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ABSTRACTS

CSIRO
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001

WRITING SCIENTIFIC PAPERS

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If you haven't written about your scientific work you might as well not have done it. But, if you haven't written it well, you won't have done much better because your scientific colleagues probably won't read it. This short workshop illustrates an approach to structuring scientific papers that encourages readers to find and retain the important information that you wish them to read. It demonstrates that logical structure is even more important than style and shows how to organize the structure of a paper around the scientific hypothesis on which the work was based. The hypothesis is a statement of what might plausibly have been expected from the experiment before it was done and that expectation can be used to describe the experiment in a way that induces readers to anticipate what they are about to read. This anticipation not only persuades them to read on but enhances their chances of retaining the information that they pick up when they do.

002

SERTOLI CELL TERMINAL DIFFERENTIATION: DOING A 'U' TURN ON A ONE WAY STREET

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The concept of terminal differentiation of Sertoli cells has been challenged and this new information has important implications for male fertility. The mammalian Sertoli cell has two distinct functions: (i) formation of the seminiferous cords and (ii) provision of nutritional and structural support to the developing germ cells. For these to occur successfully, Sertoli cells must undergo numerous maturational changes between foetal and adult life, the main switches occur around the onset of puberty, coincident with the rise in serum gonadotrophins. These switches include the loss of proliferative activity and the formation of the blood testis barrier. Follicle stimulating hormone (FSH) plays a key role in supporting Sertoli cell proliferation in early postnatal life and thus is critical in establishing sperm output in adulthood. After puberty, the size of the Sertoli cell population is considered to be stable and unmodifiable by hormones. This accepted view has been contested as data shows that the size of the adult Sertoli cell population is modifiable by hormone suppression, and that Sertoli cells can regain proliferative activity when stimulated by FSH in the Djungarian hamster[1]. The molecular mechanism(s) by which Sertoli cells re-enter proliferation are not known in this model however a study demonstrated that helix-loop-inhibitor of differentiation proteins can induce terminally differentiated Sertoli cells to re-enter the cell cycle and proliferate[2]. Thyroid hormone and testosterone may be involved in the cessation of Sertoli cell proliferation. Gonadotrophin suppression in the adult Djungarian hamster also results in the disruption of the blood testis barrier and spatial organisation of the inter Sertoli cell tight junction proteins and as a consequence the loss of all germ cells that reside inside the blood testis barrier. FSH restores the organisation of these tight junction proteins, which is associated with the appearance of more mature germ cells. It is expected that the integrity of the blood testis barrier is also re-established. It is suggested that this demonstrated plasticity of the adult Sertoli cell may be relevant in clinical settings, particularly to some types of infertility and testicular malignancies where Sertoli cells have failed to undergo these important maturational switches.

(1) Chaudhary *et al*. (2005) *Biol. Reprod.* **72**, 1205.

(2) Meachem *et al*. (2005) *Biol. Reprod.* **72**, 1187.

003

EXPRESSION OF SECRETED FRIZZLED RELATED PROTEIN-4 (SFRP-4) AND ASSOCIATED WNT SIGNALLING IN CANCER AND APOPTOSIS

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We examined the interplay between Wnt and secreted frizzled related protein-4 (sFRP4) in estradiol induced cell growth in breast cancer cells (MCF-7), and also determined the *in vivo* distribution of sFRP-4 in human breast cancer. MCF-7 cells were treated with estradiol, sFRP-4 conditioned media and a combination of the two. Real-time RT-PCR and Western blot analysis were used to determine the expression of the sFRP-4 and its associated Wnt signalling molecules following treatment. Immunohistochemistry was performed to examine sFRP-4 expression patterns in human breast cancers. Estradiol treatment up-regulated the expression of the Wnt signalling genes Wnt-10b, beta-catenin and fz-4 ($P < 0.001$ for all genes). This up-regulation was not associated with an increase in the Wnt signalling pathway as measured by the levels of active beta-catenin. sFRP-4 conditioned media reduced MCF-7 cell proliferation, down-regulated the Wnt signalling genes beta-catenin and fz-4 as well as down-regulating wnt signalling activity. sFRP-4 was able to reduce the proliferation of estradiol stimulated MCF-7 cells. Cytoplasmic sFRP-4 protein was expressed in all breast tumours examined, with intense staining evident in the lobular carcinoma *in situ* and the ductal carcinoma. These data demonstrate that sFRP-4 is a potent inhibitor of the Wnt signalling pathway in MCF-7 cells, acting not only to down-regulate the activity of the wnt signalling pathway, but also down-regulate the transcription of Wnt signalling genes. The results of these *in vitro* and immunohistochemical experiments warrant further investigation as to whether sFRP-4 expression can be indicative of prognosis in human breast cancer. In addition to breast cancer, we have also examined the role of sFRP-4 in other cancers such as ovarian and prostate.

004

CONTROL OF SKELETAL MUSCLE CELL PROLIFERATION AND DIFFERENTIATION

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Skeletal muscle is formed by mononucleated precursor cells (myoblasts) that cease cell proliferation to start differentiation; this results in fusion between the myoblasts to form multinucleated cells (myotubes) that continue to differentiate (and fuse with more muscle cells) and mature into myofibres. Myogenesis has been widely used as a model to study *in vitro* factors controlling cell proliferation and differentiation. Condition *in vitro* may not reflect what happens in the more complex *in vivo* environment. Some of the key issues are what activates quiescent myoblasts in mature skeletal muscle *in vivo*, and what controls the switch between proliferation and differentiation? The role of the matrix, and molecules such as MyoD, p53, NFAT and IGF-1 will be considered.

005

EFFICIENCY OF NUCLEAR IMPORT OF THE CHROMATIN-REMODELLING FACTOR SRY IS CRITICAL FOR SEX DETERMINATION

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15% of cases of human XY sex reversal are due to mutations in SRY (sex determining region on the Y chromosome), many of which map to one of SRY's two independently acting nuclear localization signals (NLSs) flanking its DNA binding domain. The C-terminal NLS (C-NLS) targets SRY to the nucleus through a 'conventional' pathway dependent on the nuclear import receptor importin-β (Imp-β). No importin has been shown to bind the N-terminal NLS (N-NLS), but it is known to interact with the Ca^{2+}-binding protein calmodulin (CaM). We examined seven distinct missense mutations in the SRY NLSs from XY sex-reversed human females for effects on nuclear import and ability to interact with CaM/Imp-β1. All mutations were found to result in reduced nuclear localization in transfected testicular cells compared to wild type. The CaM antagonist, calmidazolium chloride (CDZ), was found to significantly reduce SRY nuclear accumulation, indicating a dependence of SRY nuclear import on CaM. Intriguingly, N-NLS mutants were resistant to CDZ's effects, implying a loss of interaction with CaM; this was confirmed directly by *in vitro* binding experiments using recombinantly expressed protein. Either impaired CaM or Imp-β1 binding can thus be the basis of sex-reversal in human patients. Our results implicate a CaM-dependent nuclear import pathway for SRY mediated by the N-NLS that, together with the C-NLS, is required to achieve threshold levels of SRY in the nucleus for male sex determination.

007

RECENT ADVANCES IN UNDERSTANDING AND PREVENTING PRE-TERM BIRTH - THE ORAL HEALTH CONNECTION

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Pre-term birth remains one of the major unsolved problems in human health. The incidence is increasing in many Western countries, despite several decades of research studies aimed at findings ways that early birth may be prevented. Nearly two-thirds of very early pre-term births are associated with features of inflammation in the newborn, suggesting that infection may be the origin. Many studies have focussed on the possibility that pre-labour rupture of membranes or the early onset of uterine activity may result from infection spreading upward from the vagina. Unfortunately, trials designed to identify potential pathogens in the genital tract followed by appropriate use of antibiotics have failed to prevent prematurity.

The strong association between features of intra-uterine inflammation and pre-term birth, and the ineffectiveness of antibiotics to prevent the problem, suggest that the source of inflammation may be from a distant site. We are addressing the possibility that the site may be the gums. Periodontal disease affects 15% of the adult Australian population, is often undiagnosed and is not responsive to systemic antibiotic therapy. In our pregnant population, we have shown that periodontal disease is strongly associated with low birth weight. Our sheep studies have taught us that the lipopolysaccharides (LPS) from periodontal pathogens, when injected into the amniotic cavity, have much greater lethality than enteric LPS, and similar effects in inducing inflammation. We are now investigating the effects of treating periodontal disease during mid-pregnancy in a randomised controlled trial, which aims to screen approximately 5000 pregnant women and allocates those with periodontal disease to treatment during pregnancy or soon after. This study is known as the Smile Study and commenced in February 2005. Improving oral health is an exciting, but yet unproven, strategy by which a major health problem may be prevented by a relatively simple and community-based intervention.

008

PLACENTAL INFLAMMATION AND PRETERM LABOUR: STUDIES OF PATHOPHYSIOLOGY AND INTERVENTION USING HUMAN *EX-VIVO* MODELS

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Inflammatory processes and mediators are an integral aspect of the mechanics of parturition. Leukocyte infiltration/activation of the extraplacental membranes, cervix and uterus occurs prior to term labour and is accompanied by increased cytokine expression, eicosanoid production, and extracellular matrix degradation. These processes are propagated during labour to ensure progression through to delivery, and in normal parturition they occur in concert in a timely fashion. In contrast, pathological intrauterine inflammation appears to be a causative factor in a significant proportion of preterm births. While the presence of an infective organism in the amniotic cavity is confirmed in about half of pregnancies delivered with chorioamnionitis, in the remainder the cause of the excessive inflammatory response remains undetermined.

We have established and applied various models employing human placental tissues to study the processes involved in triggering intrauterine inflammatory activation and potential pharmacological approaches for intervention. Of these, an *ex-vivo* fetal membrane perfusion model has been the most powerful, allowing multi-aspect analysis of membrane gene expression, inflammatory mediator production, histological and structural integrity in response to maternal challenge with endotoxin (lipopolysaccharide). Using a variety of techniques including oligonucleotide and protein arrays we have determined that a robust and rapid inflammatory response is manifested in both the maternal and fetal compartments after maternal (decidual) exposure to lipopolysaccharide; this is accompanied by a marked increase in apoptosis in the chorionic membrane, but no detectable changes in membrane integrity.

We have evaluated in this model several anti-inflammatory drugs that inhibit the NF-κB pathway as potential pharmacologics for treating inflammation-induced preterm labour. Surprisingly, most were completely ineffective in suppressing lipopolysaccharide-induced cytokine production. Sulfasalazine, however, administered to the maternal face, effectively and rapidly abrogated the lipopolysaccharide response in both maternal and fetal compartments, with modest membrane transfer of drug. However, chorionic apoptosis was doubled in sulfasalazine-treated membranes, raising concerns over possible toxicity. Studies using these models are continuing to further evaluate the potential of novel and existing pharmacotherapies for the prevention of inflammation-associated preterm birth.

009

THE EFFECT OF MATERNAL ASTHMA DURING PREGNANCY ON PLACENTAL FUNCTION, FETAL GROWTH AND CHILDHOOD DEVELOPMENT

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Fetal growth and neonatal birth weight are significant contributing factors to the development of adult disease states in later life. In human pregnancy, we have identified sexually dimorphic differences in fetal growth with the female fetus reducing growth in response to maternal asthma and the male fetus continuing to grow at a normal rate but being at an increased risk of *in utero* death. The physiological mechanisms that confer sex-specific differences in the fetal response to maternal asthma are unknown. However our research has identified differences in mechanisms associated with fetal glucocorticoid regulation, which are also associated with changes in childhood growth patterns. Asthmatic and control pregnant women were recruited at their first antenatal visit and followed through to delivery. Subjects were assessed for severity of asthma and their use of medication, including glucocorticoid therapy, was recorded. In addition to routine antenatal care, fetal growth was determined using Doppler ultrasound. Following delivery placentas and cord blood were collected. The children of the women followed during the study were examined by a paediatrician at 6 months of age and every 12 months after that initial visit. Our data shows that in response to maternal asthma, the female fetus has an increase in cortisol, which downregulates placental GR expression, immune and hypothalamic-pituitary-adrenal function and is associated with decreased growth. The male fetus responds to increased cortisol with an increase in GR expression and no change in HPA or immune function or growth. These data indicate that the male and female fetus have different strategies to control growth and in their response to a maternal stress, such as asthma.

010

OOCYTE CONTROL OF GRANULOSA CELL DEVELOPMENT AND FUNCTION

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Oocytes orchestrate the rate of follicular development and the patterns of gene expression by granulosa cells (GCs). There are two populations of GCs in large antral follicles: mural granulosa cells (MGCs) that line the ovarian follicle wall, and cumulus cells (CCs) closely associated with the oocyte. Subtraction hybridization was used to find transcripts more highly expressed in CCs than MGCs. Among the genes expressed more highly in CCs was one encoding an amino acid transporter (*Slc38a3*). *Slc38a3* mRNA was not detected in oocytes. Expression of *Slc38a3* mRNA was reduced in the CCs after removal of the oocyte and restored by co-culturing CCs with fully grown oocytes (FGOs). Alanine is one of the amino acids transported by SLC38A3. This amino acid is poorly transported across the oocyte plasma membrane, but gains access to the oocyte from the cumulus cells via gap junctional communication. Alanine transport into cumulus cells was promoted by paracrine factors secreted by FGOs, but not by growing oocytes (GOs) from preantral follicles. Thus FGOs promote the transport of alanine into CCs, and this amino acid is then passed on to the oocyte via gap junctions. Transcripts encoding enzymes in the glycolytic pathway were also more highly expressed in CCs than MGCs. FGOs, but not GOs, promote elevated expression of some of these transcripts. Likewise, FGOs promote both glycolysis and oxidative phosphorylation by isolated CCs and MGCs. Oocytes do not effectively utilize glucose as an energy source, and oocytes require the presence of CCs to resume meiosis when glucose is the only energy source present. In contrast, oocytes can resume meiosis in the absence of CCs when pyruvate is the sole energy source. Thus oocytes apparently promote glycolysis by their companion granulosa cells to provide energy for their own development. In addition, this may be one way that oocytes coordinate their development with that of follicular somatic components.

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020

OOCYTE SIGNALLING MOLECULES AND THEIR EFFECTS ON REPRODUCTION IN RUMINANTS

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Sheep (*Ovis aries*) are a highly diverse species with more than 900 different breeds that vary significantly in their physiological characteristics including ovulation rate and fecundity. From examination of inherited patterns of ovulation rate in sheep, several breeds have been identified with point mutations in two growth factor genes that are expressed in oocytes. Currently, five different point mutations have been identified in the BMP15 (GDF9b) gene and one in GDF9. Animals heterozygous for the GDF9 and/or the BMP15 mutations have higher ovulation rates (i.e. +0.6 to +5.0) than their wild-type contemporaries. In contrast, those homozygous for any of the aforementioned BMP15 or GDF9 mutations are sterile due to abnormal follicular development from the primary stage of growth. In bovine and ovine ovaries, GDF9 is expressed exclusively in oocytes throughout follicular growth from the primordial stage of development, whereas in sheep BMP15 is expressed exclusively in oocytes from the primary stage: no data for BMP15 are available for the cow. *In vitro*, ovine GDF9 (oGDF9) has no effect on ^{3}H-thymidine incorporation by either bovine or ovine granulosa cells, whereas oBMP15 has modest (1.2 to 1.6-fold; $P < 0.05$) stimulatory effects. GDF9 or BMP15 alone inhibited progesterone production by bovine granulosa cells, whereas with ovine cells only GDF9 was inhibitory. The effects of GDF9 and BMP15 together were often cooperative and not always the same as those observed for each factor alone. Active immunisation of ewes with BMP15 and/or GDF9 peptides affected ovarian follicular development and ovulation rate. Depending on the GDF9 and/or BMP15 vaccine formulation, ovulation rate was either increased or suppressed. For example, immunisation of ewes with a BMP15 peptide in a water based adjuvant has led to a 25% increase in lambs born per ewe lambing. Collectively the evidence suggests that oocyte signalling molecules have profound effects on reproduction in mammals including rodents, humans and ruminants. Moreover, that *in vivo* manipulation of these oocyte signalling molecules provides a new approach to managing the fertility of ruminants.

021

CURRENT PROGRESS INTO TESTIS CELL TRANSFER BETWEEN CATTLE BREEDS

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Male germline cell transfer has produced offspring in mice (Brinster and Zimmermann 1994). Recently the first livestock animal, a goat, was produced (Honaramooz *et al.* 2003), while early results in cattle are promising (Oatley *et al.* 2002; Izadyar *et al.* 2003). There is an opportunity to develop this technology for the beef industry by transferring male germ line stem cells between breeds to improve the genetics of extensive Australian beef herds. This project is a part of the CSIRO National Research Flagship program that combines expertise and facilities in divisions with complementary expertise at Monash University and the University of Sydney. The environmental constraints of Northern Australia dictate that Brahman type animals show far better survival than *Bos taurus* cattle, although the carcass value of Brahmans is lower than *Bos taurus* animals. Artificial insemination is impractical in Northern Australia and thus we aim to develop testis cell transfer technique in cattle to permit Brahman bulls to deliver semen from elite *Bos taurus* or composite bulls, thereby significantly increasing the growth rate, yield and meat quality of the northern beef herd. Experiments using cattle were performed to determine the applicability of techniques used in the mouse. Initial proof of concept has been achieved that germ cell transfer can result in the donor cells successfully colonizing areas of recipient testis. The viability of isolated testis cells following short term (24 h) culture has been demonstrated through transfer into recipient calves. We have completed >50 male germ cell transfers into recipient calves, using ultrasonographic guided injection into the rete testis. Success of this procedure has been demonstrated by persistence of PKH26 dyed donor cells in the seminiferous tubules of a majority of recipients >2 months after transfer. These recipient male calves have not been depleted of their endogenous spermatogonial populations and we thus expect the efficiency of the procedure to increase as depletion procedures (ongoing) are established. Concurrent with these developments has been research into large scale culture of male germ line stem cells to provide large numbers of stem cells for transplant. Culture of testis cell suspensions has demonstrated survival of enriched testis cells under varying media and culture conditions. Initial passaging of testis cell colonies has revealed mixed cell populations (immunohistochemistry positive for spermatogonia and somatic cells). Further studies will aim to demonstrate that these cultured donor cells are able to undergo spermatogenesis in the recipient animals.

022

ISOLATION OF STEM CELLS FROM EMBRYOS AND ADULT BOVINE TISSUES

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The isolation of stem cells has become an area of increasing interest due to their potential uses in animal reproduction, somatic cell nuclear transfer and cell therapies. The most attractive options are the isolation of stem cells from individual embryos or adult somatic tissues. In addition, for cell therapy, the use of autologous stem cells is considered to have an advantage over heterologous cell based therapies in that immune rejection issues would be circumvented. Here we report on our attempts to isolate stem cells from both these sources in a bovine model.

Bovine ES-like (bES) cells were successfully isolated from embryos and maintained *in vitro* for up to six passages. These cells retained the morphology characteristic of bES cells: small cytoplasmic/nuclear ratio, nuclei with multiple nucleoli, and multiple lipid inclusions in cytoplasm. bES cell colonies grew as monolayers, as islands of ES cells surrounded by trophectoderm (TE) cells. Immunohistochemical detection of SSEA-1 and SSEA-4 demonstrated expression of these markers in bES cells but not in TE cells. Further, the expression of the pluripotent markers Oct-4, Rex-1 and SSEA-1 by RT-PCR was also detected in bES cells but not in TE cells. On spontaneous differentiation, these cells were able to form a variety of cell types including beating muscle with the cells displaying a propensity to differentiate in a manner reminiscent of human ES cells. (2) We also report the isolation of putative stem cells from adult bovine skin biopsies, which express the stem cell markers Oct-4 and SSEA-1 analysed by RT-PCR and are capable of forming 3-dimensional colonies. These cells are obtained from a skin biopsy, a relatively non-invasive technique that makes them useful as donors for therapeutic applications.

In summary, we have identified populations of stem cells from embryonic and adult bovine tissues, which are readily isolated. Further characterization of the differentiation potential of these cells is needed to identify the suitability of this population for use in autologous stem cell therapies.

023

BIOTECHNOLOGY AND REPRODUCTION IN MAINSTREAM ANIMAL INDUSTRY – A PERSPECTIVE

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This paper considers strategies to improve the reproductive performance of small ruminants in ways that lead to production systems that are 'clean, green and ethical'. This view arises from feedback from consumers, particularly in attractive export markets, and from a need to refocus on the needs of Australian producers, most of whom operate large, extensive enterprises. These people cannot use 'high-tech' systems but need low-cost, low-labour solutions to their problems. First, to control of the timing of reproductive events, they can use the socio-sexual inputs of the 'male effect' to induce synchronised ovulation in females that would otherwise be anovulatory (seasonal, lactational, prepubertal). Second, they can use nutritional stimuli for 'focus feeding', in which short periods of nutritional supplements are precisely timed and specifically designed for individual events in the reproductive process: gamete production, embryo survival, 'fetal programming' and colostrum production. Third, they can use simple behavioural observations to genetically select for temperament – this will maximize offspring survival, product quality and animal welfare. All of these approaches involve non-pharmacological manipulation of the endogenous control systems of the animals and complement the detailed information from ultrasound that is now becoming available.[1] The use of such clean, green and ethical tools in the management of our animals can be cost-effective, increase productivity and, at the same time, greatly improve the image of meat and milk industries in society and the marketplace. This does not mean, however, that they will not benefit from the opportunities that evolve from breakthroughs in reproductive technology or gene research. On the contrary, if this 'high-tech' research is done within the context of the needs of a 'clean, green and ethical' industry, first class science can have very direct and immediate benefits to our livestock industries.

(1) Martin GB, Milton JTB, Davidson RH, Banchero Hunzicker GE, Lindsay DR and Blache D. (2004). Natural methods of increasing reproductive efficiency in sheep and goats. *Anim. Reprod. Sci.* **82–83**, 231–246.

025

PROLACTIN SIGNALING THROUGH THE SHORT FORM OF ITS COGNATE RECEPTOR CAUSES SEVERE OVARIAN DEFECT

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Extensive investigations from our laboratory have clarified the action and interaction of estradiol (E) and prolactin (PRL) on corpus luteum (CL) function. Our research has led us to discover and isolate a CL specific gene that encodes a protein we named PRAP, that associates with the intracellular domain of the short form (PRLRS) but not the long form (PRLRL) and whose expression is tightly regulated by E. Our laboratory and others have established that this protein, expressed in CL of every species investigated, is a novel 17 beta hydroxysteroid dehydrogenase (17bHSD-7) whose function is to catalyze the transformation of estrone to E. Our results with cells expressing only PRLRS revealed that PRL acting through PRLRS leads to phosphorylation of PRAP/17bHSD-7 (PRAP/17b) by JAK2 establishing for the first time that a steroidogenic enzyme can be phosphorylated through its association with a membrane bound protein. The association of PRAP/17b with the PRLRS and its phosphorylation leads to its stabilization. To further investigate the role of PRL signaling through PRLRS, we used PRLR(–/–) mice expressing the PRLRS as a transgene. The results obtained were totally unexpected and of great interest. The follicles of the ovaries, expressing PRLRS only, underwent premature development followed by severe granulosa and oocyte death leaving holes surrounding collapsed zona pellucida and premature ovarian failure. The observations that: (*1*) the expression of PRLRS in the ovaries of PRL null mice leads to inhibition in Foxo3a and of GALT, two proteins whose deletion/mutation causes similar premature ovarian failure; and (*2*) that GALT promoter activity is stimulated by Foxo3a transcription factor led us to hypothesize that PRL acting through PRLRS prevents the expression of Foxo3a, which normally stimulates GALT transcriptional activity. Absence of Foxo3a then leads to inhibition of GALT and increases in galactose and its metabolites, causing galactose toxicity and granulosa as well as oocyte cell death.

026

THE CUMULUS MATRIX IN OVULATION: INERT PACKAGING OR ACTIVE DELIVERY VEHICLE FOR THE OOCYTE?

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Preovulatory follicles respond to the LH-surge with a cascade of molecular events. The ovulatory signal initially impinges on the mural granulosa layers triggering rapid tissue morphogenesis and ultimately terminal differentiation of these cells. Mural granulosa cells transiently produce a suite of transcriptional regulators, EGF-like ligands as well the extracellular matrix (ECM) proteoglycan, versican and the protease ADAMTS-1. These act in concert with permissive oocyte signals to induce and organise a complex hyaluronan (HA) rich ECM surrounding the cumulus cells and oocyte. This expanded cumulus matrix is analogous in composition to an extensive form of pericellular matrices actively associated with cell migration. During ovulation the cumulus matrix becomes anti-adhesive to the intra-follicular environment but is strongly pro-adhesive for the oviductal fimbria. When the follicle apex is perforated the COC is released binds to the fimbria and transports into the oviduct where fertilisation occurs. Success of ovulation and fertilisation is sensitive to the appropriate production and assembly of cumulus matrix components that are in turn dependent on an appropriate balance of oocyte and granulosa derived signals. Production of these cumulus matrix components is thus a potential checkpoint that assures ovulation of competent oocytes. The HA matrix is cross-linked by organiser molecules and also is enriched in proteases ADAMTS-1, 4, 5. Although these have potentially redundant functionality, ADAMTS-1 null female mice are profoundly sub-fertile and have reduced ovulation rate. Specific components of the cumulus matrix are disorganised in ADAMTS-1 null mice and cleavage of versican in these cumulus complexes is reduced. Thus ADAMTS-1 and versican have unique roles in normal cumulus matrix expansion that is important for ovulation. Altered interaction of the cumulus complex with neighbouring tissues alters transport through the oviduct, while abnormal persistence of COC matrix structure after ovulation is also likely to impair sperm interaction and penetration. Evidence thus indicates that the expanded cumulus matrix plays several active roles in oocyte release, transport and sperm interaction.

027

IN VITRO GROWTH AND MATURATION: HOW DOES THIS TECHNOLOGY FIT FOR CLINICAL APPLICATION?

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Superovulation protocols used in IVF result in multiple eggs that can be fertilized and grown in the laboratory to allow for selection of the best embryo for return to the mother, thereby increasing the chances for a successful pregnancy. However, there are many side effects of these superovulation drug protocols, such as deep vein thrombosis and hyperstimulation. The latter is of particular concern for women with polycystic ovary syndrome. Furthermore, the use of gonadotrophins has been reported to compromise both oocyte quality and the uterine environment and may contribute to the low success rates of IVF. Therefore the ability to collect large numbers of oocytes from women and mature them *in vitro* is an attractive alternative. However, although there are reports in the literature on extended maturation/culture periods of human oocytes the pregnancy rates are significantly lower than that observed after *in vivo* maturation. The ability to offer such technology is currently limited by the lack of understanding of how the conditions for *in vitro* maturation affect the quality of the oocyte and the resulting embryo. Our research is concentrated on establishing the role of metabolic balance in the oocyte for the maintenance of subsequent viability. We have determined that disruptions to the balance between mitochondrial and cytoplasmic metabolism in animal oocytes have significant adverse consequences for the resultant embryo. Changing conditions for *in vitro* maturation were also found to alter the establishment of the metabolic settings of the oocyte. The ability to determine the role of such parameters in maturing human oocytes will be important for the prospect of adoption of this technology for routine clinical practice.

028

CYTOKINE NETWORKS AND REGULATION OF SPERMATOGENESIS – WHAT SHOULD WE REALLY BELIEVE?

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Spermatogenesis is a complex yet highly organised process involving intimate interactions between the supporting Sertoli cells and germ cells at various stages of development. The repeating pattern of the cycle of the seminiferous epithelium is due to the fact that spermatogonia enter spermatogenesis at regularly spaced intervals and proceed through the process at a species-specific rate. How this degree of coordination is maintained remains poorly understood, but recent evidence has focussed attention on the role of growth factors produced by the Sertoli cells and germ cells. Several of these growth factors, such as interleukin-1α (IL-1α), IL-6, tumour necrosis factor (TNFα) and activin A, are also inflammatory cytokines. This has led some researchers to question the physiological significance of these data with respect to normal testicular function. For example, in spite of the fact that IL-1α is produced by the Sertoli cell and regulates spermatogonial proliferation and development *in vitro*, mice lacking the IL-1R, and hence unresponsive to IL-1α, possess relatively normal fertility. So what role, if any, do these cytokines play in the normal testis, or are they only important during inflammation? It is quite evident that these cytokines have stimulatory and/or inhibitory effects on spermatogonial and spermatocyte development. These cytokines also interact at multiple levels within each other's signalling pathways and have considerable redundancy of action. Moreover, expression of these cytokines varies across the cycle of the seminiferous epithelium, with major changes in production coinciding with two key events within the cycle: the release of sperm from the epithelium, and the major peaks of DNA synthesis by spermatogonia and preleptotene spermatocytes. It is therefore possible to hypothesise that release of sperm and resorption of the residual cytoplasm triggers a self-regulating inflammatory cascade within the epithelium that initiates and then modulates the next round of spermatogenic development, ensuring that spermatogonia enter the process at the appropriately spaced intervals.

034

NUTRIENT SENSING BY THE EARLY MOUSE EMBRYO: HEXOSAMINE BIOSYNTHESIS AND GLUCOSE SIGNALLING DURING PREIMPLANTATION DEVELOPMENT

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Adequate nutrient supply prior to implantation is not only essential to early embryonic growth and development but has also been implicated in metabolic programming events that influence later stage development and the onset of adult disease. The molecular mechanisms involved in early embryonic nutrient sensing and subsequent programming however have not yet been determined.

Glucose can act as an essential molecular signal for metabolic differentiation and blastocyst formation.[1,2] Our work demonstrates that propagation of this nutrient signal involves glucose metabolism through the hexosamine biosynthetic pathway, whose end-product, uridine 5′-diphospho-N-acetylglucosamine (UDP-Glc-NAc) acts as a donor substrate adding a single O-linked β-N-acetylglucosamine (O-GlcNAc) unit to serine and threonine residues of nucleocytoplasmic proteins. The number of proteins modified by this O-linked glycosylation is large and includes transcription factors, cytoskeletal components, metabolic enzymes and other cellular signaling components. This tightly regulated and dynamic modification operates in a functionally reciprocal relationship to the more familiar phosphorylation at the same sites hence altering the activity and/or stability of targeted proteins and providing a mechanism for modulating cellular physiology in response to nutrient availability.

We show that early embryonic glucose exposure, whilst not essential for energy generation during cleavage development, is nonetheless critical for the maintenance of cellular homeostasis with perturbations in glucose levels during early development leading to decreased levels of cell survival. Furthermore, using antisera specific for O-GlcNAc we have examined levels of O-glycosylated proteins in early mouse embryos in response to the presence or absence of glucose and find dramatically reduced global levels of O-linked glycosylation as well as altered nuclear levels of key transcription factors in embryos deprived of glucose.

We believe that this is the first demonstration of a nutrient effect on levels of transcriptional regulators in early development. Elucidation of the mechanisms by which the nutrient environment influences embryonic development is of fundamental importance to our understanding of the origins of adult disease.

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CIRCADIAN RHYTHMS AND THE EARLY LIFE PROGRAMMING OF ADULT PHYSIOLOGICAL SYSTEMS

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We are all familiar with the idea that the external environment influences many diverse physiological systems. For example, the level of nutrition can not only influence adult health directly, but also fetal development and subsequently many adult functions in the offspring. Maternal stress can affect fetal outcomes as can the administration of drugs during pregnancy. Until recently, however, the daily changes in environmental light have been considered to really only influence the time that we sleep and in many other species the optimal time to mate. The impact of circadian rhythms on life trajectory has had little attention.

In the last 5 years it has become clear that circadian rhythmicity is entrenched in virtually every cell of our bodies. A suite of clock gene transcription factors that include *Clock*, *Bmal1* and the *period* and *cryptochrome* genes, generate a robust daily cycle of transcription and translation of hundreds of proteins. This cellular clock system is synchronised with the external photoperiod through retinal light perception, the hypothalamic suprachiasmatic nucleus (SCN) and neural and hormonal pathways. Most importantly when the clock system in peripheral tissues is disrupted, a growing list of detrimental consequences are being uncovered. As an example, mice with mutations in either *Clock* or *Bmal1* have non-rhythmic peripheral tissues and exhibit mild to severe reproductive failure and metabolic dysfunction. Null *per2* mice have a higher incidence of salivary gland hyperplasia, teratomas and increased susceptibility to radiation induced lymphomas. It is also apparent that intrauterine insults (e.g. cocaine administration, poor nutrition and stress) can have long term effects on the central circadian timing system in the SCN. Whether this involves alterations in neural development or gene function is not known. Nevertheless it is time we paid more attention to the temporal nature of our environment as a possible contributor to lifetime disorders and diseases.

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NUTRITIONAL PROGRAMMING, FETAL GROWTH AND COMPETENCE FOR LIFE AFTER BIRTH

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During the past decade there has been a world-wide series of epidemiological and clinical studies, which have demonstrated that there are associations between prenatal growth restriction and the risk of insulin resistance, central obesity, hypertension, type 2 diabetes and cardiovascular disease in adult life. More recently there has also been increasing interest in the consequences of exposure of the fetus to increased levels of maternal nutrition and whether maternal 'overnutrition' may program an 'intergenerational cycle of obesity'. In this presentation, we review recent experimental evidence that highlights the impact of varying levels of fetal nutrition on the structural and functional development of the adipocyte, and on expression of a range of appetite regulatory peptides within the developing brain. The importance of the timing of nutritional perturbations and the different consequences of fetal undernutrition and overnutrition on subsequent gene expression within different fat depots and on the expression of appetite stimulatory and inhibitory neuropeptides will be reviewed. The importance of defining those critical windows during an individual's lifespan when nutritional or other intervention strategies will have the maximum benefit in preventing the development of obesity and cardiovascular disease will also be considered.

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IMPACT OF GLUCOCORTICOIDS ON FETAL-PLACENTAL GROWTH AND THE POSTNATAL PHENOTYPE

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Glucocorticoids are recognised as a key fetal programming signal, with excess glucocorticoid exposure *in utero* linked to various adverse outcomes in offspring including delayed puberty onset, hyperleptinemia and hypertension. Fetal glucocorticoid exposure is controlled by the placental glucocorticoid barrier, whereby two 11β-hydroxysteroid dehydrogenase enzymes regulate transplacental passage of active glucocorticoids (cortisol and corticosterone). Fetal programming by glucocorticoids is likely due to their actions in several fetal tissues, but may also be mediated via effects exerted within the placenta. Indeed, in our model of fetal programming, treatment of pregnant rats with dexamethasone inhibits both fetal and placental growth, and dose–response experiments suggest that the placenta is more susceptible than the fetus to this growth inhibition. Moreover, glucocorticoid treatment stimulates placental apoptosis and reduces expression of several placental gene products, including PPARγ, Muc1 and VEGF. This down-regulation of gene expression occurs specifically within the labyrinth zone, the region of maternal–fetal exchange, and is associated with a marked reduction in placental vascularity. These data indicate that excess placental glucocorticoid exposure is likely to compromise fetal nutrient supply, which in turn could result in adverse fetal programming effects. Subsequent, long-term effects of fetal programming in offspring can either be amplified or attenuated by the postnatal environment. Thus, while programmed hyperphagia and adiposity are exacerbated by a high-energy diet in postnatal life, we have demonstrated that programmed hyperleptinemia and hypertension are prevented by a postnatal diet enriched with omega-3 fatty acids. These effects are mediated, in part, by changes in the adipocyte phenotype, most notably in relation to leptin mRNA expression. In conclusion, fetal programming by glucocorticoids is likely to be mediated, in part, by their detrimental effects on placental growth and vascularity. Postnatally, adverse outcomes of glucocorticoid-induced fetal programming can be prevented by dietary manipulations, thus raising the possibility of preventative, therapeutic interventions.

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CONTRIBUTION OF CLAUDIN-11 TO THE INTER-SERTOLI CELL TIGHT JUNCTION, *IN VITRO*

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The inter-Sertoli cell tight junction (TJ) forms the blood testis barrier (BTB) between Sertoli cells and is composed of three major transmembrane proteins: claudin-11, occludin and junctional adhesion molecule. Formation of the BTB occurs during puberty associating with an increase in circulating gonadotrophins. Claudin-11 and occludin are hormonally regulated *in vitro* although their importance to the function of the TJ is unknown. The aim of this study was to investigate the contribution of claudin-11 to the inter-Sertoli cell TJ *in vitro* by blocking gene expression using RNA interference. Two claudin-11-specific siRNA fragments were designed for this purpose. Sertoli cells in primary culture formed stable TJs within 5 days as measured by transepithelial electrical resistance (TER). The addition of siRNA for 2 days resulted in a significant ($P < 0.01$) 55% (mean, SD, $n = 4$ cultures) decrease in TER along with a major reduction in claudin-11 localisation to the TJ as assessed by immunocytochemistry. The specificity of the siRNA was shown by the presence of extensive immunostaining of occludin and of the adherens junction protein β-catenin in the same treatments. Similarly, claudin-11 mRNA expression significantly ($P < 0.01$) decreased by 71% (mean, SD, $n = 3$ cultures) in response to both claudin-11 siRNA fragments. Occludin mRNA expression was not affected. It is concluded that claudin-11 contributes at least 55% to the function of the rat Sertoli cell TJ *in vitro*. It is hypothesised that the remaining 45% of TJ function can be attributed to other integral proteins, such as occludin and junctional adhesion molecule. It is expected that claudin-11 and other TJ proteins play a pivotal role in the function of the BTB *in vivo* with potential implications in fertility and contraception.

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MEIOSIS ARREST IN A NEW ANIMAL MODEL – A MUTATION ON CHROMOSOME 5

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Many causes of male infertility are currently unknown and there is a great need for the identification of new genes involved in spermatogenesis. The chemical mutagen, *N*-ethyl-*N*-nitrosourea (ENU) was utilized to induce random point mutations in the germline of C57Bl-6J mice in order to generate models of male infertility. Three generation breeding programs produced mice homozygous for ~3–4 point mutations. Following a phenotype-based screen of 1650 G3 males from 155 pedigrees, 15 lines were identified with abnormal male fertility parameters consistent with a recessive mutation. One infertile line, *ENU23*, exhibited a complete meiosis arrest with no germ cells ever proceeding to become spermatozoa. No infertile females have been observed. Light microscopic examination of Bouin's fixed paraffin embedded testis sections showed that the arrest occurred post-prophase and prior to the completion of metaphase I, since normal metaphase was not observed. In cells beyond the prophase stage of development, spermatocytes appeared condensed, became enlarged and subsequently underwent cell death by apoptosis confirmed by TUNEL analysis. Electron microscopy visualized normal synaptonemal complexes. The degenerating primary spermatocytes exhibited condensed chromosomes irregularly arranged on a poorly formed microtubular spindle, indicating an abortive attempt to complete metaphase. The percentage of tubules with diplotene/metaphase-like cells was calculated for 3 abnormal and 3 normal *ENU23* mice and showed 10.8% compared to 1.37%, respectively, indicating that prior to cell death the cells undergo a lag period. To identify the *ENU23* causal mutation, the mutation was bred onto a mixed C57Bl-6J/CBA background and linkage analysis was performed, identifying a region on chromosome 5 between microsatellite markers D5Mit7 and D5Mit32. Candidate genes within this region are currently being sequenced in the search for the ENU-induced mutation causing the *ENU23* phenotype. We believe this model of metaphase arrest is unique and will provide insights into the male specific events of meiosis.

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REGULATION OF INHIBIN BINDING AND ACTION VIA BETAGLYCAN EXPRESSION IN MOUSE LEYDIG-LIKE TM3 CELLS

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The actions of inhibin and transforming growth factor (TGF)-β2 are enhanced when their respective target cells express the TGF-β/inhibin co-receptor, betaglycan. In the present studies, we investigated the effects of multiple members of the TGF-β superfamily on betaglycan expression, and examined the consequences of such regulation for inhibin binding, and inhibin and TGF-β actions in mouse Leydig-like TM3 cells.

Isoforms of activin (A and B), TGF-β (1 and 2) and BMP (2, 6 and 7) each suppressed the level of betaglycan mRNA in TM3 cells to 43–46%, 26–39% and 50–71% of control, respectively, during overnight treatment. Subsequent inhibin A binding was suppressed to 72–77%, 35–36% and 66–70% of control, respectively, with IC50s of 0.07–0.7, 0.05–0.5 and 0.4–0.6 nM, respectively. The effects of inhibiting betaglycan expression by TM3 cells on their responses to inhibin and TGF-β2 were examined by transfecting cells with a promoter construct that contains three copies of the activin-responsive sequence of the GnRHR promoter (3XpGRAS-PRL-lux) either alone or in the presence of small (21 bp) duplex siRNAs corresponding to the betaglycan gene. Activin A (0.5 nM) stimulated 3XpGRAS-PRL-lux expression 3–4 fold over control in TM3 cells, and inhibin dose-dependently abolished this stimulation, with no interference from the control siRNA (against BF-1 forkhead-like protein). However, inhibin suppression of activin-stimulated activity was antagonized in cells co-transfected with betaglycan siRNA. TGF-β (1 and 2) stimulated 3XpGRAS-PRL-lux expression 5–8 fold over control, and the action of TGF-β2, but not TGF-β1, was attenuated by the betaglycan siRNA.

In summary, activin, TGF-β and BMP isoforms inhibit betaglycan expression by Leydig-like TM3 cells, and inhibin A binding is commensurately reduced. The 'knock-down' of betaglycan expression by specific siRNA inhibits TM3 responses to inhibin and TGF-β2. Whether the inhibition of betaglycan expression by activin, TGF-β and BMP has similar consequences for inhibin and/or TGF-β2 action is yet to be determined. These studies raise the possibility that multiple members of the TGF-β superfamily participate in cross-talk via the inhibin/TGF-β co-receptor, betaglycan.

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OVER-EXPRESSION OF ACTIVIN βC *IN VIVO* REVEALS A ROLE IN MALE FERTILITY

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Introduction: Activin βC subunit antagonises the formation and bioactivity of activin A via intracellular heterodimerisation and decreases activation of the activin signalling pathway (Mellor *et al*. 2003). Therefore the activin βC subunit heterodimers provide a new mechanism of regulating activin levels. Vedja and colleagues over-expressed the activin βC subunit in malignant liver cell lines, which subsequently displayed inhibition of cell proliferation and induction of apoptosis (Vedja *et al*. 2003). Conversely, Wada *et al*. demonstrated that treatment with hr-activin C stimulates growth of a liver cell line (Wada *et al*. 2004). These recent (and contradictory) reports about the *in vitro* activity of activin βC have prompted us to examine the *in vivo* role of activin βC by creating a transgenic mouse over-expressing the βC activin subunit.

Methods: The full-length human cDNA under the control of a CMV promoter was incorporated into the genome of three founder C57/B6 mice. Genotyping was performed by both Southern and PCR. Mice were monitored weekly and culled at 14–16 weeks (adult). Blood was collected by cardiac puncture, organs were weighed and a portion fixed in Bouin's or frozen for subsequent RNA and protein extraction. Daily sperm production (DSP) was determined by standard methods. Sertoli and germ cell number will be determined using the optical disector (*sic*) stereological technique in Bouin's fixed resin sections. Proliferation and apoptosis will be examined using PCNA and TUNEL respectively. Activin A was assessed by ELISA, while FSH, LH, follistatin and total inhibin were determined by RIA.

Results and conclusions: Over-expression of activin-βC resulted in decreased circulating activin A ($P < 0.005$ TG1, $P < 0.05$ TG2 and $P = 0.08$ TG3), a progressive age-related decrease in litter sizes (9.3 WT *v.* 6.3 TG1, 5.8 TG2 and 4.5 TG3; $P < 0.005$ *v.* WT) and testicular DSP ($P < 0.05$). These data support the hypothesis that βC is a novel *in vivo* regulator and is the first indication of a role for activin-βC in male fertility. This novel mouse model will significantly advance our understanding of the *in vivo* role of activin-βC.

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REGULATION OF SOCS3 EXPRESSION BY PROSTAGLANDIN, PROLACTIN AND GROWTH HORMONE: CHALLENGING THE JAK/STAT SIGNALLING DOGMA

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SOCS3 is an inhibitor of various cytokine-receptor signalling pathways and is therefore involved in suppression of cellular responsiveness to these critical regulators. SOCS3 expression is thought to be regulated by a STAT responsive element (SRE). However, our research suggests the involvement of other signalling pathways. In T-47D breast cancer cells, we found that PGE2 induces a 3–5 fold increase in SOCS3 mRNA, as determined by real-time PCR. This effect was not due to phosphorylation of STATs, or inhibited by the Jak2 inhibitor, AG490, but was inhibited by the PI3Kinase inhibitor, LY294002, Akt Inhibitor IV and partially inhibited by the PKA inhibitor, H89. It was not affected by inhibitors of MEK, PDK1, mTOR or p38-MAPK. We concurrently examined PRL-induced SOCS3 expression, and found that although STAT1 and 5 phosphorylation was increased, SOCS3 expression was again inhibited by Akt Inhibitor IV and H89 but unaffected by AG-490. To explore this further, we used a model of GH signalling, BaF3 cells stably expressing GH receptor. GH induced a 15–20 fold increase in SOCS3 mRNA, which was accompanied by increased STAT5 phosphorylation. However the SOCS3 response was not inhibited by AG-490 or H89, but was diminished by Akt Inhibitor IV. Analysis of the SOCS3 promoter revealed a FOXO binding site. When we mutated this site in a mouse SOCS3 promoter–luciferase construct, basal and GH-induced promoter activity was significantly increased. These results are consistent with FOXO acting as a repressor, which is inactivated by Akt. We propose that in T-47D cells, SOCS3 expression involves cross-talk between PI3K/Akt and cAMP/PKA, whereas in BaF3 cells, expression is enhanced by Akt phosphorylation and subsequent FOXO inactivation. These findings contrast with the accepted Jak/STAT regulation of SOCS3 expression.

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IDENTIFICATION OF A FUNCTIONAL BINDING SITE ON BETAGLYCAN FOR INHIBIN AND TGFβ

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Betaglycan is a co-receptor that modulates signaling by TGFβ superfamily members including TGFβs and inhibins. Loss of betaglycan expression, or blocking of betaglycan function, has been implicated in several human diseases and in animal disease models. However, characterization of the superfamily ligands and receptors involved in these disease states is complicated because of the pleiotropic nature of betaglycan co-receptor action. Here we report the identification and characterization of a domain within the extracellular region of betaglycan that binds inhibin-A and TGFβ-1. We show that both ligands bind to the membrane proximal region (amino acids 591–760) of the betaglycan extracellular domain. This inhibin/TGFβ-binding region is within the ZP-domain of betaglycan, but is not integral to the conserved ZP motif. Using deletion studies and site-directed mutagenesis, we show that the inhibin and TGFβ binding sites on betaglycan overlap and identify individual amino acids essential for the binding of both ligands. In particular, point mutant V614Y abolishes inhibin and TGFβ binding to the membrane proximal domain of the betaglycan ECD. A full-length betaglycan construct containing this point mutation can still bind TGFβ-1 via a separate N-terminal binding site, but is unable to bind inhibin-A. This mutant is incapable of mediating inhibin's antagonism of activin or BMP signalling. Mutation of betaglycan V614Y thus separates the co-receptor actions of betaglycan for inhibin and TGFβ. This will allow the clarification of the role of betaglycan in human disease states such as renal cell carcinoma and endometrial adenocarcinoma.

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PROGESTERONE REGULATES CXCL14 (MACROPHAGE INFLAMMATORY PROTEIN 2γ) mRNA IN HUMAN ENDOMETRIUM

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The emergence of microarray technology has enabled a thorough study of the level of transcripts in the human body. A high density micoarray analysis revealed a comprehensive list of transcripts, which were significantly different between mid-proliferative and mid-secretory phase endometrium.[2] An EST identified from the HG_U95B chip is identical to the 3′UTR of CXCL14 or macrophage inflammatory protein 2γ (MIP 2γ). The level is 19-fold higher in the mid-secretory compared to the mid-proliferative phase of menstrual cycle. This has suggested that the transcript level of CXCL14 may be directly regulated by progesterone. Northern hybridisation and *in situ* hybridisation confirmed that the transcript level of CXCL14 (MIP 2γ) was high in the mid-to-late secretory endometrium and its mRNA was localised in the glandular epithelium of this tissue.[1] *In silico* analysis has predicted six progesterone response elements (PREs) within 2040 bp upstream from the ATG site. To investigate the possible functions of these PREs, a dual luciferase assay was performed on the ishikawa cell line transfected with five deletion constructs of the gene promoter. Cells were co-transfected with progesterone receptor B (PRB) and treated with 10^{-6} M progesterone. Luciferase activities of these constructs have localised two fragments that were most likely to contain the active PREs, i.e. PRE1 and PRE2. An electrophoretic mobility shift assay showed that PRE oligonulcleotides within these two regions were able to bind PRB that was synthesised *in vitro*, although there was a stronger signal seen in the PRE2 region. A dose competition study revealed PRE1/PRB and PRE2/PRB protein binding could be competed with different concentrations of cold wild-type competitor oligonucleotides. Mutagenesis of PRE1 and PRE2 analysed by luciferase reporter assay reduced the inductive effect of progesterone treatment. This study indicates that progestegen induced transcript encoding a chemokine in the human endometrium may likely act as a chemoattractant for leucocytes during the secretory phase of the menstrual cycle.

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PLATELET DERIVED GROWTH FACTORS AND RECEPTORS CONTRIBUTE TOWARD DEVELOPMENT OF THE CORPUS LUTEUM

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In this study the expression of the family of platelet derived growth factors (PDGF) and receptors in the ovarian corpus luteum was identified and characterized, and an effect of their activity on development of the corpus luteum revealed. Gonadotropin-stimulated immature rats were utilized as a model of induced ovulation, luteogenesis and pseudopregnancy, and levels of mRNA for platelet derived growth factors (PDGF-A, PDGF-B, PDGF-C and PDGF-D) and receptors (PDGF-Rα and PDGF-Rβ) in response to gonadotropins were investigated. Intraperitoneal injection of immature rats with pregnant mare's serum gonadotropin (PMSG) followed 54 h later with human chorionic gonadotropin (hCG) resulted in a significant increase in ovarian mRNA levels for PDGF-Rβ and its ligands, PDGF-B and PDGF-D, as early as 4 h after hCG injection. Gonadotropin regulation of PDGF-B was confirmed by *in vitro* promoter–reporter assays, which showed a 2–3-fold increase in PDGF-B promoter activity in response to luteinising hormone (LH), and inhibition studies implicated protein kinase A, phosphatidylinositol 3-kinase and mitogen activated protein kinase signaling pathways in the LH-induced upregulation. In the corpus luteum, PDGF-Rα was localized to a subset of luteal steroidogenic cells, and PDGF-Rβ to cells of the luteal microvasculature. PDGF-A, PDGF-B and PDGF-C were also identified in a population of luteal steroidogenic cells. Intraovarian injection of an inhibitor of PDGF receptor activity, the tyrphostin AG1295, prior to injection of hCG in PMSG-primed immature rats resulted in a significant 22.85 ± 10.7% decrease in corpora lutea per treated ovary in comparison to the contralateral vehicle-injected control ovary. In addition, the treated ovary of 3 of 12 rats showed widespread hemorrhage throughout the entire ovary, indicating a possible role for PDGF receptor activity in maintenance of the ovarian vasculature. In summary, these data identify expression of members of the family of platelet derived growth factors and receptors in cells within the corpus luteum and reveal a role during development of the corpus luteum.

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EFFECTS OF RELAXIN DEFICIENCY ON MATRIX METALLOPROTEINASE EXPRESSION IN THE CERVIX AND VAGINA OF PREGNANT MICE

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The major functions of relaxin are associated with female reproductive physiology, especially the regulation of biochemical processes involved in the remodelling of the reproductive tract at term. Studies in relaxin deficient mice ($Rlx^{-/-}$) demonstrate that although females give birth to live young without apparent dystocia, they have abnormal cervices and vaginae. This phenotype is attributed to an increase in stromal collagen, but the mechanism(s) by which relaxin regulates extracellular matrix (ECM) production in reproductive tissues is poorly understood. In this study, we assessed the expression of matrix metalloproteinases (MMPs) in the cervix and vagina of pregnant wild-type ($Rlx^{+/+}$) and $Rlx^{-/-}$ mice. Tissues were obtained from adult C57/Blk6J $Rlx^{+/+}$ mice on days 7.5, 14.5, 17.5, 18.5 pc and $Rlx^{-/-}$ littermates on days 7.5, 14.5 and 18.5 pc. Real-time PCR using dual-labelled fluorogenic probes was performed in an Opticon 2 cycler (MJ Research) to quantify MMP-2, -3, -7, -9 and -13 gene expression. In the cervix and vagina of $Rlx^{+/+}$ mice, only MMP-2 mRNA levels were significantly higher at term compared with earlier stages of gestation. There were significant decreases in MMP-7 and -13 expression at term, but no change in MMP-3 and -9. In contrast, MMP-3, -7, -9 and -13 mRNA levels were significantly higher in the cervix and vagina of late pregnant $Rlx^{-/-}$ mice. The expression of MMP-2 did not differ between $Rlx^{+/+}$ and $Rlx^{-/-}$ mice at term. Despite the higher expression of the majority of MMPs we examined in $Rlx^{-/-}$ mice, there was no histological evidence of increased ECM degradation in the cervix and vagina in late gestation. Although previous *in vitro* studies suggest that relaxin positively regulates MMP activity, our data demonstrate that relaxin deficiency does not result in decreased MMP expression in the mouse cervix and vagina *in vivo*.

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A DIFFERENTIAL PATTERN OF FOLLISTATIN EXPRESSION IN THE PLACENTA BETWEEN SPONTANEOUS, INDUCED AND NON-LABOURING PATIENT GROUPS

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Follistatin has been identified in human placenta, fetal membranes and fluids, with serum follistatin concentrations rising during pregnancy, particularly near term. Our laboratory has shown follistatin concentrations rise across labour in spontaneous but not induced women.[1] As the placenta is a source of follistatin, this study examined placental tissues using immunohistochemistry to determine differences in follistatin localization between groups. Placental tissue was collected immediately following delivery from three groups of women at term, spontaneous onset (n = 4), induction (n = 4) and non-labouring caesarian (n = 4), and immediately formalin fixed. Antigen-retrieval immunohistochemistry using a specific chicken polyclonal antiserum (CK20) raised against a follistatin peptide (AA 121-133) or pre-immune chicken serum was performed. Positive staining of syncytiotrophoblast cells of the chorionic villi was seen in patients undergoing spontaneous labour but not in the induced and caesarian delivery group. The two labouring groups (spontaneous and induced) both showed positive staining for the vascular endothelial cells within the chorionic villi and the stratum basale, whilst the caesarian delivery group was negative for any staining within these vessels. Positive staining of Hofbauer cells was observed in both labouring groups; however, the caesarian group showed infrequent positive staining of these cell types. The differences in expression pattern in the two labouring groups (spontaneous *v.* induced) may be due to variations in labour lengths (6.5 *v.* 4.5 h, respectively); however, we would have expected a lower level of expression in the same cell types rather than the complete absence of staining. The positive follistatin staining in the syncytiotrophoblast of spontaneous patients suggests this may be the source of the rising plasma follistatin seen in this group. These differences in staining support our hypothesis that an earlier endocrine signal is absent in the induced and caesarian patient groups.

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ISOLATION OF CAG REPEAT CONTAINING GENES FROM HUMAN PLACENTA AND DECIDUA

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Pre-eclampsia is a serious disorder of pregnancy that manifests clinically in the mother as new-onset hypertension and proteinuria. Although the precise cause remains unknown, the placenta and the decidua play a fundamental role. The worldwide incidence of pre-eclampsia is 2–5% and such a high incidence, in the face of strong negative selection, suggests that the gene(s) involved have a selective advantage and/or a high mutation rate. One class of genetic diseases that involve a high mutation rate are the trinucleotide repeat expansion diseases. In these diseases repeated trinucleotide DNA sequences within specific genes multiply or expand up to 1000-fold. The result of this gene expansion/mutation is altered gene function that confers genetic susceptibility.

Thus, the overall objective of this study was to determine whether there is an association between a trinucleotide (CAG) repeat expansion and pre-eclampsia. The specific aim of this study was to isolate CAG repeat containing genes from human placenta and decidua. An adaptation of the mRNA differential display technique and traditional cDNA library screening was used.

In total, 72 placental and 51 decidual sequences were analyzed using the BLAST nucleotide comparison program. Five cDNAs were analyzed further. The unique sequences surrounding the CAG repeat regions of these five genes will be used to generate primers to ascertain if any of these repeat DNA sequences vary in number in the normal population. If polymorphic genes are identified, the primers will be used on pre-eclamptic pedigrees to determine if pre-eclampsia is associated with a repeat expansion mutation.

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FRACTALKINE, HCC-1 AND MIP-1B PROMOTE HUMAN TROPHOBLAST MIGRATION

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Human embryo implantation is a complex process requiring the attachment of an activated blastocyst to receptive endometrial epithelium and subsequent trophoblast invasion throughout the first trimester of pregnancy. Chemokines, including fractalkine (FKN), MCP-3, HCC-1 and MIP-1β, are produced by human endometrial epithelial and decidual cells with maximal production around the time of implantation/early pregnancy.[1,2] Chemokine and receptor expression was characterized in cell types at the human maternal–trophoblast interface. Highly abundant expression of chemokine receptors CX_3CR1 and CCR1 was observed in first trimester placenta and in trophoblast cells.[3] We hypothesized that CX_3CR1 and CCR1 ligands (FKN, MCP-3, HCC-1 and MIP-1β) produced by endometrial epithelial and decidualised stromal cells at the time of implantation promote migration of human trophoblast. We aimed to localize specific chemokine receptors in human first trimester tissue, and to determine whether trophoblast migration could be stimulated by the endometrium and by chemokines. Cellular localisation of specific receptors was assessed by immunohistochemistry in human first trimester implantation sites. Using an *in vitro* assay, trophoblast migration was assessed in response to human endometrial epithelial (HEEC) and decidualised stromal cells (DESC) (serum-free) conditioned medium and to recombinant human FKN, MCP-3, HCC-1 and MIP-1β. CX_3CR1 and CCR1 protein was localised to the vascular extravillous trophoblast (EVTs), but not to the invading interstitial EVTs, with weak staining on the syncytium. Significant migration of cells occurred in response to conditioned media from HEEC and DESC. FKN, MIP-1β and HCC-1, but not MCP-3 also promoted significant trophoblast migration. Neutralizing antibodies for FKN and MIP-1β but not MCP-3 significantly reduced migration to conditioned media, indicating that at least these two chemokines contributed to the effects. These data support a role for endometrial derived chemokines in promoting human trophoblast migration.

(1) Jones *et al*. (2004). *JCEM* **89**(12), 6155–6167.
(2) Hannan *et al*. (2004). *Reprod. Fert. Devel.* **16**(Suppl.), A225, p. 78.
(3) Hannan *et al*. (2004). *JCEM* **89**(12), 6119–6129.

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EFFECT OF *IN VITRO* EMBRYO CULTURE ON PLACENTAL GENE EXPRESSION IN THE SHEEP

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In vitro culture (IVC) systems feature commonly in reproductive technologies used in livestock. However, these culture conditions impact on the metabolism and physiology of the developing embryos, as well as on fetal outcome. Culturing rodent embryos in simple defined media results in decreased postimplantation viability and fetal growth rate.[1,2] Cytokines and growth factors are present *in vivo* but are absent from culture and may be causal in the perturbed fetal growth observed. Furthermore, the occurrence of large offspring syndrome (LOS) following embryo IVC in ruminants has been reported and is associated with loss of genetic imprinting.[3,4] Abnormal placental development following IVC is also likely to involve perturbed expression of imprinted genes including insulin-like growth factor II (IGF2) and its receptor (IGF2R). The IVC system used for this study included a control embryo transfer group without culture (ET, $n = 11$), *in vitro* cultured embryos in serum free defined medium (IVC-NS, $n = 10$) and *in vitro* cultured embryos in defined medium with human serum (IVCHS, $n = 8$). The cultured embryos were transferred to recipient ewes and placentomes were collected at 144–145 days gestation. Fetal weight (kg) was increased in IVCHS (5.15 ± 0.28) compared to ET (4.12 ± 0.24, $P = 0.017$) and IVC-NS (4.36 ± 0.27). Real-time RT-PCR was used to quantify IGF2 and IGF2R mRNA expression normalized to housekeeper RpP0. Although IGF2 expression was increased in the IVCHS group (2.27 ± 0.44) when compared to ET (1.22 ± 0.37) and IVC-NS (1.17 ± 0.43) groups, this was not significant. In addition, IGF2R expression was increased in the IVCHS (0.008 ± 0.003) group compared to ET (0.003 ± 0.001) and IVC-NS (0.004 ± 0.001) groups, but this was also not significant. IGF2 and IGF2R expression were, however, positively correlated in IVCNS ($r = 0.72$) and IVCHS ($r = 0.95$) placentomes, but not control ET placentomes. The presence of serum in IVC promoted fetal growth and increased expression of IGF2 and IGF2R mRNA in placental tissue. Comparison of placental gene expression from IVCHS and naturally mated pregnancies would be valuable to assess the role of serum in placental and fetal development.

(1) Bowman & McLaren, 1970.
(2) Kaye & Gardner, 1999.
(3) Thompson *et al.*, 1995.
(4) Young *et al.*, 1998.

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THE EXPRESSION OF CASPASE-14 IN THE HUMAN PLACENTA

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Pro-apoptotic genes have a role in the differentiation process in the placenta leading to the fusion of cytotrophoblast cells to form the protective syncytiotrophoblast layer. The mechanisms of apoptosis in the human placenta are not clearly understood. However, a major placental apoptotic-signalling pathway is known to involve the caspases. Caspase-14 is the most recently discovered member of the caspase family members and has not previously been examined in the human placenta. The aim of the present study was to detect caspase-14 in the human placenta and study its role in apoptosis. Human placentae were collected from first trimester and term gestation. The study consisted of two parts. In the first part, first trimester and term placentae were assessed for caspase-14 by western blotting and mRNA analysis and localised with immunohistochemical studies. In the second part, apoptosis in first trimester placenta was inhibited in an *in-vitro* model of explant villi culture with superoxide dismutase (SOD) treatment and the genes assessed. The first study demonstrated caspase-14 to be a cytoplasmic protein localised in the cytotrophoblast cells, the mesenchyme and in the syncytiotrophoblast of the first trimester. In the term placenta, caspase-14 was expressed weakly in the syncytiotrophoblast. The immunostaining data suggest a higher expression of caspase-14 in the first trimester compared to the term placenta, and this observation was later confirmed by western blot analysis. Using the SOD *in-vitro* explant culture model, no significant difference in the caspase-14 protein levels were seen in either the SOD or control group. This novel study demonstrates for the first time that caspase-14 protein and mRNA are present in the human placenta. The function of caspase-14 in the human placenta is unclear.

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EXPOSING NECROTIC TROPHOBLASTS TO ENDOTHELIAL CELLS *IN VITRO* CAUSES INCREASED ADHESION OF MONOCYTES

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A number of studies suggest that there is a generalized endothelial cell activation and inflammatory response in preeclampsia, which may be caused by factors released from the placenta including deported trophoblasts. Trophoblasts are the placental cells that are bathed in maternal blood during pregnancy and as they become aged or damaged trophoblasts are shed from the placenta and deported into the maternal circulation. The fate of deported trophoblasts is unknown but we have found that endothelial cells can phagocytose dead trophoblasts. The aim of this study was to examine the effects of phagocytosing dead trophoblasts on endothelial cell–monocyte interactions. *Methods:* The trophoblast-derived cell lines Jar and Jeg-3 were induced to undergo necrotic death by freeze/thawing or apoptotic death by exposure to UV light. HMEC-1 endothelial cells were labeled with green fluorescent cell tracker stain and then exposed to necrotic or apoptotic trophoblasts for 3 or 24 h. U937 (monocyte) cells were labeled with red fluorescent stain and incubated with the HMEC-1 monolayers for 3 or 24 h. The adhesion of the U937 cells to the HMEC-1 monolayers was quantified by flow cytometry and compared to the adhesion of U937 cells to untreated HMEC-1 monolayers. *Results:* Exposing the HMEC-1 cells to necrotic, but not apoptotic, trophoblasts induced an approximately two-fold increase in the adhesion of U937 cells to the HMEC-1 monolayers ($P = 0.01$). The findings were consistent regardless of whether the HEMC-1 cells were exposed to the dead trophoblasts for 3 or 24 h. *Conclusions:* We have previously shown that endothelial cells phagocytose both apoptotic and necrotic trophoblasts. The results of the current study suggest that shedding necrotic trophoblasts from the placenta could induce endothelial cells to become activated resulting in increased leucocyte adhesion. Thus, dead trophoblasts may be one of the factors released from the placenta that induce preeclampsia.

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P-GLYCOPROTEIN LIMITS ACTIVATION OF THE GLUCOCORTICOID RECEPTOR IN PLACENTAL BEWO CELLS

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Inadequate placental growth and function are key determinants of fetal growth retardation. Glucocorticoids potently inhibit fetal and placental growth via activation of the glucocorticoid receptor (GR). Placental and fetal glucocorticoid exposure is minimised by the 'placental glucocorticoid barrier', which consists primarily of placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) converting maternal glucocorticoids to inactive metabolites. Recent studies in the rodent brain show that P-glycoprotein (P-gp) is also an important physiological regulator of glucocorticoid access to the GR in target cells. Therefore, we hypothesised that placental P-gp may serve to exclude maternal glucocorticoids from the placenta and fetus, and thereby augment the barrier. We have used the placental choriocarcinoma cell line BeWo, and MDR-BeWo, a daughter cell line virally transduced with P-gp, to assess whether P-gp regulates access of dexamethasone to the GR.

Quantitative PCR analysis showed that MDR-BeWo cells express ~10-fold higher levels of P-gp mRNA than BeWo cells. Syncytialisation of BeWo and MDR-BeWo cells with 20 μM forskolin also increased P-gp mRNA by ~7-fold in each cell line. The elevated P-gp expression in MDR-BeWo cells resulted in a reduced activation of the GR with 1 μM dexamethasone by ~50% ($P < 0.001$) in comparison to BeWo cells. Accordingly, dexamethasone-induced apoptosis was reduced in MDR-BeWo cells, as indicated by a lack of induction of cleaved caspase 3 protein. Additionally, the P-glycoprotein inhibitor cyclosporin A (10 μM) did not increase the level of dexamethasone-induced GR activation in the low P-gp expressing BeWo cells, but potentiated GR activation by ~2-fold in the MDR-BeWo cells, to a level comparable to that in BeWo cells.

These data support the hypothesis that P-glycoprotein contributes to the placental glucocorticoid barrier. Thus, 11β-HSD2 and P-glycoprotein are likely to act in unison to reduce fetal and placental exposure to maternal glucocorticoids and minimise their growth inhibitory actions.

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LOCALISATION OF INSULIN-LIKE GROWTH FACTOR-II (IGF-II) AND ITS RECEPTOR IN EARLY MURINE PREGNANCY: A ROLE IN PLACENTATION AND ANGIOGENESIS IN THE DECIDUA?

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The highly invasive activity of the human placenta is tightly regulated by a variety of growth factors and other molecules. Contrary to the dominant view, recent data suggests that IGF-II, upon binding to the IGF2R, can stimulate an intracellular signalling pathway.[1] Evidence in humans and mice suggests that IGF-II and the IGF2R are important regulators of placental growth; however, to date they have not yet been localised to early murine implantation sites. This study provides a photo micrographic account of early placental development and the decidual vasculature in the mouse, and localises IGF-II and the IGF2R from days 5.5 to 10.5 of pregnancy. During early pregnancy, the decidua displays a paucity of blood vessels, which appear to undergo angiogenesis, so that by day 10.5 the decidua has become a highly vascularized structure, with an extensive network of dilated vessels that presumably enable maximal blood supply to the placenta. Unlike humans, murine trophoblast cells do not invade the endometrium individually, but remain in close contact with the main giant cell layer. The trophoblast giant cells (TGCs) are the outermost cell type of the murine placenta and maternal blood spaces beneath this layer are not lined by endothelium. Due to their location, TGCs appear to play a direct role in displacing this endothelium and therefore may play a role in the transformation into trophoblast lined maternal blood spaces. IGF-II and its receptor were present throughout early pregnancy in the conceptus and maternal decidua supporting their role as regulators of fetal and placental development. Most interesting, however, was their association with the developing maternal blood vessels in the mesometrial decidua. It seems likely that in mice the maternal vessels are remodelled by a variety of locally derived molecules. By association, IGF-II and its receptor are likely candidates.

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EXPRESSION AND CELLULAR LOCALIZATION OF HTRA3 PROTEASE DURING PLACENTAL DEVELOPMENT IN MICE

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Placental development in mice involves highly regulated interactions between fetal- and maternal-derived cells. We have previously cloned a novel serine protease (HtrA3) containing an insulin-like growth factor (IGF) binding domain, which was upregulated during pregnancy, especially post-implantation in the mouse uterus.[1] The present study examined HtrA3 regulation during placental development in mice, in particular, its expression in the different compartments of the placenta. Expression of mRNA was determined by Northern blot analysis in implantation units containing the decidua, placenta and fetus (day 8.5 to near-term). A specific HtrA3 antibody was generated, affinity-purified and used for Western blot analysis and immunohistochemistry. Both mRNA and protein of HtrA3 were identified specifically in the maternal decidua. In contrast, HtrA3 expression was below detection in trophoblasts, including the giant cells that are in direct contact with the decidua. This pattern persisted from the early stages of placentation to near term. The level of decidual HtrA3 mRNA and its protein gradually decreased as the placenta matured. In the decidua, only the maternal decidual cells, but not blood vessels or uterine NK cells that are present in large numbers, were positive for HtrA3. The specific localization of HtrA3, a protease possessing an IGF binding domain at the maternal–fetal interface, suggests that this protein plays an important role in mediating maternal decidual remodelling and maintenance, probably in association with the IGF system, in placental development and function.

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FOLLISTATIN SECRETION BY THE OVARY IS NOT DIRECTLY RELATED TO PGF2α INDUCED LUTEOLYSIS IN THE EWE

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Follistatin is a monomeric glycoprotein, expressed ubiquitously, and found in greatest levels in ovarian tissue. Our previous studies have demonstrated that ovarian venous follistatin levels are elevated at the same time as spontaneous luteolysis in merino ewes, but remain constant in circulating and uterine plasma across oestrous. It was hypothesized that the follistatin elevation may be part of functional luteolysis in the ovary.

A cross-sectional study of ovarian venous follistatin during luteolysis was performed using merino ewes, which were synchronized with intravaginal sponges. Luteolysis was induced 10 days after oestrus using a PGF2α (IM) injection. Circulating (jugular and arterial), uterine venous and ovarian venous (luteinized and contralateral) blood samples were taken under general anaesthesia at times 0, 6, 12, 24, 36, 48 and >48 h (54–71 h) after the PGF2α injection. All samples were assayed for progesterone by RIA and follistatin using a specific follistatin antiserum (#204) in a competitive ELISA with rhFS-288 used as standard.

Progesterone concentrations in the venous plasma from the luteinized ovaries plasma were significantly higher than those in the contralateral ovarian and circulating plasma. As expected progesterone concentrations began declining within 6 h and continued until 24 h after PGF2α. Mean circulating plasma follistatin concentrations were similar throughout the experiment with overall means of 9.9 ± 0.5 ng/mL. Both luteinized and contralateral ovarian venous plasma follistatin concentrations (11.8 ± 3.4 ng/mL) were not significantly different from circulating concentrations. However at 48 h ovarian venous follistatin concentrations were significantly elevated (22.5 ± 2.3 ng/mL) and remained elevated for at least 71 h (29.5 ± 7.9 ng/mL).

This experiment confirms our previous work that both luteinized and contralateral ovarian venous follistatin concentrations do elevate concurrently with each other but that the elevation in follistatin is not directly related to functional luteolysis as induced by PGF2α.

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REGULATION OF TGFβ SUPERFAMILY-RELATED GENES IN THE NEWBORN MOUSE TESTIS BY ACTIVIN

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Several members of the transforming growth factor-β (TGFβ) superfamily of ligands are known to influence development of male germ cells. In our ongoing investigations of genetically modified mice with different bioactive levels of activin, we noted that testicular germ cell numbers at birth are increased in the absence of activin.[1] In this study we compared expression levels of TGFβ-related genes in mice with 0, 1 or 2 copies of the *activin βA* subunit gene. Candidate genes were identified through hybridisation of total testis RNA from newborn (Day 0) wild type (WT; 2 copies) and *activin βA* null mice (KO; 0 copies) to a TGFβ SuperArray Simplicity™ membrane. Densitometric analysis of resulting signals revealed several genes that appeared to differ between genotypes. To validate these findings, pooled testes from newborn WT (2 independent groups), heterozygous (het; 2 groups) and *activin βA* KO (3 groups) mice were collected for RNA extraction. Quantitative real-time PCR analysis was performed using the Roche Light Cycler, with β-actin mRNA level used as the housekeeping reference. The mRNAs selected for analysis were: *inhibin α*, *p15INK4b*, *Smad5*, *insulin-like growth factor 1 (IGF-1)*, *ALK6 (BMPRI-B)*, and *tissue inhibitor of metalloproteinase 1 (Timp1)*. The *inhibin α* transcript level was significantly decreased in the *activin βA* KO animal compared with WT, while the het sample showed an intermediate effect. Both *Smad5* and *IGF-1* transcripts appeared to increase in the KO animals compared with WT; however, the cyclin-dependent kinase inhibitor, p15INK4b, did not change significantly across the genotypes. Similarly, *ALK-6* and *Timp1* mRNA levels were also unaffected by genotype. These findings illustrate the impact of graded levels of activin A on specific genes in the fetal testis.

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THE ROLE OF TESTIS SPECIFIC PROTEIN 1 (TPX-1) AND GAMETOGENETIN (GGN) IN MAMMALIAN SPERMATOGENESIS

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The sperm tail has a key role in male fertility, since it is responsible for motility, driving sperm toward and into the ovum. In this study, we characterized a sperm tail protein, testis specific protein 1 (Tpx-1), which is exclusively expressed in sperm and is localized to the acrosome and the outer dense fibres (ODF) of the sperm tail. We carried out yeast two-hybrid screening using an adult testis cDNA library to identify interacting protein partners of Tpx-1. A number of putative Tpx-1 interacting proteins were identified including a novel germ cell-specific protein, gametogenetin (Ggn). Ggn is highly expressed in the adult testis, specifically in late pachytene spermatocytes through to round spermatid germ cells. Ggn has more than 10 splice variants giving rise to three proteins namely GGN1, GGN2 and GGN3.[1] The biological significance and potential for *in vivo* interactions between Tpx-1 and Ggn was assessed using Northern blot analysis, immuno-histochemistry and co-immunoprecipitation. To further investigate the nature of Tpx-1 interaction with Ggn, deletion studies were performed in yeast. The cysteine-rich carboxy terminal domain of Tpx-1 was shown to be responsible for binding a region in the last 120 amino acids of Ggn. Since the Ggn clone used for analysis encoded only this carboxy-terminal portion of the Ggn protein, it is also possible that other regions of the protein are involved in the interaction with Tpx-1. Further studies involving full-length clones and/or amino-terminal encoding Ggn clones will be needed to explore the possibility of additional interacting regions. In addition, Tpx-1 and Ggn knockout mice are also being generated. These results provide a greater understanding of the normal processes involved spermatogenesis and may suggest directed means to enhance or suppress male fertility.

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C-KIT EXPRESSION STUDY: TIMING OF ONSET IN RODENT TESTIS AND IRRADIATED RAT TESTIS MODEL

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Primordial germ cell and spermatogonial cell function is essential for normal male fertility. These cells require Sertoli–germ cell interactions, specifically somatic cell-derived stem cell factor (SCF) that acts through the *c-kit* receptor to govern primordial germ cell migration in the foetus, spermatogonial differentiation during puberty and adulthood, and Leydig cell steroidogenesis. We performed a comprehensive study of the *c-kit* mRNA expression profile in the pre- and post-pubertal mouse and rat testes by *in situ* hybridisation. Expression of *c-kit* mRNA was first visualised in germ cells after birth, with the levels concordant with the number and appearance of the differentiated spermatogonial subtypes in both the rat and the mouse. We also studied *c-kit* expression in the irradiated adult rat testis, in which only undifferentiated spermatogonia are present. After treatment with Cetrorelix, GnRH antagonist (3 days, 1, 2 and 4 weeks) germ cell maturation is re-initiated. Expression of *c-kit* messenger RNA was observed in the undifferentiated spermatogonia in both untreated and treated testes sections. In contrast, c-kit protein expression was undetectable until 4 weeks of hormone treatment. This suggests that *c-kit* mRNA and protein expression are differentially regulated and that protein expression relates to somatic cell function.

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INTERACTION OF CDYL (CHROMODOMAIN Y-CHROMOSOME LIKE) WITH THE NUCLEAR TRANSPORT PROTEIN IMPORTIN A2

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Spermatogenesis is a unique, ordered process governed by the precise expression of a specific set of genes at each stage. Progression through successive stages requires the shuttling of proteins and transcription factors into and out of the nucleus to implement changes in gene transcription. Major factors that mediate nucleocytoplasmic transport are members of the importin superfamily, of which there are five and 20 different importin α and β genes, respectively, in mouse. We have previously demonstrated that several importins display distinct mRNA and protein expression patterns in adult mouse testis[1] indicating that specific importins carry a specific cargo at discrete stages of spermatogenesis. Identification of importin cargoes in the testis should help describe the potential developmental switches critical to the spermatogenic process. We performed a yeast two-hybrid screen using full length importin α2 as bait and an adult mouse testis library, identifying nine target proteins. Some of these proteins include nuclear components that may be important in eliciting changes in the nuclear structure during spermatogenesis, as well as those involved in cell cycle regulation, homologous chromosome pairing and recombination, transcriptional regulation and guanine nucleotide biosynthesis. One key candidate is CDYL, which has been implicated in male infertility. It is a chromodomain-containing protein that is predominantly expressed during spermiogenesis and has been previously described to participate in hyperacetylation of histone H4, which is believed to facilitate protamine replacement of histones during spermiogenesis. Verification of CDYL-importin α2 interaction was demonstrated using co-immunoprecipation and co-transfection, while immunohistochemical staining of testis sections indicated colocalisation in the same cell types (mainly elongating spermatids). Importantly, preliminary experiments indicated that increasing CDYL nuclear accumulation by over-expressing importin α2 can increase histone H4 acetylation. Our hypothesis is that importin α2 is central in nuclear targeting of CDYL to facilitate its hyperacetylation role during protamine-histone exchange.

(1) Hogarth *et al.* (2005). *Dev. Dynamics* (submitted).

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EXPRESSION OF *WSB2* IN THE MOUSE TESTIS

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We present a detailed study on the expression pattern of *Wsb2* in the mouse foetal and adult gonad. *Wsb2* expression was analysed during mouse embryogenesis by whole-mount, section *in situ* hybridisation and immunohistochemistry. *Wsb2* was found to be expressed in the developing mouse gonads from 11.5 dpc to 16.5 dpc. Expression is initially equal in both sexes from 10.5 dpc until 12.0 dpc, then it persists in the male gonad. *Wsb2* expression was confined to the cords in both Sertoli cell and germ cells. Other sites of Wsb2 embryonic expression were the somites, dorsal root ganglia and the lateral mantle layer of the neural tube.

mRNA encoding *Wsb2* and Wsb2 protein has been detected in the newborn testis in both gonocytes and Sertoli cells. *Wsb2* mRNA in the adult mouse testis was observed in Sertoli cells, spermatogonia, spermatocytes and the corresponding Wsb2 protein expression was in pachytene spermatocytes, round and elongated spermatids, Sertoli cells and Leydig cells.

The differential expression of Wsb2 in male versus female embryonic gonads suggests it may play a role in mammalian sex determination during embryonic development and its expression in the first wave of spermatogenesis and in the adult suggests a later role in spermatogenesis.

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PREVALENCE OF SPERM CHROMATIN INSTABILITY AMONGST BULLS IN A SUBTROPICAL ENVIRONMENT: A PRELIMINARY INVESTIGATION

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During mammalian spermiogenesis nucleosomal histone proteins bound to DNA are replaced by protamines. The integrity and stability of the protamine-DNA association in sperm can be measured using the sperm chromatin structure assay (SCSA). The recent emergence of compelling information from assisted reproduction in humans of a relationship between sperm chromatin stability and the establishment and maintenance of pregnancy has led to considerable interest in production and recreational animals.[1] The aim in the present study was to determine the incidence of sperm chromatin instability amongst bulls in a subtropical region of northern Australia, as a first step in evaluating whether sperm chromatin instability is a contributing factor to reproductive wastage in cattle. Semen was obtained from 565 *Bos indicus* and *Bos indicus* x *taurus* bulls from northern and central Queensland aged between 20 months and 10 years. Samples were subjected to standard semen evaluation[2] and aliquots stored in liquid nitrogen until chromatin integrity was determined using the SCSA.[3] Samples exposed to the SCSA for 0.5 min revealed 4.9% of bulls had a DFI >27% and this increased to 11.5% of bulls with DFI >27% when samples were exposed to the SCSA for 5 min. DFI was significantly correlated with sperm density, mass activity, motility and morphology. Location appeared to have a greater influence on DFI than genotype. Preliminary data from a small sample of bulls would suggest that a relatively high DFI can be repeatable for individual bulls. These findings indicate that sperm chromatin instability occurs in bulls in northern Australia although the prevalence might be considered to be relatively low. The relationship of sperm chromatin instability to the contribution of bulls to embryonic mortality requires further study and likewise the impact on reproductive wastage remains to be determined.

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EQUINE GROWTH HORMONE ENHANCES MOTILITY AND EXTENDS LONGEVITY OF RAM SPERMATOZOA *IN VITRO*

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Sperm survival *in vitro* decreases with time at room temperature, but may be improved by treatment with recombinant bovine growth hormone in rats[1], bulls[2] and horses[3]. Two experiments investigated the effect of equine growth hormone (eGH) on the longevity of ram spermatozoa *in vitro.* The aim of experiment 1 was to determine if the addition eGH would improve the motility of ram spermatozoa after 24 h and identify any interaction with semen dilution rates used for ram semen preservation. Semen was collected from five mature Merino rams. Ejaculates were assessed for good quality and were diluted 1 + 50, 1 + 4, 1 + 3, 1 + 2 (semen + diluent) with a Tris-based cryoprotectant. Aliquots from each ram were mixed with eGH to achieve a final concentration of 100 ng/mL eGH and stored at 20°C for 24 h. Motility of spermatozoa was then assessed manually. eGH improved the motility of spermatozoa at all dilution rates compared to controls ($P < 0.0001$) but most markedly in the 1 + 3 and 1 + 2 samples (42.6 ± 0.8% eGH *v.* 22.7 ± 2.6% control; 40.5 ± 1.4% eGH *v.* 22.7 ± 2.2% control, respectively, $P < 0.01$). The aim of experiment 2 was to determine the optimum eGH concentration for improving sperm motility. eGH was added to aliquots of diluted semen (1+3 dilution rate) to produce samples with final concentrations of 1000, 100, 10 and 1 ng eGH/mL. The samples were placed in a water bath at 20°C for 24 h at which time the motility of sperm was assessed as before. Sperm motility was higher in the 100 ng/mL eGH sample ($P < 0.05$; 39.6 ± 0.7%) compared to other concentrations (1000 ng/mL 11.8 ± 0.7%, 10 ng/mL 21.5 ± 0.7% and 1 ng/mL 11.3 ± 0.7%). We conclude that growth hormone is effective in promoting the longevity *in vitro* of ram spermatozoa stored at room temperature, and that this effect is concentration dependent.

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(2) Sauerwein *et al.* (2000). *Dom. Anim. Endocrinol.* **18**, 145–158.

(3) Champion *et al*. (2002). *Theriogenology* **57**, 1793–1800.

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SPRASA, A SPERM PROTEIN WITH A POST-FERTILIZATION FUNCTION?

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Objectives: SPRASA is a highly conserved sperm protein that is localised to the inner acrosome membrane, and shows high homology to the alpha-lactalbumin/C-type lysozyme family.[1,2] We have previously shown that SPRASA is the antigen for antisperm antibodies in some infertile patients.[1] To date, the function of SPRASA is unknown but *in vitro*, antibodies reactive with SPRASA inhibit sperm-oocyte binding in a zona-free hamster oocyte binding assay[2]. Based on this preliminary data, we postulated that SPRASA plays an integral role in fertilization, and that binding of antibodies to SPRASA may inhibit its function leading to infertility. In this study we investigated the effect of inhibiting bovine SPRASA, *in vitro,* on fertilization and embryonic development. *Methods:* Viable, motile sperm was prepared from frozen-thawed bovine sperm by swim up. Bovine oocytes were obtained from slaughter-house killed animals and matured *in vitro*. Three different treatments were investigated. (1) Sperm were incubated for an hour with a SPRASA-reactive antiserum, washed and used to fertilize 100 oocytes in insemination droplets. (2) One hundred *in vitro* matured oocytes were incubated for an hour with the SPRASA-reactive antiserum, washed, and fertilized with untreated swim-up sperm in insemination droplets. (3) Sperm and oocytes (n = 100) were coincubated with the SPRASA-reactive antiserum in insemination droplets. Controls consisted of similar numbers of sperm and/or oocytes incubated with an irrelevant antiserum. *Results:* There was a significant reduction in the number of embryos that reached morula ($P = 0.03$) or blastocyst ($P = 0.01$) when oocytes were pre-treated with the SPRASA antiserum (treatment 2) or when sperm and oocytes were co-incubated with the antiserum ($P = 0.05$ morula; $P = 0.01$ blastocyst; treatment 3). However, there was no significant difference in the rates of embryos reaching earlier developmental stages in any of the treatment groups. *Conclusion:* These data suggest that SPRASA may be expressed by oocytes and/or preimplantation embryos.

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(2) Mandal A, Klotz KL, Shetty J, Jayes FL, Wolkowicz MJ, Bolling LC, Coonrod SA, Black MB, Diekman AB, Haystead TA, Flickinger CJ, Herr JC. (2003). SLLP1, a unique, intra-acrosomal, non-bacteriolytic, c lysozyme-like protein of human spermatozoa. *Biology of Reproduction* **68**(5), 1525–1537.

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SPERM MORPHOLOGY WITHIN THE TESTIS AND CAUDA EPIDIDYMIS OF A TASMANIAN DEVIL (*SARCOPHILUS HARRISII*)

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A male Tasmanian devil, aged 6.5 years, was euthanased with intravenous pentobarbitone overdose due to the diagnosis of squamous cell carcinoma. The testes and cauda epididymes were removed immediately after euthanasia and placed into TALP-HEPES medium containing 3% BSA and dissected. Smears of the isolated sperm were made, air-dried and stained using Diff-Quik. Sperm were classified using a modification of a system originally applied to the brush-tailed possum[1] as follows:

(a) Immature I – head is perpendicular or greater than 45° to the tail.
(b) Immature II – head forms an acute angle of 45° or less with the tail.
(c) Immature III – head and tail lie in a straight line but the sperm head still has a partly expanded acrosome.
(d) Mature –the head is aligned with and has a diameter similar to the tail.

No motility was seen in the testicular sperm, but >60% of sperm from the cauda epididymes were motile. The stained preparations showed a significant difference in the distribution of sperm between the testis and epididymis ($\chi^2 = 150.45$, df = 3, $P < 0.001$), with sperm within the testis being skewed towards the earlier stages of development whereas the epididymis was skewed towards the later stages of development.

Category of sperm morphology	Sperm source	
	Testis	Cauda epididymis
Immature I	63 (37.3%)	32 (20.3%)
Immature II	103 (60.9%)	25 (15.8%)
Immature III	3 (1.8%)	33 (20.9%)
Mature	0	68 (43.0%)
Total	169 (100%)	158 (100%)

These results confirm the similarity with several other marsupials in that sperm are formed in the testis with the head at right angles to the tail but rotation of the head to become aligned with the tail is completed during epididymal transport. Work is now required to identify optimum conditions for the culture, cryopreservation and insemination of sperm from this species.

(1) Cummins JM. (1976). Epididymal maturation of spermatozoa in the marsupial Trichsurus vulpecula: changes in motility and gross morphology. *Aust. J. Zool.* **24**, 499–511.

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THE EPIDIDYMIS SECRETES PROTEINS INVOLVED IN SPERM COMPETITION: EVIDENCE FROM THE ECHIDNA

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Although there is general recognition that the mammalian epididymis is unique among the vertebrates and is essential for male fertility, there is relatively little understanding of its role in the process of reproduction. It has been suggested that it plays an important role in the competition between males to achieve paternity (sperm competition) by accumulating and storing sperm.[1] We now provide evidence that specific protein synthesized and secreted by the epididymis of the echidna plays a major role in sperm competition by binding spermatozoa into bundles of about a 100 sperm and consequently greatly enhancing their rate of motility, particularly in viscous medium. The epididymis of the echidna is structurally differentiated into a large caput epididymidis that is structurally similar to the 'initial segment' of other mammals, and a small cauda epididymidis. Using radiolabelling and 2-D electrophoresis we have shown that the caput epididymidis synthesises and secretes a similar pattern of proteins along most of its length where sperm are not associated with one another. The sperm form into bundles as they enter the cauda epididymidis where the pattern of protein secretion changes considerably, being characterised by a new band of about 80 kDa in reduced gels. Electron microscopy of the bundles shows electron dense material binding the sperm together in the bundles. The bundles persist for more than an hour after sperm are released into physiological media.

(1) Jones, RC (1998). Evolution of the vertebrate epididymis. *J. Reprod. Fertil. Suppl.* **53**, 163–182.

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PLATELET DERIVED GROWTH FACTORS AND RECEPTORS IN THE RAT OVARY CONTRIBUTE TOWARDS PREANTRAL FOLLICLE GROWTH

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In this study, the family of platelet derived growth factors (PDGF) and receptors were identified and characterized in the rat ovary and a role in contributing towards growth of preantral follicles was revealed. Real-time polymerase chain reaction revealed the presence of mRNA for all platelet derived growth factors (PDGF-A, PDGF-B, PDGF-C and PDGF-D) and receptors (PDGF-Rα and PDGF-Rβ). *In situ* hybridization identified oocytes of primordial/primary follicles and cells of the theca layer as a source of PDGF-B, PDGF-C and PDGF-D mRNA. Protein expression was explored through immunohistochemistry. In rats aged days 0 and 4, PDGF-Rα, PDGF-A and PDGF-C immunoreactivity was observed within oocyte clusters, and PDGF-Rβ and PDGF-B immunoreactivity in cells surrounding oocyte clusters. In primordial follicles, PDGF-Rβ and PDGF-C was observed in the oocyte, and PDGF-Rα and A in the either the oocyte or pregranulosa cells. In primary follicles, PDGF-A, PDGF-C, PDGF-Rα and PDGF-Rβ are expressed in the oocyte. PDGF-Rβ is also expressed in cells surrounding primordial and primary follicles, possibly the precursors to theca cells. In secondary and antral follicles, all four PDGF isoforms and both receptors are expressed in either theca or vascular cells of the theca layer, and PDGF-Rα and A are also expressed in some granulosa cells in rats aged day 20 and older. A role in preantral follicle growth was identified by *in vitro* culture of preantral follicles. Preantral follicles cultured in serum free medium increased in diameter by 11.0 ± 1.57% over 5 days. Addition of PDGF-AA, PDGF-AB or PDGF-BB to the medium resulted in increases in follicle diameter after 5 days of 18.32 ± 2.18%, 17.72 ± 2.3% and 17.6 ± 1.81%, respectively, representing a significant increase over control diameters. In summary, this study has identified and characterized the presence and localization of all members of the family of platelet derived growth factors and receptors in the rat ovary and revealed a role for these growth factors in positively influencing early follicle growth.

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EARLY OVARIAN FOLLICLE DYSGENESIS IN A DISINTEGRIN AND METALLOPROTEINASE WITH THROMBOSPONDIN MOTIFS TYPE 1 (ADAMTS-1) NULL MICE

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ADAMTS-1 is a multi-domain, multi-functional matrix metalloproteinase expressed in the mouse ovary during folliculogenesis and ovulation. Adult ADAMTS-1 null female mice are infertile; however, the exact mechanism responsible for their reproductive failure is yet to be explained.

ADAMTS-1 null ovaries prior to and following ovulation were examined and a defect in ovarian follicle development was identified. A classification system was devised and used to identify morphological differences between normal and ADAMTS-1 null ovaries. The phenotype indicated a progressive loss of follicle structural integrity ranging from a slight loss of shape, to full structural dysgenesis. As a result, large numbers of oocytes with healthy appearance were found with no surrounding follicle structure. The numbers of preantral follicles was not altered, but type 6 (early antral) follicles in the ADAMTS-1 null ovaries were significantly reduced ($P < 0.05$) when compared to wild-type ovaries suggesting initiation of follicular degeneration coincident with antrum formation. There was also a significant decrease ($P < 0.05$) in the number of periovulatory follicles (type 7 and 8) in the ADAMTS-1 null ovaries. This suggests that late folliculogenesis, in the period of rapid growth and expansion is disrupted when ADAMTS-1 is not present and results in fewer follicles available for ovulation. Further, we have demonstrated that the active form of ADAMTS-1 is present in the thecal-granulosa boundary of the ovarian follicle suggesting a role in extracellular matrix remodeling at this boundary during follicle growth. Analyses of the basement membrane at this boundary both in growing and ovulating follicles indicate that ECM remodeling in this region is indeed disrupted.

These data demonstrate that remodeling of surrounding structural matrix is crucial to follicle growth and structural integrity. Functional ADAMTS-1 is important for matrix remodeling during the growth of the follicle, particularly during antrum formation.

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EXPRESSION OF MATRIX METALLOPROTEINASES IN BOVINE THECAL CELLS

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As follicles grow the thecal layers expand. It is likely that extracellular matrix is remodelled in this process and possibly by matrix metalloproteinases (MMPs). A promising candidate to regulate MMPs is insulin-like factor 3 (INSL3). It is produced by thecal cells, its receptor, LGR8, is expressed in the theca interna (unpublished) and a related molecule, relaxin, regulates turnover of matrix in a number of tissues. For this reason we sort to examine the role of INSL3 in matrix turnover. However, in all thecal cell culture systems examined LGR8 receptors appear to be down regulated within 24 h. We therefore examined the effects of second messenger pathway activators. Thecal and granulosa cells were isolated and cultured and the levels of RNA for MMP2 and 9 quantitated by RT-PCR. MMP2 mRNA levels in thecal tissues were >10 fold higher than in granulosa cells (n = 19 follicles >10 mm). MMP2 levels were substantially greater than MMP9. At 12 h phorbol ester (100 nM phorbol 12,13-didecanoate) increased thecal expression of MMP 9 mRNA levels 11.5 fold ($P < 0.001$) and at 48 h MMP2 mRNA was increased 5 fold ($P < 0.01$). Pieces of whole follicle wall [follicles <5 mm in diameter, classified as healthy (n = 12) or atretic (n = 6)] were cultured in serum free media. Expression of the steroidogenic enzymes 17β HSD and P450scc but not 3β HSD were detected by immunohistochemistry even after 10 days. MMP activity on day 2 was analysed by gelatin zymography. Treatment with phorbol ester increased active MMP9 19 fold ($P < 0.001$). Treatment of thecal cells or follicle walls with 1 mM dibutyryl cAMP induced additional MMP activities at sizes of 110 and 122 kDa. No effects on MMP2 activity were observed. In conclusion whilst we do not know the ligand inducers of the synthesis and activator of MMPs in thecal cells they can be regulated. Hence MMPs are candidates for remodelling the extracellular matrix of thecal layers.

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FSH RECEPTOR EXPRESSION IN SMALL HUMAN OVARIAN FOLLICLES

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Follicle-stimulating hormone (FSH) is pivotal in ovarian follicle development; the granulosa cells are the targets of FSH action in the ovary via FSH receptors. Granulosa cell growth and division mark initial follicle recruitment. The acquisition of FSH receptors on granulosa cells is regarded as a key event in hormone responsiveness and consequently follicle development. Due to the low abundance of FSH receptors and low expression of its mRNA it has been difficult to definitively characterise FSH receptor expression patterns. Here, localisation of FSH receptor in different follicle populations has been assessed with *in situ* hybridisation and real-time PCR of laser microdissected samples. We have used non-radioactive in situ hybridisation to investigate FSH receptor mRNA on a wide range of follicle stages. Biopsies from healthy fertile women (28–33 years) were frozen, embedded and cryosectioned at 10 μm. DIG-labelled RNA probes were designed to detect all splice variants. Hybridised probes were detected with NBT/BCIP in a colorimetric reaction. Secondly, follicles of different morphometric stages were isolated with a laser microscope. RNA extraction, reverse transcription and real-time PCR were used to confirm RNA presence and quantify relative expression. All follicle stages (from primordial to large antral) showed the presence of FSH receptor mRNA in their granulosa cells; sense controls were negative. Observations from real-time PCR indicate FSH receptor mRNA is present in all follicle stages observed and relative expression levels increase over early follicle development. These results challenge the existing doctrine that FSH receptor is absent in the smallest follicles. This suggests initial follicle recruitment may involve gonadotrophins. The use of sensitive molecular techniques will be crucial in elucidating this further.

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IN VIVO EVIDENCE FOR A ROLE OF BONE MORPHOGENETIC PROTEIN-4 IN OVARIAN FUNCTION

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Bone morphogenetic proteins (BMPs) were first identified on the basis of their bone inducing capacity, and later shown to be members of the transforming growth factor β (TGF β) super family. Nilsson *et al.*[1] studied the effect of BMP-4 on follicular development in rat ovaries and found that the addition of BMP-4 to whole ovary cultures led to more numbers of developing primary follicles but less numbers of primordial follicles. Their studies indicate that BMP-4 acts as a transition factor for the conversion of primordial follicles to primary follicles. To test this hypothesis *in-vivo*, we conducted passive immunization studies against BMP-4 in prepubertal female mice.

The mice were divided in to four groups (n = 5), and given daily SC injections of the following treatment: anti BMP-4 (50 μg), PMSG (10 IU) (pregnant mare serum gonadotropin) with and without anti BMP-4 (0.5 mg/mL) and PBS for 3 days. All experimentation was approved by animal ethics committee, University of New England, Armidale, NSW. On the fourth day the mice were killed and the ovaries removed and weighed. The mice treated with anti BMP-4 had significantly smaller ovaries (4.1 ± 0.4 mg) than the control group (8.6 ± 0.9 mg). PMSG stimulated ovarian weight (21.0 ± 1.2 mg) but anti BMP-4 (23.2 ± 1.3 mg) did not significantly affect the weight of the stimulated ovaries. This data confirms BMP-4 is important in ovarian function; however, it is unclear whether this effect is on the ovary directly or via FSH.

(1) Nilsson, E. E., Skinner, M.K. (2003). Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biology of Reproduction* **69**, 1265–1272.

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EFFECT OF FOLLICULAR STATUS ON SHEEP EMBRYO YIELDS IS MEDIATED BY CHANGES IN THE PREOVULATORY LH SURGE

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Follicular status at the start of superovulatory treatment (presence/absence of a dominant follicle/corpus luteum) has been reported to affect subsequent embryo yields.[1] The aim of the current study was to assess if this effect may be exerted through changes in the occurrence, or characteristics, of the preovulatory LH surge. Forty-three Manchega ewes were superovulated with eight decreasing doses of oFSH (OVAGEN™), starting on Day 12 after the insertion of a progestagen intravaginal sponge. At the first two FSH doses, diameter of both largest follicles (LF1 and LF2, respectively) were determined by ultrasonography. Blood sampling for LH determination was performed from 18 to 60 h after progestagen withdrawal. Ewes were also mated between 18 and 60 h after sponge withdrawal and embryos were recovered and evaluated on Day 21, being *in vitro* cultured until hatching. Moreover, the effect of the LH surge on superovulatory yields showed that ewes showing LH surges either with later onset or shorter duration showed higher rates of embryo viability after *in vitro* culture (r = 0.461, $P < 0.005$ and r = 0.980, $P < 0.0005$). In addition, earlier LH surges were related to a decreased number of non-viable embryos at recovery (r = 0.777, $P < 0.05$), due to a higher number of degenerated embryos, and to a lower viability rate after *in vitro* culture (r = 0.521, $P < 0.05$ and r = 0.562, $P < 0.0005$). Finally, the current study showed that ewes with a higher difference between LF1 and LF2 displayed earlier LH surges (r = 0.420, $P < 0.05$), which is related to decreased embryo yields (r = 0.777, $P < 0.05$).

(1) Gonzalez-Bulnes *et al*. (2002). *Theriogenology* **58**, 1607–1614.

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ALTERED MATRIX COMPOSITION OF CUMULUS OOCYTE COMPLEXES FOLLOWING *IN VITRO* MATURATION

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The luteinizing hormone (LH) surge initiates cumulus expansion, through synthesis of hyaluronan and cross-linking proteins including versican, which stabilise the cumulus oocyte complex (COC) matrix. Versican is a substrate for the protease ADAMTS-1 and mRNA for each are localised to granulosa cells (GCs) and greatly induced following the LH surge.

In humans, the use of *in vitro* maturation (IVM) of oocytes is an appealing option, reducing costs and risk of side effects associated with *in vitro* fertilisation. IVM oocytes are of poorer quality, likely resulting from altered gene expression and environmental conditions during oocyte maturation.

Real-time PCR showed that IVM and immature COCs from Balb/c mice have 12 and 13 fold-reduced levels of ADAMTS-1 and versican expression respectively compared to *in vivo* matured COCs (PMSG + hCG 12 h). Ovulated COCs (PMSG + hCG 15 h) had similar low levels of ADAMTS-1 and versican. Samples isolated from F1 C57Bl/6xCBA mice showed similar reduced versican and ADAMTS-1 mRNA. Western blot analysis revealed that full length and cleaved versican, from ADAMTS-1/4 activity, was not detected in immature COCs, was present in *in vivo* matured COCs isolated from follicles, but strongest in ovulated COCs. IVM COCs had no detectable versican protein, supporting the mRNA data. Full-length versican was also present in GCs after PMSG + hCG 12 h or 15 h. ADAMTS-1 protein was most abundant in *in vivo* matured COCs with reduced levels seen in ovulated COCs, but was absent from IVM and immature COCs.

These results indicate that ADAMTS-1 and versican are secreted products of granulosa cells that bind and incorporate into the COC matrix. The presence of versican and ADAMTS-1 is not essential for cumulus matrix expansion *in vitro*, but may contribute to oocyte maturation, ovulation of the COC and/or interaction with sperm during fertilisation.

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MOUSE OOCYTE PARACRINE SIGNALLING TO CUMULUS CELLS BY TGF-β SUPERFAMILY MOLECULES IS INDISPENSABLE FOR CUMULUS EXPANSION

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Oocyte-secreted factors are required for expansion of the mouse cumulus-oocyte complex (COC), which is necessary for ovulation. Members of the transforming growth factor-β (TGF-β) superfamily are prime candidates for the mouse cumulus expansion-enabling factor (CEEF), and we have recently determined that growth differentiation factor 9 (GDF9) alone is not the CEEF. This study was conducted to examine TGF-β superfamily processes regulating cumulus expansion. COCs were collected from eCG-primed mice and the oocyte microsurgically removed to generate oocytectomised (OOX) complexes. An established scoring system was used to measure FSH-induced cumulus expansion; 0 (no expansion) to +4 (maximum expansion). OOX complexes treated with FSH alone failed to expand (score: 0), whereas expansion was significantly ($P < 0.05$) induced by either GDF9 (score: mean ± SEM, 3.7 ± 0.1), activin A (2.6 ± 0.1), or co-culture with oocytes (3.2 ± 0.2). The type-I receptors for GDF9 and activin are activin receptor-like kinase 5 (ALK5) and ALK4, respectively. We tested the ability of the ALK4/5/7 kinase inhibitor, SB431542, to neutralise cumulus expansion. SB431542 completely neutralised ($P < 0.05$) the response of OOX complexes to GDF9, activin and oocyte-induced cumulus expansion. SB431542 also neutralised ($P < 0.05$) COC expansion in a dose dependent manner. Follistatin, an activin antagonist was effective at neutralising the response of OOX complexes to activin (score: 0), but had no significant effect ($P > 0.05$) on the expansion of OOX complexes co-cultured with oocytes (score: 2.7 ± 0.2). This study provides evidence that activin is not the sole CEEF, but signalling through the ALK4/5/7 pathway is indispensable for mouse cumulus expansion.

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OOCYTES PREVENT BOVINE CUMULUS CELL APOPTOSIS BY MAINTAINING A MORPHOGENIC PARACRINE GRADIENT OF BONE MORPHOGENETIC PROTEINS

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Paracrine factors secreted by the oocyte regulate a broad range of cumulus cell (CC) functions. Previously we have shown that the low incidence of apoptosis in CCs is due to unidentified oocyte-secreted factors (OSF) acting in an anti-apoptotic manner. Here we examine the nature of the paracrine network of oocyte BMP growth factors and their binding proteins regulating CC apoptosis. Bovine cumulus–oocyte complexes (COC) were aspirated from abattoir-derived ovaries and oocytes microsurgically removed to create oocytectomized (OOX) complexes. OOX were treated with denuded oocytes (DO) or various growth factors for 24 h, then CC apoptosis was assessed using TUNEL together with confocal microscopy plus image analysis and by Western blotting for Bcl-2 and Bax. CC apoptosis was significantly ($P < 0.001$) reduced by DO, bone morphogenetic protein 15 (BMP15), BMP6 or BMP7 as assessed by TUNEL. Accordingly, expression of anti-apoptotic Bcl-2 was high in OOX+DO and OOX+BMP15, and low with OOX+GDF9 and OOX alone, whereas the reverse was observed for pro-apoptotic Bax. Combined treatment of OOXs with BMP6 and BMP15 did not further decrease apoptosis levels beyond that of BMP15 alone ($P > 0.05$), suggesting no additive effect of these two BMPs. Follistatin (FS) effectively antagonized BMP15 anti-apoptotic effects, and likewise, a BMP6 neutralizing antibody (NAb) antagonized the inhibitory effect of BMP6. Gremlin blocked BMP7 anti-apoptotic effects on CCs, but had no significant effect on BMP15. FS or BMP6. NAb antagonized ~50% of the anti-apoptotic activity of oocytes; however, these effects were not additive suggesting the additional involvement of other OSF. These results indicate for the first time that OSF (BMP15 and BMP6 in particular) maintains the low incidence of CC apoptosis by establishing a localized morphogenic gradient of bone morphogenetic proteins.

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OXYGEN CONCENTRATION DURING *IN VITRO* MATURATION OF MURINE OOCYTES AFFECTS BLASTOCYST CELL LINEAGE

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Follicular antral oxygen tension is thought to influence subsequent oocyte developmental competence. Despite this, *in vitro* maturation (IVM) is routinely performed in either 5 or 20% O_2 and while low O_2 has been shown to be beneficial to embryo development in many species, the effect of altering O_2 concentration during IVM has not been adequately investigated. Here we investigated the effects of a range of O_2 concentrations during IVM on meiotic maturation and subsequent embryo development after IVF. Ovaries from eCG-stimulated CBA F1 female mice (21 days) were collected and intact cumulus oocyte complexes (COCs) cultured for 17–18 h under 2, 5, 10 or 20% O_2 (6% CO_2 and balance of N_2). Matured COCs were denuded of cumulus cells, fixed and stained (1% aceto-orcein) for visualisation of maturation status. No significant difference in maturation rates between treatment groups was observed. Following IVF (performed under 5% O_2, 6% CO_2 and balance of N_2), no difference in fertilisation rates between treatment groups was observed in a randomly selected cohort 7 h post-fertilisation. There was also no significant difference in cleavage rates after 24 h or ability to reach blastocyst stage after 96 h, with a tendency ($P = 0.079$) for more blastocysts in 2% O_2. However there was a significant increase in the number of trophectoderm cells present in the resulting blastocysts ($P < 0.05$) in the 2% O_2 group (35 ± 2.1) compared to 20% O_2 (25 ± 2.8). Our data suggests that O_2 concentration during IVM does not influence nuclear maturation or subsequent fertilisation, cleavage and blastocyst development rates. However, maturation in 2% O_2 significantly alters subsequent cell lineage within blastocysts to favour trophectoderm development. Such skewed trophectoderm cell number may influence embryo viability.

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THE UBIQUITIN-PROTEASOME PATHWAY IN BOVINE AND MURINE OOCYTES UNDERGOING MATURATION

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Mammalian oocyte maturation is governed by an interaction between protein kinases, phosphatases and proteasases. Maturation promoting factor (MPF) is a serine/threonine kinase heterodimer composed of a catalytic cdc2/cdk1 subunit and a regulatory cyclin B1 subunit.[1] Cyclin B1 undergoes rapid turnover via degradation in the ubiquitin-proteasome pathway (UPP) followed by *de novo* synthesis. A high level of MPF causes metaphase arrest, then UPP degradation of cyclin B1 allows the oocyte to exit metaphase I (MI). Proteasomes have been localised in pig and rat oocytes. Although in both species they are associated with the MI and MII meiotic spindles, immunostaining at the germinal vesicle (GV) stage is different with perinuclear staining in rat GV and intra-GV staining in the pig. This may suggest different roles for proteasomes prior to GV breakdown (GVBD) in different species. This study used confocal laser scanning immunohistochemistry with a specific antiserum against the 20S proteolytic 'core' of the 26S proteasome to reveal proteasomes in murine oocytes undergoing maturation *in vitro*. In the mouse, proteasomes were associated with the meiotic spindle, similar to observations in pig and rat oocytes and preliminary studies in the bovine suggest a similar immunolocalisation. Cyclin B1 also accumulates around the spindle during meiosis.[1] This suggests that proteasomes are prevented from degrading cyclin B1 until the MI-AI and MII-AII transitions. Immunolabelling showed Fam, a deubiquitinylating enzyme (also known as Usp9x) was localised at the spindle during MI and MII. This suggests a link between Fam, the UPP and the spindle assembly checkpoint to prevent cyclin B1 degradation until required.

(1) Huo LJ, Fan HY, Zhong ZS, Chen DY, Schatten H, Sun QY. (2004). Ubiquitin-proteasome pathway modulates mouse oocyte meiotic maturation and fertilization via regulation of MAPK cascade and cyclin B1 degradation. *Mech. Dev.* **121**, 1275–1287.

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ABBERANT MURINE EMBRYONIC DEVELOPMENT FOLLOWING GLUCOSAMINE EXPOSURE DURING IVM OR EMBRYO CULTURE

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The hexosamine biosynthesis pathway is an alternate fate for glucose metabolism providing glycosylation moieties and is significantly upregulated by addition of glucosamine, a common dietary supplement. Here we determined the impact of glucosamine addition to cumulus oocyte complex (COC) maturation or during embryo culture on subsequent embryonic development. COCs were collected from 23-day-old mice 46 h post-eCG, and matured under several conditions prior to being fertilized and cultured: (1) 10 mL/COC a MEM (5.56 mM glucose) + 0, 1.25 or 5 mM glucosamine; (2) 10 mL/COC a MEM (20 mM glucose) + 0, 1.25 or 5 mM glucosamine; (3) 100 mL/COC G2.3 (5 mM glucose) + 0, 1.25 or 2.5 mM glucosamine. One-cell embryos were also flushed from age-matched donors 24 h after mating and cultured in 0, 1.25 or 2.5 mM glucosamine in G 1.3/2.3 sequential media. No differences in rates of embryonic development were detected between COCs matured in 10 mL of media with 5.56 mM glucose with glucosamine. However, blastocyst formation was significantly impaired ($P < 0.001$) when COC maturation occurred in equivalent volumes of media that contained 20 mM glucose + 1.25 mM (49.98%) or 5 mM glucosamine (44.7%) *v.* control (86.55%). Intriguingly, embryonic viability was significantly ($P < 0.001$) reduced in COCs matured in 100 mL G2.3 containing 5 mM glucose + 1.25 mM (44.6%) or 2.5 mM glucosamine (40.1%) *v.* control (79.81%), suggesting a volume × glucose concentration interaction. In contrast, embryonic development was significantly reduced (34%, $P < 0.002$) and completely ablated when 1-cell embryos were cultured in media containing 1.25 mM and 2.5 mM glucosamine, respectively (control = 88.57%). These results suggest that glucosamine up-regulated hexosamine pathway activity in both COCs and early embryos impairs subsequent embryonic development by as yet undescribed mechanisms.

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CHARACTERISING THE STEM CELL ACTIVITY OF HUMAN ENDOMETRIAL EPITHELIAL AND STROMAL CELLS

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The human endometrium has remarkable regenerative capacity. It contains rare clonogenic epithelial and stromal cells.[1] The aims of this study were to compare the stem cell properties, self renewal, differentiation and proliferative potential, of endometrial cells initiating large clones (HPP-CFU, high proliferative potential–colony forming units) with those initiating small clones (LPP-CFU, low PP-CFU). Endometrial tissue obtained from 12 ovulating women undergoing hysterectomy was dissociated into single cell suspensions and epithelial and stromal cells were cultured at clonal density (8–20 cells/cm^2). Individual clones were harvested and serially recloned to measure self-renewal, serially passaged to assess proliferative potential and cultured in various media to assess differentiation. Secondary stromal clones were characterized for mesenchymal stem cell (MSC) markers by flow cytometry. Epithelial HPP-CFU serially cloned significantly more times than epithelial LPP-CFU ($P < 0.005$, Table 1) indicating greater self renewal capacity. Similarly, stromal HPP-CFU demonstrated greater self renewal activity compared with stromal LPP-CFU ($P < 0.005$, Table 1). Serially passaged epithelial HPP-CFU underwent significantly more population doublings (PD) before senescence than epithelial LPP-CFU ($P < 0.05$, Table 1). Likewise, stromal HPP-CFU underwent a higher number of PD compared with stromal LPP-CFU ($P < 0.005$, Table 1). Stromal HPP-CFU expressed CD29, CD44, CD73, CD90, CD105 and CD146, and were negative for haemopoietic and endothelial markers, CD45, CD34 and CD31. Stromal HPP-CFU differentiated into adipocytes, smooth muscle cells, osteoblasts and chondrocytes when cultured in appropriate differentiation media. Our studies of human endometrial cells demonstrate that the rare epithelial and stromal HPP-CFU exhibit self-renewal and high proliferative potential. Furthermore stromal HPP-CFU express markers typical of bone marrow MSC and differentiate into four mesenchymal lineages. These data suggest that epithelial and stromal HPP-CFU, but not LPP-CFU, have characteristic properties of adult epithelial stem cells and MSC respectively and are likely responsible for the remarkable cyclical, regenerative capacity of human endometrium.

Table 1. Some stem cell properties of epithelial and stromal clones

Stem cell assay	Epithelial		Stromal	
	HPP-CFU	LPP-CFU	HPP-CFU	LPP-CFU
Serial cloning (no. times)	2.8 ± 0.1(3)*	0.7 ± 0.2 (3)*	3.1 ± 0.4 (4)*	0.6 ± 0.1 (4)*
Population doublings	29.8 ± 4.4 (3)#	14.9 ± 1.7 (3)#	32.3 ± 2.3 (3)*	13.4 ± 1.1 (4)*

#$P < 0.05$, *$P < 0.005$. Numbers in parentheses indicate number of patient samples examined.

(1) Chan RWS, Schwab KE, Gargett CE (2004). *Biol. Reprod.* **70**, 1738–1750.

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AQUAPORINS IN RAT UTERINE EPITHELIAL CELLS DURING EARLY PREGNANCY AND IN RESPONSE TO PROGESTERONE

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Implantation of the rat blastocyst is a highly regulated process, involving transformation of the uterine environment into one which is receptive to an implanting blastocyst. At the time of implantation, in response to progesterone, there is a dramatic decrease in the amount of uterine luminal fluid leading to close apposition between the luminal epithelium and trophoblastic cells. The rat blastocyst also always implants at the antimesometrial pole of the uterine lumen and currently mechanisms regulating this process are unknown. Aquaporins, a family of transmembrane water channels, are involved in the regulation of water movement across epithelial barriers. We investigated several aquaporins in the rat uterus during early pregnancy using reverse transcriptase PCR. Immunofluorescence and immunogold electron microscopy techniques were then used to investigate the localisation of particular aquaporins including AQP5 in the uterine epithelium during early pregnancy and in ovariectomised rats treated with progesterone. There was an increase in AQP5 molecules in the apical plasma membrane of luminal epithelial cells at the time of implantation, with a greater increase at the mesometrial compared to antimesometrial pole. A similar result was seen in luminal epithelial cells from ovariectomised rats treated with progesterone, however there was no differential concentration between mesometrial and antimesometrial poles, as there was during early pregnancy. It is suggested that the increase in AQP5 protein expression in the apical plasma membrane of luminal epithelial cells is involved in reabsorption of luminal fluid at the time of implantation. Furthermore, the differential concentration of AQP5 on luminal epithelial cells at the time of implantation could lead to the establishment of a fluid gradient within the uterine lumen and hence lead to the asymmetrical implantation position of the rat blastocyst.

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CLAUDINS AND OCCLUDIN IN THE RAT ENDOMETRIUM

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Regulation of the uterine luminal environment is important for the successful attachment and implantation of the blastocyst. Tight junctions regulate the paracellular pathway between epithelial cells lining the uterine lumen and the uterine glands. The aims of this present study was firstly to establish the presence and cellular distribution of claudins and occludin in the luminal epithelia during early pregnancy using immunofluorescence microscopy and deconvolution, and secondly to determine the influence of ovarian hormones on their expression. Occludin and claudins -1, -3, and -4 were present in luminal epithelium. Occludin and claudin-4 showed increased expression in luminal epithelium at the time of implantation, whereas claudin-1 and -3 expression remained the same throughout early pregnancy. In ovariectomised rats administered ovarian hormones, occludin and claudin-4 showed increased expression in luminal epithelium in progesterone-dominant regimes and decreased expression when administered oestrogen alone. Expression of claudin-1 and -3 in luminal epithelium was not effected by ovarian hormones. Claudin-2 was not expressed during early pregnancy nor in ovariectomised rats. In conclusion, these results show that occludin and claudins -1, -3 and -4 are present in luminal and glandular epithelium, and provide the permeability properties needed to separate the luminal and the stromal environment at the time of implantation. Furthermore, occludin and claudin-4 expression is controlled by ovarian hormones being upregulated by progesterone.

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HORMONAL CONTROL OF VASCULAR MURAL CELL RECRUITMENT IN THE MOUSE ENDOMETRIUM

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The human endometrium undergoes regular periods of growth and regression, including concomitant changes in the vasculature, and is one of the few adult tissues where significant angiogenesis (new blood vessel formation) and arteriogenesis (recruitment of vascular smooth muscle cells (VSMC) and pericytes) occurs on a routine, physiological basis. In this study, mouse models were used to investigate the effects of oestrogen and progesterone on endometrial vascular mural cell recruitment. The aim was to quantify changes in the proportion of vessels covered by α-smooth muscle actin (α-SMA, a marker of VSMC and pericytes) in hormone-treated ovariectomised mice. We hypothesised that relative vessel α-SMA coverage would increase following progesterone treatment (in conjunction with endothelial cell (EC) proliferation), but not following oestrogen treatment (when EC proliferation also occurs). Ovariectomised mice were given a single oestradiol (100 ng) or vehicle injection, before dissection 24 h later, or three consecutive daily injections of progesterone (1 mg) or vehicle. The percentage of vessel profiles with no, minimal, extensive or complete α-SMA coverage were quantified after CD31/α-SMA double immunostaining. There was a significant decrease in the percentage of vessel profiles with no α-SMA coverage following progesterone treatment (20 ± 4.3 % [mean ± SE] *v.* 57 ± 4.6 %, $t_{(7)} = 12.5$, $P < 0.001$), and a significant increase in the percentage of vessels with minimal or extensive α-SMA coverage (44 ± 3.4 % *v.* 27 ± 3.7%, $t_{(7)} = 4.7$, $P < 0.001$ and 27 ± 4.3% *v.* 5 ± 0.5%, $t_{(7)} = 5.8$, $P < 0.001$, respectively), in comparison to vehicle-treated mice. The percentage of vessels with complete α-SMA coverage, representing vessels with a coat of VSMC, did not change significantly in comparison to vehicle-treated mice (8 ± 2.3% *v.* 10 ± 1.2%, $t_{(7)} = 0.6$, $P = 0.55$). There were no significant changes in the percentage of vessels with differing α-SMA coverage in oestrogen-treated mice. In continuing studies, we will quantify the proportion of proliferating α-SMA positive cells and examine mouse endometrial tissues using a pericyte-specific marker.

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SPATIAL AND TEMPORAL EXPRESSION PATTERN OF FURIN IN THE HUMAN ENDOMETRIUM

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Furin is a proprotein convertase (PC) implicated in the endoproteolytic maturation of inactive protein precursors of growth factors, hormones, receptors, and viral envelope glycoproteins.[1] Two functionally active forms of furin, one membrane-bound containing a C-terminal transmembrane domain (TD) and a cytoplasmic tail (CT), and one soluble without the TD and CT, have been characterised. We have previously shown that PC6, one of the PCs closely related to furin, is expressed in the human endometrium and is closely associated with decidualization of stromal cells during implantation.[2] Although furin is ubiquitously expressed, its expression in the human endometrium is unknown. In this study, we investigated the spatial and temporal expression pattern of furin in the human endometrium using RT-PCR and immunohistochemistry. While furin expression is detected throughout the menstrual cycle and during early pregnancy, lowest mRNA levels are seen during the proliferative phase. Using an antibody directed against the C-terminus of the membrane bound form, furin is detected in the stroma, glandular and luminal epithelium, as well as in endothelia and neutrophils throughout the menstrual cycle and during early pregnancy. In the stroma, highest levels of furin are present during menstruation ($n = 3$), they are also high during the proliferative phase ($n = 4$), but significantly lower levels are detected during the secretory phase ($n = 10$, $P < 0.05$, Tukey HSD). In the first trimester decidua, furin is present in well decidualised stromal cells. The overall expression pattern of furin is different to that of PC6; in particular, furin expression is associated only with well decidualized stromal cells whereas PC6 is involved in the initial stages of decidualization. These data suggest that furin and PC6 play different roles in the human endometrium, especially during embryo implantation.

(1) Nakayama K. (1997). *Biochem. J.* **327**, 625–635.

(2) Nie *et al*. (2005). *Biol. Reprod.* **72**, 1029–1036.

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CHANGES IN THE EXPRESSION OF ANNEXIN IV MRNA AND PROTEIN IN HUMAN ENDOMETRIUM

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In a previous study investigating global gene expression throughout the menstrual cycle,[1] Annexin 4 (ANXIV) was identified as having significant cyclic changes in human endometrium. ANXIV belongs to a ubiquitous family of Ca^{2+}-dependent phospholipid and membrane-binding proteins. The aims of this study were to investigate the cellular localization and regulation of ANXIV mRNA and temporal expression of ANXIV protein in human endometrium during the menstrual cycle.

mRNA Expression: The menstrual cycle was divided into seven stages by histological evaluation. Curettings of endometrium were collected from 60 cycling women. For cellular localization, tissues from eight endometrial curettings were dissociated with collagenase into single cells, separated into epithelial and stromal cell fractions and snap frozen. Total RNA was extracted and ANXIV mRNA was quantified by real-time PCR.

Immunohistochemistry: Full thickness endometrial tissue was collected from 50 reproductive age women undergoing hysterectomy. Tissue sections were formalin-fixed and paraffin-embedded. Goat polyclonal ANXIV antibody was used to localize ANXIV protein.

ANXIV mRNA was significantly upregulated in the whole tissue during mid-late secretory phase of the cycle, and was predominantly expressed in epithelial cells. ANXIV protein was detected in the luminal and glandular epithelium in high levels throughout the menstrual cycle except in early secretory (ES) phase. The intensity of immunostaining was stronger in the glands of the basalis compared to functionalis in early proliferative phase, however, by the late secretory phase the functionalis glands showed higher expression levels.

ANXIV mRNA data are consistent with a role for progesterone in upregulating the expression of ANXIV, although protein levels remain high through menstruation and into the proliferative phase. ANXIV can indirectly inhibit prostaglandin production, which is important for implantation. Hence the low levels of ANXIV protein at ES phase may relate to processes involved in implantation.

(1) Ponnampalam *et al.* (2004). *Mol. Hum. Reprod.* **10**(12), 879–893.

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LIF EXPRESSION IS INDUCED IN THE MOUSE OVIDUCT FOLLOWING ACTIVATION BY SEMINAL FACTORS

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A precisely regulated sequence of molecular and cellular changes occurs in the female reproductive tract in early pregnancy to facilitate development of the embryo and its successful implantation. Cytokine–leukocyte networks are integral in the tissue remodelling and immuno-regulatory processes underpinning successful implantation. Seminal factors are implicated in activating expression of cytokine genes and inflammatory leukocyte recruitment in the uterus, but whether semen-induced effects extend to the oviduct to influence blastocyst development has not been examined. The aim of this study was to quantitate the expression of mRNA encoding epidermal growth factor (EGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), heparin-binding epidermal growth factor-like growth factor (HB-EGF), insulin-like growth factor (IGFII), leukaemia inhibitory factor (LIF), tumour necrosis factor (TNFα), transforming growth factor (TGFα) and TGFβ from oviducts collected from mice at oestrus and on day 1 of pregnancy, after mating with intact, seminal plasma deficient (svx) and vasectomised (vas) mice. Total RNA was extracted, DNAse treated, reverse transcribed into cDNA, and quantified by real-time PCR using SYBR Green chemistry. All cytokine-specific primers were designed using GenBank sequences and data were normalised to β-actin mRNA expression. Expression of LIF mRNA was induced following mating with intact or vas males, but not svx males, showing that LIF mRNA is induced by factors present in seminal plasma. mRNAs encoding EGF, GM-CSF, HB EGF, IGFII, TNFα, TGFα and TGFβ were all detected in oviduct cDNA collected from oestrus and day 1 mice. These data support the proposal that cytokine-leukocyte networks shown previously to be operative in the uterus extend to the oviduct, and are influenced by exposure to seminal plasma at mating. The cytokines expressed in the oviduct during early pregnancy are likely to be key regulators of embryo growth and development.

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IN VITRO CULTURE SIGNIFICANTLY REDUCES DIFFERENCES IN GENE EXPRESSION PROFILES BETWEEN MYOMETRIAL AND FIBROID SMOOTH MUSCLE CELLS

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Fibroids are benign neoplasms of the smooth muscle cells of the uterus. Cultured myometrial (M) and fibroid (F) smooth muscle cells (SMC) have been widely used as a model for the study of fibroid growth. Although it has been shown that FSMC can behave differently in culture to MSMC, it is not clear how relevant the cultured cells and their responses are to the *in-vivo* situation. The aim of the present study was to compare gene expression profiles of M and F tissue to cells isolated from the same tissue and cultured for up to 3 passages. M and F were collected from hysterectomy specimens (n = 6), part was snap frozen for RNA and the rest used to isolate SMC, which were cultured for 3 passages and RNA was collected at passage 0 (P0) and 3 (P3). 36 microarrays were performed on 8K human cDNA slides, 6 per each specimen (3 for M and 3 for F: tissue, cell at P0 and P3) against reference RNA. Analysis revealed significant differences between tissues and cultured cells. Independent clustering assigned tissues versus cells into two distinct groups based on their expression profiles. Parametric ANOVA with Benjamini-Hochberg correction and post-hoc testing was used to determine similarities and differences between tissues and cells. 128 genes were found to be statistically different between M and F tissue, 66 between MSMC and FSMC at P0, and only 9 at P3. More than 1100 genes were significantly changed between tissues and cultured cells, with 648 genes common between both M and F cells at P0 and P3. Similar numbers of genes were up regulated as were down regulated. Expression profiles of genes of interest including estrogen receptor α and progesterone receptor were also validated using real-time PCR. This is the first study to compare gene expression of *in vivo* and *in vitro* fibroid and myometrial SMC. The results demonstrate that large changes occur in SMC gene expression in culture, reducing differences between myometrial and fibroid cells. This study indicates that results of *in vitro* studies should be interpreted with caution as many genes have an altered gene expression profile in culture.

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EFFECT OF BATIMASTAT, A SPECIFIC INHIBITOR OF MATRIX METALLOPROTEINASES, ON ENDOMETRIAL BREAKDOWN AND REPAIR IN A MOUSE MODEL

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Strong correlative evidence supports a role for matrix metalloproteinases (MMPs) in the tissue breakdown at menstruation. As menstruation occurs in very few species besides women, there is a lack of suitable and easily accessible animal models available to examine the functional significance of potential key mediators of this process. A mouse model of endometrial breakdown and repair has been developed,[1] which morphologically resembles that of human endometrium at menstruation. Previous studies in our laboratory showed that the expression patterns of various MMPs in the mouse model closely resembled those seen in the human.[2] Administration of doxycycline, a broad spectrum MMP inhibitor, decreased gelatinase activity, but had no effect on tissue breakdown in this model. The aim of the present study was to further examine the importance of MMPs in endometrial breakdown and repair via administration of batimistat, a highly potent and specific MMP inhibitor.

Batimistat was administered I.P to mice 24 h prior to the expected time of endometrial breakdown. The efficacy of batimistat within the uterus was proven using *in situ* zymography, which identifies MMP activity (rather than latent forms). This demonstrated that batimistat was reaching its target organ and effectively inhibiting MMP activities (both gelatinase and collagenase). Examination of gross uterine morphology revealed no apparent difference between groups, with batimistat treated uteri displaying a similar extent of tissue breakdown and repair to their control counterparts. Measurement of the breaking down area compared to total endometrial area revealed no difference between control and batimistat treatment, with the breaking down areas being 69 ±13% and 72 ± 9.8% of total endometrial cross-sectional area respectively. There was likewise no effect on endometrial repair.

The results of this study together with our previous study using doxycycline, indicate that MMPs are not the key mediators of endometrial breakdown in this model.

(1) Shen *et al.* (2004) *Reprod. Fert. Dev.* **16**(Suppl), A265, p. 97.
(2) Brasted *et al*. (2003) *Biol. Reprod.* **69**, 1273.

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ENDOMETRIAL CHANGES IN A MOUSE MODEL FOR LONG-TERM PROGESTIN EXPOSURE: IMPLICATIONS FOR BREAK-THROUGH BLEEDING

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A mouse model of menstruation[1] was modified to evaluate the effects of long-term progestagens on the endometrium. Many women using long-acting progestin (P)-only contraceptives experience break-through bleeding (BTB) and this is the main reason for discontinuation of their use by 30% of users. To understand the mechanisms involved in BTB, ovariectomised mice treated with a strict steroid hormone regime, had etonogestrel- or levonorgestrel-releasing implants inserted subcutaneously. A decidualizing stimulus (oil) was administered into one uterine horn; decidualisation was maximal 48 h later, designated day 0 (d0). The implant was left in place for a further 45 days. Uteri were collected at 5-day intervals and analysed for morphological changes, leukocyte infiltration, and matrix metalloproteinase (MMP) expression.

In uteri from mice treated with etonogestrel and examined at d0, decidualisation was maximal and very few leukocytes were present (neutrophils, NEU; Macrophages, Mac; uterine natural killer cells, uNK). Some loss of tissue integrity was seen at 5d, associated with some MMP-9 positive leukocytes. However, substantial stromal breakdown was observed at 10d and this progressed until 25d. A large infiltrate of NEU and uNKs, as well as increased MMP-9 expression, was observed at these times. At 25d there was evidence of re-epithelialisation and by 45d the tissue had fully repaired back to its pre-decidualised state. Re-epithelialisation was associated with a decline in NEU and uNKs, as well as MMP-9 positive immunostaining. The morphology of uteri from mice treated with levonorgestrel implants was compared to mice treated with etonogestrel. The changes observed were very similar with a loss of tissue integrity at 5d and tissue destruction at 10d.

This mouse model mimics the changes in leukocyte populations and MMP expression observed in endometria of some women using P-only contraceptives.

(1) Brasted M, White CA, Kennedy TG, Salamonsen LA. (2003) *Biololgy of Reproduction* **69**(4), 1273–1280.

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A NEW LOOK AT MENSTRUAL REPAIR: THE ROLE OF $CD34^+$ CELLS AND $CD56^+$ UTERINE NK CELLS

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Endometrial regeneration occurs rapidly in a low estrogen environment without observable mitosis following menstruation. We have used a combination of hysteroscopy, scanning electron microscopy and immuno-histochemistry to re-examine this poorly understood process. Full thickness endometrial biopsies from 25 cycling women were used for immuno-histochemical analysis of the distribution of $CD34^+$ and $CD56^+$ cells.

Histological appearances show that immediately preceding menstruation, a small population of $CD34^+$ stromal cells are located in the basalis but that their numbers increase dramatically following the onset of menstruation, becoming the dominant stromal cell type at this time. Mean surface stromal count during menstruation of $CD34^+$ cells was 5847/mm^2 (n = 8) compared with pre-menstrual 108/mm^2 (n = 7). Conversely $CD56^+$ uNK cells in the endometrial stroma were minimal in the proliferative phase, mean 228/mm^2 (n = 6) reaching a mean 2709/mm^2 (n = 7) immediately pre-menstrually. There is a significant inverse correlation (at 0.01 level (2-tailed)) between $CD34^+$ cells and $CD56^+$ uNK cells across the menstrual cycle. We present additional scanning electron microscope and histological images to support the hypothesis that uterine endometrial repair may occur via a blood borne supply of $CD34^+$ stem cells which differentiate in to elements of the endometrium; surface epithelium, glandular epithelium and stromal uNK cells. This hypothesis has profound clinical implications for the mechanisms and management of common gynaecological conditions resulting from abnormal endometrial repair and is contrary to the current understanding that uterine endometrium is replaced from basal remnants.

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EXPRESSION OF COMPONENTS OF THE HEDGEHOG SIGNALLING PATHWAY DURING MURINE SPERMATOGENESIS

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Hedgehog (Hh) signalling is best known for its involvement in regulating patterning, driving cell proliferation, promoting cell survival and directing differentiation during embryonic development.[1] The role that Hedgehog signalling plays in the testis is not yet clearly defined, though deletion of one Hedgehog ligand, Dhh, leads to male infertility. The Gli family of zinc finger TFs, consisting of Gli1, Gli2 and Gli3, are mediators of the Hh signalling cascade in vertebrates. We have previously shown that the mRNA transcripts encoding all three Glis in the adult mouse testis are expressed highly in spermatogonia, spermatocytes and to a lower extent in the round spermatids. To understand the potential sites of action of Hh proteins in spermatogenesis, we have extended our analysis to other genes involved in the Hh signalling pathway in the adult mouse testis. Using *in situ* hybridization, Patched2, a transmembrane receptor for Hh, was detected in spermatogonia and spermatocytes, with an apparently lower expression in the round spermatids. The mRNA of Smoothened, another transmembrane protein which forms a membrane receptor complex with Patched, is highly expressed in spermatogonia and spermatocytes, again showing lower expression in round spermatids and interstitial cells. Fused, a positive regulator of Hh signalling, is highly expressed in spermatogonia and spermatocytes with slightly lower expression in round spermatids. SuFu is a negative regulator of Hh signalling, known to repress Gli1 function in part by tethering it in the cytoplasm. The mRNA encoding SuFu is absent from spermatogonia, detected in spermatocytes and persists in round spermatids where its expression appears highest, suggesting that the SuFu protein may be acting to switch off Hh signalling at that stage of spermatogenesis. Overall, the regulated expression pattern of these genes in the adult mouse testis suggests a role for Hh signalling in the regulation of spermatogenesis.

(1) Ruiz i Altaba A. (1999) *Trends Genet.* **15**(10), 418–425.

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OESTROGEN RECEPTOR BETA IS INVOLVED IN THE REGULATION OF LEYDIG CELL NUMBER IN THE MOUSE

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Recent evidence suggests that oestrogen plays a physiological role in the testis. Both oestrogen receptor alpha and oestrogen receptor beta (ERb) are present in the testis and administration of oestrogen has been shown to inhibit the development of Sertoli, Leydig and germ cells. This study investigates the effect of ERb on the testis using ERb knockout mice (bERKO). Adult male bERKO mice ($n = 8$) and their wild-type littermates ($n = 7$) were killed at 11 weeks postpartum. One testis from each animal was fixed in Bouin's fluid and embedded. Each testis was fractionated and thick sections cut and stained with PAS. The optical disector method was used to count the number of Leydig cells, Sertoli cells, spermatogonia, spermatocytes and spermatids in each testis. Trunk blood was collected and plasma testosterone concentrations measured by radioimmunoassay. No significant differences in body or testis weight were seen between the bERKO or wild-type mice. Similar numbers of Sertoli cells, spermatogonia, spermatocytes and spermatids were also observed between the two groups. The number of Leydig cells was significantly increased in bERKO mice compared with their wild-type littermates ($P < 0.05$). Despite the increased number of Leydig cells in the bERKO mice there was no significant difference in plasma testosterone concentrations in this group compared to the wild-type mice. Oestrogen has been reported to inhibit proliferation of adult-type Leydig cells and to inhibit steroidogenesis. This study suggests that the regulation of Leydig cell proliferation may be mediated by ERb. The presence of normal circulating testosterone concentrations in bERKO mice suggests that the effects of oestrogen on steroidogenesis are not brought about by ERbeta.

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FIBROBLAST GROWTH FACTOR RECEPTOR-1 (FGFR-1) IS ESSENTIAL FOR SPERMIOGENESIS, CAPACITATION AND MALE FERTILITY

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Male infertility is often a result of irregular sperm development/function. The identification of snt-2 (Suc-1 associated Neurotrophic Factor Target 2) and Fgfr-1 to the sperm tail, lead to the hypothesis that Fgf signalling through snt-2 is involved in sperm tail development/function. To test this hypothesis, transgenic mice carrying a dominant-negative variant of Fgfr-1, driven by the protamine 1 promoter (haploid specific) were created. Breeding experiments confirmed male fertility; however, one line was significantly sub-fertile and demonstrated a significantly reduced daily sperm production (DSP, 30%↓). Transgene expression levels were up to 70 times above native mRNA levels in wt mice; however, there was a concurrent upregulation of the native receptor in transgenic mice, resulting in only a 6× over-expression in transgenic : native mRNA. To increase transgene expression, independent lines were crossed (double heterozygous, DH). DH transgene expression levels were up to 120 times above the native mRNA in wild type mice, resulting in a 20× over-expression in transgenic : native mRNA. Breeding experiments showed males from 1 cross were significantly subfertile with DSPs further reduced (41%↓). Collectively this data shows Fgfr-1 signalling is required for quantitatively normal spermiogenesis. Given the millions of sperm that mice produce, a 40%↓ in DSP is unlikely to be responsible for the sub-fertility observed i.e. 2 *v.* 9 pups/litter. Therefore, a disruption of Fgfr-1 signalling may also induce a post-testicular phenotype. Western blot analysis, using tyrosine phosphorylation as a surrogate marker of sperm capacitation, showed transgenic mice had a significantly attenuated ability to initiate capacitation. As capacitation is an absolute requirement for fertilisation, the absence of capacitating capability is probably the major contributor to the sub-fertility seen in the transgenic mice. This research demonstrates for the first time that the Fgfr-1 signalling cascade is one of several pathways associated with sperm development and function.

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ADULT EXPOSURE TO DIETARY PHYTOESTROGENS REDUCES FERTILITY OF MALE RATS

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Phytoestrogens are plant-derived compounds that are particularly abundant in soy-based foods. Exposure to exogenous oestrogenic chemicals has been implicated in declining male fertility. The aim of this study is to deduce whether adult phytoestrogen exposure affects the reproductive function of male rats, and by what mechanisms phytoestrogens may be acting.

Six male rats were transferred from a low soy diet (control) to an experimental high soy diet, while nine males remained on the control diet. On days 3, 6 and 12 all males were mated and litter sizes recorded. A second group of male rats kept on the same dietary regimen were killed after 3, 6 or 12 days on the diets. The epididymides were collected from the rats. Real-time PCR was performed to measure mRNA quantities of oestrogen receptors alpha (ERα) and beta (ERβ), and androgen receptor (AR). The TBARS assay for lipid peroxidation was performed on epididymal sperm samples from rats fed the high or low phytoestrogen diet for 3 days.

The average litter size following 3 days on the high soy diet was significantly lower than that for rats maintained on the control diet. Litter sizes returned to control levels by day 12. ERα and AR expression decreased in the cauda region of the epididymis following 3 days on the high soy diet, but returned to control levels by day 6. Lipid peroxidation of epididymal sperm was increased in rats fed the high phytoestrogen diet for 3 days.

Short-term exposure to high phytoestrogen levels transiently reduces male fertility, and alters hormone receptor expression. Endocrine disruption may impair fertility by reducing antioxidant protection of sperm stored in the epididymis.

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GROWTH DIFFERENTIATION FACTOR 9 SIGNALLING SYSTEMS REGULATE MARMOSET MONKEY GRANULOSA CELL PROLIFERATION

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This study was conducted to characterize the receptor/signalling system utilized by the oocyte-secreted growth differentiation factor 9 (GDF9) to promote granulosa cell (GC) growth in the marmoset monkey. Seven adult female marmosets were primed with hFSH for 6 days, whole ovaries were removed on day 7, follicles manually excised, and GC collected from 3 size classes: periantral (PA; 0.42–0.66 mm), small antral (SA; 0.66–1.5 mm) and large antral (LA; >1.5 mm). RNA was extracted from oocytes and GC and subjected to RT-PCR using human primers. In all follicle size classes oocytes expressed GDF9 mRNA and GC expressed mRNA for key GDF9 signalling molecules; bone morphogenetic protein receptor II, activin receptor-like kinase (ALK) 5 and Smad 3. To examine the intracellular response to GDF9, cultured GC from LA follicles were transfected with luciferase reporter constructs and treated with growth factors. CAGA-luciferase (Smad 3 pathway) in transfected GC was stimulated by TGFβ1 (18× above control), GDF9 and mouse oocytes (both ~5×), but not by BMP7. Conversely, neither TGFβ1, GDF9 nor oocytes activated BRE-luciferase (Smad 1/5/8 pathway), which was stimulated 30× by BMP7. ^{3}H-thymidine incorporation was used to determine the effects of GDF9 on GC proliferation. Basal incorporation was highly dependent on follicle size, with PA follicles ~10× higher than SA and ~30× higher than LA follicles. GDF9 stimulated ^{3}H-uptake in GC from all sized follicles, most potently in PA and SA cells. The mitogenic effect of GDF9 was amplified by IGF1; ~3× in SA GC and ~5× in LA GC. In contrast, in the presence of FSH or FSH+IGF1, GDF9 did not stimulate GC proliferation. Treatment of GC with an ALK4/5/7 kinase inhibitor, SB431542, antagonized both GDF9 and GDF9+IGF1 mitogenic effects, in a dose-dependent manner. Thus, GDF9 potently stimulates primate GC proliferation utilizing components of the TGFβ signalling system initially identified in rodents.

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MORPHOMETRIC AND HISTOLOGICAL ANALYSIS OF OVARIES FROM SHEEP HETEROZYGOUS FOR THE PROLIFIC WOODLANDS ALLELE

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Woodlands are a line of Coopworth sheep with a novel, imprinted X-linked fecundity allele resulting in ovulation rates about 0.40 higher than wild-type animals. Daughters of progeny tested sires with and without the gene were studied.

Previously, lambs heterozygous for the Woodlands allele were found to have larger ovaries and more antral (i.e. type 5) but not preantral (i.e. types 1–4) follicles than in wild-type contemporaries. The large ovary phenotype was found to be transient and was absent after puberty. However, based on follow-up studies it was evident that the large ovary phenotype was not strongly associated with the Woodlands fecundity allele. Thus, it was uncertain whether animals carrying the Woodlands gene had different follicular populations compared to wild-type controls. To address this question, follicular populations were compared in adult ewes heterozygous for the Woodlands allele with age-matched controls. Using standard morphometric methods and histological analysis, no differences were observed in the mean numbers of types 1, 1a, 2, 3 and 4 preantral follicles between the genotypes. Furthermore, no differences were observed between genotypes in follicular or oocyte diameters for any follicular type. The adult Woodlands carrier ewes had twice as many small type 5 follicles (< 1mm) when compared to wild-type contemporaries although no difference was seen in the numbers of antral follicles > 1mm in diameter. In addition, antrum formation occurred at a smaller follicular diameter in the heterozygous Woodlands animals.

Therefore, the increased number of antral follicles observed in both lambs and adult ewes suggests that this difference in pattern of follicular development is associated with the X-linked fecundity allele. This novel phenotype of early antrum formation and larger number of small preantral follicles differs from that observed in sheep with the Inverdale or Booroola mutations, suggesting that a different mechanistic pathway is involved.

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CALCIUM IONOPHORE INDUCTION OF MARMOSET OOCYTE ACTIVATION

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The marmoset monkey (*Callithrix jacchus*) is a valuable model for developing assisted reproductive technologies in humans and endangered primate species. Calcium ionophore treatments have been used to induce parthenogenetic activation in a number of species, but the effectiveness of this reagent in initiating marmoset embryo development has not yet been reported. The aim of this study was to determine the developmental potential of *in vitro* matured (IVM) marmoset oocytes, following treatment with calcium ionophore. Immature oocytes from large (LA; >1.5 mm) and small (SA; 0.67–1.5 mm) antral follicles were isolated from the ovaries of FSH-primed animals and cultured in modified G2 medium for 26–30 h at 37.0°C in 6% CO_2 in air. Meiotically mature oocytes were sequentially incubated with 5 μM ionomycin for 5 min and 2 mM 6-dimethylaminopurine for 3 h and cultured in G1/G2 sequential medium at 37.0°C in 5% O_2, 6% CO_2, 89% N_2 for 10 days. Cumulus cell expansion associated with LA oocytes ($n = 118$) was greater than that of SA oocytes ($n = 212$), as determined using well established classification criteria (2.7 ± 0.1 *v.* 1.8 ± 0.2; $P < 0.01$). A greater proportion of LA oocytes completed meiosis to the metaphase-II stage compared with SA oocytes ($85 \pm 7\%$ *v.* $63 \pm 7\%$; $P < 0.05$). Pronuclear formation was induced at similar rates in mature oocytes of both groups, but the rate of cleavage was higher for LA oocytes compared with SA oocytes ($93 \pm 6\%$ *v.* $66 \pm 5\%$; $P < 0.05$). The number of cells per embryo was not different between the groups. This is the first study to demonstrate that calcium ionophore effectively induces parthenogenetic activation in IVM marmoset oocytes. However, the development of parthenotes was limited beyond the 8-cell stage. Further studies are needed to determine the cause of the developmental block.

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EFFECT OF DONOR AGE AND FOLLICLE SIZE ON OOCYTE DEVELOPMENTAL COMPETENCE IN THE PIG

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Oocytes utilised for *in vitro* embryo production (IVP) are typically derived from 3–8 mm ovarian follicles of slaughtered pre-pubertal pigs. Following *in vitro* maturation (IVM), pre-pubertal oocytes display lower developmental competence (DC) than adult oocytes. The aim of this study was to determine the proportion of follicles 3, 4, and 5–8 mm in diameter on the surface of pre-pubertal and adult ovaries, and assess DC of corresponding oocytes. Oocytes were matured for 46 h in modified medium 199. Mature oocytes from the three follicle size cohorts were activated with calcium ionophore to assess blastocyst embryo formation rate. Data were subjected to arcsine transformation, ANOVA and the Tukey post-hoc test. Compared with adult ovaries, pre-pubertal ovaries contained a higher proportion of 3 mm follicles (46 ± 4 *v.* 72 ± 4%, $P < 0.01$), but a lower proportion of 4 mm (33 ± 3 *v.* 22 ± 3%, $P < 0.01$) and 5–8 mm follicles (21 ± 5 *v.* 6 ± 2%, $P < 0.01$). Adult oocytes from the three follicle sizes displayed similar DC (41 ± 2% to 47 ± 3%). DC of pre-pubertal oocytes improved with increasing follicle size (3 mm < 4 mm < 5–8 mm; 12 ± 4%, 27 ± 8% and 50 ± 8%, respectively; $P < 0.05$). In conclusion, the predominance of 3 mm follicles accounts for the low DC of oocytes from pre-pubertal donors compared with adult donors. Further research is required to understand DC acquisition in pre-pubertal pig oocytes from the smaller follicles <5mm in diameter.

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IN VITRO MATURATION OF BOVINE OOCYTES IN SERUM-FREE MEDIA

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Culture medium supplemented with sera is commonly used for the *in vitro* production (IVP) of livestock embryos. However, serum induced complications including batch variation, the potential risk of virus and mycoplasma contamination and the implication in the large offspring syndrome in domestic animals impels the development of a serum-free culture system. In this study, we investigated whether replacement of fetal bovine serum (FBS) with bovine serum albumin (BSA) in three maturation media, tissue culture medium-199 (TCM-199), a modified synthetic oviduct fluid (mSOF) routinely used in our laboratories and a commercially available SOF-VC (Vitro Cleave, Cook Australia). Harvested oocytes were matured, parthenogenetically activated and *in vitro* cultured (Day 7) to measure maturation efficiency, embryo development and quality with the aim of developing a simplified and defined culture medium for the *in vitro* production of bovine embryos. Abattoir derived cumulus oocyte complexes were matured in TCM-199, mSOF and SOF-VC media supplemented with LH and beta-estradiol in the presence of 15% FBS or 0.08% BSA at 39ºC in 5% CO_2 in air. Polar body extrusion was assessed twenty-two hour post maturation and selected MII occytes were activated using calcium ionophore/6-dimethylaminopurine and cultured for seven days in SOF medium supplemented with 0.8% BSA. On day seven, blastocyst development was assessed and randomly selected blastocysts were stained to determine inner cell mass (ICM), trophectoderm (TE) and total cell numbers (TCN). Supplementation with either BSA or FCS did not significantly affect the maturation efficiency, blastocyst rates or differential cell numbers within each maturation media tested. However, maturation efficiency and blastocyst rates were significantly lower ($P < 0.01$) when oocytes were matured in either mSOF or SOF-VC regardless of FBS or BSA supplementation. From this study, we conclude that BSA effectively replaces FCS and TCM-199 is superior to SOF (mSOF or SOF-VC) in terms of oocyte maturation regardless of protein source. Once matured SOF and TCM-199 parthenogenetically blastocysts were equivalent in terms of embryo development and quality.

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BOVINE OOCYTE VITRIFICATION IN SODIUM FREE MEDIUM

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Vitrification has been widely developed for the cryopreservation of mammalian embryos. Despite considerable effort, the vitrification of immature bovine oocytes is less successful with poor survival post thawing and low fertilization rates and development to offspring. The MII oocyte is highly sensitive to temperature changes with cryopreservation resulting in high incidence of aneuploidy after fertilization. Recently the concentration of sodium ions has been implicated in vitrification-induced damage. This study investigated the effect of a low-sodium choline-based medium (CJ2) supplemented with either 10% bovine fetal serum (FBS), 0.8% bovine serum albumin (BSA) or 1% polyvinylalcohol (PVA) on the ability of *in vitro* matured metaphase II (MII) oocytes to survive vitrification. Cumulus removed MII matured oocytes were equilibrated in 10% ethylene glycol (EG) and 10% dimethylsulfoxide (DMSO) in CJ2 containing FBS, BSA or PVA for 5 minutes at 37ºC and then transferred into a vitrification solution composed of 20% EG, 20% DMSO and 0.6 M sucrose in CJ2. Twenty MII stage oocytes at a time were aspirated into the Gel Loader tip (GL-tip) with approximately 20 µL of vitrification solution, equilibrated 30 sec before plunging directly into liquid nitrogen and stored for 2 h. Oocytes were thawed rapidly and cryoprotectants removed by step-wise dilution in 0.25 M, 0.15 M, 0 M sucrose in TCM-199, 5 min each. Oocytes were then incubated in TCM-199 for 2 h before being stained with Hoechst-33342 and viewed under epi-florescence to determine survival. Developmental competence was determined by parthenogenetically activating (PA) surviving oocytes using calcium ionophore/6-dimethylaminopurine and cultured for 7 days in mSOF medium supplemented with 0.8% BSA. The recovery from vitrification procedure, survival post thawing and PA rates are summarized in the Table below. Vitrification in GL-tips allows efficient processing of MII oocytes with high rates of recovery. Overall, blastocyst development in this experiment was low in the control, sham groups and treated groups and that results may have been influenced by other factors (i.e. oocyte quality). Nevertheless, results demonstrate that reducing or eliminating sodium ions from the vitrification medium may protect the immature oocytes during vitrification or thawing and allow oocytes to be cryopreserved more effectively.

	Control	Sham			Vitrified/thawed		
		Serum	BSA	PVA	Serum	BSA	PVA
Vitrified *n*	0	0	0	0	465	241	149
Recovered % (*n*)	99.8^{a}	97.2^{b}	100^{a}	97.1^{b}	99.1^{a}	100^{a}	99.8^{a}
	(504/505)	(277/285)	(131/131)	(34/35)	(461/465)	(241/241)	(148/149)
Survived % (*n*)	99.4^{a}	90.6^{b}	99.2^{a}	100^{a}	86.2^{c}	81.3^{c}	79.1^{d}
	(501/504)	(251/277)	(130/131)	(34/34)	(401/461)	(196/241)	(117/148)
Blastocyst % (*n*)	6.2^{ab}	3.2^{a}	6.2^{ab}	14.7^{b}	0.9^{c}	1.0^{c}	0^{c}
	(31/501)	(8/247)	(8/129)	(5/34)	3/341	2/196	0/116

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ADDITION OF GLYCINE TO VITRIFICATION SOLUTIONS PROTECTS OOCYTE AND EMBRYO PHYSIOLOGY AND HEALTH

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Cryopreservation procedures for oocytes result in a significant reduction in viability. Although cryopreservation procedures cause dehydration and therefore osmotic stress, the role of osmolytes in solutions has not been considered and they have therefore not been included for routine use. The aim of this study was to assess the effects of the addition of the osmolyte glycine to vitrification solutions on the health and developmental competence of mouse oocytes. Oocytes were collected from F1 female mice and cryopreserved using cryoloop vitrification with or without glycine, with fresh oocytes examined as controls (n = 2086). Mitochondrial distribution and membrane potential as well as the morphology of the spindles and chromosomes were assessed. Oocytes were fertilised to assess their ability to develop into blastocysts, which were then assessed for their expression of *Glut1, Glut3* and *IGF2* by real-time RT-PCR. Statistical analysis was performed using a generalised linear model followed by multiple comparisons using an LSD test. Vitrification without glycine perturbed mitochondrial distribution (mean pixel intensity of outer region : inner region, 1.58 ± 0.20, $P < 0.01$) and mitochondrial membrane potential (mean pixel intensity 0.56 ± 0.01, $P < 0.01$) compared to control oocytes (2.34 ± 0.24 and 0.52 ± 0.01, respectively). The addition of glycine prevented these changes (1.97 ± 0.16 and 0.53 ± 0.01, respectively). Vitrification without glycine resulted in 52% of spindles and chromosomes appearing normal while this was increased to 69% with the addition of glycine, however in both treatments these abnormalities appeared to recover after culture for 2 h. Vitrification did not affect fertilisation and blastocyst development however expression of *Glut3* was decreased 2.9 fold in blastocysts resulting from oocytes vitrified in the absence of glycine ($P < 0.01$). The data presented suggests that the addition of glycine results in fewer perturbations in oocyte physiology and gene expression of the subsequent blastocysts and should therefore be considered for routine inclusion in solutions for the cryopreservation of oocytes.

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CHARACTERISATION OF OVARIAN FOLLICULAR GROWTH IN THE BRUSHTAIL POSSUM

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The number and size of follicles selected for ovulation differ between species. The aim of this study was to characterise antral follicular growth and determine the size when selection of the ovulatory follicle occurs in the monovular brushtail possum. For this study, antral follicles ≥ 1 mm were dissected from the ovaries of 31 adult female possums at different reproductive states and follicular fluid and granulosa cells were harvested from each individual follicle. Selection of the ovulatory follicle in the brushtail possum occurred when follicles reached between 2.5 and 2.8 mm in diameter. Based on the analysis of steroids in follicular fluid, before selection, most follicles produced varying amounts of oestradiol (E2), but very few if any produced progesterone (P4). After selection, the selected follicle continued to produce increasing amounts of E2 and P4, whereas most other follicles were steroidogenically inactive. Near the time of ovulation, presumably after the LH surge, P4 became the predominant steroid produced by the selected follicle and most other follicles once again produced varying amounts of E2. The number of granulosa cells per follicle was highly variable, but tended to increase with increasing diameter. Cell viability was very high, averaging about 95%. Interestingly, the morphology of granulosa cells changed markedly after selection becoming larger and granular in appearance. The weights of the vaginal cul-de-sac and uteri correlated well with the presence of a selected follicle. In ovaries from pregnant animals (n = 3), follicles grew up to 3.5 mm, and although they reached the size of a selected follicle during the follicular phase, E2 production by the other follicles was not suppressed and the weights of the cul-de-sac were less than those from non-pregnant animals with similar sized follicles. During anoestrus (n = 4), follicles did not grow beyond 2 mm and produced very little steroids.

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REPRODUCTIVE PHENOTYPE OF THE FEMALE AROMATASE OVEREXPRESSING MOUSE

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Aromatase, the product of the C*yp* 19 gene, converts androgens to estrogens. The role of estrogens within the ovary has recently been revisited; using the aromatase knockout (ArKO) mouse, we investigated the effect of estrogen deficiency on ovarian function. We now have an aromatase overexpressing (AROM+) female mouse model with elevated levels of estrogens. These mice were fertile and bred with FVB/N wildtype (WT) males, the AROM+ male being infertile. In this study we characterised the reproductive phenotype of the female AROM+ mouse.

5 WT and 10 AROM+ mice, 22–27 weeks of age were used in the study. The mice were subject to vaginal smears and killed during estrus. The ovaries, uterine horns and gonadal fat 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 AROM+ mice exhibited an abnormal pattern of cycling that in general, alternated between estrus and post-estrus. AROM+ mice were significantly heavier than their WT counterparts (WT 35.28 ± 2.89 g *v.* AROM+ 43.38 ± 2.11 g, $P < 0.05$). Ovarian, uterine and gonadal fat pad weights were not significantly different between the 2 groups (ovary: WT 17.4 ± 1.14 mg *v.* AROM+ 17.9 ± 0.06 mg; uterine horns: WT 89.7 ± 11.40 mg *v.* AROM+ 92.1 ± 6.64 mg; gonadal fat pads: WT 2.47 ± 0.62 g *v.* AROM+ 3.46 ± 0.26 g). Histological, gene expression and hormone analyses are in progress.

Our preliminary analyses indicated no significant effect of excess estrogen on ovarian, uterine and gonadal fat pad weights, despite the AROM+ mice being heavier. It remains to be determined as to whether the ovaries and uterine horns are histologically normal.

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EFFECTS OF DIET-INDUCED OBESITY ON OVARIAN FUNCTION AND FEMALE FERTILITY

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Obesity and its related complications (metabolic syndrome, Type II diabetes and polycystic ovary syndrome) are increasingly associated with female infertility. Our research is focused on understanding how diet-induced obesity, which triggers insulin resistance and symptoms of chronic inflammation, directly impacts ovarian function and female fertility. Female mice were maintained on a "Western style" diet (22% fat, 0.15% cholesterol) or a matched control diet. Body weights were monitored weekly and after 16 weeks fasting insulin levels and glucose tolerance were assessed. Mice were then paired with males and tissues collected on day 1 on pregnancy. Blood samples were taken to determine levels of progesterone, metabolites (glucose, HDL/LDL) and inflammatory cytokines. Tissue weights (fat pads, liver, kidney, spleen, pancreas, ovary and uterus) were recorded and the reproductive tissues were fixed for analysis of histology and gene expression. Zygotes were isolated from the oviduct, cultured *in vitro* and scored for on-time development and differentially stained to assess blastocyst quality. Indices of ovarian function, including ovulation rate, steroid production and oocyte quality/blastocyst development will then be correlated with degrees of insulin resistance, dyslipidemia and inflammation. Five strains of mice were tested (CBA, Balb/c, C57, SV129 and Swiss) and showed significant differences in susceptibility to diet-induced obesity and insulin resistance. In CBA mice, the first group to be completed, the high fat diet significantly increased body weight, but did not result in overtly impaired glucose tolerance. The number of days to mating was slightly extended compared to mice on the control diet. Interestingly, the high fat diet did not affect ovulation rate but resulted in dramatically impaired blastocyst development. The results of this study will reveal how ovarian folliculogenesis, oocyte competence and ovulation are affected by obesity-induced metabolic changes, which are increasingly affecting women of reproductive age.

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RESISTANCE TO GH SIGNALLING THROUGH STAT5 IS AN EARLY EVENT IN PGF2α INDUCED LUTEOLYSIS IN THE EWE

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Recently we have shown that prostaglandin-induced luteolysis in pregnant rats involves resistance to prolactin-receptor signalling through the JAK2/STAT5 pathway.[1] In the present study, we investigate whether PGF2alpha acts similarly to inhibit GH signalling in the ovine corpus luteum. The oestrous cycle of ewes was synchronised using cloprostenol and CIDR-G devices with oestrus detected by testosterone treated wethers with raddles. Twelve days after the first recorded oestrous mark, ewes were given an intramuscular injection of either saline or cloprostenol (125 μg), followed 1 h later with an intravenous injection (jugular vein) of either vehicle or 1.5 mg recombinant bovine GH (rbGH, Monsanto). After a further 15 min ewes were killed by pentobarbitone overdose and the corpus luteum removed. Tyrosine phosphorylation of STAT5 (STAT-P) in the corpus luteum was determined by immunoprecipitation and Western blot (n = 4 ewes/treatment). STAT5-P levels were relatively low in all ewes that were not treated with rbGH. Treatment with rbGH significantly ($P < 0.01$) increased STAT5-P in the corpus luteum of ewes pretreated with saline, compared to both control groups. However the STAT5-P response to rbGH was significantly ($P < 0.01$) reduced by the pretreatment with cloprostenol, although the response remained significantly ($P < 0.05$) higher than both control groups. In summary we have shown that (1) as expected, the GH-receptor signals through STAT5 in the ovine corpus luteum and (2) cloprostenol induces resistance to this GH-receptor signalling pathway.

(1) Curlewis *et al.* (2002). *Endocrinology* **143**, 3984–3993.

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TOWARDS DERIVATION OF PRIMORDIAL GERM CELLS FROM MURINE EMBRYONIC STEM CELLS

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The process of primordial germ cell (PGC) specification begins at the earliest stages of murine embryogenesis. The mechanisms underlying this process are the strong instructive cues generated by the extra-embryonic ectoderm, which, via ligand-receptor signalling in the visceral endoderm, activate pathways in the proximal epiblast to induce the PGC phenotype. We have subjected murine embryonic stem (ES) cells to similar cues in order to drive PGC lineage specification *in vitro*. ES cells were differentiated as aggregates (embryoid bodies (EBs)), a process that is thought to recapitulate the early stages of embryogenesis by providing an environment conducive to the spontaneous emergence of multiple cell lineages. To date, we have shown that EBs can also support the spontaneous emergence of cells expressing PGC markers. Expression analysis was performed on EBs from 1 to 30 days in culture. PGC markers, including nanog, dazl, fragilis, stella and SSEA1, are expressed in undifferentiated ES cells, but rapidly become undetectable in EBs as the constituent ES cells undergo differentiation. The spontaneous emergence of cells expressing these markers occurred only following long-term EB culture. This indicates a lag in the signalling normally required for PGC specification. *In vivo*, the lack of BMP4, its receptor (ALK-2) or downstream signalling molecules (Smad 1 and 5), results in the absence of PGCs in embryos. Therefore, in order to enhance PGC specification in our *in vitro* system, we have added BMP4 into the culture media. Under these conditions, the emergence of cells expressing PGC markers occurs at both an apparently higher efficiency and in a shorter time period. This suggests that BMP4 response pathways are present within the EB context and, when activated, can direct PGC specification. Thus, by recapitulating an *in vivo* physical and biochemical environment, we are able to direct PGC lineage specification *in vitro*.

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THE SUCCESSFUL USE OF BUSULFAN TO DEPLETE ENDOGENOUS SPERMATOGONIA IN RAM TESTES

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Our research into germ cell transfer builds upon murine research[1] and is aimed at using this technique in livestock species. To increase the efficiency of colonization of transplanted germ cells, the recipient testes must be depleted of endogenous spermatogonia, without affecting the supporting cells. Three depletion methods were investigated, heat, cold and chemotherapy.

Our first investigation looked at the direct cooling (0ºC) and heating (45ºC) of the testes of 4–6 week old ram lambs. Testes were collected 7 days post treatment.

The second investigation involved the systemic injection of busulfan to ram lambs aged 3-4 months. Busulfan is used for preparing recipient mice for testes cell transfer.[1] At doses affecting the stem cells of the testes, busulfan will result in mylosuppression. Therefore a preliminary dose response trial was conducted at dose rates 4, 8 and 16mg/kg to determine the most effective dose, without threatening the survival of the animal. Testes were recovered after 3 and 6 weeks. All testes sections followed routine histology and immunochemistry with PGP 9.5.

For the heat and cold study, only gonocytes were present and there were no differences in testes weights, tubule diameters or gonocyte numbers in any of the treatment groups. For the busulfan study, dose rates of 8 and 16 mg/kg resulted in severe mylosuppression and euthanasia of 7 out of 8 animals between day 12 and 18, whereas animals in the 4mg/kg group showed only mild clinical effects, that were not life threatening.

Table 1. The average numbers of gonocytes and spermatogonia/cross section tubule for each dose rate

	Control (*n*)	4 mg/kg (*n*)	8 mg/kg (*n*)	16 mg/kg (*n*)
Gonocyte count (histology)	3.8 (1)^{a}	1.6 (1)^{b}	1.8 (2)^{bc}	2.0 (1)^{bc}
Spermatogonia count (PGP 9.5)	4.7 (3)^{a}	2.9 (3)^{b}	4.2 (1)^{a}	0.7 (1)^{c}

n = number of animals. $P < 0.05$ (differences within rows).

These results indicate that busulfan reduced endogenous spermatogonia in the pre-pubertal ram. This effect is observed at systemic doses of 4 mg/kg or higher; however, doses of 8 mg/kg and above are lethal to the survival of the animal. The use of direct heat (45ºC) or cold (0ºC) to the testes does not affect gonocyte numbers in ram lambs; however, effects on more mature stages was not studied.

(1) Brinster RL, Zimmermann JW. (1994) Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA* **91**, 11 298–11 302.

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PROTEOMIC APPROACHES TO THE STUDY OF CONCEPTUS FLUIDS FROM FIRST TRIMESTER BOVINE PREGNANCIES

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Advanced reproductive technologies using *in vitro* production systems in cattle and other livestock species offer significant opportunities for improving the reproductive performance of the female. Impeding the development is the overall inefficiency and significant costs surrounding the high incidence of embryo wastage, particularly during early pregnancy and the occurrence of foetal developmental abnormalities, placentation irregularities and late term losses.

Effective molecular interchange and communication between the conceptus and its mother is of utmost importance for the development, growth and continued viability of the foetus. We are using a proteomics approach to determine the protein profile (proteome) of the conceptus fluids (amniotic and allantoic) during the first trimester of normal, IVF and NT pregnancies. Proteomic analysis and comparison of the proteins present in these fluids may lead to the discovery of novel biomarkers which can be used to determine foetal viability status. Collections of foetal fluids from abattoir collections of Day 35 to Day 100 pregnancies were characterised with respect to volume and total protein estimates and subjected to various fractionation procedures. Protein fingerprints of conceptus fluid fractions have been generated by two-dimensional electrophoresis and the identity of proteins determined by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry and database searches. Enrichment of the low-abundance and low molecular weight proteins has been achieved by ultrafiltration under denaturing and reducing conditions.

We were able to establish the identity of the majority of high-abundance proteins and a significant proportion of low-abundance proteins in both amniotic and allantoic fluids using this method. Searching of MSDB or NCBInr mammalian protein sequence databases coupled with the putative protein database deduced from the first draft assembly of the bovine genome sequence, was achieved using Mascot software.

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GONADOTROPHIC HORMONES AFFECT THE UTERUS, IMPLANTATION AND FETAL DEVELOPMENT IN MICE

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Although gonadotrophin stimulation using equine chorionic gonadotrophin (eCG) adversely influences pregnancy and fetal development, the effects of stimulation using recombinant human follicle stimulating hormone (rhFSH) are largely unknown. Evidence from human studies indicates that rhFSH may also be detrimental to the endometrium and implantation. We investigated the effect of gonadotrophin stimulation on ovarian hormones and uterine characteristics in the peri-implantation period, and pregnancy outcomes in mice. Adult female mice were stimulated with 2.5 IU or 10 IU rhFSH or 5 IU eCG, followed by 5 IU human chorionic gonadotrophin (hCG). Control mice received saline injections. On day 4 of pseudopregnancy mice either had embryos transferred to the uterus or were sacrificed and blood and uterine samples collected. Plasma progesterone and estradiol concentrations were determined by radioimmunoassay. Uterine mRNA levels of the estrogen and progesterone receptors (*ERa* and *PR)*, leukaemia inhibitory factor *(LIF)*, homeobox gene *Hoxa10* and vascular endothelial growth factor (*VEGF*) were determined by real-time RT-PCR. Uterine protein distribution of PR was determined by immunohistochemistry. Embryo transfer recipients were sacrificed on day 15 to assess pregnancy outcomes. Gonadotrophin stimulation increased plasma progesterone concentration compared to controls, while estradiol concentrations were not affected. Stimulation also reduced total *LIF* mRNA and altered the spatial distribution of PR protein in the uterus on day 4. Embryo transfer recipients administered eCG or 10IU rhFSH had lower pregnancy rates compared to controls (11, 35 and 75% respectively) and fetuses from the rhFSH group had reduced mean weight (94 ± 6 *v.* 176 ± 8 mg), length (11 ± 0.2 *v.* 12 ± 0.1 mm) and maturity (14.1 ± 0.09 *v.* 14.6 ± 0.05 days) compared to controls. These results demonstrate that gonadotrophin stimulation induces changes to the maternal reproductive tract prior to implantation that have consequences for the establishment of pregnancy and fetal development in the mouse.

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SUPRESSOR OF CYTOKINE SIGNALLING 3 REGULATES IL-11 INDUCED HUMAN ENDOMETRIAL STROMAL CELL DECIDUALIZATION.

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Decidualization of endometrial stromal cells is critical for embryo implantation and establishment of pregnancy. Locally produced cytokines such as interleukin (IL)-11 enhance decidualization of human endometrial stromal cells (HESC). IL-11 signaling is negatively regulated by suppressor of cytokine signaling (SOCS) proteins. IL-11 stimulates SOCS3 in human pituitary cells. The aim of this study was to examine the role of SOCS3 on IL-11 induced HESC decidualization. Decidualization of HESC was assessed using an *in vitro* model in which estrogen (E)+progesterone (P) or cAMP was administered for 8 days to cells. Medium was collected for prolactin (PRL) assay (a decidual marker). Cellular protein was extracted for Western analysis and cellular RNA for real-time RT-PCR analysis. SOCS3 was overexpressed in HESC cells and the effect on decidualization assessed. HESC treated with E+P or cAMP secreted PRL from day 6. Treatment of HESC with E+P or cAMP increased the abundance of SOCS3 protein, coinciding with an increase in PRL secretion. cAMP maximally stimulated SOCS3 protein and mRNA during decidualization. Antiprogestin (onapristone) added to E+P or cAMP treated cells at day 6 reduced PRL secretion but had no influence on SOCS3 abundance suggesting that SOCS3 protein was not regulated via the P-receptor pathway. Addition of IL-11 to HESC increased SOCS3 abundance from 1 h. SOCS3 abundance returned to control levels following treatment of cells with IL-11 and IL-11 neutralising antibody. SOCS3 overexpression in HESC treated with cAMP reduced PRL secretion compared to mock- or non-transfected HESC. Furthermore, IL-11 mediated decidualization was diminished by SOCS3 overexpression. We have demonstrated for the first time that SOCS3 regulates IL-11 induced decidualization and that SOCS3 overexpression in HESC disrupts decidualization. This knowledge is important in understanding the mechanisms by which IL-11 promotes decidualization of HESC and thus the formation of decidua, an essential component of a functional placenta.

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INTERLEUKIN-10 INHIBITS TNFα SYNTHESIS AND PROTECTS AGAINST LPS-INDUCED MISCARRIAGE AND PRETERM LABOUR

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The immune-deviating and anti-inflammatory cytokine interleukin-10 (IL-10) is expressed throughout pregnancy in the decidual and placental tissues. Mice with a null mutation in the IL-10 gene mice are fertile with no reduction in litter size, although fetal growth trajectories and placental structure are altered. IL-10 is known to terminate inflammatory responses and to limit inflammation-induced tissue pathology by inhibiting macrophage synthesis of tumor necrosis factor-α (TNFα). To investigate the anti-inflammatory role of IL-10 in pregnancy, the susceptibility of null mutant mice to low dose LPS-induced miscarriage and preterm labour has been evaluated. When IL-10 null mutant C57Bl/6 (IL-$10^{-/-}$) and control (IL-$10^{+/+}$) mice were given low dose E.coli LPS on d10 of pregnancy, IL-10 deficiency was associated with greater fetal loss with fewer mated IL-$10^{-/-}$ mice carrying viable fetuses at day 18 and increased rate of fetal resorption. In mice treated with LPS on day 17, preterm delivery within 24 h occurred in a higher proportion of IL-$10^{-/-}$ mice than IL-$10^{+/+}$ mice. LPS induced very high and sustained TNFα and IL-6 content in serum, uterine and placental tissue in IL-$10^{-/-}$ mice, associated with upregulated mRNA expression of both cytokines in gestational tissues. These data show that IL-10 modulates placental resistance to inflammatory stimuli by down-regulating expression of the pro-inflammatory cytokines TNFα and IL-6. We conclude that IL-10 has a dual role in pregnancy, acting to regulate placental morphogenesis and fetal growth trajectory, and to protect against inflammation-induced miscarriage and preterm labour.

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EXPERIMENTALLY INDUCED HYPOGLYCEMIA: A MODEL TO EXAMINE THE EFFECTS OF LACTATION ON REPRODUCTIVE FUNCTION IN DAIRY COWS?

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The metabolic changes subsequent to lactogenesis have been associated with poor reproduction in high-producing dairy cows.[1,2] Periods of hypoglycaemia reflect severe energy deficit and are associated with changes in plasma levels of growth hormone (GH), insulin-like growth hormone-I (IGF-I) and insulin-like growth hormone-II (IGF-II). Somatotropic activity has been shown to influence reproductive functions.[3–5]

This study evaluated the effects of experimentally induced hypoglycaemia in seven non-lactating cows, over a 7-day period. Phloridizin treatment (8 g/d) resulted in urinary glucose loss (control: 3.5 ± 1.0 g/d and phloridizin: 468 ± 46 g/d) and decline in plasma glucose (control: 60.6 ± 0.6 mg/dL and phloridizin: 71.8 ± 0.4 mg/dL; $P < 0.001$). Treatment increased plasma beta hydroxybutyrate (BOH), non-esterified fatty acids (NEFA) and IGF-I concentrations ($P < 0.001$). Plasma insulin and GH concentrations did not differ. During treatment, expression of mRNA for total growth hormone receptor (GHR(tot); $P = 0.012$) and GHR(1A) ($P < 0.001$) in liver tissue declined. Luteal and follicle diameters in ovaries recovered after treatment did not differ. Expressions of mRNA for IGF-I ($P = 0.052$) and interleukin-1β (IL-1β) in corpus luteum and for 3β-hydroxysteroid dehydroxyenase (3β-HSD) within dominant follicles were significantly elevated, while mRNA for GHR(tot), cytochrome P450 cholesterol side chain cleavage enzyme (P450-SCC), and steroidogenic acute regulatory protein (StAR) tended to increase ($P < 0.1$) with treatment.

The treatment resulted in changes similar to those of nutritional stress or the initiation of lactogenesis. Phloridizin-induced hypoglycaemia may be a model to investigate mechanisms linking glucose metabolism, and the somatotropic axis to reproductive function. The advantages of such a model, is that it allows for strict control of the level of hypoglycaemia. The use of non-lactating cows also removes the feedback mechanisms that modulate mammary gland requirements, and therefore will minimize the between cow variance when using lactating cows.

This work was completed with the help from Dexcel Farms and the Dairy Cattle Fertility team. This study was funded by the New Zealand Foundation for Research, Science and Technology (DRCX 0202).

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OPTIMAL TESTICULAR SIZE OF DONOR AND RECIPIENT FOR TESTICULAR GERM CELL TRANSPLANTATION IN THE BOVINE

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The maturity status of donor and recipient testis appears to be important in the efficiency of testicular germ cell transplantation. When neonatal mice were used as recipients, they show 9.4 times greater colonization and 4 times larger colony size than in the adult.[1] The objective of this study in cattle was to investigate the effect of testicular maturity of donor and recipient calves on success of the testis cell transplant procedure. Testicular maturity was measured indirectly by scrotal circumference and donor testicular cells were enzymatically isolated from 8 Angus calves aged 5–7 months (scrotal circumference, SC of 18–22cm) and injected into both testes of 12 recipient calves aged 4–6 months (SC 15–21cm. Donor cells were labeled with the red fluorescent dye PKH26 then transferred into the rete testis under ultrasonographic guidance. Castration of recipients was performed 2–6 months following injection and then frozen sections were used to localize the PKH26 positive donor cells. Five sections from different 5 areas in each testis were prepared and 100 tubules were counted.

In 15 of the 24 (63%) testes, PKH positive donor cells were identified. There was no correlation between colonization rate and maturity of donor animal testis for the range of testis sizes studied. Testis cells from donors of SC 18-20 cm or of SC 21-22 did not result in different number of recipient testis with positive cells (7/10 (70%) *v.* 8/14 (57%)) or the number of positive cells per testis (1.92 ± 0.67% *v.* 2.5 ± 1.01%). Recipient maturity (SC of 15–18 cm *v.* SC of 19–21 cm) had no effect on the colonization rate (7/11 (64%) *v.* 8/13 (62%)); however, there were significantly more positive cells per testis in less mature (SC of 15–18) recipients (3.18 ± 1.21% *v.* 1.52 ± 0.64% $P < 0.05$)). In summary we have demonstrated successful testicular germ cell transplantation between calves and while donor testis cell age appeared to have little effect on the efficiency of colonization, less mature testis provided more suitable conditions for colonization.

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ANDROGENS AUGMENT THE MITOGENIC EFFECTS OF OOCYTE-SECRETED FACOTRS AND GROWTH DIFFERENTIATION FACTOR 9 ON PORCINE GRANULOSA CELLS

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Androgens, acting directly through the androgen receptor (AR), are thought to promote granulosa cell (GC) growth *in vivo*, but generally inhibit growth and promote GC differentiation *in vitro*. We hypothesised that the growth-promoting action of androgens on GC requires paracrine signalling from the oocyte. To test this hypothesis, we cultured mural GC from small antral (1–3mm) pre-pubertal pig follicles in the presence or absence of denuded oocytes (DO) from the same follicles to examine whether mitogenic responses, determined by uptake of tritiated thymidine, to combinations of FSH, insulin like growth factor 1 (IGF1) and dihydrotestosterone (DHT; 500 nM), were influenced by oocyte-secreted factors (OSFs). To further explore the identity of such factors, we performed the same experiments, substituting recombinant mouse growth differentiation factor 9 (GDF9), a known OSF, for the DO. Alone, DHT induced a small (<2-fold), but consistent increase in IGF1-stimulated DNA synthesis. OSFs stimulated DNA synthesis in all experimental combinations, most significantly in the presence of IGF1 ($P < 0.0001$), and DHT enhanced ($P < 0.05$) the stimulatory effect of OSFs in all instances. Like OSFs, GDF9 substantially increased IGF1-stimulated DNA synthesis ($P < 0.0001$), and again, DHT enhanced ($P > 0.01$) this effect. In further experiments, two AR agonists, testosterone (10-1000nM) and DHT (5–500 nM), dose-dependently augmented the mitogenic effect of OSFs or GDF9 in the presence of IGF1. Only the highest doses of androgen had an independent stimulatory effect; lower doses required the presence of an OSF(s). Antiandrogen (hydroxyflutamide) treatment, used to block AR activity, antagonized the androgen X GDF9 interaction. In conclusion, androgens, via activation of the AR, stimulate porcine GC proliferation *in vitro* by potentiating the growth-promoting effects of oocytes or GDF9. These signalling pathway interactions are likely to be important regulators of folliculogenesis *in vivo* and may cause the excess follicle growth that is observed in androgen-treated female animals.

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PROSPECTIVE ISOLATION OF HUMAN ENDOMETRIAL MESENCHYMAL STEM CELLS USING CD146 AND PLATELET-DERIVED GROWTH FACTOR RECEPTOR-β

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The human endometrium has an immense regenerative capacity. Previously we identified a small population of clonogenic endometrial stromal cells or mesenchymal stem cells (MSC).[1] Prospective isolation of MSC allows for their characterisation. We hypothesise that the expression of MSC marker, CD146, and pericyte/fibroblast marker, platelet-derived growth factor receptor-β (PDGFRβ), will enable the prospective isolation of endometrial MSCs. The aims of this study were to (1) determine if CD146 and PDGFRβ will prospectively isolate endometrial MSCs with clonogenic activity, (2) identify their location in human endometrium, and (3) determine the differentiation capacity of $CD146^+PDGFR\beta^+$ stromal cells.

Endometrial tissue from 13 ovulating women undergoing hysterectomy was digested with collagenase to produce single cell suspensions. Leukocytes and epithelial cells were removed. Stromal cells were analysed by flow cytometry, FACS sorted into enriched and depleted populations, and cultured for clonal analysis.[1] Immunohistochemistry was performed on full thickness human endometrium. Sorted populations of stromal cells were passaged for culture in various differentiation media, and analysed for adipogenic, myogenic, chondrogenic or osteogenic differentiation by histological stains and RT-PCR.

A small, consistent population of $CD146^+$ endometrial stromal cells was identified (7.8 ± 1.1%, $n = 8$). In contrast, PDGFRβ expression varied (34.1 ± 9.7%, $n = 5$), and 2.5% of cells were $CD146^+PDGFR\beta^+$. Clonogenicity of $CD146^+$ stromal cells was significantly higher than $CD146^-$ stromal cells, 2.5 ± 1.1% and 1.2 ± 0.6%, respectively ($n = 6$, $P = 0.03$). $CD146^+$ stromal cells were located perivascularly, similar to bone marrow MSCs, whereas PDGFRβ weakly stained the stroma, with stronger staining observed around the blood vessels. $CD146^+$ cells differentiated into adipocytes, smooth muscle cells, chondrocytes and osteoblasts.

This study identified CD146 as a marker of clonogenic endometrial stromal cells, and supports the perivascular location of endometrial MSCs. It also demonstrated that $CD146^+$ cells can differentiate into four mesenchymal lineages. These data suggest that CD146 can be used for the prospective isolation of endometrial MSCs, which may be further enriched by PDGFRβ co-expression.

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PROGESTERONE STIMULATES ENDOTHELIAL CELL PROLIFERATION, BUT NOT STROMAL CELL PROLIFERATION, IN MOUSE ENDOMETRIUM

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Previous studies have suggested that progesterone stimulates stromal cell (SC) proliferation in the mouse endometrium[1]. However, these studies have not differentiated endothelial cells (EC) from other SC. In this study, we investigated the effects of progesterone on cellular proliferation in ovariectomised mouse endometrium. We hypothesised that progesterone would stimulate both SC and EC proliferation. One group of CBA × C57 mice (n = 6) were treated with a single injection of 100 ng of estradiol on day eight following ovariectomy, followed by a day with no treatment and three consecutive daily injections of 1 mg progesterone. Other groups were treated with either the vehicle (n = 5), estradiol (n = 4) or progesterone (n = 5) injections only. All groups were dissected on day 13 after ovariectomy, 4 h following a BrdU injection. CD31/BrdU double staining immunohistochemistry allowed proliferating EC to be differentiated from proliferating SC. Mice treated with progesterone only had significantly higher EC proliferation in comparison to females treated with progesterone following oestrogen priming (P = 0.05) or vehicle only (P = 0.01) (progesterone only: median = 97.3 proliferating EC (PEC)/mm^2 [range = 60.8–203.4]; oestrogen plus progesterone: 41.0 PEC/mm^2 [8.9–86.9]; vehicle only: 0.0 PEC/mm^2 [0.0–3.1]). Unexpectedly, there was no significant difference in SC proliferation among the treatment groups (progesterone only: 50.1 PSC/mm^2 [39.2–102.6]; oestrogen plus progesterone: 46.1 PSC/mm^2 [12.6–120.8]; vehicle only: 44.8 PSC/mm^2 [17.3–68.4]). To determine if VEGF had a role in the progesterone-induced EC proliferation, the previous experiment was repeated with the inclusion of mice treated with VEGF anti-serum. The addition of VEGF anti-serum significantly inhibited progesterone-induced EC proliferation (46.8 PEC/mm^2 [38.9–128.0]; P = 0.04], but had no effect on SC proliferation (P = 0.3). These results demonstrate that progesterone stimulates endometrial EC proliferation, but not SC proliferation as reported by earlier studies[1]. Studies are currently underway to further investigate the role of VEGF in mediating progesterone effects on endometrial EC.

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SEMINAL PLASMA INFLUENCES PREGNANCY OUTCOME THROUGH EFFECTS ON BOTH UTERINE RECEPTIVITY AND THE PRE-IMPLANTATION EMBRYO

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Optimal uterine receptivity and viability of the preimplantation embryo are key requirements for successful initiation of a pregnancy. The molecular and cellular events activated by insemination are thought to play a role in establishing an environment conducive to positive pregnancy outcomes. Previously, we have employed a blastocyst transfer model to demonstrate that lack of uterine exposure to seminal plasma at the time of conception results in fetal growth restriction and impaired postnatal development of progeny. Here we utilise a 2-cell embryo transfer technique to determine the effects of uterine seminal plasma exposure on embryo development and its subsequent effects on pregnancy outcomes at term in the mouse. Two-cell embryos were collected from donor mice and transferred into the oviduct of day 1 pseudopregnant recipient females primed either by mating with vasectomised (vas) males or vasectomised males from which the seminal vesicle glands, the major source of seminal plasma, had been surgically removed (vas + svx). At day 18 of pregnancy there was no difference in the number of total implantations, however fetal weight was reduced by approximately 5% (P < 0.05). Placental weight at day 18 was significantly increased, resulting in an increase in the fetal weight: placental weight ratio, an indicative measure of placental function. At term, progeny from pregnancies initiated in the absence of semen exhibited a 10% reduction in birth weight and remained smaller through the pre-weaning period. Fetal growth retardation was more severe when embryos were transferred at 2-cell stage than in comparable experiments where blastocysts were transferred after development in a normal donor tract. In combination, these experiments demonstrate that seminal plasma exposure at the time of conception impacts on pregnancy outcome through independent actions on both uterine receptivity and embryo development. These findings are important when considering current practises of assisted reproductive technologies, as well as having implications in pregnancy outcomes in the general population where seminal plasma signalling may be compromised.

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PLACENTAL EXPRESSION OF SECRETED FRIZZLED RELATED PROTEIN (SFRP4) IN THE RAT: ASSOCIATION WITH β-CATENIN LOCALIZATION AND REGULATION BY GLUCOCORTICOIDS

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Wnt genes regulate a diverse range of developmental processes including placental development. Activation of the wnt pathway results in nuclear translocation of β-catenin and activation of the TCF/Lef family of transcription factors. The secreted frizzled related proteins (sFRPs) modulate wnt signaling by binding to either the wnt ligand or its transmembrane frizzled receptor. The current study examined the spatial and temporal expression of one of these secreted molecules, sFRP4, in the rat placenta over the final third of pregnancy, and whether associated changes occurred in the expression and localization of β-catenin. The expression of sFRP4 and β-catenin was also analysed in a model of glucocorticoid-induced placental growth restriction.

Analysis by quantitative RT-PCR over the final third of pregnancy demonstrated a dramatic increase in sFRP4 mRNA (14-fold, $P < 0.001$) specifically within the basal zone of the placenta near term. *In situ* hybridization and immunohistochemistry localized sFRP4 expression primarily to giant trophoblasts of the basal zone. In addition, sFRP4 protein was notably upregulated in association with a restricted nuclear translocation of β-catenin. Maternal dexamethasone treatment (1 μg/mL in drinking water; day 13–22) further increased the expression of sFRP4 mRNA in both the basal (120%, $P < 0.05$) and labyrinth (285%, $P < 0.01$) zones of the placenta at day 22 (term = 23 days) compared to untreated controls.

These data indicate that sFRP4 expression is increased in the basal zone of the rat placenta, the major site of apoptosis in late pregnancy, and is further stimulated by glucocorticoids. Moreover, the observed inhibition of β-catenin expression and its nuclear translocation suggest that sFRP4 inhibition of wnt signaling in the placenta may contribute to placental apoptosis and ultimately fetal growth restriction.

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NEURONAL REGENERATION PEPTIDE (NRP): A NOVEL TROPHOBLAST MIGRATION AND SURVIVAL ENHANCING FACTOR

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Autocrine and paracrine factors regulate survival, proliferation, migration and invasion of placental cytotrophoblasts cells. While trophoblast migration appears to be tightly controlled, the nature of the chemoattractive factors that facilitate and direct trophoblast invasion remains undefined. Our group recently discovered a chemottractive factor (NRP) that exerts its biological activities on the CNS. Studies of NRP actions reveal extensive influences on postnatal neuronal migration, differentiation and survival. Based on the neuronal activities of NRPs and parallels between neuronal and placental cell behavior patterns, we speculated that NRPs could be involved in placental development.

Migration assays were performed over 22 h using Boyden chambers pre-coated with NRP and laminin ($n = 6$ chambers per condition), to test the effect of NRPs on trophoblast migration. CTBs were isolated from term placentas by trypsin digestion and Percoll purification, and experiments were conducted within 6 h. Trophoblasts were seeded into the inner chamber (50 000 cells/well) in M199 media supplemented with 10% FBS and antibiotics. Total number of cells migrating was counted. Migration was increased by 95 ± 14 % (mean ± SEM) in the presence of 100 fM NRP ($P = 0.0016$, *t*-test) compared to controls (bovine serum albumin). Survival assays were also performed using both primary trophoblasts and Jar choriocarcinoma cells. Apoptosis over 48 h, induced by treatment with TNF-α (5 ng/mL) and IFN-γ (100 U/mL), was completely abrogated by 10 pM NRP in Jar cells and 1 pM in primary trophoblasts, as judged by MTT assay (mitochondrial activity).

Inadequate placentation is implicated in the pathogenesis of a number of serious pregnancy disorders such as preeclampsia. Our findings suggest important roles for NRPs in regulating trophoblast migration and survival. The possibility that defective NRP actions may be involved in various placental pathologies, or that NRPs could be used pharmacologically to augment placentation, remain to be explored.

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SEX DIFFERENCE IN THE EFFECT OF CORTISOL ON THE LH RESPONSE OF THE PITUITARY TO EXOGENOUS GNRH IN HYPOTHALAMO-PITUITARY DISCONNECTED GONADECTOMISED SHEEP

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We have used the hypothalamo-pituitary disconnected (HPD) sheep model to investigate direct pituitary actions of cortisol to suppress LH secretion in response to exogenous GnRH. We previously observed that, during the non-breeding season, treatment with cortisol did not suppress the LH response to GnRH in HPD gonadectomised rams or ewes.[1] In the present experiment, we tested the effect of cortisol on the LH response to exogenous GnRH in gonadectomised HPD sheep during the breeding season. Using a cross-over design, HPD gonadectomised Romney Marsh rams (n = 6) and ewes (n = 5) received a saline or cortisol (250 μg/kg/h) infusion for 30 h on each of two days, one week apart. All animals were treated with 125 ng i.v. injections of GnRH every 2 h during a 6 h control period preceding the infusion and during the infusion. Jugular blood samples were taken during the control period and the first 6 h and last 6 h of the infusion (over 3 LH pulses). Mean plasma concentrations of LH and LH pulse amplitudes, driven by programmed GnRH injections, were similar in gonadectomised rams and ewes and there were no significant effects of saline infusion between the control periods or the saline infusion in either sex. The amplitude of LH pulses was significantly ($P < 0.05$) reduced in rams during the first 6 h of the cortisol infusion compared to the control period, but there were no effects of the cortisol infusion in ewes. These data show that, in the absence of sex steroids, there is a sex difference in the mechanism by which cortisol acts at the pituitary to reduce LH secretion in response to exogenous GnRH in HPD gonadectomized sheep during the breeding season. We conclude that the effect of cortisol to reduce secretion of LH involves an action on the pituitary, at least in gonadectomised rams.

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IS THE HIGH LEVEL OF CIRCULATING FSH IN BOOROOLA EWES DUE TO THE BMP R1B MUTATION?

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Booroola ewes are characterised by having high ovulation rate and litter size caused by the action of the Booroola fecundity gene (*FecB*), which encodes the bone morphogenetic protein receptor (BMPR) type 1b mutation (Q249R). The product of the *FecB* gene influences both pituitary and ovarian activity. In both neonatal and adult Booroola ewes carrying a double copy of the FecB allele, plasma concentrations of the pituitary-derived follicle stimulating hormone (FSH) are higher than in non-carrier females. BMPR-1b is expressed in the sheep pituitary gland and recent reports have shown that in addition to GnRH, TGF-β family members such as BMP 6, 7, and 15 induce pituitary FSHβ expression. Thus BMPs may function in regulating FSH synthesis in the pituitary. We are investigating whether we can detect induction/secretion of FSH in response to BMP treatment of ovine primary pituitary cell cultures, with the aim of elucidating whether the Booroola mutation is the cause of the increased FSH secretion. Due to the high variance between cultures from different animals, and between replicates from the same animal, we are attempting to determine the best method for approaching the question. Some results from these investigations will be presented.

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REPEATED 24-HOUR EXPOSURE OF EWES TO RAMS DURING THE TRANSITION INTO BREEDING SEASON COMPACTS THE MATING OF THE FLOCK

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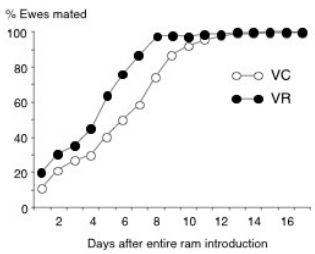
Synchronisation of oestrus during the breeding season is currently only possible with exogenous hormones, but these practices are not sustainable with growing consumer demand for 'clean, green and ethical' products.[1] Therefore non-pharmacological methods of oestrus control need to be developed. Exposure of anoestrous ewes to rams induces a synchronous ovulation, but this 'ram effect' is not thought to work in the breeding season. This experiment tested whether repeated 24-h exposure of ewes to rams during the transition into the breeding season would compact the subsequent distribution of mating. This experiment used two groups of ewes: Group VR (n = 103) was exposed to 3 vasectomised rams for 24 h on Days 0 (September 10th), 17 and 34 of the study, while Group VC (n = 106) remained isolated from rams. On Day 50, raddled entire rams were introduced (ERI) and marks recorded daily. Blood was sampled from a subgroup of 35 ewes per treatment twice weekly for progesterone. At mating, synchrony scores were calculated for each group (lower score = greater synchrony). VR ewes mated 5 days (median) after ERI whereas VC ewes mated after 6 days (interquartile range: 2–6 and 3–8; $P < 0.001$). VR ewes had a lower synchrony score than VC ewes (2.34 *v.* 3.08; $P < 0.01$) and less variance around the median time of mating (Levene' test; $P < 0.05$) indicating a more compact mating (Fig. 1). The median onset of the breeding season was earlier in VR ewes (median: October 7th; interquartile range: 3rd–7th) than VC ewes (median: October 10th; interquartile range 7th–10th; $P < 0.01$). The enhanced synchrony in VR ewes was not solely due to a more synchronous onset of the breeding season. In conclusion, repeated short-term ram exposure during the transition into the breeding season is a potential non-pharmacological method of oestrus synchronisation during the breeding season.



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A DYNAMIC MODEL OF THE CONTROL OF PULSATILE LUTEINIZING HORMONE SECRETION BY GONADOTROPHIN-RELEASING HORMONE

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Infusion of GnRH in a continuous manner or with a very high pulse frequency initially stimulates but then downregulates LH secretion.[1,2] This phenomenon is caused by the slow rate of internalisation of the GnRH receptor (GnRH-R) and the subsequent slow return of receptors to the plasma membrane of the gonadotroph.[3] Pulsatile release of GnRH overcomes this problem by allowing a delay between successive stimulations. It is difficult to determine the relative importance of critical control points in this process in an animal model because GnRH activity reflects integrated inputs from many internal and external factors. We are therefore using SAAM II software to develop a compartmental model of the relationship between GnRH-R availability and LH responses following changes in GnRH pulse frequency. The model has three receptor states (free, bound, and internalised) and one LH compartment (Fig. 1). We assumed LH release is a function of the amount of receptor that binds GnRH. Following GnRH binding, receptors are rapidly lost as they enter the internalised state and then slowly returned to the membrane surface. We further assumed that the slow rate of receptor return explains the decrease in LH response with very high frequencies of GnRH pulses. The values for parameters were based on data obtained from experiments with sheep. In our current version of the model, downregulation is observed when gonadotrophs are stimulated with GnRH pulses every 15 min (Fig. 1), but not with pulses every 30 or 60 min, at a slow recycling rate (0.004 min^{-1}). In contrast, LH secretion increases when GnRH is pulsed every 15 min and recycling rate is increased to 0.04 min^{-1}. This suggests that, in sheep, a recycling rate between 0.004 and 0.04 min^{-1} is a critical aspect of the intracellular control of the process. Future work will include steroid feedback loops.

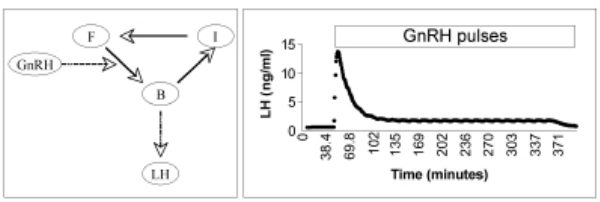

Figure. 1. Left: relationships between compartments for GnRH, LH and the GnRH receptors that are free (F), bound (B), and internalised (I). Right: simulation with a pulsatile infusion that induces down-regulation of receptor numbers and loss of LH response.

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PHOTO INHIBITED HEAT SHOCK PROTEIN 108 GENE EXPRESSION IN THE CHICKEN HYPOTHALAMUS

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In domestic juvenile chickens kept on short days, photoinduced luteinsing hormone (LH) release, and by inference gonadotrophin-releasing hormone (GnRH) release, are readily detectable within 4 days of photostimulation.[2] The molecular mechanisms responsible for the rapid photoinduced release of LH and GnRH in avian species are unknown. It has been suggested that it might involve a cascade of gene expression associated with an increase in cfos in the basal hypothalamus and glial cells in the median eminence.[1] A microarray was made consisting of known genes of interest and clones obtained from a hypothalamic short day/long day subtractive library. An experiment was undertaken to determine if this reproductive neuroendocrine microarray could detect new targets for study in the chicken model of photostimulated GnRH release. The microarray was interrogated with hypothalamic RNA from juvenile chickens showing an increase in plasma LH after 4 days of photostimulation. Six genes were identified as showing changes in expression after photostimulation on the microarray. However, only one gene, encoding heat shock protein 108 (HSP108), could be confirmed by quantitative competitive RT-PCR. The expression of this gene decreased both in the hypothalamus and the optic tectum. Treatment of short day juvenile chickens with thyroxine, to mimic the effects of photostimulation, resulted in LH release and depression of HSP108 expression in the anterior but not the basal hypothalamus. Immunocytochemical analyses showed that HSP108 is widely distributed in the brain including glial-like cells with terminals in the median eminence. HSP108 is suggested as a candidate protein involved in the regulation of gonadotrophin release from the median eminence by glial cells.

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THE ROLE OF INSULIN IN MILK PROTEIN SYNTHESIS

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The mammary explant culture model has been frequently used to mimic lactation and to examine the endocrine control of milk protein gene expression. Studies in the mouse show the expression of the milk protein genes in explants requires insulin in the presence of prolactin and cortisol. The role of insulin in milk protein synthesis in the dairy cow is not as clear. The bovine mammary explant culture model has been utilised to show that insulin is essential for alpha-s1-casein gene expression and the synthesis of the casein proteins. In addition, mouse culture experiments were undertaken to provide an insight into the underlying molecular mechanisms of insulin action in hte mammary gland. A global analysis of the genes induced in the cultured explants was done using Affymetrix microarray and showed 132 genes, including the major milk protein genes, required the complement of insulin, cortisol and prolactin for maximal expression. Twenty-seven genes showed a 3-fold change in gene expression in response to insulin. The function of these genes can be largely categorised into maintenance of cell integrity, signal transduction, transport mechanisms, cellular metabolism and a direct effect on gene transcription in the nucleus. The requirement for insulin in milk protein synthesis is highlighted by its role in inducing the STAT5 gene, known to be a key transcription factor for the milk protein genes. Interestingly, dairy cows of high genetic merit have unusually low serum concentrations of insulin. This has occured in association with a high selection pressure for milk volume that has altered the regulation of blood glucose homeostasis. Our study indicates that this intensity of selection for high milk volume could be compromising the dairy cow's potential for milk protein production: Has selecting for milk volume in many populations of dairy cows been achieved by lowering circulating insulin levels with consequent effects on the efficiency for milk protein yield as well as compromised reproductive performance.

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ANTI-APOPTOTIC EFFECTS OF GLUCOCORTICOIDS AND PROGESTERONE IN A HUMAN MAMMARY EPITHELIAL CELL LINE

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Previous studies in the lactating rat mammary gland have shown that removal of both progesterone and glucocorticoids can induce apoptosis, and that replacement with either steroid alone can prevent this effect.[1] These results raised the possibility that progesterone may exert its anti-apoptotic effects in the mammary gland via the glucocorticoid receptor (GR). A similar mechanism has previously been proposed to account for the action of progesterone in the corpus luteum.[2] To assess this possibility we examined the effects of glucocorticoids and progesterone on apoptosis in the non-tumorigenic human mammary epithelial cell line, MCF-10A.

Initial PCR analysis demonstrated that MCF-10A cells do not express progesterone receptor mRNA (PR-A or PR-B). MCF-10A cells were incubated in serum-free DMEM:F12 medium with additives in the presence or absence of cortisol (10^{-6}M) or progesterone (10^{-5}M to 10^{-8}M). DNA fragmentation (an index of apoptosis) was quantitated by either 3′-end labelling or by a novel method of image analysis of SYBR® gold-stained gels. DNA fragmentation was clearly increased after 24 h in the absence of cortisol, consistent with previous reports for this cell line.[3] In a separate experiment, replacement of cortisol with progesterone also reduced apoptosis by 48 h, although this protection was evident only at the two highest progesterone doses (10^{-5}M and 10^{-6}M).

These results suggest that despite the absence of a progesterone receptor, progesterone is able to prevent apoptosis in MCF-10A cells, albeit at a physiologically high doses. This supports the hypothesis that some actions of progesterone may be mediated via the GR. Further studies are required to determine the precise molecular pathways by which cortisol and progesterone prevent apoptosis.

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REGULATED EXPRESSION OF THE C-FMS RECEPTOR CHARACTERISES DISTINCT OVARIAN MACROPHAGE POPULATIONS

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Macrophages represent a major immune cell type in reproductive tissues and are thought to regulate multiple aspects of reproduction, including ovarian function. We have previously shown a distinctive phenotype of ovarian thecal macrophages present around the preovulatory follicle, such that secreted cytokines are uniquely regulated within these cells across the oestrus cycle. C-fms is a macrophage-specific gene that encodes the receptor for colony-stimulating factor-1 (CSF-1), and that regulates macrophage proliferation, differentiation and migration, as well as pro-inflammatory responses.[1,2] We acquired transgenic mice (from DA Hume, Institute for Molecular Bioscience, University of Queensland) that express green fluorescent protein (GFP) exclusively in macrophages under direction of the *c-fms* gene promoter.[3] In ovaries from these animals we have previously reported that macrophages constitutively positive for macrophage markers, F4/80 and MHCII, exhibited spatially regulated expression of GFP (c-fms); being GFP+ within the stroma surrounding small follicles, particularly atretic follicles, but GFP– in theca surrounding preovulatory follicles and healthy corpora lutea (CL), further reinforcing the concept that these macrophages are not classically activated but have a unique resident phenotype. Further examination of the GFP+ ovarian macrophage population has revealed that the highest levels of GFP expression were in macrophages associated with TUNEL+ regressing CL and, even though CSF-1 typically induces proliferation, the GFP+ macrophages within the regressing CL did not incorporate BrdU label nor express cyclin D1. This indicates that in the murine ovary c-fms expression may not regulate ovarian macrophage proliferation or migration but more likely represents a subset of classically activated ovarian macrophages that are actively differentiating or phagocytically active.

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LEUKOCYTE TRAFFICKING IN THE OVARY OF MICE IMMUNISED WITH RECOMBINANT MURINE CYCLOMEGALOVIRUS EXPRESSING MURINE ZONA PELLUCIDA 3

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Inoculation of female BALB/c mice with recombinant murine cytomegalovirus encoding murine zona pellucida antigen (MCMV-ZP3) confers infertility characterised by depletion in ovarian tertiary follicles by day 21 post inoculation followed by a progressive depletion in primordial follicles.[1] Cell mediated immune responses begin as early as day 10 post immunisation with MCMV-ZP3[2] with the recruitment of leukocytes before serum antibody can be clearly detected in mice. The physiological mechanisms leading to infertility in inoculated mice are being progressively delineated with the role of leukocyte subsets implicated in early pathological changes in ovarian architecture. The aim of this study was to investigate the effect of MCMV-ZP3 infection on leukocytes including T cells recruited into the ovary following infection with recombinant virus. Fifteen BALB/c female mice were randomly allocated into three groups of five animals at 6 weeks of age. Group one received an injection of PBS, group two and three received intraperitoneal inoculations of 2×10^4 pfu of MCMV and MCMV-ZP3 respectively. Ovaries were retrieved at day 10, 21 and 35 post inoculation and one ovary from each mouse was sectioned for immunohistochemical analysis of resident leukocytes using mAb CD45 reactive with all leukocyte lineages and mAb for CD4 and CD8 positive T cells. MCMV-ZP3 inoculation increased the abundance of ovarian leukocytes including CD4 and CD8 positive T cells for all time points post immunisation except for CD8 positive T cells 21 days post infection (Table 1).

Table 1. Abundance of positively stained leukocytes (CD45) and T cells (CD4 and CD8) in mouse ovarian sections at day 10, 21 and 35 post immunisation with MCMV-ZP3

Inoculant	Day 10			Day 21			Day 35		
	CD45	CD4	CD8	CD45	CD4	CD8	CD45	CD4	CD8
MCMV	6.82	ND	ND	4.89	ND	ND	18	3.79	10.35
MCMV-ZP3	40.33	59.65	60.35	17.41	25.17	ND	58	67	35

ND, not detected

These results suggest that leukocytes, including T cells, are involved in causing early changes in the ovary post infection with MCMV-ZP3 that lead to the depletion of existing ovarian follicles leading to life long infertility in mice. Further experiments are underway to investigate the role of antibody and changes in leukocyte populations in the ovary as the course of infection with recombinant virus progresses.

This study is funded by the Cooperative Research Centre for Pest Animal Control.

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ENDOMETRIAL LYMPHATICS ARE REDUCED IN THE FUNCTIONALIS COMPARED TO BASALIS AND MYOMETRIUM

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Information on uterine lymphatics is limited. The aim of this study was to characterise uterine lymphatic vessels and the corresponding growth factors in the endometrium and myometrium across the normal human menstrual cycle. Uterine tissues were collected from patients undergoing hysterectomy. Lymphatic and microvascular density (MVD/mm^2) was determined on serial sections of full thickness uterus (n = 45) with antibodies to D2-40, CD31, CD34 and FVIII. Lymphangiogenic growth factors VEGF-C and VEGF-D immunolocalisation was also determined on serial sections. VEGF-C, VEGF-D and lymphatic endothelial cell markers VEGF-R3 and D2-40 protein expression was determined on protein extracted from myometrium and endometrium and separated by SDS-PAGE from proliferative (n = 5) and secretory (n = 5) hysterectomy specimens. The lymphatic vessels were closely associated with smooth muscle cells of spiral arterioles and VEGF-C and VEGF-D were primarily localised in the endometrial glands, luminal epithelium and myometrial smooth muscle bundles. The lymphatic MVD was significantly reduced in the functionalis (15.1 ± 2.3mm^2) compared to basalis (80 ± 11.4mm^2) and myometrium (63 ± 9.2mm^2). Overall, lymphatics constituted 12% of all vessels in the functionalis, 60 % in the basalis and 30% of the myometrium. D2-40 positive uterine lymphatics showed considerable heterogeneity, with 88% co-localisation with the blood vessel marker CD31, but only 46% expressing CD34 and 31% with FVIII. Protein expression of VEGF-C, VEGF-D, VEGF-R3 and D2-40 were significantly reduced during the proliferative phase compared to the secretory phase and were also significantly reduced in the endometrium compared to the myometrium across the cycle ($P \leq 0.05$). Endometrial functionalis lymphatics are reduced in conjunction with a reduction in lymphangiogenic growth factors compared with the myometrium.

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THE ROLE OF UTERINE NATURAL KILLER CELLS IN CAUSING IRREGULAR BLEEDING IN HT USERS

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Menopausal hormone therapy (HT) causes irregular bleeding in up to 60% of user. This is extremely unpopular with patients, and commonly leads to invasive and expensive investigations to rule out underlying pelvic pathology. In most cases no cause is found. The aim of this study was to further elucidate the mechanisms of vascular fragility. Uterine NK cells are known to increase vascular fragility during the normal menstrual cycle. We hypothesise that HT is associated with an increase in uterine natural killer (uNK) cells. Eighty six endometrial biopsies were obtained from 59 postmenopausal users of continuous combined HT. Uterine NK cells were identified using immunohistochemistry as being CD56+. Image analysis was used to identify absolute number of CD56+ cells and their distribution within the stroma. Endometrial histology was classified using Noyes criteria. A statistically significant increase in endometrial uNK cell density was observed in HT users compared to postmenopausal women not using HT ($P < 0.001$). uNK cell populations were more marked in biopsies taken during a bleeding episode compared to those HT users with amenorrhoea ($P = 031$). uNK cells are a major regulator of endometrial vascular integrity and are known to be disrupted in irregular bleeding with progestin only contraceptives. This is the first study to report the presence of uNK cells in postmenopausal endometrium and the first to report a significant association between bleeding patterns and uNK cell density. We postulate that HT induces an increase in endometrial uNK cell populations and that their presence stimulates endometrial vascular fragility leading to bleeding.

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LPS INTRODUCED AT MATING INDUCES KC PRODUCTION IN THE MURINE UTERUS DURING EARLY PREGNANCY

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An inflammatory cascade is elicited in the female reproductive tract following mating in mice. The recruited leukocytes and cytokines have roles in facilitating implantation through activating immunological tolerance and endometrial tissue remodelling. We have previously shown that seminal plasma acts to induce synthesis of GM-CSF and IL-6 in the female reproductive tract in response to $TGF\beta_1$ present in seminal fluid. Recently we have shown that chemokines, specifically the neutrophil chemoattractant KC, is dramatically upregulated after mating. The purpose of this study was to identify the active constituent of semen responsible for KC induction. Female mice were mated with either intact, seminal vesicle deficient or vasectomized males and uterine flushings were collected approximately 8 h later, when KC content was measured by specific ELISA. KC production was increased 13-fold, 6-fold and 10-fold respectively, indicating that neither the seminal plasma nor the sperm fraction of semen was necessary for induction. To investigate more precisely the identity of the KC inducing factor, an *in vitro* primary uterine epithelial cell culture system was employed. Uterine epithelial cells were harvested from estrous female mice and exposed to a range of doses of seminal vesicle fluid, $TGF\beta_1$or lipopolysaccharide (LPS). Following addition of seminal vesicle fluid or $TGF\beta_1$ KC production was decreased by 100% and 58% respectively whereas it was increased ~2-fold in response to LPS. Together these data indicate that LPS derived from the male or lower female reproductive tract accessing the uterus after insemination is required for KC induction, and implicate commensal bacteria as having a key regulatory role in the cellular and molecular quality of the uterine immune environment during early pregnancy.

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THE POST-INSEMINATION INFLAMMATORY RESPONSE IN THE EWE

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Insemination causes an inflammatory response in the female reproductive tract of many species. The cytokine/leukocyte network initiated during this reaction is believed to enhance reproductive success.[1] This study investigated the post-insemination inflammatory response in the ewe. Fifteen nonparous ewes were mated with the same ram for 1 h and their reproductive tracts were collected 3, 6, 18, 24 or 48 h later. Another fifteen ewes were used as controls. Tissue samples and luminal mucus were collected from 10 sites in each reproductive tract and stained with haematoxylin and eosin, Diffquik and immunohistochemically using a monoclonal CD68 antibody to quantify neutrophils, eosinophils and macrophages. Presence of interleukin-8 (IL-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF) was investigated using immunohistochemistry and enzyme-linked immunosorbent assay. Neutrophils and macrophages increased in reproductive tissues following insemination. Mean cell counts in 1.5-mm^2 tissue of mated (M) and control (C) ewes demonstrated a peak in neutrophils at 6–18 h post-insemination with significant differences ($P < 0.05$) between mated and controls in the posterior cervix (M = 23.7; C = 4.1) and uterine body (M = 34.5; C = 11.5). Macrophages peaked at 18–24 h with significant differences ($P < 0.05$) between mated and controls in the vagina (M = 13.4; C = 4.6), posterior cervix (M = 10.4; C = 2.7), mid-cervix (M = 8.5; C = 3.0) and ipsilateral mid-uterine horn (M = 14.2; C = 7.9). Neutrophils increased in the lumen of the cervix and uterine body following insemination but macrophage numbers did not change. Insemination did not affect eosinophils. IL-8 and GM-CSF were detected in endometrial epithelial cells in mated and non-mated ewes. Highest concentrations of IL-8 were found in vaginal mucus. Small quantities of GM-CSF were detected in occasional mucus samples. No difference between mated and non-mated ewes was demonstrated for either cytokine. In conclusion, the post-insemination inflammatory reaction in the ewe involves an increase in neutrophils and macrophages in reproductive tissues, with neutrophils crossing the epithelium into the lumen. There was no apparent increase in IL-8 or GM-CSF in response to insemination.

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COMPARISON OF LYMPHOCYTE SUBSETS AND FUNCTION IN THE RAT AND MOUSE TESTIS

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Testicular leukocytes are assumed to be involved in immunological surveillance against infection and tumours as well as regulation of local immune responses. They are implicated in mechanisms that make the testis a successful site for tissue transplantation in both rats and mice. Our previous studies using multi-colour fluorescence flow cytometric analysis to examine isolated testicular leukocytes in the rat testis have established the existence of a significant population of predominantly $CD8^+$ T cells and a comparable number of lymphocytes expressing natural killer (NK) cell markers (NK and NKT cells). The functional activity of these testicular NK and NKT cells subsequently has been confirmed by a standard flow cytometric cytotoxicity assay using an NK-sensitive tumour cell line (YAC-1) and an NKT-sensitive tumour cell line (U937). Similar analyses of mouse testicular leukocytes have shown a slightly different pattern. The data indicate that mouse testicular lymphocytes comprise T cells, NK cells, and NKT cells, similar to the rat testis. However, while the apparent numerical densities of T cells in rat and mouse testes were similar, the numbers of NK and NKT cells were considerably lower in the mouse. Mouse testicular NKT cells were positive for staining with the tetramer CD1d/αGC, which is used to identify classical NKT cells, whereas rat NKT cells did not stain for this marker. Moreover, the CD8/CD4 T cell ratio in the mouse testis displayed a skewing towards the $CD4^+$ subset. These data highlight the possibility that the immunological environment, and hence the course of immunological events, might be quite different in the testes of the two species. The reasons for these differences are not clear, however they should be taken into account when considering studies of testicular immune processes. Finally, comparative studies of immunological process in the testes of rats and mice may be very informative.

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CALMODULIN-DEPENDENT PROTEIN KINASE II, AND NOT PROTEIN KINASE C, TRANSDUCES THE CA^{2+} SIGNAL AT FERTILIZATION

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Mouse eggs arrest at metaphase II following ovulation and are only triggered to complete meiosis when fertilized. Sperm break the cell cycle arrest by a long-lasting series of Ca^{2+} spikes that lead to an activation of the anaphase-promoting complex. The signal transduction pathway is not fully resolved but both protein kinase C (PKC) and calmodulin-dependent protein kinase II (CamKII) activities increase at fertilization and previous pharmacological studies have implicated both in cell cycle resumption. Here we used a combination of pharmacological inhibitors and constitutively-active cRNA constructs of PKCα and CamKII microinjected into mouse eggs, to show that it is CamKII and not PKC that is the sufficient trigger for cell cycle resumption from metaphase II arrest.

Constitutively active PKC constructs had no effect on meiotic resumption but caused an immediate and persistent elevation in intracellular Ca^{2+} when store-operated Ca^{2+} entry was stimulated. With respect to meiotic resumption, the effects of constitutively-active CamKII on eggs were the same as sperm. Eggs underwent second polar body extrusion and pronucleus formation with normal timings; while both securin and cyclin B1 destruction, visualised by coupling to fluorescent protein tags, were complete by the time of polar body extrusion. Induction of a spindle checkpoint by overexpression of Mad2 or by spindle poisons blocked CamKII-induced meiotic resumption but the Ca^{2+} chelator BAPTA did not. Furthermore direct measurement of Ca^{2+} levels showed that CamKII did not induce exit from metaphase II arrest by raising Ca^{2+}. Therefore we conclude that PKCs may play an important role in maintaining Ca^{2+} spiking at fertilization by promoting store-operated Ca^{2+} entry, while CamKII transduces cell cycle resumption, and lies downstream of sperm-induced Ca^{2+} release but upstream of a spindle checkpoint. These data, combined with the knowledge that CamKII activity increase at fertilization, suggest that mouse eggs undergo cell cycle resumption through stimulation of CamKII.

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POTENTIAL ROLE OF GLYCODELIN FOR FERTILIZATION SUCCESS IN ART

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Background: Glycodelin (Gd), a dimeric glycoprotein, appears in the female and male reproductive tract in isoforms like GdA from amniotic fluid and endometrium, GdF from follicular fluid, GdS from seminal plasma and on sperm surface as well. Little is known about the role of Gd in IVF. The aim of our study was to investigate the influence of the Gd-amount in supernatants of IVF-cultures to fertilization success. *Methods:* Employing monoclonal antibody (mAb) M4f8 soluble Gd levels were evaluated by an enzyme-linked immunosorbent assay (ELISA). We assayed 107 supernatants after 18 h *in-vitro* fertilisation of single cultured oocytes (S18) with 100 000 motile spermatozoa derived from 11 couples undergoing conventional IVF procedure. The results have been compared to blanks comprised of sperm suspensions incubated without any oocytes. The Gd-content in S18 were correlated to fertilization success and characterized by PN-scoring 18 h after insemination. Furthermore we evaluated supernatants (n = 21) of oocytes after ICSI. Total protein (TP) of all supernatants was assessed for calculating the Gd/TP-ratio. *Results:* The soluble Gd values were calculated for the cultures belonging to each couple. The levels of Gd differed interindividually by a wide range from 4.9 up to 69.2 ng/mL (0.1–3.0% Gd/TP) and in the blanks 2.0–59.5 ng/mL (0.1–4.5% Gd/TP) as well. We associated a couple specific Gd level from 10.0–60.0 ng/mL with a high fertilisation rate (FR = 88%). Both a soluble Gd at a lower level in sperm–oocyte suspensions and a S18 > 60.0 ng/mL were accompanied by a decreased FR (25%). The supernatants (n = 21) after ICSI (FR = 14%) were found Gd-free. *Conclusions:* Gd-amounts in S18 fluctuated both between the different patients and within their individual sperm–egg cultures. Therefore the protein is suggested not being introduced by spermatozoa only but rather by cumulus cell effects. Beside other parameters one of the fertilisation-dependant factors may be the patient-specific Gd concentration in the surrounding medium.

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INFLUENCE OF THE *IN VITRO* ENVIRONMENT ON RAT GAMETES AND PRE-IMPLANTATION EMBRYOS

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Rat *in vitro* fertilization (IVF) and culture (IVC) is attempted by few because of its reputation for difficulty. Currently very few functional rat *in vitro* systems (IVS) exist for sperm–oocyte interaction research. Successful fertilization of rat metaphase II (MII) oocytes was achieved with two different media, Enriched Krebs Ringer Bicarbonate (EKRB) (70.2%) and M16 (57.4%). Using this IVS we have shown that the rat germinal vesicle-intact (GV-i) oocyte lacks the necessary maturity to interact with capacitated caudal epididymal spermatozoa, whether zona pellucida intact (ZP-i) or free (ZP-f). Proteomic analysis of the protein profile of the oolemma from the GV-i stage through to the MII stage in oocytes is being conducted to characterize any maturational changes that may occur. In addition we provide initial evidence to suggest that an acrosome-intact spermatozoa can fuse with the oolemma of a ZP-f MII oocyte during IVF. Although high percentages of polyspermic embryos in ZP-f IVF (64.8–100%) were observed, the possibility that the rat oolemma may undergo a post-fusion block to polyspermy was implied by a small proportion of normally fertilized embryos (3.8–17.0%) in M16 supplemented with different ratios of hyperactived spermatozoa. Despite successful culture to the blastocyst stage for *in vivo* fertilized zygotes (33.73%) and 2-cell stage embryos (79.3%), IVF embryos have repeatedly failed to develop in culture. Two dimensional analyses of the protein profile of oocytes/embryos immediately prior to fertilization (MII oocyte–101 spots) and the maternal to zygotic transition (MZT) (zygotes–59 spots and 2-cell embryos–84 spots) has shown a difference in patterns of protein expressed. Comparison of IVF zygotes (41 spots) obtained from EKRB displayed reduced protein expression suggesting that nuclear maturation and/or MZT is not being adequately supported. These data illustrate that rat IVF and IVC require suitable media if its problematic reputation is finally to be shed.

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GAMETES ALTER THE OVIDUCTAL SECRETORY PROTEOME *IN VIVO*

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We sought to identify altered oviductal protein secretions in response to the presence of gametes in the oviduct *in vivo* at the time of ovulation. Experiments were designed to compare oviductal fluid from a gamete-stimulated oviduct to a non-gamete-stimulated oviduct within the same animal. Clips were introduced at the infundibulum of both oviducts to prevent oocytes from entering the oviducts and one uterine horn was cut to prevent sperm access to that oviduct. Sows were artificially inseminated the next day with diluted boar semen. Control sows that had undergone the same surgical procedures were inseminated with diluent (no sperm). The day after insemination oviducts were removed and oviductal fluid was collected. Two-dimensional gel electrophoresis, isotope coded affinity tag (ICAT) technology and LC-ESI-MS/MS were used to identify and quantify proteins regulated by presence of spermatozoa in the oviduct. To identify the effect of oocyte presence in the oviduct, only one oviduct was clipped at the infundibulum. This prevented oocytes from entering that oviduct. Sows were monitored for ovulation using sonography and 24 h after ovulation oviducts were removed and oviductal fluid was analysed as described above. Results indicated that *in vivo*, the oviduct responded to the presence of spermatozoa and oocytes by altering its secretory proteomic profile. Our surgical interventions were not responsible for any of these alterations. Many of the identified proteins are known to be involved in oocyte maturation, maintenance of sperm viability, fertilisation, and embryonic development. Our findings suggest that the oviduct responds to gametes by providing specific molecules to sustain, regulate or enhance events preceding and during fertilisation, and early embryonic development. Furthermore, it seems a gamete recognition system is present in the oviduct.

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INSULIN RECEPTOR INTERNALIZATION IN MOUSE PREIMPLANTATION EMBRYOS

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The insulin receptor (IR) mediates the actions of insulin and insulin-like growth factors (IGF-I and II). Two IR isoforms result from alternate splicing of exon 11, IR-A (without exon 11) and IR-B (with exon 11). Exon 11 is 36 bp and encodes 12 amino acids (717–729) in the COOH-terminus of the IR alpha-subunit. IR-A has higher binding affinity for insulin and IGF-II than IR-B. Interestingly, IR-A is predominantly expressed in fetal tissues, peripheral nerve, brain and tumours whilst IR-B is expressed primarily in classical insulin sensitive tissues such as adult liver and muscle. Our previous studies showed that in mice, like other species, the IR is expressed throughout preimplantation development. IR-B is expressed throughout the preimplantation period, whilst IR-A is expressed following compaction. Immunofluorescent confocal microsopy using an exon11 specific antiserum revealed IR-B immunoreactivity in cell membranes of zygotes and embryos to the morula stage and concentrated in the trophectoderm of blastocysts. Previous studies have shown that insulin can have proliferative effects prior to compaction.[1] Consistent with a functional IR at the 2-cell stage, insulin treatment rapidly increased cytoplasmic staining for IR-B within 5-15 min suggesting IR internalization on binding of insulin, which may be either trafficking to the nucleus for regulation of transcription or bound for degradation. Further investigations are underway to address these two options.

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PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-ALPHA IS INVOLVED IN H^+-MONOCARBOXYLATE TRANSPORTER 2 AND CATALASE PROTEIN EXPRESSION IN CULTURED PREIMPLANTATION MOUSE EMBRYOS

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Cleavage stage embryos consume pyruvate before switching to glucose as the major energy substrate for blastocyst formation. This switch is conditional, because freshly collected two-cell embryos form blastocysts without glucose by increasing pyruvate consumption. Zygotes cultured without glucose cannot adapt in this way and degenerate, but paradoxically demonstrate upregulation of the H^+-monocarboxylate transporter protein, MCT2, in morulae. MCT2 is a high affinity transporter implicated in redox shuttling for peroxisomal beta-oxidation of fatty acids.[3] Fatty acids may provide energy for embryos[2] but peroxisomal beta-oxidation has not been explored in preimplantation development. Rat oocytes possess a primitive peroxisomal system.[1] The possibility therefore exists that MCT2 may also be linked to fatty acid metabolism in embryos. Peroxisome proliferator activated receptor (PPAR)-alpha is a transcriptional regulator of fatty acid transport and beta-oxidation, and controls expression of catalase, a major peroxisomal enzyme. This investigation explores the role of PPAR-α in the glucose-driven control of MCT2 expression in mouse embryos. Zygotes (18 h post-hCG) were cultured in KSOM in the presence or absence of glucose, or KSOM with selective agonists of PPAR-α, fenofibrate and WY 14643. Expression of MCT2 and catalase was analysed by confocal laser scanning immunohistochemistry and western blot. Results confirm the presence of catalase throughout preimplantation development. With glucose, cytoplasmic immunoreactivity for catalase was punctate and diffuse, while MCT2 was localised to apical membranes of outer blastomeres in morulae. Without glucose, catalase and MCT2 expression were increased with notable localisation of catalase to nuclei. This response was reflected in morulae cultured in the presence of glucose and PPAR-α agonists. These data suggest that PPAR-α plays a role in controlling catalase and MCT2 expression in embryos, and that conditions in the absence of glucose are more conducive for PPAR-α activation.

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CHARACTERIZATION OF E74 LIKE FACTOR 3 IN THE MURINE PRE-IMPLANTATION EMBRYO

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The E26 transformation specific (ETS) family of transcription factors is defined by a highly conserved DNA binding domain containing a hydrophobic core motif 5′-GGA[A/T]-3′. A subfamily of ETS factors, epithelium-specific ETS factor (ESE), includes E74-like factor 3 (Elf3). Elf3 is expressed in many organs including the eye, skin and gastrointestinal tract. By upregulating specific gene transcription, Elf3 is instrumental in causing these organs to become fully differentiated. The presence of Elf3 in the pre-implantation embryo was first reported in 2002.[1] Data mining has suggested that the expression of Elf3 increases at the 16-cell embryo and blastocyst stages of pre-implantation development compared to the preceding stages. Reverse transcriptase–polymerase chain reaction (RT-PCR) was used to amplify the 3′ untranslated region (3′UTR) of Elf3. These results suggest that there are two isoforms of Elf3 present in the pre-implantation embryo, which differ by an 88 bp deletion/insertion. The expression and location of these isoforms was investigated using RT-PCR and *in-situ* hybridisation.

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REPRODUCTION IN THE ARRHYTHMIC *BMAL1* KNOCKOUT MOUSE

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There is strong epidemiological evidence indicating that disruption of the endogenous circadian rhythms can cause a range of health problems ranging from metabolic and cardiovascular disorders to reproductive failure. Circadian rhythmicity is generated by a suite of genes called 'clock genes' that are cyclically expressed in the brain and peripheral tissues. The CLOCK and BMAL1 transcription factors regulate the expression of many genes involved in cell growth, angiogenesis and development. The *Bmal1* knockout mouse provides an interesting model to analyse the impact of arrhythmicity on reproductive physiology. Female *Bmal1*$^{-/-}$ mice show a delay in the onset of puberty (WT = 32.7 d, KO = 38.6 d, n = 8–16). Female *Bmal1*$^{-/-}$ mice reproductive tissues are significantly smaller than in WT mice (Ovaries –40%, Oviduct –25%, Uterus –60%, n = 10). Female *Bmal1*$^{-/-}$ mice have essentially normal estrus cycles (cycle length WT = 4.2 d, KO = 4.8 d, n = 8) and are able to ovulate and mate but are unable to establish viable pregnancies. They are as responsive to a standard superovulation protocol as their wild type littermates (ovulated oocytes WT = 23.8, KO = 22.8, n = 7–10), suggesting the ovaries are developmentally competent.

These results suggest disruption of circadian rhythmicity in the mouse affects fertility at multiple sites. Further investigation into the importance of rhythmicity, particularly post ovulation and post fertilisation is required.

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DIETARY PROTEIN DOES NOT INFLUENCE MITOCHONDRIAL DISTRIBUTION IN THE 2-CELL MOUSE EMBRYO

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Excess dietary protein can negatively influence fertility. The underlying mechanisms remain to be completely elucidated; however, variations in reproductive tract pH, and ammonium and urea concentrations have been implicated. Mouse embryos cultured in the presence of ammonium showed a shift in mitochondrial distribution away from the nucleus towards the cell cortex, suggestive of reduced mitochondrial activity, ATP production and embryo viability. In this study we determined the effect of dietary protein *in vivo*, on mitochondrial distribution in the 2-cell mouse embryo. Five-week-old Swiss female mice (n = 10) were fed low (9%), medium (14%) or high (25%) dietary protein for 3 weeks; feed intake and body weight were recorded weekly. At 8 weeks of age mice were primed with 5 IU of PMSG, then 5 IU hCG 48 h later, and mated overnight with males of proven fertility. Forty hours post-hCG females were sacrificed, their oviducts collected and flushed with media. The total number of 2-cell embryos and oocytes retrieved were recorded. Active mitochondria were stained in the 2-cell embryos using Mitotracker Green (Molecular Probes), and were visualised using confocal microscopy. Density of perinuclear and cortical staining was determined in Photoshop 7.0, using an established method. Females fed the medium diet consumed significantly less and gained less weight than those fed the low or high diet (Table 1), despite similar final body weights (data not shown). Females fed the low diet tended to have a lower ovulation rate and fewer 2-cell embryos than females consuming the other diets (Table 1, $P > 0.05$). There was no significant effect of dietary protein on the distribution of mitochondria between the perinuclear and cortical region of the embryo, which may be reflective of lower *in vivo* ammonium levels compared to those described in culture.

Table 1. Dietary protein effects on feed intake, body weight and reproductive parameters

	Dietary protein				
	Low	Med	High	SEM	Signif.
Intake (g/day)	3.66[b]	3.23[a]	3.38[b]	0.057	$P < 0.05$
Body weight gain (mg/day)	180[b]	130[a]	211[b]	16.53	$P < 0.05$
Ovulation rate	7	17	17	3.18	ns
No. 2-cell embryos	4	13	14	3.20	ns
Mitochondrial distribution (perinuclear : cortical)	0.71	0.80	0.82	0.044	ns

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SENSITIVITY OF EMBRYOS TO AN ENVIRONMENTAL STRESSOR, AMMONIUM, IS DEPENDENT ON STAGE OF TEMPORAL EXPOSURE

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Extended embryo culture *in vitro* may cause increased cellular perturbations resulting in poorer developmental outcomes. Exposure of embryos to ammonium throughout the entire pre-implantation period decreased cell number and ICM development, increased apoptosis and perturbs glucose metabolism. The aim of this study was to examine the relative susceptibility of the pre- and post-compaction stage embryo to these perturbations resulting from temporal exposure to ammonium. Mouse embryos (n = 350 per treatment) were collected from F1 female mice. Embryos were exposed to either control medium or medium with 300 μM ammonium for the entire culture period. Temporal treatments involved culture with or without ammonium, from the zygote to 2-cell stage, 2-cell to 8-cell stage, or the 8-cell to the blastocyst stage. At the blastocyst stage, ICM development, apoptosis, gene expression and glucose metabolism were assessed. Differences between treatments were determined using generalised linear modelling and LSD post-hoc tests. Exposure to ammonium at any stage did not affect blastocyst development. Exposure to ammonium pre-compaction significantly decreased both blastocyst and ICM cell number while these were unaffected when exposure occurred post-compaction. Levels of apoptosis were significantly increased when exposure to ammonium was continual to the blastocyst stage (6.5% compared to control 2.4%, $P < 0.05$) or from the zygote to the 2-cell stage (5.8%, $P < 0.05$). However, apoptosis was not altered during post-compaction exposure (2.8%). Glucose uptake was decreased by culture with ammonium at all stages of development ($P < 0.001$). Gene expression of GLUT1 in the blastocyst was not altered by ammonium while GLUT3 expression was significantly reduced by exposure at all stages of development ($P < 0.01$). The data presented suggests that the pre-compaction stage embryo is most susceptible to ammonium stress and the effects of this early stage exposure appear irreversible. Intriguingly, glucose uptake and GLUT3 expression at the blastocyst stage appear to be markers of ammonium exposure.

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DIFFERENTIAL EXPRESSION PATTERNS OF GENES WITH IMMUNE AND DEVELOPMENTAL RELEVANCE IN INDIVIDUAL BOVINE PREIMPLANTATION EMBRYOS PRODUCED BY NUCLEAR TRANSFER

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The overall efficiency of cloning by somatic cell nuclear transfer (NT) remains low when compared to *in-vitro* fertilisation (IVF). Inefficient reprogramming of the donor nucleus and epigenetic effects are hypothesized to lead to aberrant gene expression in cloned embryos. In this work differences in gene expression, between blastocysts from IVF and NT derived pre-implantation bovine embryos, were explored using a bovine cDNA array. 439 array elements were identified as being differentially expressed. 102 elements showing upregulation and 337 elements downregulation in NT blastocysts in a pairwise comparison to IVF-derived blastocysts. A major subset (65 elements) of differentially expressed elements comprised immune-related genes, possibly a reflection of the immune-focused cDNA microarray used in this study. For example, several cytokine genes, as well as elements of the innate immune system, were showing elevated expression in NT blastocysts. In addition, two regulatory elements of the same endocrine pathway exhibited a remarkable expression pattern, where the gene for one inhibitor showed upregulation and the gene for another inhibitor showed downregulation in NT blastocysts. Of specific interest is the observation that genes belonging to two growth factor pathways are upregulated in NT embryos. These results suggest an important role for immune-related genes during embryogenesis and indicate that specific cell growth and differentiation factors are of significant interest as targets in defining the abnormalities of preimplantation NT embryo development.

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EFFECT OF EXOGENOUS TRANSFORMING GROWTH FACTOR BETA 1 ON REPRODUCTIVE PERFORMANCE IN MALE TGFβ1 NULL MICE

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The transforming growth factor beta 1 (TGFβ1) family are potent cytokines that regulate tissue development, inflammation and immunity. Our studies in null mutant mice implicate a key role for TGFβ1 in male reproductive function. The TGFβ1 null mutation results in profound infertility due to inability to copulate successfully associated with reduced testosterone synthesis, although penile erection and sperm production do occur. To investigate whether fertility status can be improved in TGFβ1 null mutant mice by exogenous cytokine replacement, we used Alzet mini-pumps implanted subcutaneously to deliver a constant supply of recombinant latent TGFβ1 to TGFβ–/– mice (n = 7, 2.1 μg/day over 2 weeks). Control TGFβ–/– mice (n = 6) and +/+ mice (n = 10) received pumps containing BSA carrier only. Circulating levels of TGFß1 were increased in TGFβ–/– mice and reached levels comparable to those seen in fertile heterozygote littermates. Increased circulating testosterone was evident in a proportion of TGFβ–/– mice after exogenous TGFβ replacement compared with untreated control mice. However, serum testosterone content was widely variable within all groups, so statistical significance was not achieved. Videotaping of nocturnal mating behaviour while caging treated males with normal receptive female mice showed that unlike TGFβ+/+ mice, which successfully mounted and intromitted, untreated TGFβ–/– mice failed to engage in normal mating behaviour. TGFβ–/– mice treated with exogenous cytokine were occasionally seen to intromit but less frequently than TGFβ+/+ controls. Ejaculation did not occur in any of the TGFβ–/– mice regardless of TGFβ replacement, compared with TGFβ+/+ mice where 8/10 mice ejaculated during the 2 h observation period. The trend towards improvement in both testosterone levels and copulation activity of the TGFβ1 null mice treated with exogenous cytokine suggests that systemic TGFβ1 availability may influence reproductive performance in male mice. However, since fertility was not restored by cytokine replacement, locally produced TGFβ in the reproductive tract and/or hypothalamic pituitary axis are also implicated in regulating fertility.

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CHARACTERISATION OF LIPOPOLYSACCHARIDE (LPS) RECEPTOR EXPRESSION AND THE INFLAMMATORY RESPONSE OF THE RAT TESTIS

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Inflammation in the testis is disastrous for the developing spermatogenic cells, leading to temporary and sometimes permanent sterility. The majority of testicular macrophages display a unique protective phenotype whereby production of some key inflammatory mediators, specifically interleukin-1β (IL-1β), tumour necrosis factor-α (TNFα) and NO, in response to stimulation with LPS is relatively poor. Leydig cells and Sertoli cells also respond to high doses of LPS, producing the inflammatory cytokines, particularly IL-1α and IL-6. Although these data suggest that the LPS receptor (toll-like receptor 4, TLR4) and its associated binding proteins, CD14 and MD2, are expressed on several testicular cell types, expression of these proteins in the testis has not been described previously.

Using real-time PCR and Western blotting, we established that TLR4, CD14 and MD2 are all expressed by testicular macrophages, Leydig cells, Sertoli cells, spermatocytes and round spermatids. Unexpectedly, the spermatogenic cells displayed the highest level of TLR4 surface expression as determined by flow cytometry. There was no response of the spermatogenic cells to LPS stimulation *in vitro*, at least in terms of mRNA expression for the inflammatory cytokines, IL-1α, IL-1β, TNFα, IL-6, activin A and the chemoattractants, CXCL-1 and CXCL-2. Although production of several cytokines was relatively diminished, testicular macrophages responded to LPS with a significant increase in mRNA expression for all of these inflammatory regulators. These data indicate that the protective phenotype of the testicular macrophages is not due to insensitivity to LPS or absence of the receptor, but may involve downstream regulation of specific inflammatory responses. The data also suggest that spermatogenic cells are capable of responding to TLR4 ligands, although not by producing inflammatory mediators. The actual function of the LPS receptor on the spermatogenic cells remains to be discovered.

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FURTHER CHARACTERISATION OF LYMPHOCYTE-SUPPRESSING ACTIVITY IN GONADAL FLUIDS

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Protection of the developing gametes from an autoimmune response within the testis and ovary is essential for reproductive success, and autoimmune infertility represents a failure of this protection. The gonads also represent favorable sites for grafts of foreign tissue, that is, they are 'immunologically privileged'. The actual mechanisms responsible for testicular and ovarian immune privilege are poorly understood. However, it has been well established that testicular interstitial fluid and ovarian fluid have profound inhibitory effects on T-cell activation and proliferation *in vitro*. We have established previously that a partially purified preparation of the inhibitor, isolated from bovine follicular fluid, suppresses proliferation in an *in vitro* T-cell activation assay, through induction of T-cell anergy and/or atypical apoptosis. Addition of increasing doses of normal fetal calf serum and/or bovine serum albumin blocks the actions of the inhibitor and progressively increases the ED_{50} of the assay. It has also been shown that stimulating the T-cells with phorbol-12-myristate-13-acetate (PMA) in place of a polyclonal mitogenic stimulus such as phytohaemagglutinin bypasses the anergic effects of the inhibitor. These results suggest that the activity of the inhibitor may be negatively regulated in the circulation and tissues by serum-derived proteins and other factors. These data also indicate that the inhibitor's activity is mediated through a specific cellular pathway, most likely involving protein kinase C isotypes, which are activated by PMA. Further work will delineate the molecular pathways and mechanisms of serum regulation of the gonadal lymphocyte-suppressing activity, which may be exploited in the treatment of autoimmune diseases and for prevention of transplant rejection.

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MURINE HIF-1α LOCALISATION BY IMMUNOHISTOCHEMISTRY IN A MOUSE REPRODUCTIVE TISSUE

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Hypoxia-inducible factors (HIFs) are heterodimeric transcription factors consisting of an α and β subunit. The level of O_2 within cells regulates the stability of HIF-1α and HIF-1 is considered the primary mediator of cellular responses to hypoxia, helping restore O_2 homeostasis by promoting the expression of hypoxia-sensitive genes involved in cell survival, angiogenesis, cell proliferation and metabolism.

There are few published studies investigating the role of HIF-1 protein in mouse tissues using immunohistochemistry, due to the lack of a reliable protocol and the inability of many commercially available antibodies to detect murine HIF-1α protein. We have developed a protocol that has allowed us to analyse the presence and location of HIF-1α protein in the mouse epididymis and found that it was predominantly located in the nucleus of discrete principal cells in the epididymis of mice housed under normoxic conditions and sacrificed by cervical dislocation. Interestingly, a 2.5× increase ($P < 0.05$) of HIF-1α protein staining intensity in both the nucleus and cytoplasm of principal cells in the epididymis was detected in mice housed under normoxic conditions but sacrificed with CO_2 gas, compared to mice sacrificed by cervical dislocation. HIF-1α protein detection was 3-fold increased in the nucleus and cytoplasm of principal cells when mice were exposed to hypoxic conditions (6% O_2 for 1 h). Our results demonstrate that murine HIF-1α can be detected in discrete cells under normoxic conditions, suggesting local differences in O_2. Acute hypoxic responses, via deliberate exposure or even CO_2 euthanasia can significantly upregulate HIF-1α protein levels. Further studies will investigate the role of HIFs and hypoxia in male and female reproductive function.

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EFFECTS OF MODERATE SPINAL CORD INJURY ON THE EXPRESSION OF A BARRIER MARKER IN ENDOTHELIAL CELLS OF THE TESTIS AND IN THE PROSTATE OF RATS

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It was recently shown that the endothelial barrier antigen (EBA), previously thought to be specific to endothelial cells in the central nervous system, was also present in endothelial cells in the testis and in epithelial cells in the dorsolateral prostate of adult rats.[1] In the present study, we examined the effect of moderate spinal cord injury (SCI), produced by compression for 5 min of the cord at T 10/11.

There was a slight reduction in EBA in the testis and prostate 24 h after SCI, and this became more obvious after 3 days. EBA was completely absent from the prostate and testis at 1 week. By 2 and 4 weeks some expression of EBA returned, and at these times EBA was also detected in the ventral prostate. Brain endothelial cells remained positive throughout.

We cannot yet say whether these changes are due directly to interference with the nerve supply, or involve changes in androgen status.

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TESTICULAR GROWTH FACTOR EXPRESSION AFTER HEMICASTRATION IN THE NEONATAL BOAR

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The molecular mechanisms associated with testicular hypertrophy after hemicastration are poorly understood. Sertoli cells in culture underwent increased proliferation when exposed to fibroblast growth factor-2 (FGF2)[1] and transforming growth factor-α (TGFα).[2] To determine whether FGF2 and TGFα expression is upregulated during accelerated testicular growth after hemicastration in the boar animals were assigned to (a) control (n = 12), no treatment; (b) hemicastrated (n = 14), left testis removed on Day 10 after birth. The right testis was removed from half the control and hemicastrated boars, respectively, on Days 15 and 20 after birth. RNA was extracted from sections of frozen testis, cDNA synthesised using TaqMan® Reverse Transcription, and real-time PCR performed. FGF2 expression was determined using forward (5′GTG TTA CAG ACG AGT GTT TCT TTT TTG3′), internal (5′acg act gga atc taa t3′) and reverse (5′TTC CTC GAC CGG TAA GTA TTG TAG T3′) primers. TGFα expression was similarly determined using forward (5′GGC TGT CCT CAT CAT CAC ATG T3′), internal (5′tgc tga tac act gct gc3′) and reverse (5′CGG CAC CAC TCA CAG TGT TT3′) primers. Data were analysed by ANOVA and LSD test (testis weight) and unpaired two-tailed t-tests assuming equal variance (FGF2, TGFα). There was no difference ($P > 0.05$) in testis weight between hemicastrated (3.9 ± 0.3 g; mean ± SEM) and control (3.6 ± 0.5 g) boars on Day 5 but on Day 10 hemicastrated boars had a greater (P = 0.01) testis weight (6.2 ± 0.8 g) than controls (4.3 ± 0.4 g). There were no differences ($P > 0.05$) between control and hemicastrated boars in TGFα or FGF2 expression on Days 5 and 10. It is concluded from the findings that upregulation of TGFα or FGF2 gene expression is not required for testicular hypertrophy subsequent to hemicastration in the neonatal boar.

(1) *Biol Reprod* (1987); **37**: 665–674.
(2) *Mol Cell Endocrinol* (2001); **181**: 221–227.

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THE EFFECT OF OXYTOCIN ON CELL GROWTH AND STEROID PRODUCTION IN NORMAL HUMAN PROSTATE CELLS *IN VITRO*

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Oxytocin (OT) is present in reproductive tissues of male mammals including human prostate tissue. OT increases prostatic muscle tone and prostatic growth. OT is increased in benign prostatic hyperplasia (BPH), an androgen dependent condition that develops with age. Dihydrotestosterone (DHT) is the active hormone in the prostate and is converted from testosterone by the enzyme 5 a reductase. Conversion has been shown to be augmented in the presence of OT. The aim of this study was to investigate the effect of oxytocin on cell growth and steroid production in cultured normal human prostate cells.

Normal human prostate stromal and epithelial cells (Clonetics) were cultured with OT, oxytocin antagonist (OTA) or oxytocin/oxytocin antagonist combination (10 ng/mL, 1 ng/mL or 0.1 ng/mL) in media containing 10 nmol of testosterone. Media was changed daily over the 5 day growth period and frozen. Cell proliferation assay was performed at harvest on day 5 to ascertain cell numbers. Media from days 1, 3 and 5 were extracted and radioimmunoassayed for testosterone and DHT.

OT increased stromal cell number in a dose dependent manner ($P < 0.001$). Treatment with OT or OTA had no significant effect on epithelial cell numbers. In stromal cell media from Day 1, DHT concentrations were higher in cells treated with OT than control cells ($P < 0.05$). By Day 5 the concentration of DHT was low in all treatment groups except OT (10 ng/mL). No effect of OT or OTA was seen on DHT concentrations of media from epithelial cells.

OT may increase cell growth in prostate stromal cells but not epithelial cells grown *in vitro*. This effect may be related to the conversion of testosterone to DHT and DHT to its metabolites. These results demonstrate that OT may play a role in the regulation of cell growth, steroid production and steroid metabolism in the human prostate.

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CHARACTERIZATION OF SIV INFECTION IN THE MALE GENITAL TRACT OF JUVENILE MACAQUES

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Reproductive organs contribute infected cells and free viral particles to semen in human immunodeficiency type-1 (HIV-1) infected individuals, increasing the risk of infection from the HIV-1 positive male to the mother and ultimately to the offspring. The majority of information gathered with respect to the HIV-1 burden in the male reproductive tract (MGT) have been conducted in tissues obtained on autopsies of testis, prostate and epididymis of individuals that die from AIDS. Therefore, little is known about the progression and pathogenesis of the infection within these organs. Investigating the mechanism of the spread of HIV-1 in the cells and tissues of the MGT, particularly during the asymptomatic stage, remains a critical task.

Infection of macaques with simian immunodeficiency virus (SIV) is a useful animal model for studies of mucosal transmission and viral transmission via breastfeeding. In this study eight juvenile macaques (2.5 yo) were infected with SIVmac for a period of 3–6 months and testis and epididymis tissue were collected in two intervals, 3 and 6 months post-infection. To determine SIV progression and pathogenesis in the MGT we have used EM, immunohistochemistry, confocal microscopy and immunoblotting.

Our preliminary EM obtained via analysis of testis and epididymis tissue of SIV infected macaques show the presence of elongated spermatids in the epididymis. Scattered viral like SIV particles were observed in the testis and epididymal lumen, principally associated with aberrant germ cells. Necrosis of epididymal tissue was also observed, potentially due to the SIV burden in this organ.

The data indicate that SIV infected juvenile macaques are a potential model for studying HIV-1 pathogenesis and its effect in spermatogenesis as well as the immune response of testis in a species that is closely related to humans.

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NUTRITION, INSULIN, LEPTIN AND PUBERTY IN MERINO RAM LAMBS

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Merino sheep developed in Mediterranean regions so are well adapted to acute changes in food availability. However, restricted intake during pregnancy, especially in animals that are pregnant over the dry summer, could limit the positive effects of a winter rainy season on fetal development. In this study, we tested whether the level of nutrition during pregnancy and during pre-pubertal development affected blood concentrations of insulin and leptin, scrotal circumference and age of puberty in male Merino lambs fed with pasture. During dry weather, pregnant sheep were supplemented *ad libitum* with hay and lupin grain (*Lupinus angustifolius*) to compensate for decreases in pasture supply. Puberty was detected using a standardised behavioural test with oestrous ewes. Lambs were considered pubertal if they displayed mounting in two successive weekly tests. There were no differences in plasma concentrations of insulin or leptin. The values for both hormones simply displayed the same pattern, with a rise after feeding and a fall during non-feeding periods. There was no difference among treatments in either scrotal growth or age to puberty (Table 1). This might be because the dietary treatments, being administered by food restriction under field conditions, would not have the same effects as severe undernutrition that has been used in laboratory studies. Alternatively, Merino sheep may have a greater capacity to cope with mild nutritional stress.

Table 1. Growth rate in scrotal circumference (GRSC) and age to puberty in ram lambs fed under different dietary regimes during the last 55 days of pregnancy and during the first year after parturition

Quality of pasture during pregnancy	Quality of pasture after parturition	Treatment group	GRSC (cm/d)	Days to puberty
Low (n = 29)	Low (n = 15)	LL	0.067	291
	High (n = 14)	LH	0.054	290
High (n =27)	Low (n = 15)	HL	0.056	298
	High (n = 12)	HH	0.053	291

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OESTROGEN RECEPTOR ALPHA AND BETA IN THE PROSTATE OF THE BRUSHTAIL POSSUM (*TRICHOSURUS VULPECULA*)

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Oestrogen plays a physiological role in the growth of the prostate and has been implicated in the development of prostate disease in man. Both oestrogen receptors are present in the normal human prostate with oestrogen receptor alpha (ERα) localised in stromal cells and oestrogen receptor beta (ERβ) in epithelial cells. However, in benign prostatic hyperplasia (BPH) ERα is found in both stromal and epithelial cells. The study of human prostate growth is complicated by the lack of a suitable animal model. The brushtail possum (*Trichosurus vulpecula*), however, may provide a novel model for such studies. It has a prostate that is structurally similar to that in man and also exhibits seasonal hyperplasia and regression. This study investigated the localisation of ERα and ERβ in the possum prostate. Male possums were killed throughout the year including the breeding season when the prostate is enlarged and the non-breeding season. Each prostate was divided into central and posterior regions and either fixed in Bouins fluid or snap frozen. The presence of both ERα and ERβ were determined using Western blotting with the Novocastra antisera NCL-ER-6F11 (ERα) and NCL-ER-beta (ERβ). Immunohistochemical localisation of the receptors was determined using the same antisera for ERα on paraffin sections, and for ERβ on frozen sections. Western blotting demonstrated immunoreactive bands of ~60 kDa for ERα and ERβ protein in the central and posterior prostate in the breeding and non-breeding periods. ERα was localised to the nuclei of both epithelial and stromal cells in central and posterior regions of the prostate throughout the year. ERβ was localised to the nuclei of stromal cells in the central and posterior prostate throughout the year. This study confirms the presence of ERα and ERβ in the possum prostate. The distribution of ERα is similar to that seen in prostatic tissue from men with BPH.

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ADULT SERTOLI CELLS PROLIFERATE IN RESPONSE TO EXOGENOUS FOLLICLE STIMULATING HORMONE IN THE ADULT PHOTO-INHIBITED DJUNGARIAN HAMSTER

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Sperm production relies on nutritional and structural support from Sertoli cells. Sertoli cells undergo maturational changes (e.g. cessation of proliferation and formation of the blood–testis barrier) around the onset of puberty in higher mammals[1] and maturational failure has been associated with some infertility syndromes and testicular malignancies[2]. The Sertoli cell population is considered to be stable and unmodifiable by hormones after puberty in mammals, although recent data using the adult Djungarian hamster showed that Sertoli cell numbers decreased by 35% in the absence of serum gonadotrophins, and returned to control levels by short-term replacement of follicle stimulating hormone (FSH)[3]. Therefore, the aims of this study were to (i) quantify the proliferative activity of Sertoli cells in the hormonally manipulated Djungarian hamster, and (ii) examine the localisation of several tight junction proteins as markers of the blood–testis barrier. Long day (LD) photoperiod (16L : 8D) adult hamsters were exposed to short day (SD) photoperiod (8L : 16D) for 11 weeks to suppress gonadotrophins and then received FSH for up to 10 days. Sertoli cell proliferation was assessed immunohistochemically by the colocalisation of GATA-4 and PCNA, and quantified by stereology. Tight junction proteins (occludin and ZO-1) were colocalised using confocal microscopy. Sertoli cell proliferation in both the LD and SD controls was minimal; however, in response to FSH treatment proliferation was upregulated within 4 days compared with SD controls (98% *v.* 2%, $P < 0.001$, respectively). Tight junction proteins colocalised at the blood–testis barrier in LD hamsters, but were disorganised within the Sertoli cell cytoplasm in SD animals. FSH treatment restores colocalisation in a time-dependent manner. It is concluded that FSH contributes to the regulation of Sertoli cell proliferation and tight junction formation in the adult Djungarian hamster. This data provides definitive evidence that the adult Sertoli cell population in this model is modifiable by hormones.

(1) Meachem *et al.* (2005). *Biol Reprod* **72**, 1187.
(2) Allan *et al.* (2004). *Endocrinol* **145**, 1587.
(3) Russell and Peterson (1985). *Int Rev Cytol* **94**, 177.

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TESTOSTERONE SECRETION IN THE AUSTRALIAN SEA LION

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The objectives in this study were to characterize the pattern of testosterone secretion over 24 h in the captive Australian sea lion and to establish whether exogenous hormone stimulation could be used to obtain an index of testosterone secretion in this species. Blood samples were collected from three males at 3-h intervals for 24 h on three occasions at 2-week intervals. Exogenous GnRH, hCG and LH were administered at the end of each 24-h sampling period and changes in circulating testosterone monitored over 5 h. Circulating concentrations of testosterone during 24-h sampling periods were greater ($P < 0.01$) during darkness than during daylight. Exogenous hCG administered induced a significant rise in circulating concentrations of testosterone. In the 5 h following GnRH, hCG and LH administration, circulating testosterone concentrations reached similar levels to the highest concentrations observed during the respective 24-h sampling periods. These findings have provided evidence of a 24-h pattern of testosterone secretion in the Australian sea lion. This study has also shown that the testis response to exogenous GnRH, hCG and LH in the Australian sea lion can provide an index of the prevailing testosterone biosynthetic capacity of the testes.