investigated differed significantly between R1 ES cells and hatching blastocysts (ANOVA, $P<0.05$, n=3 experiments). We conclude that histone variant expression reflects preimplantation developmental demands. Further, histone code expression profiles show significant change upon extended cell culture and maintenance of pluripotency as indicated by comparing *in vivo* hatching blastocysts to the R1 ES cell line.

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Pluripotency genes in a marsupial, the tammar wallaby

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Markers of pluripotency and early differentiation in the early embryo have been extensively characterised in eutherian species, most notably the mouse. By comparison, mechanisms controlling pluripotency and early lineage specification have received surprisingly little attention in marsupials, which represent the second major infraclass of mammals. Early marsupial embryogenesis exhibits overt morphological differences to that of eutherians, however the underlying developmental mechanisms may be conserved. In order to characterise early marsupial development at the molecular level, we have identified, cloned and analysed expression of orthologues of a number of eutherian genes encoding transcription factors and signaling molecules involved in regulating pluripotency and early lineage specification. These genes include *POU5F1* (*OCT4*), *SOX2*, *NANOG*, *FGF4*, *FGFR2*, *CDX2*, *EOMES*, *TEAD4*, *GATA6* and *KITL* and are all expressed at early stages of development in the tammar. In addition, we have identified and cloned tammar *POU2*, which has orthologues in non-mammalian vertebrates. *POU2* is a paralogue of *POU5F1* – a master regulator of pluripotency in eutherians. Genomic analysis indicates that *POU5F1* arose via gene duplication of *POU2* prior to the monotreme-therian divergence. Both genes have persisted in marsupials and monotremes, while *POU2* was lost early during eutherian evolution. Similar expression profiles of tammar *POU5F1* and *POU2* in early embryos and gonadal tissues suggest possible overlapping roles in the maintenance of pluripotency.

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Synergistic effects of cAMP modulating agents in pre-IVM and in IVM on bovine cumulus and oocyte functions

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cAMP plays a crucial role in oocyte maturation. It has been shown that prolonging spontaneous maturation by modulating oocyte cAMP levels during in vitro maturation (IVM) improves developmental outcomes. This study sought to assess the effect of inclusion of cAMP modulators during a pre-IVM phase and during IVM on cumulus cell (CC) and oocyte function. Bovine cumulus-oocyte complexes (COCs) were precultured for 2h in medium supplemented with/without cAMP modulators: 0.5mM IBMX and 0.1mM forskolin (FK). cAMP in control COCs dropped significantly from 15±4 to 2±1 fmol/COC within 30 min, whereas FK+IBMX during pre-IVM increased levels to 180±19 fmol/COC, which had notable persistent effects on a number of COC functions throughout IVM. cAMP modulators delayed oocyte GV progression; after 2h of pre-IVM, 67±5% were at GVII stage compared ($p<0.0001$) to 12±3% in controls, 66±5% of which had progressed to GVIII. This was associated with a significantly higher level of oocyte-CC gap junction mediated communication (1000±148 vs. 340±73 units). We next assessed the interaction of cAMP modulators in pre-IVM and IVM (20uM cilostamide) phases. After 9h of IVM, significantly more oocytes were arrested at GV stage when modulators were present in both pre-IVM and IVM phases, compared to in IVM alone (96±1 vs. 74±2%, $P<0.05$). Moreover, progression to MII was delayed by modulators in pre-IVM and IVM (24±1, 75±2, 92±1% MII at 20, 24 and 28 hours, respectively), compared to in IVM alone (78±1, 95±1, 98±2% MII). After 24h of IVM, intra-oocyte cAMP levels remained 15 fold higher ($P<0.05$) with modulators in both phases compared to in IVM alone. These results show that the efficacy of cAMP modulators during IVM is substantially improved by management of COC cAMP levels during the pre-IVM phase. Such an approach has profound effects on CC and oocyte function, including increasing oocyte-CC communication and delaying meiotic resumption.

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Disruption of mitochondrial function in the blastocyst alters expression of the chromatin remodeler ATRX

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Exposure of an embryo to sub-optimal environments, including poor embryo culture media or inadequate maternal diet, can disrupt fetal and placental development and whilst the exact mechanisms responsible remain unknown, perturbed embryo metabolism has been implicated. We propose that stress applied to an early embryo causes mitochondrial dysfunction, resulting in a permanent epigenetic change. Thus the aim of this study was to determine the affect of directly perturbing mitochondria in the embryo, on development, metabolism and expression of the ATP-dependant chromatin remodeling protein, ATRX.

Zygotes collected from gonadotrophin stimulated C57BL/6xCBA mice were cultured to the two-cell stage and then exposed to one of three treatments; control medium (C), medium lacking pyruvate (-P; embryos dependant on the mitochondrial Malate Aspartate Shuttle, MAS) or medium lacking pyruvate plus 5µM amino-oxyacetate (AOA), a specific MAS inhibitor (-P+AOA). Blastocyst development and metabolism were assessed by determining cell number and allocation, glycolysis, and ATP:ADP ratio. Relative gene expression of ATRX, was examined using RT PCR.

Embryos dependant on the MAS alone (-P) had significantly decreased blastocyst development (87.1% vs 98.2%, $p<0.05$), with a compensatory increase in glycolysis (0.20 vs 0.07 pmol/cell/hr, $p<0.001$) despite a decrease in ATP:ADP (0.10 vs 0.13, $p<0.06$), relative to the control. Inhibition of the MAS (-P+AOA) further reduced blastocyst development,(77.3%, $p<0.001$) and decreased ATP:ADP (0.08, $p<0.004$), but there was no change in glycolysis relative to control embryos (0.09 pmol/cell/hr, $p=0.3$). Expression of ATRX was significantly increased for –P+AOA embryos relative to the control (1.63 vs 1.0, $p<0.007$) but did not differ for –P embryos (1.1).

This study demonstrates that direct perturbations of mitochondrial function in the embryo compromises its metabolic regulation and blastocyst development, and the expression of the epigenetic modulator ATRX. Further studies are underway to elucidate the implications of disrupted metabolic control and this epigenetic modulator on pregnancy outcomes.

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Human cumulus cell gene expression as a marker of clinical embryo grade

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In Australia, Assisted Reproductive Technology (ART) accounts for approximately 3% of births. However, the success rate remains around 65% for women under 35 years of age, hence multiple embryo transfer is frequently preferred to improve the probabiity of achieving a term pregnancy. A biochemical marker for oocyte and embryo developmental potential would augment successful pregnancy outcomes following IVF/ICSI by optimising oocyte and embryo selection, therefore increasing the number of single embryo transfers (SET) performed in ART cycles. Changes in expression levels in human cumulus cells may reflect the quality of their enclosed oocyte. We investigated cumulus cell gene expression and subsequent embryo development to find a marker of embryo quality. Paired samples of cumulus cells were collected from oocytes that progressed to embryos of either high or low grade from eleven IVF/ICSI patients. Following cumulus oocyte complex retrieval cumulus cells were trimmed from the oocyte, and all oocytes and resulting embryos were cultured and tracked individually. Cumulus cell gene expression was assessed using a real-time RT-PCR assay, measuring expression of cyclooxygenase 2 (COX2; PTGS2), Pentraxin 3 (PTX3), Versican (VCAN), Tumour Necrosis Factor Alpha Induced protein 6 (TNAIFP6; TSG6), Lactate Dehydrogenase A (LDHA), Phosphofructokinase Platelet (PFKP), Gremlin (GREM1), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and 18S ribosomal RNA. Standard curves using plasmid subclones for each target were run to assess copy numbers of genes. Embryo morphology was assessed by an embryologist and correlated to relative gene expression. Cumulus cell gene expression was altered in cumulus cells from oocytes which subsequently developed into higher quality (Grade 1 and 2) embryos compared with cumulus cells from oocytes which developed into lower quality (Grade 3 and 4) embryos. This may lead to establishment of markers prognostic for developmental outcome, facillitating more reliable selection of higher quality embryos, increasing single embryo transfers and improving health outcomes from ART.

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Substitution of skim milk with bovine serum albumin in a stallion semen diluent

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Skim milk has long been utilised as a source of protective proteins in stallion semen diluents. However, skim milk is also thought to contain components that are toxic to sperm and reduces the clarity of sperm suspensions, which impedes sperm assessments. This may also reduce the effectiveness of staining procedures used to process sperm for flow cytometric sex-sorting. The aim of this study was to ascertain the optimal concentration of bovine serum albumin (BSA) to replace skim milk in a traditional stallion semen diluent, Kenney's Modified Tyrode's (KMT) Medium[1], for handling and processing stallion sperm prior to flow cytometric sex-sorting.

Two ejaculates were collected from each of three pony stallions. Each ejaculate was divided into five aliquots and diluted in either KMT with skim milk or KMT supplemented with 0, 0.25, 0.5 or 1% BSA. Diluted samples were further divided into two aliquots and either stored at 15 º C for 18 hours prior to incubation and assessment, or incubated and assessed immediately upon arrival. Samples were incubated at 34 º C and evaluated at 0, 45 and 90 minutes for objective motility and acrosome integrity.

No interactions were observed between any treatments over time. There was a lower percentage of intact and a higher percentage of detached acrosomes for sperm incubated in KMT containing 0% BSA than all other treatments. A greater proportion of sperm incubated in KMT with skim milk had partial acrosome damage compared with other treatments. There was no difference in % total motility for sperm incubated in KMT with skim milk, and KMT containing 0.5 and 1% BSA.

KMT diluent supplement	% acrosome intact	% partial acrosome damage	% detached acrosomes	% total motility
Skim milk	61.3 (a)	16.3 (a)	22.4 (a)	49.3 (a)
0% BSA	55.6 (b)	12.9 (b)	31.5 (b)	27.4 (b)
0.25% BSA	63.1 (a)	10.1 (b)	26.9 (c)	38.4 (c)
0.5% BSA	64.5 (a)	10.6 (b)	24.9 (ac)	44.1 (a)
1% BSA	65.0 (a)	9.9 (b)	25.1 (ac)	47.5 (a)

Percentages in columns with different letters differ significantly ($P<0.001$)

These results indicate that BSA may be suitable as an alternative protein source in stallion semen diluents. Further studies are required to compare sex-sorting rates and sperm quality after sex-sorting, incubation and staining in skim milk compared with BSA-based media.

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Primordial germ cell specification in a marsupial, the tammar wallaby

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Primordial germ cells (PGCs) are the precursors of the gametes. In the mouse, PGCs are specified within the proximal epiblast in response to signals from the extraembryonic membranes during early gastrulation. Epiblast cells competent to form PGCs express *Ifitm3*. A subset of these cells then express *Blimp1*, a marker of PGC precursors. Once lineage-restricted, PGCs express *Stella*. Germ cells entering the gonads express VASA protein, which is a component of the germ plasm in animals in which germ cells are specified by the inheritance of maternal determinatives.

Almost all of the research on mammalian PGC specification has used the mouse as a model and it is tacitly assumed that findings in the mouse will apply to mammals in general. We are using the tammar wallaby as a marsupial model for PGC specification. Eutherians and marsupials diverged 125-148 million years ago, so comparisons between the two will provide insights into the evolution of the control of mammalian PGC specification.

There are IFITM clusters in both the human (chromosome 11) and mouse (chromosome 7). In the mouse, *IFITM1, 2* and *3* are expressed in PGCs, whereas *IFITM4* and *5* are not (1). Only one *IFITM* member, *IFITM5*, is annotated in the opossum Ensemble database. We have cloned one tammar *IFITM* member and identified at least one other putative member in the tammar trace archive database. We have also cloned tammar *BLIMP1* and *VASA*, both of which show high sequence conservation with other mammals. RT-PCR profiles for both genes during tammar gastrulation are similar to those for the mouse. In contrast, no marsupial *STELLA* orthologue has been identified in either the opossum or tammar genomes.

These findings suggest that some but not all of the signals and mechanisms involved in eutherian PGC specification are also applicable to marsupials.

(1) Lange UC, Saitou M, Western P, Barton SC and Surani MA (2003) BMC Dev. Biol. Epub 2003 Mar 19

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Increased FSH activity increases primordial follicle reserve and enhances preovulatory follicle survival in transgenic FSH female mice

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The mammalian female reproductive lifespan is determined by the depletion rate of the finite ovarian follicle reserve established prior to or shortly after birth. Follicle formation, initiation and early growth are thought to be independent of follicle-stimulating hormone (FSH), whereas antral follicle development requires FSH stimulation. Rising serum FSH is one of the earliest signs of reproductive ageing in women, coinciding with declining fecundity and an accelerated decline in remaining follicle reserves, but whether or not increased FSH plays a direct or feed-forward role in accelerating reproductive ageing remains undetermined. We previously described transgenic (Tg) mice with rising serum human FSH that produced larger litter sizes <20 weeks of age, then rapidly declining litter size from 20-40 weeks old (wo) culminating in premature infertility[1]. Despite declining fertility, ageing TgFSH females maintained ovulation rates ~ 3-fold higher than wt females. Follicle quantitation revealed that ovarian antral follicle numbers at diestrus were equivalent in 26 wo TgFSH and wt females. The elevated ovulation rates in TgFSH females may reflect increased preovulatory follicle survival during proestrus, as ~70% of large antral follicles go on to ovulate in TgFSH females, compared to only 30% in wt females. In contrast to the view that higher FSH may increase follicle development and consequently accelerate follicle depletion, examination of follicle reserve revealed that subfertile or infertile 26-52 wo TgFSH females exhibited increased total ovarian primordial follicle numbers (60%, $P < 0.05$) with no significant change in primary follicle numbers compared to age-matched wt females. Therefore, increased FSH activity appeared to act as a survival factor for primordial follicles. Our current analysis of increased FSH actions in female mice suggests that FSH may enhance the survival of both early (primordial) and late (preovulatory) follicle populations.

(1) McTavish KJ, et al. Endocrinology. 2007 Sep;148(9):4432-9.

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Loss of betaglycan expression contributes to malignant properties of human granulosa tumour cells

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Betaglycan is a type III TGF-beta receptor that binds to both inhibin and TGF-beta with high affinity and determines cellular sensitivity to these ligands. Previous studies have suggested that betaglycan acts as a tumour suppressor in certain human epithelial cancers. However, the roles of betaglycan in ovarian granulosa cell tumours (GCTs) are poorly understood. The objective of this study was to determine whether human GCTs exhibit betaglycan expression, and if so, what impact this receptor has on tumour biology. Real-time PCR was used to quantify betaglycan transcripts in human GCTs ($n = 18$) and normal premenopausal ovaries ($n = 11$). This analysis established that GCTs exhibited a significant two-fold reduction in mean betaglycan mRNA levels as compared to the normal ovary ($p<0.05$). Similarly, two human GCT cell lines, the KGN and COV434, exhibited low betaglycan expression and poor responsiveness to TGF-beta and inhibin in luciferase reporter assays. Stable transfection of GCT cell lines with a wildtype betaglycan (WT-BG) expression plasmid conveyed ligand responsiveness . FACS analysis was used to examine cell cycle progression and cell death in the GCT cell lines. This analysis revealed that WT-BG had no effect on the number of cells cycling or undergoing apoptosis. However, WT-BG significantly increased the adhesion of COV434 ($P<0.05$) and KGN ($p<0.0001$) cells to collagen IV and fibronectin, decreased cellular invasion through Matrigel COV434 (50%) and KGN (75%), and inhibited wound healing COV434 (70%) and KGN (80%). Collectively, the data establish that betaglycan is an important regulator of granulosa cell biology and suggest that a deficiency in betaglycan contributes to the pathogenesis of granulosa cell cancer. The work further suggests a role for betaglycan in the prevention of tumour invasion, possibly by increasing the adhesion of granulosa cells to matrix components. Supported by: the NHMRC of Australia (RegKeys 338516; 241000; 441101; 388904).

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A role for transforming growth factor-β1 (TGF-β1) during the establishment of folliculogenesis

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A group of structurally related proteins, known as the transforming growth factor-β (TGF- β) superfamily, have been implicated in the local regulation of ovarian function. It is unclear what role TGF-β1-3 plays in folliculogenesis during the period after birth in the rat. We investigated whether the TGF-β ligands and their receptors were present during this period of development and the effects of TGF-β1 on granulosa cell function (proliferation, apoptosis, steroidogenesis).

Ovaries from rats 4, 8 and 12 days of age were isolated and RNA extracted and reverse transcribed for real-time PCR. The expression of the TGF-β ligands and TGFβRI and TGFβRII were measured. Granulosa cells isolated from DES treated immature rats were treated with FSH (100ng/ml) and TGF-β1 (1 or 10ng/ml) for 2hr, n=4 replicates. The RNA was extracted and prepared for RT-PCR. The expression of cyclin D2, FKHR, SCC, 3βHSD and StAR were measured. TGFβRI and TGFβRII proteins were localised to postnatal rat ovary by immunohistochemistry.

TGF-β1-3, TGFβRI and TGFβRII were present in rat ovaries as early as 4 days after birth. Expression of TGF-β1 mRNA increased 2-fold between day 4 and 12. TGF-β2 and TGF-β3 mRNAs declined between day 4 and 8 and remained low at day 12. The type I and II TGF-β receptors were differentially regulated with TGFβRI expression high at day 4, declining at day 8. In contrast, TGFβRII appeared to be ubiquitously expressed. Cyclin D2 mRNA expression was enhanced in the presence of both TGF-β1 and FSH, whereas FKHR mRNA expression declined. TGF-β1 had no impact on the steroidogenic mRNAs. TGFβRI and TGFβRII proteins were localised to the cytoplasm of oocytes, granulosa cells and theca cells.

These studies indicate that TGF-β1 can exert effects on ovarian folliculogenesis as it is established during the postnatal period. Proliferation and apoptosis appear to be targets of TGF-β1 action.

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Suppressors of cytokine signalling (SOCS): roles in ovarian follicle activation

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Oocytes are sequestered in primordial follicles before birth and remain quiescent in the ovary for decades, until recruited into the growing pool throughout the reproductive years. Therefore activation of follicle growth is a major biological checkpoint that controls female reproductive potential. However we are only just beginning to elucidate the cellular mechanisms required, for either maintenance of the quiescent primordial pool, or initiation of follicle growth. Analysis of microarray data derived from neonatal mouse ovaries indicated that members of the Suppressors of Cytokine Signalling SOCS family of proteins may play pivotal roles in folliculogenesis. We undertook a detailed analysis of gene and protein expression patterns of the eight members of the SOCS family, namely CIS and SOCS1-7, within adult and neonatal mouse ovaries. Quantitative real time PCR and immunohistochemistry was performed to determine mRNA levels and cellular localisation in the ovaries of cycling and new born animals. SOCS proteins were expressed largely within the oocytes of developing follicles and in the granulosa cells of the larger preovulatory follicles. Expression of SOCS4 in the granulosa cells and SOCS5 within the oocyte was coincident with the activation of oocyte growth and the differentiation of squamous pregranulosa to cuboidal granulosa cells. Our investigation has identified a role for the SOCS family proteins within the ovary and SOCS4 and SOCS5 as major regulators of cytokine signalling pathways in follicle activation and development.

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CD44 signaling in mouse ovulatory cumulus oocyte complexes

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Oocytes grow and develop within ovarian follicles, providing a nurturing environment prior to their release (ovulation) into the oviduct for fertilisation. For ovulation to occur the ovarian follicle responds to LH from the pituitary, leading to a cascade of regulated gene expression and formation of the hyaluronan rich cumulus matrix around the oocyte. This COC (cumulus oocyte complex) matrix is composed of high concentrations of the ECM glycosaminoglycan hyaluronan (HA) cross-linked by a number of HA-binding proteins. Several of the COC matrix components are essential for ovulation, since null gene mutations in mice lead to ovulation defects. Mechanisms by which the COC matrix controls ovulation however, are unknown. We have investigated cellular signaling and cellular phenotypes that occur as part of the formation of the COC matrix.

The transmembrane HA receptor CD44 was significantly upregulated in cumulus cells from 6h after hCG (LH analog) treatment (9.8±1.5 fold) until ovulation at 12h post hCG (11.8±2.9-fold). In many cell types CD44 activates the intracellular Rho-family GTPase Rac1 and its activator, the guanine exchange factor Tiam1, pleiotropic regulators of cytoskeletal function, cell-cell adhesion and migration. We found both Rac1 and Tiam1 were strongly detected in cumulus cells, but not regulated by hCG. These observations show that at the time of ovulation a macro-molecular complex associated with cell motility is assembled through the extracellular interaction of the COC matrix and cell surface proteins. We investigated the migratory and invasive activity of COCs from hormonally stimulated mice. Migration of cumulus cells from hCG treated mice was significantly increased compared with untreated COCs. Furthermore the hCG-stimulated cumulus cells were able to invade a range of ECM substrates including collagen and laminin. These results suggest the cumulus cells in the expanded COC transition to a motile cell phenotype that may play a key role in promoting ovulation.

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TGF-β and ovarian follicle development

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The role TGF-β plays in ovarian follicular growth and differentiation was investigated using a 'physiological' culture system. TGF-β ligand and receptors are present in the rat ovary from 4 days after birth. Therefore we established organ cultures with these ovaries in order to assess the potential impact of TGF- β1 on follicle growth and transition from the primordial through to the primary and preantral stages of development. Whole ovaries were isolated and cultured for 10 days on floating filters with the addition of supplemented DMEM/Hams F-12 media and either FSH (100ng/ml), TGF- β1 (10ng/ml), or a combination of the two. Media as well as treatments were refreshed every second day. At the end of the culture period, ovaries were fixed in 10% formalin, embedded in paraffin and sectioned at 5µm. Sections were used for morphological assessment and ovarian follicle counting with three serial sections mounted/slide and every alternate slide used for counting of primordial, primary and preantral follicles. An evaluation of atresia by the detection of apoptotic cells was undertaken using terminal deoxynucleotidyl transferase (TdT) mediated dUTP-biotin nick-end labelling (TUNEL) via the ApopTag® Peroxidase *in situ* apoptosis detection kit.

Results gathered from this study show preantral follicle numbers declined significantly when treated with the combination of FSH and TGF- β1, consistent with our morphological appraisal of atresia where the combined treatment appeared to produce more apoptotic follicles than healthy follicles, suggesting an increase in atretic primary and preantral follicles. These preliminary findings suggest an inhibitory role for TGF- β1 in the presence of FSH, resulting in fewer follicles making the transition from the primary to the preantral stage. Further studies are required to test the effects of other TGF-β superfamily members on follicle transition *in vitro*.

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Effect of season on sow ovarian morphology

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Reduced farrowing rate caused by embryonic mortality is a manifestation of seasonal infertility in pigs. The ability of the oocyte to mature, be fertilised and sustain embryonic development is acquired gradually by the oocyte throughout folliculogenesis. This study was undertaken to determine if seasonal differences in ovarian morphology are associated with reduced reproductive performance displayed during seasonal infertility. Sows culled after weaning were sourced from two genetically distinct herds (Farms A and B). Pairs of ovaries were collected from sows 4 days post-weaning during winter (n = 131) and summer (n = 275). Ovarian weight (Farm A only) and the numbers of small (3-4 mm) and large (5-8 mm) follicles were assessed (Farms A and B). Data did not follow normality and was analysed using the Mann-Whitney test. Mean ovarian weight per sow during winter (20.0 ± 1.3 g) was significantly heavier than that during summer (15.3 ± 0.8 g; $P < 0.05$). Farm A ovaries had a greater total number of antral follicles in winter compared with summer, and a greater number of antral follicles in winter compared with Farm B ovaries ($P < 0.05$). In Farm A ovaries, the proportion of follicles that were large was greater in summer compared with winter (78% vs 66%; $P < 0.05$), but the follicular distribution did not change with season in Farm B ovaries. While the findings demonstrate that ovarian weight was greater in winter compared with summer, they suggest that this difference was not the result of changes in the number or distribution of surface antral follicles. The difference in ovarian weight is possibly due to differences in ovarian tissue resulting from regressed corpora lutea. Further studies are being undertaken to assess the effect of season on oocyte developmental competence and the steroid content of follicular fluid isolated from small and large follicles.

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Signalling pathways involved in mouse GDF9 and BMP15 stimulated thymidine uptake by rat granulosa cells

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The oocyte-secreted factors growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are essential for ovarian follicular growth and development. Understanding the molecular mechanisms of these factors could assist with the development of future products for fertility control. Thymidine uptake by rat granulosa cells is stimulated cooperatively by GDF9 and BMP15. Inhibitors of the activin receptor-like kinase (ALK) 4,5,7 and the nuclear factor kappaB (NF_KB) second messenger pathways block ovine GDF9 and BMP15 stimulated thymidine incorporation. The ALK 4,5,7 receptor pathway is known to be essential for the cooperative effects of mouse (m)GDF9 and mBMP15 on thymidine incorporation but the role of other pathways has yet to be determined, which was the focus of this study. Inhibitors of NF_KB (Sn50; 10µg/ml), ALK 2,3,6 receptor (Dorsomorphin; 1µM), p38 mitogen-activated protein kinase (p38 MAPK; SB239063; 5 µM) and c-Jun-N-terminal kinase (JNK; TAT-TI-JIP153-163; 5 µM) pathways were each cultured with recombinant mGDF9 (25 ng/ml) and mBMP15 (6 ng/ml) in a rat granulosa cell [^{3}H]-thymidine bioassay. The p38 MAPK inhibitor caused partial inhibition of thymidine uptake but this appeared to be non-specific as a similar level of suppression was observed in the control cultures. Neither the ALK 2,3,6 receptor nor the NF_KB pathway inhibitors had any effect on mGDF9 and mBMP15 stimulated thymidine uptake. The JNK inhibitor showed a 1.7-fold increase in stimulation above the mGDF9 and mBMP15 effect ($p<0.01$) but a similar stimulation was also observed in some controls. This differs from the results observed with ovine GDF9 and BMP15 where thymidine uptake was completely blocked by the NF_KB inhibitor and the JNK inhibitor had no effect. In conclusion, the molecular mechanisms of GDF9 and BMP15 function are dependent on the species of origin of the growth factor and therefore caution is needed when extrapolating findings from one species to another.

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The development of an immunoassay to measure progestogen using printed biosensors, and its application to the assessment of ovarian function in the Numbat (*Myrmecobius fasciatus*)

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A biosensor system using screen printed sensors was developed to measure progesterone as an index of ovarian function, and compared with a standard enzymeimmunoassay (EIA). The sensors were coated with a monoclonal progesterone antibody which cross-reacts with a wide range of progestogens, and incubated in a mixture of sample/standard and progesterone-3-CMO-horseradish peroxidise (Prog/HRP). The endpoint was the change in potential read following the addition of sodium perborate. The assay was optimised in terms of the Prog/HRP concentration, the antibody dilution and incubation times. It was then used to measure progestogen in the urine of five female Numbats (*Myrmecobius fasciata*). Results were available using the sensors within 20 minutes compared to the standard EIA protocol of 2 hrs. The serial dilution of a urine sample taken at the diestrus stage showed parallelism with the serially diluted standard. There was a significant rise in progesterone (mean ± sem) after mating compared to that seen before for both the EIA (1.31 ± 0.20 to 3.70 ± 0.13 ng/ml) and the sensor (1.83 ± 0.33 to 4.02 ± 0.61 ng/ml), and there were no significant differences between the sensor and EIA results at either stage (all $p>0.1$). A comparison of the values obtained with the sensors to those obtained with the conventional EIA showed a significant correlation for each of the animals ($r=0.82$ to 0.99). It is concluded that the biosensor system is a viable alternative to conventional EIA, and provides the advantage of (a) a shorter assay time and (b) greater potential for use in the field.

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Brain gene expression changes in MHC Class II genes and Neuropilin 2 associated with the transition from acyclic to cyclic ovarian function in postpartum beef cows

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The aim was to identify hypothalamic genes associated with transition from acyclic to cyclic ovarian function in postpartum cows. Ovarian status was recorded at slaughter for primiparous Brahman cows (Zebu, *Bos indicus*) at 27-34 days postpartum and hypothalamic tissue was obtained at the same time. Ovaries were classified as: Ov1 (n=4), follicles 4-5 mm; Ov2 (n=4), follicles equal or larger 10 mm without ovulation; Ov3 (n=4), corpus haemorrhagicum or corpus luteum. Hypothalamic regions were: H1 (SC-POA, APVN, anterior hypothalamic nucleus, anterior portion of the arcuate nucleus, nearby areas of the diagonal band of Broca, and medial septum); H2 (basal hypothalamus-median eminence, ventromedial hypothalamus, posterior portion of the arcuate nucleus, and anterior part of the mammillary body). Gene expression was determined using the Agilent bovine 44k DNA microarray and differential expression (DE) was ascertained by mixed model analysis. Functional clustering of DE genes using DAVID (www.david.abcc.ncifcrf.gov) revealed an enrichment of genes linked with synaptic transmission in H1 and H3 comparing Ov1 and Ov3. Three members of the major histocompatibility complex (MHC) class II gene family (BOLA-DQA1, BOLA-DQA2 and BOLA-DQB) showed a higher expression level in Ov2 than Ov1 in H1 and H2. BOLA-DGA2 and BOLA-DQB were down regulated in Ov3 when comparing Ov2 and Ov3. MHC family members have been associated with plasticity in the brain[1]. Functional clusters of genes involved in cell-cell adhesion were differentially expressed when comparing Ov1 and Ov2 (H2), and Neutropilin-2 which promotes GnRH neuronal migration[2] was up regulated in Ov1. The findings provide preliminary evidence that gene expression related to cellular plasticity within the basal hypothalamus-median eminence is associated with the transition from acyclic to cyclic ovarian function in the postpartum cow.

(1) Håvik B et al 2007 Neuroscience 148:925–36
(2) Cariboni A et al 2007 TRENDS in Neurosciences 30:638-44

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The endocrine disruptor, bisphenol A, alters gene expression in the hypothalamus of the ram lamb but does not alter LH secretion

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Bisphenol A (BPA) is an endocrine disruptor, which has both oestrogenic and anti-oestrogenic actions. It has profound effects on development, especially of sex organs but also on the brain. The actions on the brain are poorly understood and little is known about males. Most work has been conducted in fish & rodents, but the lifespan of domestic ruminants means that BPA may accumulate in tissues over many years. This study tested the hypothesis that treatment of ram lambs with BPA would alter LH secretion as well as gene activity in various hypothalamic regions. Ram lambs were treated from 1 month old with thrice weekly injections (im) of BPA (3.5mg/kg) or vehicle (n=10/group) for 80days. Weekly bodyweights and jugular blood samples were collected and on day 79, blood samples were taken every 15min for 8h for LH RIA. The sheep were killed, body & testicular weights collected and the hypothalamus dissected into anterior, dorsal and ventral blocks, then frozen in liquid nitrogen. Bodyweight (Cont. 26.0±0.5kg vs BPA 25.2±0.7kg) and paired testicular weight (Cont. 31.6±1.2g vs BPA 29.3±1.4g) did not significantly differ (P>0.05). LH secretion also did not differ, in either mean LH concentrations (Cont 0.31±0.14 vs BPA 0.29±0.12ng/ml) or pulses/8h (Cont 1.8±0.4 vs BPA 2.0±0.4). Total RNA was extracted from the basal hypothalamus (ventral block) and RNA from 2 treated and 2 control animals was hybridised on 44K Agilent bovine microarrays. Preliminary analysis suggests 118 probes were differentially expressed. Gene ontology analysis of these differentially expressed probes revealed involvement in a broad range of biological processes and cellular components. This study suggests that gene array analysis is a suitable tool with which to study BPA actions in the sheep hypothalamus. Future work will expand this pilot microarray study to include all animals and all hypothalamic regions.

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The technical and biological validation of an LH assay for use with the Western Grey Kangaroo (*Macropus fuliginosis*) and Black-flanked Rock Wallaby (*Petrogale lateralis lateralis*)

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Methods for the measurement of marsupial LH invariably rely upon the similarity of the LH molecule between different species and usually use anti-ovine or anti-bovine LH antibody and an ovine or bovine labelled LH preparation. Initial attempts to measure plasma LH in the Western Grey Kangaroo with assays using antibodies to 4 different isoforms of ovine LH raised in 7 different rabbits were unsuccessful. An enzymeimmunoassay (EIA) developed for the Asian elephant (Zoo Biology 23:45-63) was then applied to the Western Grey Kangaroo and the Black-flanked Rock Wallaby. This EIA has an anti-bovine-LH monoclonal antibody (518B7 provided by Dr Jan Roser, University of California, Davis, USA), biotinylated ovine LH label and bovine LH standard (NIADDK-oLH-26 and NIH-bLH-B10, both provided by Dr Janine Brown and Nicole Abbondanza, Smithsonian Institute, Front Royal, Virginia USA). Technical validation showed that serial dilution down to 1:8 of plasma from 7 individuals of each species showed parallelism to the assay standard curve, and control samples (1.24-5.30 ng/ml) had between-assay coefficients of variation <9%. Biological validation was achieved by challenging animals with intramuscular GnRH (Fertagyl®, 2.5 μg/kg) and measuring LH before and 25 minutes after the injection. Significant increases in plasma concentrations of LH (mean ± sem; all p>0.0005) were seen after GnRH for both the Western Grey Kangaroo (from 5.0 ± 0.8 ng/ml to 9.4 ± 1.2 ng/ml; n=19) and the Black-flanked Rock Wallaby (from 6.0 ± 0.7 ng/ml to 10.6 ± 0.6 ng/ml; n=28). In conclusion, this assay can be successfully used to measure LH in these two species.

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Equine chorionic gonadotrophin isoform composition in commercial products compared to isoform composition in pregnant mare plasma

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It is well documented that there is considerable batch to batch variation in the activity of commercial preparations of gonadotrophins. These products are used in a variety of assisted reproductive procedures in the livestock industry, consequently this high degree of variation between products and batches adds to the already considerable between animal variations in response to the treatment. Equine chorionic gonadotrophin (eCG) is a heterodimeric glycoprotein hormone secreted by the placental endometrial cups during the first third of gestation in the horse. Plasma is harvested from pregnant mares between 40-90 days of gestation and the eCG isolated and used to formulate commercial preparations. Previous research has shown that eCG like the other gonadotrophins is a highly heterogeneous molecule with significant differences in bioactivity between isoforms. The aim of this study was to determine whether significant differences in isoform composition exist between various commercial preparations of eCG (n=15), and how this compares to the isoform composition found in plasma (n=23). Concentrations of eCG were determined using a competitive eCG ELISA. Liquid phase iso-electric focusing was used to fractionate plasma and the commercial preparations into 10 pH ranges from pH 3.0 to pH 10.0. Data from the 10 fractions were grouped into acidic (pH 3.0-5.1), intermediate (pH 5.2-7.9), or basic (pH 8.0-10.0) isoform categories for analysis. Immunoactivity between commercial eCG products ranged from 44% to 362% of stated bioactivity. Iso-electric focusing showed that the majority of the immunoactivity (92%) of the commercial preparations was found in the acidic fractions (pH 3.0-5.1), and in particular in the pH range 3.0-3.8. This contrasted starkly with isoform profiles found in pregnant mare plasma samples which showed a much greater spread across all 3 pH ranges. In summary, the isolation processes of commercial eCG preparations appears to selectively favour the acidic isoforms of eCG.

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IGF2 polymorphisms predict pregnancy outcome

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IGF-II is an important determinant of placental trophoblast invasion and subsequent placental function. IGF-II can act in autocrine/paracrine and endocrine fashions to promote placental invasion and differentiation and within both the fetus and placenta to promote fetal growth. We aimed to determine whether polymorphisms in *Igf2* are associated with common pregnancy complications associated with uteroplacental insufficiency. Pregnant women were recruited in early pregnancy for a prospective case control study at the Women's and Children's Hospital and Lyell McEwin Health Service, Adelaide, Australia. Buffy coats were retrieved from maternal blood sampled at 15 weeks gestation, paternal blood and cord blood collected following delivery. Pregnancy outcomes were classified into normal (n=126), preeclampsia (PE, n=31), gestational hypertension (GH, n=18), small-for-gestational-age (SGA, n=50), PE+SGA (n=16) or pre-term birth (PTB, n=19) by an experienced obstetrician. Three polymorphisms in *Igf2*, *Igf2 ApaI* and *Igf2 MspI* single nucleotide polymorphisms, and *INS+2336* deletion/insertion polymorphism were selected for investigation. DNA was extracted from buffy coats and genotyping was performed by PCR followed by High Resolution Melt analyses. Data were analysed by Chi Square and Fisher's Exact Test and Likelihood Ratios (LR) were calculated. In normal pregnancies, all polymorphisms were in Hardy-Weinberg Equilibrium. *Igf2 ApaI* in the neonate, and hence placenta, was associated with PE (P=0.016, LR=7.46) and PTB (P=0.024, LR=8.61). Neonatal *Igf2 MspI* was associated with SGA (P=0.007, LR=9.81). Gestational age was associated with maternal *Igf2 ApaI* (P=0.0004) and *INS+2336* (P=0.0021), as well as neonatal *INS+2336* (P=0.0046). Birthweight was associated with paternal *Igf2 MspI* (P=0.044) when corrected for gestational age. Although this work is ongoing, data thus far suggest polymorphisms in the gene encoding IGF-II, primarily in the placenta, are associated with a range of pregnancy complications which have been associated with impaired placental function. Ongoing research will determine whether these polymorphisms are associated with aberrant placental *Igf2* expression.

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Expression of oestrogen receptor-α and modulators of steroid receptor signalling, proline-rich nuclear receptor-2 and peptidylprolyl isomerade-D, in the hypothalamus of suckled and weaned postpartum beef cows

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The aim was to characterise gene expression in the hypothalamus of suckled and weaned postpartum beef cows. The hypothalamus was obtained at slaughter from 12 primiparous Brahman cows (Zebu, *Bos indicus*) at 27 and 34 days postpartum. Six cows were weaned 7 days or 14 days before slaughter. Hypothalamic regions used for gene expression were: H1 (SC-POA, APVN, anterior hypothalamic nucleus, anterior portion of the arcuate nucleus, nearby areas of the diagonal band of Broca, and medial septum); H2 (basal hypothalamus-median eminence, ventromedial hypothalamus, posterior portion of the arcuate nucleus, and anterior part of the mammillary body). Gene expression was determined using the Agilent bovine 44k DNA microarray and differential expression (DE) was ascertained by mixed model analysis. A total of 122 genes were DE in H1 and 84 genes were DE in H2; 41 DE genes were common to H1 and H2. Functional clustering of DE genes using DAVID (www.david.abcc.ncifcrf.gov) revealed DE gene clusters in H1 associated with signalling events and ion binding, and DE gene clusters in H2 associated with hormone activity and ligand-receptor interactions. Of the DE genes, approximately 25% were linked with oestrogen signalling. This included oestrogen receptor-α (ESR1) that showed lower DE in H2 for weaned cows. Two modulators of steroid receptor signaling, proline-rich nuclear receptor coactivator-2 (PNRC2)[1] and peptidylprolyl isomerase D (PPID)[2], showed altered expression. In weaned cows, expression level of PNRC2 was lower in H1 and H2, while that of PPID was decreased in H1. The overlapped hypothalamic regions H1 and H2 are known to contain GnRH neuron terminals and kisspeptin neurons. Weaning promotes the resumption of cyclic ovarian function in postpartum cows, and the similar shifts in DE of ESR1, PNRC2 and PPID provided further evidence of a role for oestradiol at the hypothalamus in regulating postpartum reproduction.

(1) Zhou D et al 2006 Nucleic Acids Res 34:5974-86
(2) Kumar P et al 2001 Biochem Biophys Res Commun 284:219-25

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INSL3 is a measure of human Leydig cell functionality both during fetal and adult life

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Insulin like factor 3 (INSL3) and testosterone are the two major secretory products of the testis, both produced by the interstitial Leydig cells. The Leydig cells of the testis have two distinct generations, one developing before birth (fetal Leydig cells, FLC) and an adult type (adult Leydig cells, ALC) that become differentiated and functional at puberty. Although these two types of Leydig cells represent distinct populations, rodent studies show that both types produce testosterone and INSL3. Both are presumed to have evolved from a common stem cell pool. We measured INSL3 levels in human amniotic fluids collected at various times of gestation and show for the first time that the human male fetus indeed generates INSL3 at a time appropriate for the first transabdominal phase of testicular descent, which appears to be the primary physiological role for the fetal hormone. INSL3 appears to be independent of androgen production. The adult type Leydig cells (in adult men) secrete INSL3 that can be measured in the peripheral circulation at levels ranging from 0.5 to 2.5 ng/ml. We studied a large randomly recruited cohort of 1183 men from South Australia, comparing serum INSL3 concentrations with age, and a variety of endocrine, cognitive and morphological parameters. INSL3 concentration was observed to decline significantly with age. This however, had no correlation with testosterone or components of the HPG axis. INSL3 is an independent measure of Leydig cell function (quality and number), which appears to be independent of acute control via the HPG axis. Its decline with age reflects a decline in the properties of the Leydig cell population only, and emphasizes a gonadal component in the age-related decrease in androgen production. *Research supported by ARC Discovery grant DP0773315.*

SRB POSTERS

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In vivo development of embryos derived from sex-sorted and non-sorted ram sperm

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The use of sex-sorted ram sperm results in pregnancy rates similar or superior to that of non-sorted sperm after laparoscopic insemination of synchronised ewes under defined conditions [1, 2]. To further assess the fertility of sex-sorted ram sperm, embryo production and development rates were examined after the insemination of superovulated ewes with either sex-sorted or non-sorted sperm. Merino ewes (n = 30) were synchronised in oestrus using progestagen sponges inserted for 14 d and hormonally stimulated with PMSG (600 i.u.) on sponge removal (SR), FSH (133mg) at decreasing doses every 12 h for 4 d before insemination, and GnRH 24 h before insemination. Each ewe was inseminated in the uterus by laparoscopy 42-44 h after SR with 15 x 10^6 motile X- or Y-chromosome bearing or non-sorted spermatozoa. On day 6 after insemination, antegrade flushing of both uterine horns was performed. Overall, the fertilisation rate was higher using X-chromosome bearing sperm (70%) compared to both Y-chromosome enriched (59%) and non-sorted treatments (64%). Embryo development was more advanced after the insemination of non-sorted sperm with more hatching (hg) and expanded blastocysts (exb) recovered per animal (hg: 3.37 ±1.19; exb: 5.00 ± 1.68) compared with sorted groups (X hg: 1.71 ± 0.92; exb: 3.28 ± 1.80; Y hg: 1.67 ± 1.67; exb: 1.50 ± 1.02). Moreover, embryos were recovered at earlier developmental stages after insemination with Y- compared with X-chromosome bearing sperm. In conclusion, the use of sex-sorted sperm did not affect the efficiency of embryo production. However, the development of embryos derived from sex-sorted sperm was delayed compared with those from non-sorted sperm.

(1) de Graaf et al. 2007. Reproduction in Domestic Animals 42, 648-653

(2) Beilby et al. 2008. Theriogenology [IN PRESS]

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Cryopreservation of oocytes and follicular cells of the cane toad *Bufo Marinus*

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Amphibians are currently the most threatened of all vertebrate groups with more than 30% of all known species in decline, facing extinction or recently extinct. Cryobanking of amphibian germ cells and reproductive tissues could be used to manage threatened species and provide insurance against extinction. However, cryopreservation of fully developed amphibian oocytes and whole embryos has not been achieved due to technical problems freezing such large cellular structures. As an alternative approach, we investigated the feasibility of developing protocols for the slow-cool freezing, storage and retrieval of developmentally competent amphibian ovarian follicles containing Stage I and II oocytes which are much smaller in size than later developmental stages. Ovarian follicles from euthanased Cane Toads were incubated in cryodiluents containing either glycerol or DMSO to assess cryoprotectant toxicity and response to slow cooling freezing protocols. The fluorescent live cell stain SYBR 14 and its counter stain propidium iodide was used to score the proportion of viable follicle cells before and after cryopreservation. Cryoprotectant type, concentration and exposure time all had significant effects ($P < 0.05$) on the viability of follicle cells, with significant interactions between these variables. Overall, glycerol was less toxic to follicle cells than DMSO. At higher concentrations, glycerol exerted high osmotic stress on oocytes, and there was evidence that DMSO triggered apoptosis in oocytes. The most effective cryopreservation protocol for stage I and II oocyte follicles resulted in a post-thaw recovery of a mean 70% of viable follicular cells. This protocol involved cryopreservation in 15% v/v glycerol, inclusion of seeding and temperature holding periods during cryopreservation, coupled with rapid thawing in a 30°C water bath. The successful cryopreservation of intact follicles in this study indicates the potential to recover functional ovarian tissues post cryopreservation for continuation of amphibian oogenesis *in vitro* or *in vivo*.

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Differential effect of hexoses on sperm metabolism and function in culture

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Currently there is lack information regarding how human spermatozoa regulate their energy metabolism. This is surprising considering that carbohydrate metabolism is a vital point for the understanding of sperm function. This coupled with the increased use of assisted reproductive technology and the importance of a well-balanced culture media has led us to hypothesise that an imbalance of carbohydrate presence in the media may alter sperm function, particularly in relation to oxidative stress, DNA damage and lipid peroxidation.

Sperm samples were obtained from three healthy normospermic donors for this study. Motile sperm were separated from semen samples using density gradient separation. Samples were incubated at different media conditions with varying glucose or fructose concentrations (0, 2.5, 25mM) for 6-24hrs. Reactive oxygen species (ROS) were measured using 5-(and-6)-carboxy-2', 7'-dichlorofluorescein diacetate (DCFDA). Sperm DNA damage was determined using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling (TUNEL). Lipid peroxidation was assessed using the probe BODIPY (581/591) C_{11}. Carbohydrate uptake from the media was measured using a fluorometric procedure. Statistical differences between treatments were assessed by ANOVA and Bonferroni post-hoc test.

No significant motility differences were found following treatments. Results showed an increased level of ROS production as glucose concentration increased ($p<0.05$). This was accompanied by an increased number of TUNEL positive cells ($p<0.05$). Furthermore, lipid peroxidation of spermatozoa was significantly increased when incubated under high glucose concentrations ($p<0.01$). In contrast, increases in fructose concentrations did not alter ROS levels or the number of TUNEL positive cells. Sperm metabolised both glucose and fructose in vitro and the removal of one carbohydrate resulted in a compensatory increase in the metabolism of the other.

To our knowledge, this is the first report providing evidence that altered carbohydrate metabolism may induce ROS production, lipid peroxidation and increase the number of sperm exhibiting DNA damage.

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Repercussions of a transient decrease in pH on embryo viability and subsequent fetal development

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Changes in the environment to which the pre-implantation embryo is exposed can significantly influence fetal outcomes, indicative of 'embryo programming'. Although many previous in vitro stress models have demonstrated programming changes using high dose stress effects, it is possible to induce similar effects with a physiologically relevant stress model.

This study investigates the effect of a subtle transient pH change, during the first cleavage division, on blastocyst viability and fetal outcomes after embryo transfer.

Zygotes from F1 hybrid mice (C57BL6xCBA F1) were cultured to the 2-cell stage (19h) in either control G1 medium or in G1 containing a weak acid, 2mM DMO (5,5-Dimethyl-2,4-oxazolidinedione), then cultured to the blastocyst stage in control media G1/G2 (72h) . Exposure to DMO induced a decrease in intracellular pH from 7.25 (control) to 7.10 (DMO). At the blastocyst stage, inner cell mass (ICM) cell number and cellular apoptosis were assessed, or embryos were transferred to pseudopregnant recipients to assess implantation and fetal outcomes. Differences were assessed using Student's t-test or generalised linear modelling followed by post-hoc tests.

Exposure to DMO during the first cleavage division significantly reduced total blastocyst cell number from 83.0 ± 6.4 to 63.6 ± 3.8 ($P<0.05$), reduced ICM number from 30.6 ± 3.6 to 20.2 ± 1.8 ($P<0.05$) and significantly increased the apoptotic cell index from 1.9% to 3.2% (for control verses DMO embryos respectively)($P<0.05$). Blastocyst development was unchanged. Exposure to DMO during the first cleavage division did not alter implantation rates however fetal weight was decreased from 1058.9mg ± 25.2 (control) to 949.1mg ± 26.7 (DMO) ($P<0.05$) and crown rump length decreased from 21.9mm ± 0.4 (control) to 20.6mm ± 0.5 (DMO) ($P<0.05$).

In conclusion, this study demonstrates that a transient reduction in pH of only 0.15 units during early pre-implantation embryo development significantly reduces resultant blastocyst viability and perturbs fetal growth, indicative of altered embryo programming. The mechanism behind this permanent change however is currently unknown.

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Isolation and preliminary characterisation of putative sheep embryonic stem cells

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The establishment of true, fully characterised embryonic stem (ES) cells from livestock, (eg sheep) has yet to be reported. Such cells could make a significant impact on assisted reproductive technologies, vaccine delivery, and animal health and well-being in the livestock industries. To date in sheep, there is a single report of putative ES cells which were maintained in an undifferentiated state for only 2 passages. Here we report the isolation and culture of pluripotent ES-like cells from *in vivo* derived, vitrified, sheep blastocysts. The inner cell mass of blastocysts were isolated by immunosurgery, cultured in Stem Cell Sciences' (SCS) novel inhibitor-based media, on a feeder layer of mouse embryonic fibroblasts (MEFs), resulting in putative ovine ES cells proliferating to at least passage 5. One cell line, BMCOV002, was established out of four thaw-recovered embryos. The colonies formed compact, near homogenous, small cell, multilayered, and well defined dome shaped masses that were morphologically similar to both mouse and human ES cell colonies. A peripheral halo of filamentous differentiated cells was detected in selected colonies from passage 3 to passage 5. The putative ovine ES-like cells were passaged by mechanical excision between days 6-7, and these colonies stained positive for alkaline phosphatase at both passage 3 and passage 5. Expression levels of genes encoding the pluripotent transcription factors *OCT4*, *SOX2*, *REX1* and *NANOG* are shown using RT PCR in cells from passage 3. An important first step in studying the properties of ovine ES-like cells is the ability shown here to isolate and culture cells. Our attention now is focused on maintaining these cells for some months in an undifferentiated state, and on being able to successfully cryopreserve and regenerate these cell lines.

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Regulation of insulin receptor and its substrates by follicle stimulating hormone in granulosa cells

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Insulin receptor signaling receptor regulates the process of follicular development and maturation, however the regulation of insulin receptor (IR) and its signaling component during folliculogenesis is not well understood. This study demonstrates FSH mediated regulation of insulin receptor isoforms specially IR-B, its substrates and some of the insulin related responses like glycogen synthesis and cell proliferation. There was significant increase in mRNAs expression for insulin receptor (IR) isoforms, IR-A and IR-B in ovary in response to PMSG and in granulosa cells (GCs) in response to FSH. IR protein increased in GCs in response to FSH in a dose and time dependent manner. Interestingly, the expression of mRNAs and proteins for IRS-1 and IRS-2 increased significantly in GCs by FSH. Serine phosphorylation (Ser 636/639) of IRS-1 was decreased by FSH, thus facilitating IRS-1 activation. FSH stimulated glycogen synthesis in a dose dependent manner both by PI 3 kinase dependent and independent pathways. Insulin regulated the amount of FSH stimulated glycogen synthesis by granulosa cells. In contrast, FSH and insulin synergistically stimulated glycogen synthesis GC proliferation which was completely inhibited by LY294002. Knockdown of IRS-1 mRNA by siRNA inhibited FSH stimulated GC proliferation indicating an important role of IRS-1 downstream of FSH. Further research is required to delineate the signaling components involving IRS-1 and IRS-2 in response to FSH and thus involved in the cross-talk between FSH and insulin in GCs. Results thus demonstrate that pituitary FSH regulates insulin receptor and its substrates in rat GCs which might be important for follicular growth and oocyte development.

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Effects of vitrification and slow-cooling on the structure of lamb and mouse ovarian tissues

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Follicular dynamics of ovarian tissue varies with species and age. The morphology and distribution of primordial, primary and secondary follicles were examined using mouse and lamb ovarian tissue before and after cryopreservation. Ovarian cortical tissue was processed from mouse (C57BL6J×CBA) and abattoir-sourced lamb ovaries and sliced into fragments (1mm^3). The fragments were randomly divided into three experimental groups. For vitrification, the tissue fragments were equilibrated (10% v/v ethylene glycol (EG) and DMSO, 20 mins) and transferred to vitrification solution (17% v/v EG and DMSO and 0.75M sucrose, 3 mins), and loaded onto a Fibreplug (CVM kit, Cryologic). For slow-cooling (SC), up to ten ovarian fragments were placed in slow cooling solution (10% v/v DMSO and 0.1M sucrose, 5 mins, RT), and then loaded in straws before placed in a programmable freezer (Cryologic CL8800i). For histological comparison, fresh and cryopreserved-thawed ovarian tissues were fixed, embedded and sectioned (5µm) and stained with haematoxylin and eosin. Primordial, primary and secondary follicles were evaluated and the normality of follicular structures was scored. Differences between treatment groups were examined using a Chi Square test. In lamb, 83% of follicles assessed were primordial, 16.4% primary and 0.6% secondary whereas in mouse the ratios were 60%, 27% and 13% respectively. In lamb, proportion of follicles with normal morphology after thawing for vitrification (77.2%) and SC (79.3%) was not significantly different from fresh controls (83.1%). Similarly, in mouse, the proportion of normal follicles after warming for vitrification (92.3%) and SC (90.5%) was not significantly different from fresh controls (91.4%). No obvious detrimental effects on morphology of follicles derived from either vitrification or slow-cooling of lamb and mouse ovarian tissue was observed.

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Liver gene expression in suckled postpartum beef cows maintained on moderate and improved subtropical pasture

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The liver is the first organ to receive absorbed nutrients from the splanchnic vasculature1 which places the liver at an important juncture between nutrition, metabolic homeostasis and reproductive function. The best described component of this interface is secretion of IGF-1 by the liver and dependence of ovarian follicular maturation on hepatic-derived IGF-12. The aim in the present study was to ascertain the expression of hepatic genes associated with metabolic regulation in postpartum beef cows maintained on moderate or improved pasture, and with contrasting live weight and body condition. Multiparous Droughtmaster cows (*Bos taurus* x *Bos indicus*) were placed on moderate (MP, n=7) or improved (IP, n=7) pasture at 6-7 months of gestation and remained on the treatments after calving. Liver biopsies were taken 6 weeks after calving and gene expression was determined by quantitative PCR using SYBR Green3. Week 6 represents attainment of the plateau in nutrient demand for lactation. Data were analysed by ANOVA using SAS STAT. One week after calving, IP cows had greater ($P < 0.01$) live weight and body condition score (BCS) than MP cows (585±12 kg and 528±21 kg; 3.7±0.2 BCS and 2.3±0.2 BCS). IP cows had greater ($P < 0.05$) relative expression of hepatic genes associated with cholesterol biosynthesis (sterol regulatory element binding factor, SREBF2; peroxisome proliferators-activated receptor delta, PPARD), fatty acid biosynthesis (carnitine palmitoyl-transferase, CPT1A; acyl-CoA oxidase, ACOX1; fatty acid desaturase 1, FADS1; sterol regulatory element binding factor 1, SREBF1) and insulin signalling (hepatic insulin receptor substrate 1, IRS1). IP cows resumed cyclic ovarian function between 11-16 weeks postpartum and one MP cow had cycled by 16 weeks. The differences in expression of metabolic genes between MP and IP cows may have partly contributed to differences in reproductive function postpartum.

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Differentially expressed genes in endometroid endometrial carcinoma using microarray: a preliminary report

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Background: Endometrial cancer is the commonest cancer among women in the western countries. It ranks the fourth in gynaecological cancers incidence in Malaysia. Endometriod carcinoma is the commonest subtype of endometrial cancer. The etiology of this disease is still not fully understood. Previous theories have suggested the disease is caused by the activation of oncogenes and inactivation of the tumor suppressor genes. The aim of this study is to identify expressing genes involved in the endometrial carcinoma compared with the normal endometrium.

Materials and Methods: All endometrial tissues were obtained from patients that undergo total hysterectomy. The pathologist confirms the histolopathological examination. Total RNA was isolated and confirmed using the bioanalyzer 2100. Gene list were profiled using Affymetrix Human Genome Gene Chip 1.0 ST array. The results were analyzed using GeneSpring 9.0 GX software.

Results: The software analysis showed 237 differentially expressed genes (2-fold change) between normal and tumor. Among of these genes, 28 were found to be up regulated and 209 were down regulated in tumor compared with the normal ($p<0.01$). There are several genes that are differentially expressed between endometrial carcinoma and normal tissues and found to be associated with the transforming growth factor-beta (TGF-beta) pathway. A mong the genes include CD44, CAV1 and TGFβR3.

Conclusion: This preliminary finding has suggested the molecular difference of endometrial carcinoma and normal endometrium. These transcripts may be participated in the pathogenesis of this disease.

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Gene expression analysis in endometriotic lesions using laser capture microdissection

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Previous studies examining gene expression profiles in normal endometrium and endometriotic lesions have used RNA extracted from whole tissue samples. Results from these studies can be difficult to interpret as they reflect expression averaged across several different cell types that may be functionally quite different. The aim of this study was to establish laser capture microdissection (LCM) as a technique to examine gene expression in stromal and epithelial cells from normal and ectopic endometrium. We hypothesised that genes associated with inflammation would be elevated in cells from endometriotic lesions. Full thickness uterine samples were collected during abdominal hysterectomy from normal cycling premenopausal women. Endometriotic lesions were collected during abdominal laparoscopy. Samples were either frozen in OCT or stored in RNAlater for 12h before freezing. Tissues were immunostained with an antibody against CD10 to identify ectopic endometrial stromal cells prior to LCM. Endometrial epithelial and stromal cells were collected using the PALM MicroLaser System. RNA quality was accessed using Experion. TGFβ1, MMP1, αSMA, SMAD2 and NFκB mRNA was analysed using real-time RT-PCR. Of the endometriotic samples stored in OCT (n=58), only 14% (n=8) had visible endometrial glands. Of these, only 37% (n=3) had RNA of an acceptable quality for further analysis. However, RNA quality and quantity were dramatically improved in 3 of 5 samples collected in RNAlater. In preliminary studies, expression of TGFβ1 and αSMA mRNA was elevated in endometriotic lesions in comparison to the normal endometrium, whereas NFκB expression did not change. We have shown that RNAlater solution is useful to preserve RNA quality for small clinical endometriotic samples and that immuno-guided LCM-generated homogenous cell populations coupled with real-time RT-PCR can provide valuable insights into cell and disease-specific gene expression in endometriotic lesions.

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Macrophage abundance and phenotype in the mammary gland fluctuates throughout the estrous cycle

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Macrophages are versatile cells with roles in the generation and execution of immune responses, tissue breakdown and remodeling and production of regulatory cytokines. These cells are implicated in development of reproductive tract tissues that mature after birth. Descriptive and knockout studies indicates that macrophages play multiple roles in epithelial cell proliferation, phagocytosis and tissue remodeling during pubertal mammary gland development, pregnancy, lactation, and involution. However, the role of macrophages in remodeling over the course of the ovarian cycle has not been previously investigated. Estrus cycles were monitored in C57/Bl6 mice by daily vaginal smears, and mammary glands were analysed for the abundance and location of macrophages in all four stages of the cycle (n=6-8 per group). Absolute number of macrophages was quantified by FACs analysis of digested mammary gland tissue stained with macrophage-specific antibody F4/80. The location of macrophages was investigated in fixed, paraffin embedded tissue by immunohistochemistry using F4/80 antibody. H&E staining revealed that the most differentiated epithelial ducts and alveoli structures appeared in diestrus and proestrus. Whilst there was no difference in the absolute number of macrophages at any stage, the location of these macrophages varied. Macrophages accumulated around the more highly differentiated epithelial ducts, leading to significantly more macrophages around the ducts during proestrus compared with metestrus ($P<0.05$). These macrophages were in close contact with the epithelium, and displayed long projections and widespread cytoplasm. In contrast, macrophages on the edge of the gland within the collagen-rich connective tissue were characteristically round in shape. These results indicate that macrophages are present in the adult virgin mammary gland, and fluctuate in location and phenotype over the course of the estrous cycle. Further analysis of the phenotypes of macrophages present in these different locations is required for a more comprehensive understanding of the role of macrophages in mammary tissue homeostasis.

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The role of macrophages in regulating uterine epithelial cell proliferation

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During the oestrous cycle, uterine epithelial cells respond to ovarian steroid hormones by producing an array of cytokines and chemokines that cause macrophage recruitment into the uterus and regulate macrophage activation phenotype. In turn, growth factors and cytokines synthesised by macrophages potentially impact epithelial cell proliferation, secretory function and receptivity to embryo attachment. To investigate the hypothesis that uterine macrophages are essential contributors to the proliferation of uterine epithelial cells, we have used an ovariectomy and steroid replacement model in CD11b-DTR 'Mac-terminator' mice. These mice are engineered for CD11b promoter-driven expression of the monkey diphtheria toxin (DT) receptor, allowing acute systemic ablation of macrophages by administration of human diphtheria toxin (DT). CD11b-DTR mice were ovariectomised, then 2-4 weeks later were primed with E_2, followed by administration of DT (25 ng/g, ip) to effect macrophage depletion, and BrDU to label proliferating cells. Control mice were given PBS instead of DT. Uterine tissues were stained with F4/80 to detect macrophages, and anti-BrDU to detect BrDU+ epithelial cell nuclei. DT treatment was associated with a depletion of >90% of F4/80+ uterine macrophages. However, the numbers of BrDU+ epithelial cells and the architecture of the luminal epithelial surface and abundance of epithelial glands were similar in control and DT-treated uterine tissues. These data suggest that resident macrophages may not be essential for estrogen-driven uterine epithelial cell proliferation. In ongoing experiments we are assessing the effect of macrophage depletion on epithelial cell expression of functional markers including those involved in regulation of embryo attachment.

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Genetic diversity of the major histocompatibility complex and response to immunocontraceptives in the brushtail possum

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The brushtail possum (*Trichosurus vulpecula*) is a major invasive pest in New Zealand. One option for its control is the use of an immunocontraceptive vaccine, a method of fertility control that employs the immune system to attack reproductive cells or proteins. Initial trials of immunocontraceptive vaccines have shown individual variation in immune response. Concerns have been raised that the use of such a vaccine on wild populations could result in selection for possums that remain fertile because of low or no response, and subsequently negate the efficacy of the vaccine. Therefore, it is important to establish the basis of this variation. The major histocompatibility complex (MHC) is an important component of the immune system which influences the nature of immune responses. This study aimed to document genetic variation in MHC loci of New Zealand possums, and investigate whether there was a relationship between MHC haplotypes and individual immune responses to immunocontraceptive vaccines. We used known marsupial (possum, red-necked wallaby, tammar wallaby, opossum) MHC sequences to design PCR primers for possum MHC loci. The variability of these loci was screened in populations of possums from locations throughout New Zealand, and between individuals with known responses to immunocontraceptive vaccines. We identified 71 novel class I and class II MHC alleles and observed significant variation in allele identity and frequency between geographically separate possum populations in New Zealand. Comparisons of MHC haplotype and immunocontraceptive vaccine response showed that some of these alleles differ between high-responding and low-responding possums. The considerable variation we have found in the possum MHC, combined with differences in the occurrence of MHC genes at locations around New Zealand, provide a potential basis for the observed variability of individual possum's responses to immunocontraceptive vaccines.

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The contraceptive potential of a long-acting IL-11 inhibitor

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Interleukin 11 (IL-11) signalling is essential for the establishment of pregnancy in mice, through its action on the differentiation of uterine endometrial stromal cells (decidualization), a critical process during embryo implantation. IL-11Rα deficient mice are infertile due to defective decidualization[1]. IL-11 expression peaks between days (D) 4.5-9.5 of pregnancy (D0: day of plug) in mouse decidua. We examined the effect of administering (intraperitoneal [IP] injection or vaginal gel) a PEGylated IL-11 antagonist (PEGIL-11A) on decidualization and pregnancy outcome in mice. The sera half-life of PEGIL-11A (IC_{50} 2.8nM) following IP injection was 24h, compared to <1h for the non-PEGylated antagonist (IC_{50} 0.26nM). Following IP injection, PEGIL-11A localised to decidual cells and blocked the IL-11 decidual target protein, cyclin D3. IP injection of 600µg/application PEGIL-11A (or PEG control) at 1000h and 1600h on D3 and 1000h on D4 (n=4/group), resulted in smaller implantation sites than controls on D6 due to retarded mesometrial decidual formation. On D10, severe decidual destruction was visible: implantation sites contained regions of haemorrhage and the uterine luminal epithelium had reformed, suggesting a return to oestrous cycling. Following vaginal application in aqueous placebo gel, PEGIL-11A localised to decidual cells. Vaginal application of 200µg/application PEGIL-11A (or control) twice daily from D2 to D5 (n=4/group), resulted in smaller implantation sites than controls on D6 due to partial inhibition of mesometrial decidual formation. This study demonstrates that PEGIL-11A blocked IL-11 action in the uterus, resulting in total pregnancy loss, equivalent to the IL-11Rα deficient mouse. In women, IL-11 and its receptor are produced by the uterine luminal and glandular epithelium during the period of uterine receptivity[2], suggesting that IL-11 may act during initial blastocyst attachment to the luminal epithelium as well as stromal decidualization. This study provides proof-of-principle for the development of a novel, non-hormonal contraceptive for women.

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(2) Dimitriadis E et al. Molecular Human Reproduction 2000; 6: 907-914.

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Increased expression of an androgen receptor regulated gene, kit ligand, in polycystic ovaries

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Polycystic ovaries (PCO) are induced by pathological or pharmacological female androgen excess, but the role of the androgen receptor (AR) in the pathogenesis of PCO is unknown. We therefore tested the hypothesis that PCO have increased expression of AR or kit ligand (KITL), a cytokine that was recently identified as a candidate AR-regulated gene in the ovary (1). Immunohistochemical analysis of AR and KITL expression was performed on archival paraffin-embedded sections of 8 morphologically normal and 8 polycystic ovaries from women under the age of 40 years. Stained sections were scanned with a NanoZoomer Digital Pathology System and immunoreactivity was qualitatively assessed using a 0-3+ scale, where 3+ represents the most intense staining. Electronic images of follicles at different stages of folliculogenesis were assessed by two independent observers who were blinded to the morphology of the source ovary. Each individual ovary contributed a minimum of 1 follicle per size class and a minimum of 10 follicles per size class were analysed. AR immunoreactivity was present in granulosa cells at all stages of folliculogenesis, in thecal cells of large antral follicles, and in the ovarian stroma. Staining intensity for AR did not differ between normal and polycystic ovaries. KITL expression, summarised in Table 1, was found to be significantly elevated in the oocytes of primordial and primary follicles and in the granulosa cells of follicles at all stages of folliculogenesis. These results show that AR expression is normal in PCO but expression of an AR-regulated gene is increased, potentially due to an excess of androgen hormone that is characteristic of women with PCO. Based on the roles of KITL established by murine studies, increased expression of KITL could explain many of the features of PCO including follicle excess, hyperthecosis and abnormal androgen secretion.

Table 1. Expression of KITL protein in normal and polycystic ovaries

	Primordial		Primary		Large Antral	
	ooctyes	GC	ooctyes	GC	theca	GC
Normal ovaries	- / +	- / +	- / +	- / +	++	+
Polycystic ovaries	+ / ++[a]	+ / ++[a]	+/++[a]	++ / +++[b]	++	++ / +++[b]

GC = granulosa cells; [a]≤0.05; [b]≤0.001 as compared to normal ovaries.

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A xenotransplantation model for investigating the role of human endometrial stem/progenitor cells in endometriosis

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Endometriosis is a major cause of infertility in women. Recent evidence suggests that stem/progenitor cells are present in human endometrium which may be responsible for its remarkable regenerative capacity.[1] In mouse endometrium, candidate epithelial and stromal stem/progenitor cells have been identified as label retaining cells (LRC).[2] We hypothesised that endometrial stem/progenitor cells gain access to the peritoneal cavity in menstrual debris where they establish ectopic endometriosis lesions in women who develop endometriosis[3]. Our aim was to identify LRC in human endometrial tissues transplanted into an ectopic site in immunocompromised mice. Endometrium was dissected into pieces (1x1mm x depth of endometrium) from hysterectomy tissues from ovulating women (n=7) and transplanted beneath the kidney capsule of ovariectomised NOD/SCID mice. One week later, mice were administered a single 100 ng estradiol valerate (E2) injection and 50 ug/g BrdU (6 ip injections over 3 days) to label proliferating endometrial cells with BrdU. E2 injections were given fortnightly to induce weekly cycles of endometrial growth and regression and to chase out the BrdU. Mice were sacrificed after 6-12 weeks chase and the explants examined for LRC and other markers by immunofluorescence. Endometrial explants underwent major remodeling during the BrdU pulse-chase, and cells in both glands and stroma underwent proliferation ($PCNA^+$). Rare LRC were identified in the human endometrial epithelium and stroma. Epithelial LRC were ER-alpha negative while some stromal LRC were ER-$alpha^+$ by confocal microscopy. Immunostaining of the LRC for ER-beta, alpha-smooth muscle actin and human endometrial stromal stem/progenitor cell markers (CD146, PDGFR-beta)[4] will further characterise these candidate stem/progenitor cell populations in ectopic endometrium. This preliminary study suggests that combining the LRC technique with xenotransplantation of human endometrial tissue into a well vascularised ectopic site may provide a novel model for investigating the role of endometrial epithelial and stromal stem/progenitor cells in the pathogenesis of endometriosis.

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In vivo and *in vitro* evidence for cancer stem cells in human endometrial cancer

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Cancer stem cells (CSCs) have been identified in solid human cancers, including breast, colon, and ovary. Recent evidence suggests that the highly regenerative human endometrium harbors rare populations of epithelial stem/progenitor cells[1]. We hypothesised that CSCs are responsible for the epithelial neoplasia associated with endometrial carcinoma (EC), the most common gynaecological malignancy in women. The aim of this study was to demonstrate that a rare population of EC cells posses CSC properties.

Stem cell characteristics were assessed in 25 EC and 2 endometrial hyperplasia tissues obtained from women aged 62 ± 9 yrs. Samples were cultured at clonal densities (100-500 cells/cm^2) for 3-5 wks to determine cloning efficiency. Individual clones were serially subcloned (<10 cells/cm^2) every 2-4 wks to determine self renewal capacity. Isolated cells in serial dilution (10^3–10^6 cells) were placed under the kidney capsule of immunocompromised mice for 12-16 wks to examine for the presence of tumour initiating cells (TIC). Resulting tumours and original parent tumours were examined for markers by immunohistochemistry.

Most samples (23/26) contained rare colony forming cells. The cloning efficiency was 0.23% ± 0.28% (n=11) in G1, 0.78% ± 0.67% (n=8) in G2, 0.22% ± 0.21% (n=3) in G3, 0.03% (n=2) in type II tumours, and 0.14% (n=2) in hyperplasia samples, and did not differ significantly between grades or between type I EC and normal endometrial epithelial samples [1] . Single cell derived clones subcloned 2.5 ± 1.4 (n=11), 3.2 ± 0.4 (n=5), 3.5 (n=2), 3.0 ± 1.7 (n=3), and 2.5 (n=2) times in G1, G2, G3, type II tumours and hyperplasia samples respectively, indicating increasing self renewal capacity with increasing tumour grade. Transplanted EC single cell suspensions initiated tumour growth with similar morphology, ERα, PR, EpCAM, cytokeratin, and vimentin expression as the parent tumour, indicating the presence of TIC.

This evidence suggests that rare cells possessing the CSC properties of clonogenicty, self renewal, and tumorigenicity, may be responsible for the initiation and progression of EC.

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Proteomic analysis of proliferative and secretory phase endometrium

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The human endometrium undergoes marked proliferation and differentiation between the proliferative and secretory phases of the menstrual cycle; however the underlying biology is poorly understood. This aims of this study were to identify proteins differentially expressed in the human endometrium between the proliferative and secretory phases of normal menstrual cycles.

2D SDS-PAGE analysis with differential fluorescent Cydye labelling was conducted across the pH range 4–7 on endometrial tissue extracts from mid-proliferative and mid-secretory phases (n=4/group). Profiles were quantitatively assessed using SameSpots image analysis software. Differentially expressed proteins were identified using MALDI-TOF MS and were used to generate biological network by Ingenuity Pathways Analysis. Protein expression changes for three of the proteins were validated by immunohistochemistry.

Of 1017 protein spots detected in the proteome of endometrial tissue, 196 were significantly differentially expressed ($p < 0.05$) between the proliferative and secretory phases. 157 proteins increased in expression, whilst 39 were decreased compared to the proliferative endometrium. Mass spectrometry identified 76 proteins representing 42 unique gene products. The identity and expression change for 3 proteins (Rho-GD1α, CLIC1, PGRMC-1) was confirmed using immunohistochemical staining of tissue section. Using pathway profiling software, the identified proteins were broadly grouped into seven major functional categories (cell architecture, transcription regulation, transport, membranes, enzymes and regulatory proteins), with the majority showing clear dependence on the Jnk signalling pathway. Comparison of the proteomic data with published mRNA expression array data clearly demonstrated discrepancies between the protein changes and gene expression, suggesting that a large majority of proteomic changes occurring at the transition of proliferative to secretory phase were due to post-translational modifications.

These studies enable the understanding of the complex dynamics of protein expression and the possible involvement of post translational modifications in cyclic changes of human endometrium.

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Rational design of bifunctional siRNAs that silence genes and recruit the innate immune system to treat ectopic pregnancies

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RNA interference (RNAi) is a new therapeutic approach, silencing genes to disrupt diseases. However, short interfering siRNAs (molecule used in RNAi) can have off-target effects, activating the immune system through RNA sensing toll-like receptors (TLR) 3, 7 and 8. We have previously proposed that in some diseases (cancers, ectopic pregnancies) it may be useful to enhance the immune response. A novel class of immunostimulatory siRNAs could be developed, silencing genes important to disease *and* recruiting the immune system to further aid disease clearance.

We set out to develop a rational design strategy that enhances immunostimulatory properties to any siRNA sequence but maintains effective gene silencing. We screened a set of siRNAs targeting lamin. All were of the same sequence, except for different immunostimulatory motifs on the 3' end of the sense strand. We also investigated a different design where we added a small micro-RNA like poly-uridine bulge (potentially immunostimulatory) on the sense strand. We used human peripheral blood mononuclear cells (PBMCs) to test for immunostimulation, and HEK 293-T cells to test for lamin gene knockdown.

Of all strategies tested, the poly-uridine bulge was best. It silenced the lamin gene as effectively as control, but caused a 2-3 fold increase of IFN-α and TNF-α. We verified this approach by adding the poly-uridine bulge onto an siRNA of low immunostimulatory potential targeting GFP. The bulge markedly enhanced immunostimulation in a dose response manner, and did not compromise gene knockdown.

The addition of a poly-uridine bulge to siRNAs can increase immunostimulation without affecting gene silencing efficacy. Immunostimulatory siRNAs might be particularly efficacious to treat ectopic pregnancies where there are abundant immune cells, and functional TLR 7/8 in the trophoblast (unpublished observations). We now plan to test this immunostimulatory siRNA approach in an *in vivo* ectopic pregnancy model using JEG-3 cells xenographted in NOD-SCID mice.

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The effect of maternal dietary protein on the expression of genes involved in TGF-β superfamily bioavailability in the bovine ovarian cortex of female offspring

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Although numerous familial studies have indicated a genetic component to polycystic ovary syndrome (PCOS), a consistent and repeatable association with any particular gene has not been definitively established. However, one genetic marker, the microsatellite D19S884, has shown repeatable and consistent association with PCOS. D19S884 maps to intron 55 of the fibrillin-3 gene. The family of fibrillin extracellular matrix proteins includes the family of latent TGF-β binding proteins which are known to have roles in ovarian function. These provide structural support to tissues and regulate TGF-β bioavailability and are candidates for involvement in clinical manifestation of PCOS. Many of the risk factors associated with PCOS including type II diabetes, heart disease, and obesity are also known to be affected by in utero experience. In addition, although diet has not been examined in the PCOS outcome of offspring, some fetal experiences including androgen exposure have been linked to PCOS outcome.

We therefore investigated the effect of protein content of maternal diet on expression of fibrillins-1, -2, and -3 and LTBPs-1 and -2 in the tunica albuginea of ovaries from female offspring by QRT-PCR. Diets were either high (13% protein per total dry weight) or a low protein (5%) during the first two trimesters. All three fibrillins were expressed in the tunica albuginea with the highest level of expression observed for fibrillin-1 followed by fibrillin-2, with low levels of expression of fibrillin-3. Both LTBP-1 and LTBP-2 were expressed, with the highest expression observed for LTBP-2. No significant ($P > 0.05$) effects of diet upon gene expression were observed. Expression levels of all three fibrillins were positively correlated with each other, and fibrillins-1 and 2 were positively correlated with LTBP-2. This is the first demonstration of the expression of fibrillins and LTBPs in the ovary, however these were unaffected by the maternal diets examined.

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Seminal fluid TGFβ regulates follistatin mRNA expression human Ect1 cervical epithelial cells

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Introduction of seminal fluid into the female reproductive tract following coitus stimulates a local inflammatory response. Inflammatory leukocyte recruitment is regulated by induction of cytokine and chemokine synthesis in female tract epithelial cells by seminal fluid signalling agents. Affymetrix microarray analysis in immortalised ectocervical epithelial (Ect1) cells identified the potent anti-inflammatory cytokine follistatin (*FST*) as the most strongly differentially expressed gene, with a ~12-fold increase in mRNA expression induced by seminal fluid. Follistatin has recently been implicated as a key cytokine in early pregnancy by studies in female follistatin-null mice, which exhibit infertility as a consequence of failure to resolve the uterine post-mating inflammatory response. The aim of this study was to investigate seminal plasma regulation of follistatin in human Ect1 cervical cells, and to examine the role of the major active seminal fluid constituent, TGFβ, in controlling Ect1 cells follistatin mRNA expression. To confirm Affymetrix findings, qRT-PCR experiments were undertaken in Ect1 cells incubated with 10% pooled human seminal plasma (SP). Primers specific for the tissue bound isoform of follistatin (*FST*288) as well as both *FST*288 and the circulating 315 isoforms (*FST*all) were used. Ect1 cell incubation with 10%SP elicited 3.8-fold and 4-fold increases in *FST*288 and *FST*all respectively. Incubation of Ect1 cells with TGFβ1, TGFβ2 and TGFβ3 showed differential effects of the three isoforms, with rTGFβ2 inducing FST288 and FSTall, while rTGFβ1 and TGFβ3 exerted little effect. . These results suggest that seminal plasma induces follistatin synthesis after coitus and that TGFβ2 is at least partly responsible for this effect. Follistatin induced by seminal fluid may act to limit the course of inflammation after intercourse, and thereby prevent uncontrolled inflammatory damage. Follistatin induced in the female tissues would be augmented by follistatin delivered from the male, since human seminal plasma also contains a high concentration of this cytokine.

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Data mining: relationship of sperm kinetics and DNA integrity

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Intracytoplasmic sperm injection (ICSI) is a popular technique in treating infertile male that bypasses sperm natural selection. Due to this, the occasional and unintentional use of spermatozoa in ICSI with high amount of DNA fragmentation seems to be unpreventable. The objective of this study was to develop single sperm selection technique and in the process to determine the relationship between sperm kinetic parameters and sperm DNA damage. Semen from sexually matured male Boer buck cross species were collected and cryo-preserved. After taking into consideration semen sperm count, sperm were isolated individually in an ELISA plate by diluting semen in extender. Then every sperm's kinetics was assessed by computer-assisted sperm analyzer (CASA) while neutral comet assay was used to quantitate and categorize its DNA damage condition. DNA damage was categorized from minimal damage (category 0) to extensive damage (category 4). Relationship between CASA parameters and DNA damage category of 490 sperms was determined using a Classification and Regression modelling (C&R). A total of 208 sperm data was used to generate a suitable C&R model. A further 250 sperm data was then used to determine accuracy of the model. Results obtained indicated that VSL, WOB and VCL were important factors in determining the overall condition of a particular sperm. A low value of VSL would indicate minimal DNA damage. Identification of higher category of DNA damage would require combination assessment of VSL, WOB and VCL. Accuracy of the developed C&R model was at 83.6%. Based on the above procedure it has been shown that sperm kinetics and DNA integrity can be considered together in selecting potential sperm for ICSI procedure.

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Effects of *Eurycoma longifolia* Jack on spermatogenesis and sperm counts of male rats treated with oestradiol

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Eurycoma longifolia has been known for its aphrodisiac effects in male. Our previous study showed that with the dosage of 8 mg/kg body weight (BW) *Eurycoma longifolia* root extract increased plasma total testosterone levels in male rats when given for 14 days. Oestrogen is a potent inhibitor for testosterone production and spermatogenesis. The aim of the present study is to determine the effects of *Eurycoma longifolia* (8 mg/kg BW) on the spermatogenic cell count and sperm count of testosterone-suppressed male rats. Adult male Sprague-Dawley healthy male rats weighed 200-250 g were treated with, either control vehicle (no active ingredients given), oestradiol (500mg/kg BW), *Eurycoma longifolia* (8 mg/kg BW) or combination of *Eurycoma longifolia* and oestradiol for fourteen consecutive days. Results showed that sperm count and spermatogenic cell count were increased in *Eurycoma longifolia* treated group compared to control group ($P<0.05$) and to oestradiol treated group ($P<0.05$). While in oestradiol treated group, sperm count and spermatogenic cell count were reduced significantly ($P<0.05$) compared to control group. Combination of *Eurycoma longifolia* and oestradiol did not affect sperm count and spermatogenic cell count when compared to control group but they were significantly increased compared to oestradiol treated group ($P<0.05$). Thus, the study has shown that *Eurycoma longifolia* is potentially capable to suppress harmful effects of oestradiol on spermatogenesis and sperm counts in healthy male rats when given for fourteen consecutive days.

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Polyunsaturated fatty acids and mitochondrial reactive oxygen species production in human spermatozoa and male infertility

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Reactive oxygen species (ROS) are traditionally considered detrimental by-products of cellular metabolism. However, ROS have conflicting roles in human spermatozoa, either as a functional mediator of sperm capacitation or generating a state of oxidative stress that is associated with male infertility. Using the probe MitoSOX Red, we have shown that defective human spermatozoa generate mitochondrial ROS in manner that was negatively correlated with motility ($R^2 = 0.8048$). Previous research has shown higher levels of polyunsaturated fatty acids (PUFAs) in defective spermatozoa. However, the addition of PUFA to normal human spermatozoa results in increased mitochondrial ROS production ($P < 0.001$) and lipid peroxidation ($P<0.001$) determined by MitoSOX Red and BODIPY C_{11} assays, as a consequence human spermatozoa also exhibited decreased sperm motility ($P<0.001$). Ongoing research is currently evaluating the relationship between cellular levels of PUFAs in human spermatozoa and mitochondrial ROS generation and decreased sperm motility. This research demonstrates that mitochondrial ROS generation in human spermatozoa may have significant consequences for their function and we propose that elevated PUFA content may be a primary cause of increased oxidative stress and therefore male infertility.

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Expression of mammalian cysteine-rich secretory proteins in the mouse model

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Mammalian cysteine rich secretory proteins are a family of four proteins exhibiting a high amino acid sequence similarity and belonging to the CAP (Cysteine rich secretory proteins, Antigen-5 proteins and the plant Pathogenesis related-1 proteins) superfamily of proteins. They are designated CRISP 1, 2, 3 and 4. Structurally, mammalian CRISP's are characterised by 16 cysteine residues involved in intra-molecular di-sulphide bonds and the formation of 2 domains, ie., the CRISP domain (CD) and CAP domain. Whilst studies on mouse CRISP2 suggest that the CD is involved in ion channel regulation, studies on non-mammalian CAP superfamily members suggest that the CAP domain is involved in proteolytic activity.

They are predominantly expressed and localised in the male reproductive tract, however, the EST expression databases suggest that mammalian CRISPs are expressed more widely than in the male reproductive tract. The objective of this study was therefore to conclusively define the expression and localisation of each CRISP protein in a mammalian system.

A reverse transcription PCR expression profile and immunohistochemical analysis of 16 mouse tissue was conducted to establish the expression and localisation of each of the four CRISPs. These data showed that although the CRISPs have a strong expression and localisation bias to the male reproductive tract, they are widely distributed throughout the body in mice, including the ovary, uterus, and mammary gland. Whilst each CRISP has a clear expression profile, there was a striking localisation of androgen regulated CRISPs (1, 3, 4) in immune tissue including the spleen and thymus. Such a localization raises the spectre of a role for CRISPs in the normal physiology and disease of several organs.

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The role of sperm mitochondria in the process of epididymal maturation

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On leaving the testis, spermatozoa can neither swim nor achieve fertilization of the oocyte. These functional properties are acquired as spermatozoa engage a process of post-testicular maturation in the epididymis. Research into the biochemical basis of sperm maturation has revealed that this process is associated with activation of sperm mitochondria. Immature spermatozoa from the caput epididymis displayed a low mitochondrial membrane potential (MMP) whereas mature spermatozoa from the caudal epididymis actively maintained a high MMP. Moreover mitochondrial generation of reactive oxygen species could be triggered by antimycin in mature caudal epididymal spermatozoa but not in immature cells recovered from the caput epididymis. The molecular mechanisms responsible for regulating mitochondrial function were reversible since washing the cells free of epididymal fluid allowed immature spermatozoa to acquire a high MMP while incubating mature caudal cells in caput epididymal fluid, suppressed MMP. These results strongly suggested that fluid from the caput epididymis contains a mitochondrial inhibitor and that activation of mitochondrial activity is due to the removal or inactivation of this inhibitor during epididymal transit. The causative factor is not species specific because incubation of ejaculated human spermatozoa in murine epididymal fluid effectively suppressed their MMP. Caput epididymal fluid was fractionated using FPLC and each of the fractions was tested for their bioactivity. A major protein band with a molecular mass approximately 150 kDa was present in the active fraction. Further characterisation of this reversible mitochondrial inhibitor may reveal the mechanisms by which epididymal spermatozoa control mitochondrial function during maturation. It may also contribute to our understanding of human male infertility and potentially serve as a novel target for male fertility regulation.

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MDM2 maintains the latency of expression of TRP53 in the mouse preimplantation embryo

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TRP53 is a tumour suppressor that causes cell-cycle arrest or cell death in response to a range of stressors. Normal preimplantation embryo development requires that TRP53 is maintained in a labile state[1]. Culture of mouse C57BL6 preimplantation embryos causes this latency to be breached and this is a cause of the low embryo viability following culture. MDM2 is an ubiquitin ligase that targets TRP53 for degradation by the proteosome. MDM2 is activated by Serine 166 phosphorylation (pMDM2). This is commonly catalysed by the phosphatidylinositol-3 kinase (PI3K) and AKT signalling pathway. A range of embryotrophins activate the PI3K and AKT pathway. This study tested the hypothesis that TRP53 is maintained in a latent state in the normal embryo by the activation of MDM2 by the actions of embryotrophins via a PI3K and AKT signalling pathway.

Genetic deletion of *Mdm2* prevents normal preimplantation development in a *Trp53* dependent manner[2]. Addition of an MDM2 inhibitor (Nutlin-3) to culture medium caused a dose-dependent inhibition of zygote development ($p < 0.001$) that did not occur in *Trp53*$^{-/-}$embryos. Immunofluorescence and Western blot analysis detected pMDM2 throughout mouse preimplantation development. Zygote culture reduced the levels of pMDM2 formation. Furthermore, blocking the actions of Paf, PI3K or AKT in vitro reduced in the expression of pMDM2, and also resulted in higher levels of TRP53 expression in embryos. The embryopathy resulting from increased TRP53 could be partially ameliorated by the addition of the TRP53 antagonist α-pifithrin to media ($p < 0.05$).

The results show MDM2 was activated by an embryotrophin (Paf), PI3K and AKT signalling pathway and was required for the latency of TRP53 expression in the preimplantation embryos.

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Normal follicle growth and maturation in a three-dimensional *in vitro* culture system: follicular environment manipulation and assessment of oocyte outcomes

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In vivo, the oocyte matures in a niche environment surrounded by somatic cells, and later in ovarian follicular development, by follicular fluid. Maternal diet influences the environment in which an oocyte matures but the mechanisms by which an altered metabolic profile, such as hyperinsulinemia, affects oocyte quality are not known. We investigated the use of a three dimensional follicle culture system allowing direct manipulation of the follicular environment thus circumventing systemic hormonal and metabolic effects. Secondary follicles (113.4±1.02µm, n=54) were isolated from mice at d12, encapsulated individually in 2µl of alginate matrix, and cultured in aMEM/5%FCS/10mIU/ml LH/100mIU FSH at 37^{o}C/5%CO_2, with media sampling and replacement every second day. Following 12 days of culture there was a significant 3-fold increase in follicle diameter (320±10.1µm, n=51). Histological analysis showed normal follicular morphology and antrum formation. Analysis of estradiol (15.0ng/ml), androstenedione (7.8ng/ml) and progesterone (23.7ng/ml) in the media at d12 confirmed normal steroidogenesis and differentiation. Treatment of follicles with an ovulatory stimulus (1.5IU/ml hCG/5ng/ml Egf), resulted in cumulus expansion and hyaluronan localising to the cumulus oocyte complex (COC) and follicular basement membrane. These analyses were consistent with follicle growth and induction of ovulation *in vivo*. Further, COCs isolated from follicles and matured *in vitro* (IVM) in the presence of Egf and FSH, underwent cumulus expansion (CEI 2.8±0.2) and were capable of fertilisation and blastocyst development. LH did not induce IVM COC expansion (CEI 1.36±0.2), reflecting the normal *in vivo* differentiation process. However, culturing follicles in high insulin (5ug/ml) led to a significant increase in the degree of IVM cumulus expansion in response to LH (CEI 2.1±0.3) indicating inappropriate cumulus cell differentiation, which may lead to poorer oocyte quality. These results demonstrate that this technique recapitulates normal *in vivo* folliculogenesis and is useful for manipulation of the follicular environment and assessment of oocyte outcomes.

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Culture of mouse zygotes altered epigenetic states of the *agouti viable yellow* allele in the resulting progeny

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Epigenetic modification of the genome is one potential mechanism for the persistence of altered gene expression. Recent evidence indicates that the growth environment of the preimplantation embryo may have long-term consequences in the gene expression of progeny (Mahsoudi *et al.*, 2007; Watkins *et al.*, 2008). This study assessed whether culture of zygotes induced persistent epigenetic changes in gene expression. The *agouti viable yellow* (*Avy*) allele is epigenetically sensitive, and is a widely used model for studying epigenetic phenomena. It has active (hypomethyalted) and inactive (hypermethylated) epialleles. Progeny with an active epiallele have yellow fur; those with an inactive epiallele have an agouti coat. Some progeny are mosaics for the two epigenetic states and have a mottled coat color.

Males (*Avy*/*a* C57BL/6) were mated to wildtype females (*A*/*A* FVB). Embryo treatments were: (1) zygotes collected and cultured for 96 hours in Sydney IVF Media suite and transferred to day 3 pseudopregnant Swiss females; (2) blastocysts collected and transferred to recipients without being subjected to culture; (3) naturally mated females left to give birth without interventioin. The number, sex, genotype, and epi-phenotype of the resulting progeny were recorded.

The culture of zygotes caused a significant shift in the expression of the epialleles compared to embryos that were transferred without culture (P=0.014), or those born following natural matings (P<0.001). Offspring that resulted from culture are more likely to have active epialleles, resulting in a higher proportion of progeny with yellow fur. The results of this study show that culture of mouse zygotes changed the expected expression of the *Avy* allele. This study provides evidence for the epigenetic basis for the long-term ‘memory' of preimplantation embryo's growth environment, and for robust and readily accessible methodology for addressing the underlying molecular bases for this phenomenon.

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Investigation of pluripotency in derived embryonic stem cell (ESC) lines

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To produce autologous ESCs for a bovine model of cell therapy, we activated oocytes by calcium ionophore (CI) and 6 dimethylaminopurine (6 DMAP) and isolated ESCs from the resulting parthenotes. Parthenote ESC lines (pbESC) would also provide a valuable tool for epigenetic studies on ESCs. Five pbESC like-cell lines were expanded for 12 passages over 120 days and differentiated to form embryoid bodies by suspension culture. The pbESC lines demonstrated typical ESC morphology and expressed ESC markers including alkaline phosphate and stage-specific embryonic antigen, SSEA1 and SSAE4 asssessed by histochemical and immuno-fluorescence staining, respectively. In addition, gene expression of Oct4, Rex1, SSEA1 and ALP was confirmed using RT-PCR. These cells had a normal karyotype. The cells formed EBs and showed expression of the markers of three embryonic germ layers.

In summary, we show that ESCs can be derived from bovine parthenogenetic blastocysts and that these cells express pluripotent markers and have ability to form EBs and differentiate into cells indicative of the three embryonic germ layers. Additional work will focus on imprinted gene expression and will provide further evidence of the parthenogenetic origin of the pbESC lines.

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Importin proteins and their role in maintenance of the stem cell state

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The importin (IMP) family of proteins mediates transport into the nucleus for many proteins larger than 40 kD. Through differential cargo recognition, IMPs regulate cellular events by controlling nuclear access of transcription factors and chromatin remodeling agents. During spermatogenesis, many IMPs change expression and localization in a manner concordant with specific stages of spermatogenic development. To assess the potential role of IMPs in the transition between the stem cell and subsequent differentiation, we undertook analysis of the expression and subcellular localization of several key murine IMPs in both pluripotent embryonic stem cells (mESCs) and embryoid bodies (EBs). All of the IMPs analysed (IMPα2, 3, 4, IMPβ1 and IMP5) were detected in undifferentiated mESCs by immunofluorescence, and each exhibited distinctive nucleocytoplasmic distribution patterns. Subcellular localisation of most IMPs altered after 10 days of mESC differentiation as EBs. This was paralleled by changes in the mRNA levels of IMPα1-4, IMPα6, IMPβ1 and IMP5, concomitant with alterations in the expression level of the pluripotency marker, Oct3/4. Reducing IMP-dependent nuclear import through overexpression of specific dominant-negative IMP constructs led to alterations in import or production of Oct3/4 protein, depending on the specific IMP. These findings indicate that IMPs may play very specific but distinct roles in cell fate choice between maintenance of pluri/multipotency and commitment to differentiation in ESCs and potentially in spermatogenesis or other organs that contain stem cells.

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How do antiphospholipid antibodies contribute to preeclampsia?

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Preeclampsia is characterised by elevated maternal blood pressure which is preceded by endothelial activation. The cause of this endothelial cell dysfunction is unclear but it appears to be triggered by a placental factor. One of the risk factors for developing preeclampsia is the presence of antiphospholipid antibodies (aPL) in the maternal blood but exactly how aPL predispose women to developing preeclampsia is unclear. A second feature known to be associated with preeclampsia is excessive shedding and deportation of dead trophoblasts. We have previously shown that shed trophoblasts are phagocytosed by endothelial cells and that phagocytosis of necrotic trophoblasts leads to endothelial cell activation[1] . In this study we examined the hypothesis that aPL alter the number or nature of trophoblasts shed from the placenta resulting in endothelial cell activation. Using our published model of trophoblast shedding [2] human first trimester placental explants were treated with monoclonal aPL, IIC5 or ID2, or control antibody CD45 for 72 hours. Shed trophoblasts then were harvested and counted using a Cellometer AutoT4 automated cell counter. The activity of caspases 3&7 was analysed in all treated shed trophoblasts using a FLICA™ kit. The treated shed trophoblasts also were exposed to the endothelial cell line HMEC-1 for 24 hours. The level of ICAM-1 by HMEC-1 was determined by cell-based ELISA. The number of trophoblasts shed from placental explants was increased 2 fold following aPL treatment whereas, treatment with CD45 resulted in only a 1.3 fold increase in shedding. Trophoblasts shed from aPL-treated explants contained less active caspases 3 & 7 compared to control shed trophoblasts. Moreover, phagocytosis of trophoblasts shed from aPL-treated explants induced significantly increased expression of ICAM-1 compared to controls. aPL treatment affected the number and nature of trophoblasts shed from placentae in such a way that phagocytosing endothelium become activated. These findings suggest that aPL treatment may have shifted the type of cell death that shed trophoblasts are undergoing from apoptosis to a more necrotic or aponecrotic mechanism. This type of shedding of trophoblasts *in vivo* might contribute to the endothelial cell activation which is a hallmark feature of preeclampsia.

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Identifying novel biomarkers predictive of impending human labour

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Human labour is characterised by structural remodelling of the cervix and overlying fetal membranes, myometrial activation and parturition. We hypothesise that temporal biochemical alterations of the cervix and supracervical fetal membranes associated with impending labour may be reflected in the cervico-vaginal fluid (CVF). 2D PAGE proteomic analysis performed on serial CVF samples collected from women (n=9) during late pregnancy and in spontaneous labour demonstrated 9 significantly altered protein spots (p<0.05) in association with term labour. Seven different proteins were identified using electrospray ion-trap mass spectrometry (interleukin-1 receptor antagonist (IL-1ra), cystatin-A, glutathione *S*-transferase P, peroxiredoxin-2, thioredoxin, Cu,Zn superoxide dismutase, and epidermal fatty-acid binding protein). These proteins are involved in anti-inflammatory activity, protease inhibition, and oxidative stress defence. Validation of these potential biomarkers using ELISA is currently underway. Findings for the anti-inflammatory cytokine, IL-1ra are discussed. CVF was collected weekly from 106 women at 36 weeks' gestation up to and including spontaneous labour. The concentration of IL-1ra was 4-fold lower during labour compared to 15-21 and 22-28 days from labour (p<0.05) and was also significantly lower (p<0.05) at 0-7 days compared to 15-21 days before labour. After subdividing the women, the concentration of IL-1ra at 8-14 and 15-21 days before labour was 6-fold lower in women who had prelabour rupture of membranes at term followed by regular contractions compared to women who had spontaneous labour with intact membranes. Receiver-operator characteristic curve analysis indicated that IL-1ra best predicted term labor within 3 days of sampling with a cut-off value of 0.4 m g/ml (sensitivity 50.7%, specificity 72.2%, positive predictive value 36.1%, negative predictive value 82.6%). This IL-1ra validation study suggests that the decrease in IL-1ra in CVF during spontaneous term labour may be associated with proinflammatory-mediated remodelling of the fetal membranes leading to their rupture.

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Identification of differentially expressed proteins in ovine chorion rupture sites at preterm and term using proteomics

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Introduction: A significant number of babies are delivered preterm and many of these deliveries are due to spontaneous rupture of foetal membranes. However, the mechanisms underlying foetal membrane rupture are not well defined. The sheep has proved to be a valuable model for elucidating the physiology of birth, with many of the findings clearly relevant to human parturition. In the current study we have used the sheep model to investigate changes in protein expression in the chorion in relation to the onset of spontaneous full term labour.

Methods: Proteomic analysis was used to compare the protein profiles at the chorion rupture site between mid/late gestation (136 days, n=6) and term delivery (145days, n=6). Proteins were solubilised and separated into soluble and insoluble fractions which were separated by 2D-electrophoresis. Relative protein expression was quantified using the PROGENESIS software and protein spots were picked and identified using Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) mass spectrometry. Data-mining used gene ontology (GO terms) for each protein and these were clustered into networks using DAVID bioinformatics resource (http://david.abcc.ncifcrf.gov/home.jsp).

Results: A total of 150 protein spots were identified of which 60 proteins were found to differ by more than two-fold between preterm and term samples. This group was significantly enriched for proteins from the several functional GO categories including; Oxidoreductase activity (7 proteins); negative regulation of apoptosis (4); structural molecule activity (7); protease inhibitor activity (7); Carbohydrate metabolism (10); Glucose metabolism (5); Glycolysis (5); response to stress (8) and heat shock (3).

Of the differentially regulated proteins, ten were found to be significantly up-regulated at term (ANOVA p<0.05) including isocitrate dehydrogenase, glutamate dehydrogenase 1, malate dehydrogenase 2. A further ten proteins, including serpin peptidase inhibitor SERPINA12, serpin peptidase inhibitor SERPINH1 precursor and heat shock protein 90 kDa beta were found to be down regulated.

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Follicle differentiation and luteinisation in the mouse is associated with hypoxia inducible factor activity

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Hypoxia inducible factors (HIF) are transcription factors that mediate the response to hypoxic stress. Under hypoxic conditions, HIF is stabilized, translocates to the nucleus, and binds to the Hypoxia Response Elements (HRE) upstream of numerous target genes involved in angiogenesis and glycolysis, including Vegf, Glut-1 and Ldha. Little is known about the role of HIFs in regulating ovarian function. In rat granulosa cells, FSH stimulates HIF 1α via the PI3K/Akt pathway, demonstrating a role for HIFs during follicular development. In contrast, there is limited information regarding the role of HIFs during corpus luteum formation.

In this study we investigated whether HIFs play a role in follicle differentiation and luteinisation. Prepubertal C57Bl6 females were stimulated with eCG (5 iu) followed 46 h later by hCG (5 iu). Mice were sacrificed at 0, 4, 8, 12, 16 and 24 h post hCG and granulosa cells were collected for Western analysis of HIF-1a protein. To investigate HIF activation in the ovary, a transgenic reporter mouse line was developed by lenti-viral incorporation of an HRE (4)-SV40-eGFP construct. Ovaries were collected from mice plugged day 1, 4 and 8 for CL analysis in vivo.

A time- dependent increase of HIF 1α protein levels in granulosa cells, maximal around time of ovulation, was observed. Ovaries from cycling HRE-eGFP transgenic mice exhibited no eGFP in primordial, primary or preantral follicles. Upon antrum formation, eGFP was evident in occasional sections in antral follicles but HIF signaling was restricted within the theca. In contrast, corpora lutea on pregnancy day 1, 4 and 8 readily expressed eGFP and eGFP expression increases as luteinisation progresses.

These results demonstrate that in vivo HIFs may play a role in folliculogenesis, but this is restricted to theca cells of antral follicles prior to hCG stimulation. Following hCG, maximal HIF activity is associated with the time of ovulation. In addition, HIF activity is maintained during luteinisation.

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Investigation of the role of SRC in capacitation associated tyrosine phosphorylation of human spermatozoa

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Capacitation is a pre-requisite for mammalian spermatozoa allowing them to gain the ability to fertilize an oocyte. A fundamental part of this mechanism is a dramatic increase in tyrosine phosphorylation. Implicated in this process in the mouse is a unique cAMP/PKA-mediated pathway involving a PKA-activated tyrosine kinase suggested to be $pp60^{c\text{-}src}$ (SRC). The Src kinases examined were predominantly expressed in the human sperm tail, a site compatible with a role in mediating the capacitation-associated tyrosine phosphorylation cascade. Co-immunoprecipitation revealed that PKA-c could be isolated from sperm and this interaction was restricted to capacitated cells, suggesting PKA-mediated activation of SRC forms an integral part of the signaling cascade assembled during capacitation. Upon activation, SRC undergoes autophosphorylation of Y416 and thus phosphorylation of this residue indicates the presence of active SRC kinase. The phosphorylation status of SRC was compared using both 2D-immunoblotting and immunocytochemical studies, both revealing a significant increase in SRC activation during capacitation. Furthermore, suppression of PKA and SRC through application of SU6656, or H89, a PKA inhibitor, led to a dramatic decrease in tyrosine phosphorylation and SRC activity. In conclusion, this study has provided evidence for the involvement of non-receptor tyrosine kinase, SRC, in regulating tyrosine phosphorylation associated with capacitation. Inhibition of SRC did not completely suppress tyrosine phosphorylation suggesting this complex signal transduction pathway exhibits a degree of functional redundancy.

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Whole body heat stress induces selective germ cell apoptosis in mice

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Introduction: In scrotal mammals, heat stress (43°C/ 20 min) to the scrotum results in germ cell death in the testes[1], abnormal spermatozoa, and infertility[2] whereas two days of whole body heating (36°C, 12 hrs/ day) reduces testes weight, sperm numbers and fertility[3]. The aim of the present study was to determine the intratesticular effects of whole body heating on germ cell maturation and apoptosis. Methods: C57BL/6 mice (n=16) were housed at 37-38°C for 8 hrs/ day for 3 days while controls (n=4) were kept at 23-24°C. Animals from heat treated (n=4), and control groups (n=1) were sacrificed at 16 hrs, 7, 14 and 21 days post exposure to heat. Testes were weighed and analysed by t-test. In testes from each animal, two sections 70µm apart were end labelled for TdT-mediated-dUTP nick (TUNEL). Apoptosis was determined in 200 seminiferous tubules by a colour threshold set in the particle analysis program (Olympus).The tubules were staged as I-VI (early), VII-VIII, IX-X and XI-XII (late) and results analysed using Wilcoxon test. Results: The weights of testes were significantly reduced in heat-treated animals ($p< 0.05$) at 16 hours, 7 and 14 days with no significant difference at 21 days. Apoptosis was significantly higher in the heat-treated group in stages I-VI and XI-XII at 16 hrs, 7 and 14 days ($p< 0.05$). In addition, in stages VII-VIII and IX-X apoptosis was significantly higher at 16 hrs ($p< 0.05$) with no statistical difference between other time intervals. By day 21, the levels of apoptosis did not differ significantly from the controls in any of the stages ($p>0.05$).Conclusion: Whole body heat stress can induce stage and cell specific degeneration of the germ cells in the seminiferous epithelium. The germ cells undergoing apoptosis are spermatogonia, primary spermatocytes and early spermatids. In addition, heat stress produces significant apoptosis of germ cells in the hormone dependent stages VII-VIII immediately after heat stress.

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PLZF is a spermatogonia stem cell-specific marker in the sheep testis: application to enrichment of ovine spermatogonial stem cell

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Identification and isolation of spermatogonial stem cells (SSCs) are prerequisite for long term culture, genetic manipulation, and transplantation research. The promyelocytic leukemia zinc-finger (PLZF) has been identified as a spermatogonia stem cell marker in rodent and other species, however its expression in sheep testis has not been reported yet. In this study, we validated an antibody that specifically binds to spermatogonia stem cell in sheep testis, thus demonstrated that PLZF is a spermatogonia stem cell marker and can be used for its identification . Testes from 12 Merino rams were selected to represent four stages of testis development at testis weights of 3-5 g (neonatal), 30 g (peripubertal), 50 g (prepubertal) and 100 g (mature). Three testes sections from 4 different developmental stage were stained with PLZF antibody and 25 individual tubules in each section were counted. In the sections, the percentage of PLZF positive cells/per tubule was increased nearly two fold from neonatal (6. 4 ± 0. 4%) to peripubertal (1 2.2 ± 2.8%), and then the percentage begin to decline in prepubertal (4.6 ± 0. 7%) and mature testes (3.1 ± 0.6%).

A single cell suspension of testicular cells was generated by a two step enzymic digestion (n=4) and spermatogonia stem cells were enriched by overnight differential plating with 0.2 % gelatine coated flask . The percentages of spermatogonia stem cells in the single cell suspensions were assessed by PLZF antibody staining of smears. Compared to the initial isolation (3.1 ± 0.6%), spermatogonia were enriched 11 fold in overnight differential plating (34.0 ± 5.7,%) ($P < 0.05$). These data provide the basis for future studies aimed at refining conditions of spermatogonial stem cell culture and manipulation prior to male germ stem cell transplantation in sheep.

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GDF9 and BMP15 are germ-free regulators of Sertoli cell function

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Oocyte-secreted Growth Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15) are critical regulatory factors in female reproduction. Together they promote granulosa cell proliferation and stimulate the maturation of preovulatory follicles. Despite their importance in female fertility, GDF9 and BMP15 expression patterns and function during spermatogenesis have not been investigated.

In this study, we show that the expression and stage-specific localisation of both factors are limited to the germ cells of the rat seminiferous epithelium; with GDF9 being principally localised in round spermatids and BMP15 in gonocytes and pachytene spermatocytes. To identify potential cellular targets for GDF9 actions, cells of the seminiferous tubule were isolated and screened for the expression of the GDF9 and BMP15 signalling receptors (ALK5, ALK6, and BMPRII). Individual receptor types were expressed throughout the seminiferous epithelium, but co-expression of type I and type II receptors was limited to Sertoli cells and round spermatids.

Based on the reproductive actions of related TGFβ ligands in the ovary and testis, GDF9 was assessed for its ability to regulate tight junction formation and inhibin B production in rat Sertoli cell cultures. When recombinant mouse GDF9 was added to immature Sertoli cell cultures, it inhibited membrane localisation of the junctional proteins claudin-11, occludin and ZO-1, thereby disrupting tight junction integrity. Concomitantly, GDF9 up-regulated inhibin subunit expression and significantly stimulated dimeric inhibin B protein production. Together these results demonstrate that GDF9 and BMP15 are germ cell specific factors in the rat testis, and that GDF9 can modulate key Sertoli cell functions.

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A-kinase anchoring protein 4 in the marsupial

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A-Kinase Anchor Protein 4 (AKAP4) is an X-linked member of the AKAP family of scaffold proteins that anchor cAMP-dependent protein kinases and play an essential role in fibrous sheath assembly during spermatogenesis and flagellar function in spermatozoa. Marsupial spermatozoa differ in structural organization from those of eutherian mammals but data on the molecular control of their structure and function are limited. We therefore cloned and characterized the *AKAP4* gene in a marsupial, the tammar wallaby (*Macropus eugenii*). The gene structure, sequence and predicted protein of *AKAP4* were highly conserved with that of eutherian orthologues and it mapped to the marsupial X-chromosome. There was no *AKAP4* expression detected in the developing young and in the adult, expression was limited to the testis with a major transcript of 2.9kb identified by Northern blotting. *AKAP4* mRNA was detected by in situ hybridization in the cytoplasm of round and elongated spermatids in the adult testis while its protein was found in the sperm tail from principal piece of the flagellum. This is consistent with its expression in other mammals. Thus this gene appears to have a conserved role in spermatogenesis for at least the last 166 million years of mammalian evolution.

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Renin angiotensin system polymorphisms are associated with pregnancy complications

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Preeclampsia (PE), small for gestational age (SGA) and pre-term birth (PTB) together affect 20% of first pregnancies. Currently there is no reliable way to identify women at risk. Polymorphims in genes in the renin angiotensin system (RAS) may be associated with impaired placentation and poor maternal response to pregnancy and hence predict risk for pregnancy complications. We aimed to determine if three functional polymorphisms in RAS genes, namely, AGT M235T, ACE (I/D) and AT1R A1166C are associated with pregnancy complications. Pregnancy trios were prospectively recruited from two public hospitals in Adelaide . Pregnancies were classified into normal (n=110), PE (n=26), SGA (n=47), PE+SGA (n=12), gestational hypertension (GH, n=17), PTB (n=20) . PE , PE +SGA and GH were also grouped together as hypertension. Parental blood and maternal blood pressure was sampled or measured at 15 weeks gestation. Cord blood was sampled after delivery. DNA was extracted from buffy coats and genotyped using high resolution melt analyses. Maternal plasma [ACE] was measured by ELISA. Data were analysed by ANOVA and Fisher's exact test. Likelihood ratios (LR) were calculated where appropriate. Maternal ACE I/D was associated with hypertension ($p= 0.001$, LR = 14.8) and SGA ($p= 0.019$, LR = 10). Paternal AT1R A1166C was associated with PTB ($p = 0.01$, LR = 7.4). For ACE I/D, plasma [ACE] in women with DD was 33% and 62% higher than ID and II, respectively (both $p<0.001$). Systolic blood pressure in women with DD was 6% higher than II ($p = 0.04$). Our data suggest that RAS polymorphisms are associated with pregnancy complications. Furthermore, maternal ACE genotype determines plasma [ACE] and affects blood pressure at 15 weeks gestation, well before symptoms manifest. Our data also suggest that paternal genotype may be important in determining risk for pregnancy complications, consistent with the role of paternity in their aetiology.

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Functional perspectives of caspase-14 in the human placenta

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The functional barrier for exchange between the mother and fetus in the placenta is created by the fusion of cytotrophoblasts with one another to form a continuous, multinuclear syncytiotrophoblast, which is maintained by the incorporation of underlying proliferative cytotrophoblasts. Disruption to this process has been hypothesised to be involved in the aetiology of preeclampsia. Recently we investigated caspase-14 in the context of trophoblast differentiation as it is crucial to epidermal differentiation and keratin stabilisation, revealing disparate expression of caspase-14 in the differentiating BeWo cell line. Consequently, further examination as to its functional role in trophoblast differentiation was conducted using RNA Interference (RNAi), with the hypothesis that differentiation would be suppressed following caspase-14 silencing. 100nM siRNA were delivered into the BeWo cell line for 16 hours prior to the addition of 20μM Forskolin. Cultures were incubated for a further 24, 48 or 72 hours prior to the extraction of RNA and protein. Transcription of the trophoblast hormone β-hCG, the endothelial mediator of eNOS, and cytokeratin 18 were found to be increased after both 24 and 48 hours of differentiation following silencing, implicating caspase-14 in the regulation of these pathways. As both β-hCG and eNOS are significantly increased with trophoblast differentiation, this indicates that caspase-14 functionally suppresses BeWo differentiation. Furthermore, the differential expression of cytokeratin 18 indicates a conserved role for caspase-14 in keratin homeostasis in barrier formation. In conclusion, the suppression of caspase-14 in the BeWo cell line resulted in augmented differentiation, a trait often observed in preeclampsia. Further investigation of caspase-14 activity would provide important insight into mechanisms of trophoblast differentiation, particularly in relation to preeclampsia.

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The fibroblast – smooth muscle cell relationship is altered in uterine leiomyoma

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Uterine leiomyoma (fibroids) are benign neoplasms of the myometrium. Their etiology is still poorly understood, but they are known to be sex steroid hormone dependant. Although these tumors are thought to consist of smooth muscle cells (SMC), fibroblasts constitute a major cellular component. Genes that are central to fibroblast activation and function, including transforming growth factor-β (TGFβ), as well as many extracellular matrix (ECM) genes have altered expression in leiomyoma. Despite this, the role of fibroblasts in these tumors has not been investigated in leiomyoma before.

The aims of the present study were to isolate fibroblasts from myometrium (MAFs) and leiomyoma (LAFs); and to compare gene expression of fibroblasts and SMC from these 2 different sources. Myometrium and leiomyoma were dissociated into single cell suspensions, stained with fluorescently labelled CD31, CD90 and 1B10 antibodies and separated into different cell populations using fluorescence- activated cell sorting. Endothelial cells were removed from the analysis; remaining cells were sorted into 1B10-CD90 +ve (fibroblasts) and 1B10-CD90 –ve (SMC) populations and used for RNA isolation and quantitative real-time PCR. Significantly higher levels of expression of aldehyde dehydrogenase1 (ALDH1), cellular retinoic acid binding protein 2 (CRABP2), estrogen receptor (ERα) and progesterone receptor (PR) were observed in MAFs compared to MSMC, while no equivalent differences were observed between LAFs and LSMC; and lower levels of TGFβ were observed in fibroblasts compared to SMC in both tissues. Results for ALDH1, ERα and PR were also confirmed using immunohistochemistry.

This is the first study to successfully isolate LAFs and MAFs and demonstrate an altered fibroblast-SMC relationship in uterine leiomyoma. This work provides the first evidence that LAFs might play an important role in leiomyoma pathophysiology. Investigation of fibroblast function in this disease may provide important new fundamental knowledge on its etiology, ultimately leading to the development of new medical treatments.

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Embryonic survival in prolific ewes

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Introduction: A significant proportion of potential lambs are lost (commonly 15-20%) between ovulation and birth. Little is currently known about factors associated with multiple birth capacity of the uterus which are essential to convert gains in ovulation rate to live lambs. The relationship between maternal uterine and hormonal environment as well as the heritability of embryonic survival (ES) in prolific ewes is investigated.

Methods: Litter size (LS) from known ovulation rate (OR) records (n=6393) collected over 16 years were assessed for heritability with ASREML analysis and to identify the pedigree of outliers. From this flock, closely related high ovulation rate ewes with significantly different litter sizes (High ES; OR2.6/LS2.4 versus Low ES; OR2.9/LS1.6) were selected. Uterine anatomy collected from Day 14 cyclic (n=5 High and n=5 Low ES) and Day 16 pregnant ewes (n=14 high and n=10 Low) as well as systemic concentrations of hormones indicative of uterine (activin-A, follistatin) and ovarian (inhibin-{α}, progesterone) function were compared.

Results and Discussion: ASREML analysis reported ES to be a trait of low repeatability (r=0.10) and even lower heritability (h^2=0.04). However, pedigrees of outlier animals indicated a segregation pattern consistent with a single autosomal gene with a major effect on enhanced ES.

No anatomical differences between high and low ES ewes were discerned. However, significant differences for circulating levels of activin-A (depressed), time of activin-A peak (earlier), follistatin (elevated), progesterone (early rise) and inhibin-{α} (depressed) concentrations were found around the time of oestrus in High versus Low ES ewes. Given the involvement of activin-A in injury-induced inflammation, these results suggest a role for the activin-follistatin system, possibly through increased residency of immune cells and cytokine activation, for increasing ES in prolific ewes.

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Focal adhesions disassemble during early pregnancy in rat uterine epithelial cells

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Successful blastocyst implantation require uterine epithelial cells (UECs) to undergo the 'plasma membrane transformation' followed by the removal of these cells around the implantation sites. The present study investigated the distribution and expression of two principal focal adhesion proteins talin and paxillin in rat UECs during early pregnancy and their role in the loss of these cells at the time of implantation. Results from immunofluorescence microscopy have demonstrated a major distributional change of talin and paxillin in UECs where an intense basal staining of these proteins on day 1 of pregnancy was lost at time of implantation. This was consistent with the significant decrease in paxillin seen through western blotting analysis. Interestingly the amount of talin was not significantly different between day 1 of pregnancy and at the time of implantation with a calpain 2 mediated proteolytic fragment of talin seen on both of these days of pregnancy. Calpain 2 activity was further investigated through western blotting analysis and increases in the active form of calpain 2 at the time of implantation. These observations suggest that talin and paxillin have disassembled from the site of focal adhesions where talin is undergoing cleavage by active calpain 2 at the time of implantation. This allows UECs to become less adherent to the underlying basal lamina facilitating their removal during blastocyst invasion. Hence, disassembly of focal adhesions at the time of implantation is a critical event for successful implantation and placentation.

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Ezrin and EBP50 relocate apically in rat uterine epithelial cells during contact with opposing cells, except cells contacting the blastocyst

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Uterine epithelial cells are important in constantly maintaining a tissue protective barrier, and only under the specific hormonal conditioning of pregnancy which involves a remodeling of the cell ultrastructure termed the 'plasma membrane transformation'. This allows for the successful invasion of the blastocyst. Indirect immunofluorescence microscopy in rat uterine epithelial cells during pregnancy shows that ezrin and EBP50 are relocated to the apical membrane upon lumen closure at the time of implantation, and on average results in 90% colocalisation. Ezrin and EBP50 function as a linked protein complex at the time of implantation seen through immunoprecipitation results from day 6 of pregnancy. The ezrin-EBP50 complex is also associated with the membrane, shown using cell fractionation and western blotting analysis in which ezrin increased dramatically in the membrane concentrated fraction, and correspondingly decreased in the cytosolic fraction leading up to implantation. At the apical membrane these proteins are likely associating with intra-membranous signaling molecules which allow communication between contacting cells. The same protein complex is also relocated to the apical membrane when cells contact an inanimate filament inserted into the uterus of a non-pregnant rat. The only unique contacting circumstance in which these proteins are not seen at the apical membrane is within the implantation chamber itself, more specifically the cells in direct contact with the implanting blastocyst. These results highlight the unique situation that is implantation, which involves specific blastocyst signaling and influence upon the uterine epithelial cells lining the implantation chamber. It may be that the ezrin-EBP50 protein complex is critical at the earlier stage of apposition, and through blastocyst influence, are subsequently lost from the apical membrane to allow for successful invasion.