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ABSTRACTS

002

HUMAN SPERMATOZOA: FRUITS OF CREATION, SEEDS OF DOUBT

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Defective sperm function is the largest defined cause of human infertility, affecting one in twenty Australian males. Despite its prevalence, we are only just beginning to understand the underlying mechanisms. The past decade has seen two major advances in this field: *(1)* the discovery that Y chromosome deletions play a key role in the aetiology of non-obstructive azoospermia/oligozoospermia; and *(2)* recognition that oxidative stress can impact upon the functional competence of human spermatozoa through peroxidative damage to the sperm plasma membrane. Oxidative stress has also been found to disrupt the integrity of DNA in the male germ line and may represent an important mechanism by which environmental impacts on human health are mediated. Thus, paternal exposure to various toxicants (cigarette smoke, organic solvents, heavy metals) has been linked with oxidative DNA damage in spermatozoa and developmental defects, including cancer, in the F1 generation. The male germ line becomes particularly vulnerable to such factors during the post meiotic stages of differentiation. Pre-meiotic germ cells always have the option of undergoing apoptosis if DNA damage is severe. However, post meiotic germ cells have lost both the ability to mount an apoptotic response and the capacity for DNA repair. As a result, germ cells are particularly vulnerable to genotoxic agents during spermiogenesis and epididymal maturation. If the fertilizing capacity of the spermatozoa is retained following toxicant exposure, then DNA damage will be transferred to the zygote and must be repaired subsequently by the oocyte and/or early embryo. Aberrant DNA repair at this stage has the potential to create mutations that will compromise embryonic development and, ultimately, the normality of the offspring. Elucidating the causes of oxidative damage in spermatozoa should help resolve the aetiology of conditions such as male infertility, early pregnancy loss and childhood disease, including cancer.

003

UTERINE AND PLACENTAL FACTORS REGULATING CONCEPTUS GROWTH: INSIGHTS FROM THE EWE

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Uterine adenogenesis is the process whereby endometrial glands differentiate and develop and is primarily a postnatal event in all mammals. In domestic animals and humans, adenogenesis involves initial differentiation and budding of glandular epithelium followed by invagination and extensive tubular coiling and branching morphogenesis through the endometrial stroma to the myometrium. Transient exposure of the neonatal ewe to a progestin from birth to postnatal Day 56 resulted in a uterine gland knock out (UGKO) phenotype in the adult. UGKO ewes exhibit a peri-implantation defect in conceptus (embryo/fetus and associated extraembryonic membranes) survival, indicating the functional importance of uterine glands and their secretions. Genomic and proteomic analysis of uterine endometrium from UGKO ewes has identified many candidate genes that regulate conceptus development and implantation, including endogenous Jaagsiekte sheep retroviruses (enJSRVs), glycosylated cell adhesion molecule one (GlyCAM-1), osteopontin and galectin-15. Galectin-15, also known as OVGAL11, and a previously uncharacterised member of the galectin family of secreted β-galactoside lectins, was discovered in the endometrium of sheep. In endometria of cyclic and pregnant sheep, galectin-15 mRNA was expressed specifically in the endometrial luminal epithelium but not in the conceptus. In pregnant sheep, galectin-15 mRNA expression appeared in the epithelia between Days 10 and 12 and increased between Days 12 and 16. Progesterone induced and interferon tau stimulated galectin-15 mRNA in the endometrial epithelium. Galectin-15 protein was concentrated near and on the apical surface of the endometrial luminal epithelia and localised within discrete cytoplasmic crystalline structures of conceptus trophectoderm. Galectin-15 is hypothesised to function extracellularly to regulate trophectoderm migration and adhesion to the endometrial epithelium and intracellularly to regulate cell survival, growth and differentiation. In sheep, the sequential actions of ovarian steroid hormones (oestrogen and progesterone), interferon tau, placental lactogen and placental growth hormone constitute a servomechanism that directly regulates endometrial gland morphogenesis and terminal differentiated function to provide increasing histotrophic nutrition for conceptus growth and development. Knowledge gained from this research will be used to prevent or treat infertility, fetal growth retardation and disease in domestic animals and humans.

004

FROM MOLECULES TO MIND: STRESS, ALLOSTASIS AND INTEGRATION OF BRAIN AND BODY

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The mind involves the whole body and two-way communication between the brain and the cardiovascular, immune and other systems via neural and endocrine mechanisms. Stress is a condition of the mind and a factor in the expression of disease that differs among individuals. A broader view is that it is not just the dramatic stressful events that exact their toll but rather the many events of daily life that elevates activities of physiological systems so as to cause some measure of wear and tear. We call this wear and tear 'allostatic load', and it reflects not only the impact of life experiences but also genetic load; individual life-style habits reflecting items such as diet, exercise and substance abuse; and developmental experiences that set life-long patterns of behavior and physiological reactivity (1).

Hormones associated with stress and allostatic load protect the body in the short-run and promote adaptation, but the long run allostatic load causes changes in the body that lead to disease. This will be illustrated for the immune system and brain regions involved in stress, fear and cognition (e.g. hippocampus, amygdala and prefrontal cortex). Besides developmental influences associated with mother–infant interactions, the most potent of stressors in adult life are those arising from competitive interactions between animals of the same species, leading to the formation of dominance hierarchies. Psychosocial stress of this type not only impairs cognitive function of lower ranking animals, but it can also promote disease (e.g. atherosclerosis) among those vying for the dominant position, as well as depressive illness. Social ordering in human society is also associated with gradients of disease, with an increasing frequency or mortality and morbidity as one descends the scale of socioeconomic status (SES) that reflects both income and education. Although the causes of these gradients of health are very complex, they are likely to reflect, with increasing frequency at the lower end of the scale, the cumulative burden of coping with limited resources and negative life events as well as differences in life style, and the allostatic load that this burden places on the physiological systems involved in adaptation and coping.

(1) McEwen, B.S. (1998) Protective and damaging effects of stress mediators. *New England J. Med.* **238**, 171–179.

010

COORDINATING THE TRANSITION FROM EGG TO EMBRYO IN MAMMALS

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At fertilization of mammalian oocytes, the sperm induces a series of increases in the concentration of intracellular Ca^{2+}. These Ca^{2+} oscillations trigger all the events of egg activation, including cortical granule exocytosis, completion of meiosis and entry into the first mitotic division. Thus, intracellular Ca^{2+} plays a pivotal role in coordinating the transition from egg to embryo. Our work is focussed on understanding how the oocyte prepares for fertilisation, how the Ca^{2+} oscillations are controlled and how Ca^{2+} stimulates signalling pathways that lead to optimal early embryonic development. In this lecture I will focus on the downstream pathways of Ca^{2+} signalling at fertilisation. Conventional Protein Kinase C (cPKC) is the major downstream target of Ca^{2+} in many cell functions. Using PKC-GFP fusion proteins we have found that cPKC is recruited to the membrane in a manner that is dependent on the frequency and amplitude of the Ca^{2+} oscillations. Recruitment of cPKC appears to promote the Ca^{2+} influx necessary to sustain the generation of long lasting Ca^{2+} oscillations. In other cell types cytosolic Ca^{2+} increases are known to stimulate mitochondrial respiration. We have found that maintenance of resting Ca^{2+} levels and sperm-induced Ca^{2+} oscillations are critically dependent on mitochondrial ATP production: a feature not shared by many cell types. Since Ca^{2+} release increases ATP consumption we investigated whether the Ca^{2+} transients increase mitochondrial activity so as to meet this increase in demand. Monitoring autofluorescence from NADH and flavoproteins reveals that Ca^{2+} transients stimulate a change in redox state of mitochondria, presumably by activating Ca^{2+}-sensitive dehydrogenases of the TCA cycle. Thus, through activation of downstream pathways, including PKC, cyclin B degradation and mitochondrial activity, intracellular Ca^{2+} provides a signal that orchestrates the activation of early mammalian development.

011

WAKING UP THE EGG. HOW THE SPERM REGULATES EXIT OUT OF THE MEIOTIC CELL CYCLE

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A series of calcium spikes are induced in the mammalian egg cytoplasm at fertilisation. These calcium spikes, which last for several hours, are the necessary and sufficient signal that stimulates the egg to escape from arrest at metaphase of the second meiotic division. Metaphase arrest is achieved by preventing the destruction of cyclin B1, the regulatory component of Maturation (M-Phase) Promoting Factor, and securin, which prevents segregation of sister chromatids. Both these proteins are destroyed by tagging with ubiquitin, using an E3 ligase the Anaphase-Promoting Complex (APC). Ubiquitination tags them for proteolysis by the 26S proteasome. Work from my lab has demonstrated that the sperm calcium signal works through activating the APC, not the 26S proteasome. Although we do not know which APC component is affected by calcium, this activation appears specific to a metaphase-arrested cell cycle state. More recently we have found that the APC is differently regulated at specific points during exit from meiosis II. Before extrusion of the second polar body it is the APC activator cdc20 that regulates APC activity. However, following extrusion of the second polar body cdh1 appears the major regulator. It is probable, therefore, that the calcium spiking affects the activity of both APC^{cdc20} and APC^{cdh1}. This swap in APC activator at the time of second polar body extrusion has not been reported in eggs of other species, in fact non-mammalian eggs all lack cdh1. Since APC^{cdc20} and APC^{cdh1} have different substrate specificities, the function of APC^{cdh1} in mammalian eggs warrants further investigation.

012

METABOLIC DETERMINANTS OF IMPLANTATION SUCCESS AND PROGRAMMING LONG TERM VIABILITY IN EMBRYOS

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It has long been recognised that energy substrate supply and metabolism are key determinants of early embryo development during *in vitro* culture. Recently it has been revealed that exposure to suboptimal metabolic environments during early embryo development can 'programme' subsequent development, leading to perturbed fetal development. For example, amino acid uptake profiles during early cleavage have been found to predict subsequent embryo development and potentially implantation success. However, the by-product of amino acid metabolism, ammonium, has also been found to significantly alter development, possibly through perturbed methylation of imprinted genes. Our own work has focussed on the role of oxygen availability and subsequent embryo development. Somatic cells respond to changing oxygen concentration by altering intracellular REDOX state (the balance between oxidative and reductive power within a cell), which in turn can alter transcription via REDOX-sensitive transcription factor activity. Furthermore, oxygen is known to have direct effects on transcriptional activity via the hypoxia-inducible factors (HIFs), transcription factors whose stability and DNA-binding activity are directly regulated by pO_2, in particular under hypoxic conditions. Using a mouse model, we have demonstrated that reducing pO_2 from 50 mmHg to 15 mmHg during the compaction and blastulation periods alone significantly alters expression patterns of oxygen-sensitive genes (such as glucose transporters), without significantly altering developmental progression to the blastocyst stage. Following transfer, embryos cultured under 15 mmHg O_2, despite similar implantation rates, produced fewer viable and lighter fetuses than *in vivo*-derived control embryos or those cultured in either atmospheric or 50 mmHg pO_2. This demonstrates that mouse embryos are sensitive to changes in their metabolic state during the post compaction period and that operating through causal pathways, the environment during this period of development can significantly affect subsequent developmental potential. Ironically, bovine embryo development appears to benefit under a low O_2 concentration. Furthermore, HIF protein stability appears to differ between the two species, which may be the underlying cause for the differences in gene expression and developmental competence.

013

THE REGULATION OF SURVIVAL OF THE PRE-IMPLANTATION EMBRYO

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There are many aspects of the regulation of the growth and survival of the pre-implantation embryo that remain enigmatic. The increasing production of such embryos by assisted reproductive technologies (ART) in human medicine, animal production and conservation biology has highlighted the relatively poor viability of such embryos. Many embryos fail to survive past the normal time of implantation. Population biology theory predicts that any circumstance that results in high death rates within a population creates a potential for genetic selection. This occurs if the surviving individuals have a genetic make up that preferentially favours survival.

Since ART clearly favours the survival of some embryos over others, it is a high priority to develop a sound understanding of those factors that normally govern embryo survival and how they may be affected by ART. It raises the question, do embryos that survive ART have a genetic make up that favours their survival compared to the proportion of the population that does not survive?

It is now demonstrated that autocrine and paracrine factors are essential for embryo survival and that these act via the 1-o-phosphatidylinositol-3-kinase (PI3K) survival signalling pathway (1). PI3K activates many downstream pro-survival and anti-apoptotic mediators.

ART changes the expression of some of these mediators. Pharmacological and genetic moderation of their expression can influence embryo survival, highlighting potential targets for genetic selection through ART. Studies in appropriate models will allow rational approaches to safety assessment of ART and spawn new strategies for media and procedural design.

(1) Lu, D. P., Chandrakanthan, V., Cahana, A., Ishii, S., O'Neill, C. (2004) *J. Cell Sci.* **117**, 1567–1576.

014

NOVEL UTERINE GENES IN REGULATION OF EMBRYO IMPLANTATION

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Implantation of the embryo into the maternal endometrium is the first and critical step leading to the establishment of a pregnancy. It has been well established that only during the 'window' of implantation, a limited time span when the uterine environment is receptive, can a blastocyst successfully implant into the uterus. The development of uterine receptivity is accompanied by remarkable morphological and physiological changes in the endometrium, and this is primarily driven by the coordinated effects of the ovarian steroid hormones. Uterine tissue remodelling during implantation also contributes significantly to the development of the placenta. Insufficient uterine remodelling causes implantation failure and infertility. To date, the exact molecular events occurring in the uterus during the establishment of receptivity and at the actual site of implantation are still not well understood. We used the mouse as a model and identified a number of previously unrecognised molecules that are uniquely regulated in the early stages of implantation: one of these is proprotein convertase 6 (PC6). The potential importance of these genes and their products in modulating fertility in the primate, including the human, was demonstrated by their unique spatial and temporal expression in the endometrium of human and rhesus monkey during the phase of uterine receptivity and at implantation. The importance of the genes for implantation was ultimately confirmed by functional studies *in vivo* using morpholino antisense oligonucleotides. These molecules will be discussed in terms of their identity, expression and functions.

015

DISRUPTED DECIDUALISATION IN *SOCS3* GENE MUTANT MICE

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Cytokines comprise a large family of secreted glycoproteins that regulate many fundamental biological processes. Cytokine signals are relayed to target cells via binding to cell surface receptors. The receptors signal via members of the Janus kinases (JAKs) and signal transduction and activators of transcription family (STATs). The SOCS proteins negatively regulate cytokine signalling by inhibiting components of the JAK/STAT pathway. Genetically modified mice in which individual SOCS genes are ablated have revealed key biological roles for these proteins. SOCS3 null mice die at mid gestation due to placental insufficiency. By embryonic Day (E) 9.5 there is a marked decrease in the spongiotrophoblast layer and an increase in trophoblast giant cells in *SOCS3* null placentae. With increasing gestational age, there is progressive disorganisation of the *SOCS3* null placental labyrinth. Takahashi *et al.* (1) used tetraploid aggregation to demonstrate that the placental defect was attributable to intrinsic defects in the *SOCS3*-deficient trophoblast cells or yolk sac endoderm. Based on evidence from *in vitro* assays, *SOCS3* has a role in downstream negative regulation of signalling via a large number of cytokines. To identify the cytokine responsible for the placental phenotype, we crossed *SOCS3* null embryos with mice lacking leukaemia inhibitory factor (LIF). This rescued the placental phenotype of the *SOCS3* null mice, thereby demonstrating that alterations in LIF signalling are responsible for profound abnormalities of the murine placenta.

(1) Takahashi *et al.* (2003) *EMBO J.* **22**, 372–384.

016

ROLE OF CITED GENES IN PLACENTAL MORPHOGENESIS: STUDIES IN NULL MUTANT MICE

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Cited1 and Cited2 interact with CBP and p300. CBP/p300 bind numerous proteins and evidence exists, for Cited2 at least, that Cited binding prevents the binding of other proteins to CBP/p300. Since CBP/p300 interact with many proteins, can acetylate protein and DNA, and act as a ubiquitin ligase, it is likely that Cited1 and Cited2 function at a number of sites during development. We have generated mice that carry a null mutant allele for each of these genes. Analysis of null mutant embryos demonstrates that both Cited1 and Cited2 are required for normal embryonic development and survival. Although both Cited1 and Cited2 are expressed in the developing embryo and placenta, it appears that abnormal placental development and function is the cause of embryonic death.

The defect that develops in the placentas of Cited1 null mutants is not apparent until late in gestation (16.5dpc). Cited1 null mutants are smaller than controls at birth and die during the early postnatal period. The placentas of these mutants are disorganised, with spongiotrophoblasts projecting in to the labyrinthine layer. In addition, resin casts of the maternal blood spaces within these placentas revealed extremely enlarged blood sinuses. We are searching for factors that could result in the increased size of the maternal blood sinuses.

Cited2 null placentas and embryos are significantly smaller than controls; mutants die 3/4 the way through gestation (15.5dpc). The null mutant placentas have proportionally fewer spongiotrophoblasts, trophoblast giant cells and invasive trophoblasts. In addition, resin casts of fetal vasculature of the placenta reveal that the capillary network is underdeveloped. Through the isolation of trophoblast stem (TS) cells we are exploring the possibility that TS cell proliferation and/or differentiation is impaired due to a lack of Cited2. We suspect that the development of the phenotype may relate to the Hypoxia Inducible Factor-1a (HIF1a) transcription factor as Cited2 expression is induced by HIF1 and it acts to negatively regulate its activity.

021

IDENTIFICATION AND STUDY OF GENES IMPORTANT FOR FETAL GERM CELL BIOLOGY IN MICE

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We are using a multi-pronged approach to discovering genes and proteins that regulate the allocation, proliferation, migration, differentiation and apoptosis of primordial germ cells (PGCs) in the developing mouse embryo. First, we are using suppression PCR and microarray screening methods to identify genes whose expression is restricted to, or enriched in, gonads of a specific sex or developmental stage. In this way several genes were identified whose expression is restricted to germ cells. Second, we are using a proteomic approach to identify important proteins and the genes that encode them. Protein expression profiles are being compared between different sexes and stages of mouse fetal gonad development. Data so far indicate that this method is a useful adjunct to transcriptional profiling, capable of identifying not only proteins that are differentially expressed, but also those that are differentially modified, for example by phosphorylation. Third, *in silico* screening of mouse EST databases identified 23 new candidate genes whose expression appears to be limited to pluripotent cells and the germline. Many of these genes are novel uncharacterised transcripts. Preliminary *in situ* expression analyses show that eight of these genes are indeed limited to the germline and to pluripotent cells. These genes may have important functions in germline specification and function. We are currently developing approaches, including inducible RNAi-based methods, for examining the function of these genes, initially *in vitro* but also ultimately *in vivo*.

022

SPERMATOGONIAL STEM CELLS: FROM BASIC RESEARCH TO CLINICAL APPLICATIONS

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The testis contains undifferentiated spermatogonia and is therefore the only adult organ populated with proliferating germline cells. Whereas the biology of these cells is quite well understood in rodents, their modes of mitotic expansion and differentiation are poorly understood in primates. The existence of these cells offers clinically relevant options for preservation and restoration of male fertility. New approaches based on male germ cell transplantation and testicular tissue grafting can be applied to generate a limited number of sperm and could therefore be considered as important new avenues applicable to a variety of disciplines like animal conservation, genetic germline modification or restoration of fertility in oncological patients. In principle, germ cell transplantation presents a removal of the stem cell from the donor's niche and a transfer into the niche of a recipient. Grafting can be considered as a transplantation of the stem cell in conjunction with its niche. Germ cell transplantation of human spermatogonia into mouse testes revealed that the stem cells survive and expand but are not able to differentiate and complete spermatogenesis. We have developed an approach to infuse germ cells into monkey and human testes and showed that germ cell transplantation is feasible as an autologous approach in primates. Furthermore, we applied germ cell transplantation in the monkey model mimicking a gonadal protection strategy for oncological patients. Ectopic xenografting of testicular tissue was applied to generate fertile sperm from a variety of species. Newborn testicular tissue was grafted into the back skin of immunodeficient mice and developed up to qualitatively complete spermatogenesis. The rapid progress in the development of novel experimental strategies to generate sperm from cryopreserved spermatogonial stem cells or immature testicular tissue will lead to many new options for germline manipulation and fertility preservation.

023

DRIVERS OF GERM CELL DIFFERENTIATION

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Spermatogenesis requires progression of a self-renewing male germline stem cell population through a precisely timed and ordered developmental sequence to form spermatozoa. For several years, we have been investigating the functional impact of signals on this progression by members of the TGFβ superfamily, follicle stimulating hormone and the bcl-2 family. For example, our lab and others have shown that activin A, bone morphogenetic protein (BMP)-4 and glial-derived neurotropic factor (GDNF) all modulate stem cell development and spermatogonial differentiation at the onset of spermatogenesis after birth. We are trying to understand how germ cells 'interpret' this plethora of competing signals to mediate maturation.

Progression through successive maturation states in response to such signals requires the movement of proteins, including transcription factors, into the nucleus to implement changes in gene transcription. We are investigating the concept that regulated transport of proteins into the nucleus is one mechanism that governs spermatogenic differentiation, and we have focused on analysis of the major class of nuclear transport factors, the importins (IMPs). A diverse family comprising at least six different α forms and 20 different β forms in the mouse, the IMP proteins selectively bind a diverse range of cargo proteins and mediate their passage through the nuclear pore complex and into the nucleus. Our immunohistochemical and *in situ* hybridisation studies have demonstrated developmentally regulated expression of many IMPs in germ cells, in the fetus, in the neonate and in the adult. This suggests that they function to transport cargo required for discrete stages of spermatogenesis. Our recent studies examined the mouse embryonic gonad around the time when specification to form either a testis or an ovary occurs. The IMPβ3 protein is present in both male and female germ cells, but the subcellular localisation and expression patterns within these cell types is gender- and age-specific. We are currently exploring the functional significance of our observations.

024

POST MEIOTIC GENE TRANSCRIPTS – SENSE, ANTISENSE, OR NONSENSE

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The mature spermatid is a highly specialised cell type whose structure and physiology have evolved to convey a haploid genome intact through a relatively inimical environment to find, interact with and achieve fertilisation of a mature female gamete, thus to produce a healthy diploid embryo. It is logical to assume that these highly specialist functions are the product of a unique set of genes or transcripts, expressed in late germ cell stages. It is equally logical to see the protein products of these genes as likely targets for a post meiotic approach to male contraception. Indeed many such spermatocyte- and spermatid-specific transcripts have been identified by differential cloning approaches. Some transcripts appear to represent novel sperm-specific genes, some represent sperm-specific alternative splice products, or alternatively initiated transcripts. However, for many such transcripts, there are features that lend doubt to the notion that they are truly functional in the context of sperm physiology. Many transcripts derive from undefined, TATA-less promoters. Some gene products have no legible open reading frame. Some transcripts are even produced as antisense molecules. Some appear as functional transcripts, but are not translated. Some appear to be highly species specific. Some appear to be functionally redundant, when tested in gene ablation experiments. The male gamete is under extreme selection pressure. It is therefore plausible that these apparently aberrant transcripts may have a function beyond that of conventionally generating physiologically relevant proteins, as in most somatic cells. This presentation reviews current ideas about the sperm transcriptome and presents various hypotheses to help us understand the mechanisms and purpose of post meiotic gene expression.

025

OVARIES: UP IN A POF OF SMOKE

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Premature ovarian failure (POF) or premature menopause is a common disorder, defined by the occurrence of menopause under the age of 40 years and is characterised by amenorrhoea, hypoestrogenism and elevated gonadotrophins. Worldwide it affects 1% of all women and occurs in 0.1% before the age of 30 years. The major problems associated with POF are the loss of fertility at an early age and the psychological problems associated with this. In addition there are the physiological effects of reduced oestrogen, which include an increased risk of osteoporosis. POF is a heterogeneous disorder and the cause of most cases is unknown. A significant proportion (20-30%) of women with POF have a genetic predisposition. Our primary goal is to identify genes involved in POF. In most cases, the menopause is due to the loss of follicles, and it stands to reason that suitable candidate genes for POF development would be genes that regulate the rate of follicle loss. We have identified two common gene mutations, a 769G>A transition in the inhibin alpha gene in approximately 5% of POF patients associated with POF at a very early age, and mutations in FOXL2 in approximately 5% of patients. FOXL2 is thought to act downstream of inhibin which suggests that other candidate genes may arise from the analysis of the activin signalling pathway. Functional studies will help us to understand more about the molecular basis of POF. Each mutation has been associated with less than 10% of POF cases and POF is likely to be caused by mutations at many different loci. It is hoped that determining the molecular basis of POF will lead to the development of genetic tests to predict the development of POF, and eventually lead to treatment that will return fertility to these women.

026

ENDOMETRIOSIS – LINKAGE, POSITIONAL CLONING AND GENOME WIDE ASSOCIATION

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Endometriosis is a complex disease which affects up to 10% of women in their reproductive years. Common symptoms include severe dysmenorrhea and pelvic pain. The disease is associated with subfertility and some malignancies. Genetic and environmental factors both influence endometriosis. The aim of our studies is to identify genetic variation contributing to endometriosis and define pathways leading to disease. We recruited a large cohort of affected sister pair (ASP) families where two sisters have had surgically confirmed disease and conducted a 10 cM genome scan. The results of the linkage analysis identified one chromosomal region with significant linkage and one region of suggestive linkage. The regions implicated by these studies are generally of the order of 20–30 cM and include several hundred genes. Locating the gene or genes contributing to disease within the region is a challenging task. The best approach to the problem is association studies using a high density of SNP markers. The recent development of human SNP maps and high throughput SNP genotyping platforms makes this task easier. We have developed high throughput SNP typing at QIMR using the Sequenom MassARRAY platform. The method allows multiple SNP assays to be genotyped on the same sample in a single experiment. Throughput and genotyping costs depend critically on this level of multiplexing and we routinely genotype 6–8 SNPs in a single assay. We are using bioinformatics and functional approaches to develop a priority list of genes to screen early in the project. SNP markers in these genes are being genotyped using the MassARRAY platform to search for genes contributing to endometriosis. In the future, genome wide association studies with our families may locate additional genes contributing to endometriosis.

027

MAPPING NOVEL BREAST CANCER SUSCEPTIBILITY GENES BY LINKAGE ANALYSIS OF AUSTRALIAN MULTIPLE CASE KINDREDS

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We have been using the resources of the Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer (kConFab) and of the Australian Breast Cancer Family Study (ABCFS) to identify kindreds suitable for mapping high penetrance breast cancer susceptibility loci other than BRCA1 and BRCA2. A 10 cM genomewide search was carried out in 40 families in which BRCA1 and BRCA2 mutations had been excluded with high probability. The highest LOD score under heterogeneity (HLOD) was 2.16 (non-parametric LOD 1.83, $P = 0.04$) at the 11p telomere; several other regions with HLODs = 1.5–2.0 also merited investigation using fine mapping but have so far neither been confirmed or rejected by these analyses. Subsets based on age of onset and presence of other cancers correlated to some extent with particular linkage peaks and several regions (notably 2q and 13q) corresponded to areas of suggestive linkage reported recently in more limited studies of other cohorts. A large collaborative analysis of these data together with those from similar studies undertaken by members of the international Breast Cancer Linkage Consortium (BCLC) is under way. It is therefore likely that further major breast cancer susceptibility loci will be localised in the near future. The complementarity of these studies with genetic association, candidate gene and tumour-based approaches will be discussed.

040

OVARIAN RESEARCH IN THE POST GENOMIC ERA: ANALYSES OF GDF-9 AND OTHER PARACRINE SIGNALING PATHWAYS

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Recent publication of the genomic sequences for human and multiple model organisms allows the elucidation of the evolutionary origins of human genes. Because of the coevolution of polypeptide ligands and their cognate receptors, analysis of human genomic sequences allows one to predict the pairing of these elements. Based on a genomic approach, we identified the known BMPRII and ALK5 as the receptors for GDF-9 (1,2) and elucidated downstream signaling Smad molecules in ovarian follicles. We also identified LGR7 and LGR8 as receptors for relaxin and INSL3 respectively (3,4). Based on the known production of INLS3 by testicular Leydig cells and ovarian theca cells, we investigated the expression of the INSL3 receptor, LGR8, in male and female gonads. Of interest, LGR8 expression was found exclusively in oocytes in the ovary and male germ cells in the testis. We further found that LH stimulates INSL3 transcripts in ovarian theca and testicular Leydig cells. INSL3, in turn, binds LGR8 expressed in germ cells to initiate meiotic progression of arrested oocytes in preovulatory follicles *in vitro* and *in vivo* and to suppress male germ cell apoptosis *in vivo* (5). In contrast to its stimulation of cAMP production by somatic cells, INSL3 interacts with germ cells to activate the inhibitory G protein, thus leading to decreases in cAMP production. Our data demonstrate the importance of the INSL3–LGR8 paracrine system in mediating gonadotropin actions in gonads. We have set up an ovarian gene database (Ovarian Kaleidoscope database at http://ovary.stanford.edu) in which more than 1 600 ovarian gene pages can be accessed online and searched by gene name, function, mutation phenotype, expression pattern, cellular location and other parameters. Database analysis of polypeptide ligand and receptor genes provides a functional genomic paradigm for the identification of novel ligands and receptors in the ovary. To assist ovarian researchers in the analyses of their DNA array datasets, a Microarray Data Interpreter has been set up in the OKdb to facilitate searches and comparisons of known and novel genes in the ovary.

(1) *Biol. Reprod.* 2002. (2) *Mol. Endocrinol.* 2004. (3) *Science* 2002. (3) *J. Biol. Chem.* 2002 (4) Kawamura *et al. PNAS* 2004.

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ACTIVIN A: FROM REPRODUCTIVE FACTOR TO INFLAMMATORY CYTOKINE

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Activin A was originally isolated and characterised as a reproductive feedback regulator of follicle-stimulating hormone. While potent paracrine networks involving activin and its binding protein, follistatin, are present in the gonad and pituitary, recent focus has been on emerging roles in a number of other systems, such as erythropoiesis, neuronal survival, embryonic development and inflammatory processes. The latter relatively new property was first suggested by us when follistatin in the circulation was elevated in sheep undergoing surgical trauma. We have since focussed on a model of acute inflammatory challenge using the bacterial cell wall component lipopolysaccharide (LPS) or endotoxin. This has highlighted that the release of activin into the bloodstream occurs extremely rapidly, within about 50 min. The response appears to be biphasic and precedes or is at least coincident with the release of a number of key pro-inflammatory cytokines, such as tumour necrosis factor α and interleukin-6. The mechanisms of this release are still being delineated, but it is fever- and prostaglandin-independent, and largely unaffected by blocking other key cytokine responses. Nevertheless, it is directly downstream of the LPS receptor and its activation pathway. Importantly, activin's property as an inflammatory cytokine appears to be borne out in a number of clinical inflammatory syndromes such as septicemia, suggesting that it is a hitherto undescribed component of the organism's innate immune response to infection.

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THE ROLE OF TGF-β IN NORMAL AND PATHOLOGICAL LENS DEVELOPMENT

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How the lens develops its highly ordered architecture and growth patterns is a major question in developmental biology. During embryogenesis, cells in the anterior and posterior segments of the lens vesicle, differentiate into the epithelial and fibre cells, respectively. Our research has aimed to identify the molecules and mechanisms that regulate the divergent fates of lens cells. We have studied the roles of various growth factors in regulating lens cell fates using rat lens epithelial explant cultures and transgenic and mutant mouse models. Our research has shown that members of the FGF growth factor family are key initiators of lens fibre differentiation in mammals and there is now compelling evidence that a gradient of FGF in the eye controls lens polarity and growth patterns. Recent evidence also supports a role for TGF-β signalling in this process and indicates that a cascade of growth factor signalling may be required for normal fibre differentiation. Less is known about the anterior segment; however, our recent studies point to an important role for the Wnt growth factor family in epithelial differentiation. Growth factor signalling can also cause pathological changes; e.g. TGF-β can destabilise the normal epithelial phenotype and induce aberrant growth and differentiation that mimics the epithelial-mesenchymal transition characteristic of some forms of cataract. These studies highlight the importance of growth factor signalling in regulating the ordered growth and differentiation of the lens. It is also clear that the bioavailabity of some growth factors needs to be tightly regulated so that they act in the appropriate cellular compartment. Some cataracts may be a consequence of disturbed growth factor, particularly TGF-β, regulatory mechanisms.

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ROLE OF TGFβ IN ADRENAL STEROIDOGENESIS BEFORE BIRTH

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During mammalian development there are periods when the fetal adrenal is either relatively refractory or increasingly sensitive to trophic stimulation. This pattern of regulation of adrenal growth and function ensures that the fetal lungs, liver, brain and kidney are exposed in a programmed temporal sequence to the genomic actions of circulating glucocorticoids. A range of studies in the rat and sheep have also demonstrated that exposure to excess glucocorticoids at inappropriate times in fetal life inhibits fetal growth and permanently reprograms the development of the cardiovascular and metabolic systems resulting in postnatal hypertension, abnormal hepatic glucose production and poor glucose tolerance In most mammalian species, there are therefore a range of mechanisms that protect the fetus from exposure to glucocorticoids of either maternal or fetal origin at inappropriate times in gestation. The factors that act to maintain periods of adrenal quiescence are not known. There is evidence that intra-adrenal transforming growth factor beta 1 (TGFβ1) is an inhibitor of adrenocortical steroidogenesis in the adult. In recent studies, we have demonstrated that expression of TGFβ1 is high in the fetal sheep adrenal at around 100 days gestation and that adrenal TGFβ1 expression then falls with increasing gestational age and is lowest immediately after birth. Following the activation of adrenal cytochrome P450 C17 (CYP17), there is an inverse relationship between adrenal TGFβ1 and CYP17 expression and TGFβ1 may therefore play a novel inhibitory role in the regulation of adrenal steroidogenesis during mid and late gestation. Whilst functional activation of the fetal adrenal is dependent on the fetal hypothalamo–pituitary axis, adrenal TGFβ1 mRNA expression is not altered by disconnection of the fetal hypothalamus and pituitary in late gestation. It therefore appears unlikely that TGFβ1 mRNA expression is regulated directly by either bioactive ACTH or cortisol in late gestation. The mechanism by which TGFβ1 expression is upregulated in mid gestation remains to be determined.

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TETRAHYDROGESTRINONE (THG) IS A POTENT ANDROGEN AND PROGESTIN

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Tetrahydrogestrinone (THG) is a novel steroid recently identified by a sports doping laboratory as an illicit agent sold to improve elite athletic performance. While its structure is closely related to gestrinone, a 19-nor progestin, and resembles that of trenbolone, a potent banned synthetic androgen, THG was never marketed, so no information on its hormonal properties are known. We therefore examined THG for steroidal bioactivity using yeast transformed with a steroid receptor-reporter system, comparing its bioactivity to other known androgens, nandrolone, 7α nandrolone (MENT), norbolethone, 5α-norbolethone, norethandrolone and trenbolone, as well as THG's parent compound, gestrinone. Yeast were stably transformed with human androgen receptor (AR) or progesterone receptor A (PR) cDNA, together with a reporter plasmid containing a β-galactosidase gene under the transcriptional control of an androgen (ARE) or progestin (PRE) reporter element. Bioassays were established by culturing transformed yeast in the presence of the steroids over the range of 1.2×10^{-6} to 5.9×10^{-10} M.The bioassay end-point was β-galactosidase activity in yeast cell lysates. THG showed dose-dependent highly potent activation of AR activity with an EC_{50} of 0.29 nM compared with other steroids nandrolone (0.12 nM), norbolethone (0.3 nM), 5α-norbolethone (0.026 nM), gestrinone (0.59 nM), trenbolone (0.78 nM), norethandrolone (0.19 nM) and MENT (0.01 nM). THG also activated PR (EC_{50} 0.7 nM) with much higher potency than its parent steroid, gestrinone (EC_{50} 30 nM). We conclude that THG is a potent androgen and progestin. It shows similar potency to the comparator androgens, nandrolone, norbolethone, 5α-norbolethone and trenbolone. The discovery of this illicit designer androgen raises concern about the possibility of other novel androgens being produced from other marketed synthetic sex steroids.

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DIFFERENTIAL REGULATION OF INHIBIN BINDING VIA BETAGLYCAN EXPRESSION IN SEVERAL MOUSE CELL LINES

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Inhibin A, a member of the transforming growth factor (TGF)-β superfamily, binds to mouse adrenocortical (AC), Leydig (TM3) and Sertoli (TM4) cell lines with high affinity via at least eight membrane protein species, two of which are forms of betaglycan. Inhibin A has been proposed to inhibit the actions of activin and BMP by sequestering their type II receptors in high affinity complexes with betaglycan (1). We previously found that BMPs appear to counteract inhibin action in AC cells by selectively suppressing the expression of endogenous betaglycan, consequently reducing inhibin binding. In the present studies, we have examined how factors that stimulate betaglycan expression in other systems modify the binding of radiolabelled inhibin A at the surface of AC, TM3 and TM4 cells.

AC, TM3 and TM4 cells were treated overnight with glucocorticoid, membrane-permeable cAMP analogue or retinoic acid, after which the levels of betaglycan mRNA, corrected for GAPDH content, were measured using real-time RT-PCR, and [^{125}I]inhibin A binding was determined. Treatment of AC cell cultures with 8Br-cAMP (1 mM), glucocorticoid (RU28362, 100 nM) or retinoic acid (30 mM) increased betaglycan mRNA levels 120-150%, and increased subsequent inhibin A binding to 146 ± 12, 132 ± 13 and 125 ± 18% of control (mean ± SD, n=6–12). The glucocorticoid and cAMP treatments also increased inhibin binding to TM3 and TM4 cells by similar amounts, but retinoic acid was less effective. Affinity labelled protein species of deduced sizes 115 and >170 kDa, consistent in size with betaglycan forms, were the primary target for stimulation by these agents, whereas species of 65 and 75 kDa were selectively increased by retinoic acid in the AC cells.

In summary, glucocorticoids, retinoids and hormones that stimulate cAMP production may increase the expression of betaglycan in inhibin target cells, increase their binding of inhibin, and thereby promote inhibin action. These studies confirm that betaglycan is a primary determinant of inhibin binding and action. The protein species other than betaglycan that are selectively upregulated by retinoic acid in AC cells are yet to be identified. *Funded by the NH&MRC of Australia (RegKey 241000 & 198705).*

(1) Wiater & Vale (2003) *J. Biol. Chem.* **278**, 7934.

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A REPOSITORY OF ENU MUTANT MOUSE LINES AND THEIR POTENTIAL FOR MALE FERTILITY RESEARCH

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One in 25 western men are infertile and the causal factor is frequently unknown, although it is expected that many are genetic in origin. My project aims to identify genes critical to mouse spermatogenesis using ENU mutagenesis. A further aim was to develop a repository of mutant mice and data on their fertility parameters for use by the reproductive biology community. This research will aid the diagnosis and development of specific treatments for human infertility and the development of contraceptive agents. The potent mutagen *N-ethyl-N-nitrosourea* (ENU) was utilized to generate libraries of C57BL/6 mice with random point mutations throughout their genomes. A 3 generational breeding program produced mice that were homozygous for a number of mutations. I subsequently performed a number of large scale screens on 3rd generation males, identifying lines carrying recessive mutations specifically affecting male fertility. Thus far we have observed a wide range of abnormal testis phenotypes including Sertoli Cell only, hypospermatogenesis, meiosis arrest, abnormal sperm morphology and abnormal hormone levels. From these analyses a repository including all data and tissues collected from 1200 3rd generation male mice from 122 different lines has been developed and will become publicly available. This includes testis and epididymal histology and serum levels of FSH, LH, activin A and inhibin. Further, I have stored gDNA long term and cryopreserved sperm to enable regeneration of lines in the future. In addition, I have developed a high throughput mutation screening protocol for the detection of mutations within genes of interest using denaturing high performance liquid chromatography (DHPLC). Collectively, our repository and gene screening techniques can be used in conjunction with artificial reproductive technologies to generate mouse models reflective of human conditions and altered specific gene function.

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AN $\alpha_6\beta_1$-INTEGRIN/FOCAL ADHESION KINASE COMPLEX MAY REGULATE SPERMIATION AND SPERMIATION FAILURE

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Spermiation is the final step of spermatogenesis (sperm production) where mature spermatids are released from the somatic Sertoli cells. Spermiation is hormone sensitive; testosterone (T) and FSH withdrawal causes a disruption to the disengagement of spermatids, which are instead retained by Sertoli cells. The mechanisms involved with spermatid release and retention are not understood. We showed previously that an unknown adhesion junction containing β_1-integrin persisted on retained spermatids suggesting that a defect in this adhesion complex at disengagement may underlie spermiation failure. The aim of this study is to identify the α-integrin dimerised with β_1-integrin and investigate the role of phosphorylated FAK, a kinase that is involved with integrin-mediated cell adhesion, during spermiation and spermiation failure. Four adult Sprague-Dawley rats received T and oestradiol implants and FSH antibody for 7 days to suppress testicular T and FSH and induce spermiation failure. Using immunohistochemistry, α_6-integrin (but not α_4-integrin) and FAK-Tyr^{397} were localised on the Sertoli cell plasma membrane adjacent to mature spermatids. This localisation was observed until the point of spermatid release and remained on the Sertoli cell that surrounded retained spermatids after hormone suppression. A similar localisation has been previously observed with β_1-integrin, suggesting that all three form a complex at the site of disengagement. To look at the function of FAK-Tyr^{397}, comparative Western blot analysis is currently being undertaken on seminiferous tubules specific for spermiation from control and treated animals. Preliminary studies suggest that FAK-Tyr^{397} remains phosphorylated during spermiation failure, suggesting that FAK dephosphorylation may be important for the function of spermatid-associated adhesion complexes, as has been demonstrated in other adhesion systems. In conclusion, $\alpha_6\beta_1$-integrin/FAK-containing adhesion complexes are associated with spermatids during spermiation, and the function of such complexes are likely to be perturbed during spermiation failure.

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EFFECTS OF PHYTOESTROGENS ON THE OVARIAN AND PITUITARY PHENOTYPES OF OESTROGEN DEFICIENT FEMALE AROMATASE KNOCKOUT MICE

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Phytoestrogens can induce both estrogen agonistic and antagonistic effects, depending on the tissue, estrogen receptor content and endogenous levels of estrogen. Dietary phytoestrogens are promoted as alternatives to synthetic estrogens for hormone replacement therapy, however their effects on the reproductive axis have not been exhaustively studied in vivo. Female aromatase knockout mouse (ArKO) mice are estrogen-free, and anovulatory with a block in folliculogenesis, hemorrhagic cysts and development of Sertoli cells within their ovaries. We evaluated the ArKO mouse as a model to test the effects of phytoestrogen-supplemented diets on the reproductive organ weights, ovarian morphology, gonadotropin levels and the transcript levels of ovarian somatic cell and steroidogenic markers. The extent to which phytoestrogens either as soyfree or soymeal diet or genistein added to a soyfree diet, exert estrogenic effects varied with the type of phytoestrogen and the parameter being tested. The genistein diet significantly increased uterine and ovarian weights of ArKO compared to wildtype mice, whilst both the soy, and to a larger extent the genistein diet, improved ovarian morphology compared to the soyfree diet. Transformation to morphological Sertoli cells in ArKO mice was decreased by both phytoestrogen diets, whilst the gene expression of Sertoli cell markers was not affected. The soy diet increased both LH and FSH in both genotypes compared to animals on the soyfree diet. The genistein diet reduced FSH levels in ArKO mice, correlating with increased ovarian inhibin subunit expression. In conclusion, phytoestrogens are estrogenic in ArKO mice. Specifically, they can affect serum gonadotropin levels, and offset the development of Sertoli cells and hemorrhagic cysts within the ovaries, depending on the type of dietary phytoestrogen. Further studies are required to determine the effective doses and treatment regimes for phytoestrogens as endocrine modulators. *Supported by NH&MRC Reg Key#198705.*

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POLYCYSTIC OVARY SYNDROME: ARE ENDOCRINOLOGISTS AND GYNECOLOGISTS TREATING THE SAME PATIENTS?

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Women with polycystic ovary syndrome commonly consult endocrinologists or gynaecologists. The diagnosis and management of this disorder are controversial, and it is not known if these specialty groups differ in their approach. Our objective was to compare the investigation, diagnosis and treatment of polycystic ovary syndrome by endocrinologists and gynaecologists. A questionnaire containing a hypothetical patient case history with varying presentations was sent to endocrinologists and gynaecologists in teaching hospitals and private practice. Evaluable responses were obtained from 138 endocrinologists and 172 gynaecologists. The two specialty groups differed markedly in their choice of essential diagnostic criteria. Endocrinologists regarded androgenisation (81%) and menstrual irregularity (70%) as essential for diagnosis, whereas gynaecologists cited polycystic ovaries on ultrasound (61%), androgenisation (59%), menstrual irregularity (47%) and elevated LH : FSH ratio (47%). (All P values <0.001.) Gynaecologists were more likely to request ovarian ultrasound (91% *v.* 44%, $P < 0.001$) whereas endocrinologists were more likely to measure adrenal androgens (80% *v.* 58%, $P < 0.001$) and fasting lipids (67% *v.* 34%, $P < 0.001$). Gynaecologists were less likely to assess glucose homeostasis but were more likely to use a glucose tolerance test to do so. Diet and exercise were chosen by most respondents as first-line treatment for oligomenorrhoea, hirsutism, infertility and obesity. Endocrinologists were more likely to use insulin sensitisers, particularly metformin, for these indications. In particular, for infertility, endocrinologists favoured metformin treatment whereas gynaecologists recommended clomiphene. There is a lack of consensus between endocrinologists and gynaecologists in the definition, diagnosis and treatment of polycystic ovary syndrome. Women may receive different diagnostic advice and treatment depending on the type of specialist consulted.

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OVARIAN LEUKOCYTES AND CYTOKINES IN POLYCYSTIC OVARY SYNDROME

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Polycystic ovary syndrome (PCOS) is associated with anovulatory infertility and metabolic disturbances. PCOS has an unknown aetiology but is involved with aberration of substances that lead to follicular growth. The ovarian leukocyte/cytokine network is important in the ovary and has not been adequately examined in PCOS. The aim of the study was to look at the distribution of leukocytes in the ovaries of women with PCOS and to look at expression of cytokine and chemokine mRNA in follicular cells from these patients. Ovaries were obtained from PCOS ($n = 5$) and non-PCOS ($n = 4$) women undergoing gynaecological surgery for non-ovarian conditions prior to the menopause. They were immunostained for a wide variety of leukocyte markers and distribution counted using visual imaging software. Luteinising granulosa cells were obtained prior to ovulation in women undergoing *in vitro* fertilization with ($n = 11$) and without ($n = 22$) PCOS and mRNA studied using quantitative RT-PCR for various cytokines and chemokines. The CD45RO subset of leukocytes (principally activated/memory T-lymphocytes) were significantly decreased in ovaries from PCOS women compared to non-PCOS women. The other leukocytes were not different in distribution and numbers. Transcripts for CSF-1, IL-1β, IL-6, IL-8, IL-10, MCP-1 and TNFα were not different between PCOS and non-PCOS women whilst GM-CSF mRNA was not detectable in either group. There was an association between high testosterone levels and high IL1β and low TNFα transcripts. Women who became pregnant following IVF had higher levels of IL-10 mRNA. The role of T-lymphocytes in PCOS needs further examination, and if the leukocyte/cytokine network in PCOS is important, other cells and cytokines need examination. This is the first study to definitively describe the leukocyte/cytokine network within polycystic ovaries. While other cells and substances may be important in PCOS and intervention procedures such as ovarian drilling, it does not appear as though macrophages, neutrophils, B-lymphocytes and a variety of cytokines are involved in the aetiology of PCOS.

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MEASUREMENT OF TOTAL TESTOSTERONE IN WOMEN: COMPARISON OF A DIRECT RADIOIMMUNOASSAY VERSUS RADIOIMMUNOASSAY AFTER ORGANIC SOLVENT EXTRACTION AND CELITE PARTITION COLUMN CHROMATOGRAPHY

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Testosterone (T) has multiple significant physiological effects in women. To date no rapid, simple assay of total T has been shown to produce reliable results in women at the low end of the normal female range. The aim of this study was to evaluate the accuracy of a direct radioimmunoassay (dRIA) for total T by comparing values for total T measured by this assay with values determined by a conventional RIA (cRIA) method that utilizes extraction and chromatographic steps prior to quantification. Methods: Fasting serum samples were obtained from a sub-group of 259 healthy women, aged 18-75 years, randomly recruited from the community and stored at -80°C. Total T was measured by the dRIA method using antibody coated tubes and iodine-labeled T tracer. For comparison, total T levels were also measured using the cRIA after organic solvent (ethylacetate : hexane (3 : 2)) extraction and Celite column partition chromatography prior to RIA. Results: The mean T level by dRIA was 0.76 nmol/L (median 0.70, SD 0.54, min 0.10, max 3.2). The mean difference between the two measurements (dRIA-cRIA) was –0.28 (SD 0.3). The limits of agreement using the Bland-Altman approach on log transformed data showed that, on average, the dRIA value was 63% of the cRIA value and that 95% of the time the dRIA estimate lay between 26% and 155% of the cRIA estimate. However, with respect to clinical application, for classification of values in the lowest 10th centile, agreement between assays was seen in 245/259 women (Kappa = 0.68) Conclusion: The dRIA is a clinically useful assay that provides precise measurements of total T in women, particularly when values are low, and is appropriate for the study of the issue of 'low' T within the female population.

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REGULATION OF MOUSE MURAL GRANULOSA CELL PROGESTERONE SYNTHESIS BY OOCYTE PARACRINE FACTORS

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Oocytes secrete soluble paracrine factors that play an important role in the growth and development of surrounding follicular cells. It is known that oocytes suppress FSH-induced progesterone production by mural granulosa cells (MGC), however it is unclear which growth factor(s) are involved. Some candidate molecules include growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and BMP6. The aim of this study was to examine the role of these factors in the regulation of FSH-induced MGC progesterone production. Prepubertal 129/SV mice were primed with eCG and ovaries were collected ~46 h later. MGC from large antral follicles were cultured with either denuded oocytes (DO; 0.25/µl), GDF9 or BMP15 (0.25-4% v/v) or BMP6 (10-200ng/ml), in the absence or presence of FSH. Cells were cultured for 18 h followed by a further 6 h pulse of ^{3}H-thymidine. After 24 h cells and media were harvested for assessment of MGC DNA and progesterone synthesis, respectively. Treatment with FSH increased MGC progesterone production 9-fold, which, as expected, was antagonised by coculture with DO (by 73%). GDF9 and BMP15 both decreased FSH-induced MGC progesterone in a dose dependent manner, significantly reducing control levels (100%) to 17% and 30%, respectively, at doses of 2%v/v. All doses of BMP6 abolished FSH-stimulated progesterone. Even though all treatments inhibited progesterone production, only two of these, GDF9 (0.25%v/v) and DO (0.25DO/µl), stimulated MGC DNA synthesis (2.1 and 3.3 fold above controls, respectively). The BMP receptor type-II (BMPR-II) is a known receptor of several oocyte factors. Treatment with a BMPR-II ectodomain completely antagonised DO- and GDF9-stimualted MGC DNA synthesis, but progesterone levels were only partially restored (by 50%). These data indicate that BMP15 and BMP6 mimic the progesterone-regulating, but not the growth-promoting, activities of oocytes, whereas GDF9 does both. Although the BMPR-II ECD antagonises these oocyte factors, this receptor-signalling system may not necessarily be the means by which oocytes regulate MGC progesterone synthesis.

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ESTROGEN ACTIONS ON FOLLICLE FORMATION AND EARLY FOLLICLE DEVELOPMENT

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Estradiol 17 beta (E2) effects late follicular development whilst primordial follicle formation and early activation are thought to be independent of E2. To test this hypothesis we compared numbers of primordial and primary follicles in wildtype and E2 deficient ArKO mice, and the immunohistochemical staining or mRNA expression of Mullerian inhibiting substance (MIS), Wilms tumour 1 (WT-1), and growth differentiation factor (GDF9), known to effect early follicular differentiation. Proliferating cell nuclear antigen (PCNA) staining was a marker of proliferative index. The effects of E2 replacement for 3 wk in 7 wk old ArKO and wildtype mice on these parameters were also tested. We used unbiased, assumption-free stereological methods for quantification of early follicular numbers in the mouse ovary (1). ArKO mice had reduced numbers of primordial and primary follicles compared to wildtype (63%, $p<0.001$ and 60%, $p=0.062$ of Wt respectively). This reduction was not corrected by E2 treatment, suggesting that E2 effects the initial formation or activation of primordial follicles. There was a significant increase in the diameters of the oocytes in primordial follicles of ArKO mice compared to wildtype. There were no differences in the immunostaining of MIS, WT-1 and PCNA in primordial and primary follicles between wildtype and ArKO mice. The only difference was as a consequence of Sertoli and Leydig cells in ovaries of ArKO mice. GDF9 mRNA expression was markedly increased in ArKO ovaries. E2 treatment restored the ovarian follicular morphology, and consequently the immunostaining patterns, but had no effect on early follicle numbers. In conclusion, E2 has a role in controlling the size of the oocyte and primordial follicle pools in mice. *Supported by NH&MRC RegKey #241000 and 198705.*

(1) Britt and Myers (2004) *Reproduction* **127**,:569–580.

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STUDIES OF THE ACTIVIN PATHWAY IN DOMINANT AND SUBORDINATE BOVINE FOLLICLES BEFORE, DURING AND AFTER SELECTION

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In monovulatory species such as cattle, one of a cohort of developing follicles assumes dominancy and continues to grow in each follicular wave. After dominant follicle selection, pituitary-derived FSH levels decrease through a negative feedback loop mediated by oestradiol and inhibin A produced by the dominant follicle. The dominant follicle itself only requires very low basal levels of FSH, thus escaping atresia which is the fate of the subordinate follicles. The mechanisms involved in dominant follicle (DF) selection remain unclear. Most studies have focused on the stages following selection. To investigate what roles activin and inhibin play in DF selection we looked at the quantitative changes in the expression of the genes coding for the activin/inhibin subunits (*Inhibin α, βA* and *βB*) as well as other genes in the activin pathway (*SMAD2, ActRIIA/B, follistatin (FST), FSHR*). We examined mRNA levels in follicular granulosa cells (GCs) before (d1.5), during (d2.5) and after (d3.5 and 7) DF selection using real-time RT-PCR. Prior to DF selection, highest levels of *inhibin βA, FST and SMAD2* transcripts converged on the largest follicles. *Inhibin α*, *ActRIIA/B* and *FSHR* levels did not correlate with follicular size at this stage. At Day 2.5, highest levels of *inhibin βA, inhibin α, FST and SMAD2* transcripts were seen in a single putative DF. *ActRIIA/B* and *FSHR* did not show any difference between follicles. By Days 3.5 and 7, a dramatic difference in expression levels of *inhibin βA, inhibin α and FST* were seen in DF compared to SF. Yet in absolute terms *inhibin βA* levels decreased after selection, whereas *inhibin α* levels increased. *Inhibin βB* expression was only detected in Day 7 GCs and was significantly higher in the DF. These results suggest a shift from an activin environment during the pre and peri-DF selection period, to an inhibin environment following DF selection. Inhibin/activin protein levels in the follicular fluid using western ligand blotting confirmed this. We postulate that the higher activin activity within DF influences the selection mechanism as activin and inhibin have been shown to play a role in gonadotropin regulation in the ovary around the time of selection.

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REGULATION OF BOVINE OOCYTE MEIOTIC AND DEVELOPMENTAL CAPACITY BY GLUCOSE AND GLUCOSAMINE

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Glucose is an important substrate for *in vitro* oocyte maturation (IVM) and is metabolised by cumulus oocyte complexes (COCs) via glycolysis or is used for extracellular matrix (ECM) synthesis. Follicular glucose concentration is significantly lower than commonly used IVM media (2.3 mM *v.* 5.6 mM in TCM199). Glucosamine is an alternative substrate for ECM and supplementation to IVM media reduces glucose uptake by COCs. The aim of this study was to determine the effect of glucose and glucosamine supplementation during IVM on bovine oocytes. First, bovine COCs ($n = 400$) were matured in TCM199 (containing pyruvate, BSA, hCG and FSH), or synthetic follicular fluid medium (SFFM; a defined medium based on bovine follicular fluid composition) with 2.3 mM or 5.6 mM glucose ± 5 mM glucosamine and nuclear maturation was assessed after 24 and 30 h. Significantly less COCs matured in 2.3 mM glucose completed nuclear maturation compared to COCs matured in 5.6 mM glucose ($P < 0.05$), whereas glucosamine had no effect on meiotic maturation. We then compared oocyte developmental capacity following IVM ($n = 600$) in TCM199 or SFFM + 5.6 mM glucose ± 5mM glucosamine. Blastocyst production was severely perturbed when COCs were matured in the presence of glucosamine (–glucosamine 32% *v.* +glucosamine 4%; $P < 0.001$). To determine the cause of this reduction in oocyte developmental competence, we investigated oocyte protein synthesis by maturing COCs ($n = 100$) in SFFM + 5.6 mM glucose ± 5mM glucosamine + 1 mM L-[2,3,4,5,6–^{3}H] phenylalanine. In the presence of glucosamine, oocyte protein synthesis was reduced 40% compared to oocytes matured in control medium ($P < 0.05$).

These results demonstrate that while glucosamine supplementation has no effect on oocyte nuclear maturation, cytoplasmic maturation is compromised, as demonstrated by perturbed oocyte protein synthesis and embryo development. In contrast, glucose concentration has a significant influence on meiotic progression. This provides a useful model to investigate the mechanisms of establishment of developmental competence in oocytes following maturation.

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THE EFFECT OF FSH CONCENTRATION DURING IVM AND GAMETE CO-INCUBATION LENGTH DURING IVF ON THE DEVELOPMENT OF UNSTIMULATED PREPUBERTAL EWE OOCYTES.

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The developmental competence of prepubertal oocytes can be increased by the administration of gonadotrophins prior to oocyte collection (1); but this is not possible with abattoir-sourced oocytes, and modifications to the IVP system may increase in vitro development. Experiments were conducted to determine the effects of FSH concentration (10, 20 or 60 μg mL^{-1}) during IVM (5 replicates) and gamete co-incubation length (short: 2-3 h, long: 18-20 h) during IVF (6 replicates) on subsequent embryonic development. For both experiments ovaries were collected from prepubertal lambs (16-24 weeks) slaughtered at an abattoir and embryos produced in vitro (1). Data were analysed by chi-squared test. Oocyte cleavage at 48 hours post-insemination (hpi) was higher for oocytes matured in medium containing 20 (60/77; 77.9%) and 60 (56/73; 76.7%) than 10 μg mL^{-1} (40/67; 59.7%) FSH. Blastocyst formation (% cultured oocytes) on Day 7 (Day 0 = IVF) was higher for oocytes matured with 20 (31/77; 40.3%) than 10 (16/67; 23.9%) or 60 μg mL^{-1} (20/73; 27.4%). Oocyte cleavage at 48 hpi was reduced for short (36/57; 63.2%) compared with long (49/55; 89.1%) co-incubation, although blastocyst formation (% cultured oocytes; Day 7) did not differ between groups (22/57; 38.6% and 23/55; 41.8%, respectively). These results demonstrate that increasing the FSH concentration above normal levels during IVM of prepubertal lamb oocytes improves development in vitro. Gamete co-incubation length did not influence the proportion of oocytes progressing to the blastocyst stage.

(1) Morton *et al.* (2003) *Proc. Soc. Reprod. Fert.* P18.

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CELL DEATH OF THE THECA INTERNA DURING BOVINE OVARIAN FOLLICULAR ATRESIA

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It is generally accepted that death of cells within the theca interna occurs late during ovarian follicular atresia. Histological classifications of atresia are usually based solely upon the morphology of the membrana granulosa. Atresia of bovine antral follicles less than 5 mm in diameter has been redefined as either antral or basal atresia depending on where in the membrana granulosa cell death is initiated. The aim of present study was to investigate changes within the theca interna during both antral and basal atresia. Bovine ovaries were collected and processed for light microscopy and immunohistochemistry. Each follicle less then 5 mm was classified as either healthy, antral atretic or basal atretic, with antral atresia being further classified either early-mid or late stage. Sections were labelled by TUNEL to identify dead cells combined with lectin from *Bandeiraea simplificifolia* to identify endothelial cells or with an antibody to cytochrome P450 cholesterol side-chain cleavage to identify steroidogenic cells. The numerical density of steroidogenic cells within the theca interna was significantly reduced ($P < 0.001$) in basal atretic follicles compared to healthy and antral atretic follicles. In both antral and basal atresia there was death of endothelial cells and steroidogenic cells. However cell death was greater in endothelial cells ($P < 0.05$) and steroidogenic cells ($P < 0.001$) of the theca interna of basal atretic follicles. There was no significant difference in the amount of cell death in the membrana granulosa between early-mid antral atresia and basal atresia while death of the membrana granulosa was significantly increased in late antral atresia compared to basal atresia ($P < 0.01$). Therefore we conclude that basal atresia is not a progression of antral atresia and that the theca interna can be susceptible to cell death early in atresia in basal atretic follicles.

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OOLEMMAL PROTEOMICS: IDENTIFICATION OF OOCYTE CELL SURFACE PROTEIN COMPLEXES INVOLVED IN SPERM–EGG INTERACTION

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While the molecular basis of sperm-oolemma interaction is of considerable biological significance, the protein(s) involved in this process are yet to be identified. In particular, the point at which developing mammalian oocytes acquire the capacity to bind to, and fuse with, capacitated, acrosome-reacted spermatozoa is yet to be clearly defined. Previous reports suggested that mature metaphase II (MII) mouse oocytes possessed the capacity to bind and fuse with spermatozoa while, in contrast, immature germinal vesicle (GV) phase oocytes did not. In this study oocytes were cultured in α-MEM containing 5% fetal calf serum. For GV oocytes, α-MEM was also supplemented with 1 μM Milrinone to prevent GV breakdown. Standard murine IVF was performed in 100 μL α-MEM media droplets containing 10 to 15 oocytes and 2.5×10^4 capacitated spermatozoa.

These studies found that no significant difference existed in the levels of sperm-oocyte binding or fusion between freshly isolated GV oocytes (14 sperm/egg bound, 7 sperm/egg fused), cultured arrested GV oocytes (16 sperm/egg bound, 7 sperm/egg fused) or cultured MII phase oocytes (17 sperm/egg bound, 8 sperm/egg fused). Interestingly, upon fusion MII oocytes commenced sperm nuclear decondensation whereas arrested GV oocytes did not.

Comparison of biotinylated oolemmal surface proteins revealed markedly different protein profiles between the GV oolemma and the MII oolemma. The MII oolemma revealed a multitude of proteins ranging in size from approximately 20 to 200 kDa, whilst the GV oolemma revealed only six middle range molecular weight proteins ranging from approximately 50 to 90 kDa. The protein(s) implicated in sperm-egg interaction must be one or more of those proteins found in both oocyte populations. Comparison of these two profiles has greatly reduced the number of possible candidates, allowing possible identification of the proposed GPI-linked sperm receptor.

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ZONA PELLUCIDA VACCINES FOR FERTILITY CONTROL OF BRUSHTAIL POSSUMS IN NEW ZEALAND

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Introduced marsupial brushtail possums (*Trichosurus vulpecula*) are a major pest in New Zealand because of their impacts on conservation values and agricultural production. Immunologically-based fertility control (immunocontraception) offers an effective and humane alternative approach to possum management. The zona pellucida (ZP) is an extracellular coat around all mammalian eggs and an attractive target for the development of immunocontraceptive vaccines. Antibodies against ZP are ovary-specific and act by preventing sperm from binding and penetrating the ova and/or by disrupting the development of follicles or early embryo. The aim of these studies was to test the efficacy of possum-derived ZP antigens for their ability to elicit sustained immune responses and cause infertility, and to assess a range of options for development of a bait-delivered contraceptive vaccine. Alloimmunisation with possum ZP2 and ZP3 proteins showed that self-ZP antigens elicited strong humoral immune responses and reduced the fertility of female possums by 72-80%. Several potentially possum-specific immunocontraceptive peptides have been identified by linear epitope mapping and amino acid alignment and are being tested for their ability to reduce fertility. Recent trials have demonstrated that possums mount immune responses against ZP antigens delivered in transgenic plants and bacterial ghosts. Research on antigen and specific peptide identification, non-target effects and delivery systems is ongoing. *Research supported by NZ Foundation for Research Science & Technology, Marsupial CRC and NZ Animal Health Board.*

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A PROTEOMIC ANALYSIS OF RAT CAPUT AND CAUDA SPERM USING USING DIFFERENCE IN 2D-GEL ELECTROPHORESIS

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When testicular spermatozoa migrate to the epididymis they are still functionally incompetent. Having lost the cellular machinery to support gene transcription and protein translation, these cells acquire the gamut of biological functions needed to achieve fertilization under the influence of factors provided by the epididymal microenvironment. Although the biological changes exhibited by spermatozoa during epididymal transit have been well established, the molecular basis for these changes is still poorly understood. Difference in 2D Gel electrophoresis (DIGE) is a powerful new technology for comparing up to three different protein samples in the same 2D gel, thus eliminating the variation that occurs with more traditional proteomic approaches. Using a combination of this procedure and matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry, we have unambiguously identified 8 proteins that change significantly during epididymal maturation including a-enolase, hsp60, endoplasmin, phosphatidyl-ethanolamine binding protein, testis lipid binding protein and the b-subunit of the F1 ATPase. The nature of these changes (80 kDa mass shift and increase electronegative charge) suggested a series of phosphorylation events. In order to further characterize these changes, Western blot studies were conducted using anti-phosphoserine antibodies. This analysis revealed a dramatic increase in serine phosphorylation for two major proteins (54 and 73 kDa) during epididymal transit, one of which (54 kDa) and confirmed by MALDI-TOF analysis to be the b-subunit of the mitochondrial ATPase. The phosphorylation of this protein was associated with a 3-fold increase in the ATP content of epididymal spermatozoa as they pass from the caput to the cauda epididymis. This change clearly identifies mitochondrial ATP production as a key component of the epididymal changes that lead to the generation of vigorously motile functional spermatozoa. The kinase responsible for the F1-ATPase phosphorylation appears to be PKA regulated and a clear potential target for contraceptive intervention.

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REGULATED EXPRESSION OF mRNAs ENCODING NUCLEAR TRANSPORT PROTEINS DURING SPERMATOGENESIS

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During spermatogenesis, the germ line stem cells undergo a complex series of cellular transitions to form mature male gametes. These transitions require regulated nucleocytoplasmic shuttling of transcription factors and cell cycle regulators, mediated in part by proteins known as importins. The importins bind to specific cargo proteins in the cytoplasm and transport them into the nucleus via interactions with components of the nuclear pore. There are two families of importins, termed the αs and the βs, with five different importin α members identified in the mouse genome. In this study, we examined the mRNA expression patterns of importin α s in the rodent testis using *in situ* hybridization and Northern blotting. Each *importin α* displayed a distinct expression pattern in the adult mouse testis. *Imp α1* mRNA was detected in spermatogonia through to early pachytene spermatocytes. *Imp α4* mRNA was detected in pachytene spermatocytes, α 6 in round spermatids and *α2* in both of these cell types. Northern blotting with *in situ* hybridization probes on total testis RNA from adult rat and mouse and 10 dpp (days postpartum) rat revealed distinct transcript sizes for *imp α 1, 2, 4* and *6*. For all importin αs, the mRNA signal level in the 10 dpp rat sample was lower than in the corresponding adult sample. The distinct expression patterns for each *importin α* family member in germ cells of the adult rodent testis suggests these importins are required to carry specific cargo at distinct stages of spermatogenesis. These data extend our previous analysis of importin β 1 and β 3 expression in the fetal and adult testis, which also demonstrate developmentally regulated importin expression. Ongoing studies are examining the cellular localization of importin α proteins and investigating their specific functions. We predict they each carry cargo required for distinct transitions in spermatogenesis.

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SUBCELLULAR LOCALISATION OF TESTIS FORMS OF MAP2 IS A PRODUCT OF COMPETING TARGETING SIGNALS/DOMAINS

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The process by which mitotic spermatogonia develop to form spermatozoa requires fine control of microtubule-based structures. Assembly, stabilization and disassembly of these structures are regulated by a heterogeneous group of proteins known as Microtubule Associated Proteins (MAPs). One of the best characterized MAPs is MAP2, which is found in both brain and testis. Different MAP2 isoforms arise from alternative splicing of the same gene. These have been categorized into high and low molecular weight (HMW and LMW), depending on the presence/absence of an extension arm encoded by exon 9. Exon 10 encodes for a nuclear localization signal (NLS), and in the brain, this exon is found exclusively on HMW isoforms, which localize to the cytoplasm of neurons. In the testis, however, exon 10 can be found on LMW isoforms, which localize within the nucleus of germ cells. Transfection of GFP-tagged MAP2 constructs containing exons 10 and 11 into mammalian cell lines (GC-1, GC-2 and COS-7) reveal that the NLS is capable of localizing the entire protein within the nucleus of transfected cells, and this effect is partially dependent on the presence of the protein's tubulin binding domains. Experiments utilizing an exon 10/11 specific probe to hybridise to MAP2 mRNA in mouse and rat testis sections show that mRNA containing exon 10/11 is present in both 15 day post partum (dpp) and adult testis in spermatogonia and spermatocytes. Expression of exon 10/11 MAP2 mRNAs were not detected at 5dpp, indicating that a switch in mRNA expression occurs sometime in the 5-15dpp interval. The expression pattern of exon 10/11 MAP2 mRNA and the NLS's ability to localize LMW MAP2 protein into the nucleus indicate a novel role for MAP2 in germ cell development.

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THE MOLECULAR BASIS OF EPIDIDYMAL SPERM MATURATION

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The mammalian epididymis represents the site where functionally incompetent spermatozoa originating from the testes undergo their final maturation enabling them to engage in the complex cascade of sperm-egg interactions that culminate in fertilization. The extent to which this process is actively driven by the epididymis or reflects properties intrinsic to the gamete remains largely unknown. However, recent studies within our laboratory have demonstrated that sperm transit through region 4 (corpus) of the mouse epididymis is associated with an acquired ability to exhibit coordinated movement, capacitate and engage in sperm-zona binding. Furthermore, we have demonstrated that immature mouse sperm recovered from region 3 (caput) of the epididymis are able to display similar attributes following brief co-culture with region 4 epididymal plasma. Interestingly, immunohistological studies of the epididymal lumen within region 4 have revealed the presence of a number of dense bodies containing the molecular chaperones, heat shock protein 60 (HSP60) and endoplasmin (GRP94). Although the nature and origin of such inclusions remains to be resolved, these collective findings raise the intriguing possibility that the molecular chaperones are involved in the delivery of critical signaling molecules to the surface of spermatozoa. In light of this data, we have commenced a proteomic analysis of region 4 epididymal fluid with a view to identifying proteins which interact with the chaperones, HSP60 and GRP94. Such studies will provide an extremely important insight into the molecular basis of sperm maturation.

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CULTURE OF MOUSE MALE GERM CELLS FOR GENETIC MANIPULATIONS

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The ability to maintain germ cells in culture offers the opportunity to manipulate their differentiation and to deliver new genes through the germline. However the study of male germ cell biology is hindered by the lack of an *in vitro* culture system that supports germ cell maintenance and differentiation, and the lack of efficient gene transfer technologies. To address this, we developed a short term (1-6 day) *in vitro* germ cell-Sertoli cell co-culture system using testes of 6 day old mice, in which the only germ cell type present is the spermatogonium. To establish culture conditions favourable for maintaining germ cells in culture, we compared different substrates (plastic, laminin, poly-L-lysine, Matrigel) and cell culture additives (insulin/transferrin, retinoic acid, lactic acid, pyruvic acid). Using immunocytochemistry, we observed that germ cell numbers varied depending on the substrate, and was greatly improved on laminin or Matrigel. The culture additives tested had no effect. We noted a striking difference in germ cell growth depending on the mouse strain used, with C57BlXCBA F1 cross > Swiss > BalbC >> C57Bl/6J. To investigate gene transfer into germ cells, constructs were generated using promoters identified as exhibiting germ cell-specific expression in transgenic animals (EF1-α, Oct4, Stra8) with EGFP and lacz reporter genes. From this baseline, we have started gene transfer experiments. Initial transfection experiments were performed in mouse testis cell lines (GC1, GC2, TM3, TM4) and the P19 mouse embryonal carcinoma cell line. Several commercial transfection agents and a non-commercial product (1) were used, as was electroporation, however these did not yield reporter expression in germ cells. As these techniques require proliferating cells, we are now undertaking a study with the addition of growth factors in culture (BMP4, activin, GDNF, FSH) to enhance germ cell proliferation and therefore improve transfection efficiencies. We will also employ lentiviral transduction, as this is reportedly 100% efficient and independent of proliferation.

(1) Celebi *et al*. (2002) *Mol. Reprod. Dev.* **62**,477–482.

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IDENTIFICATION OF POTENTIAL ZONA PELLUCIDA-BINDING SPERM PROTEINS USING HEAT SHOCK PROTEIN 60

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Sperm transit through the female tract is correlated with a number of biochemical and physical changes, termed capacitation, which culminate in acquisition of the ability to fertilise an egg. One of the important correlates of capacitation is an increase in tyrosine phosphorylation of multiple sperm proteins. Recent studies of the sperm surface proteome have demonstrated that, following capacitation, a number of tyrosine-phosphorylated proteins become expressed on the sperm surface. Analysing these proteins, we have identified at least three members of the chaperone family including heat shock protein 60 (HSP60), heat shock protein 90 (HSP90) and Endoplasmin (ERP99). Although these proteins are not directly implicated in zona binding, we hypothesise that they are involved in the assembly of a multimeric zona receptor on the sperm surface. To investigate this hypothesis, we have initiated studies to purify sperm surface proteins which associate with these chaperones. This has involved the cross-linking of sperm surface proteins, using a membrane impermeable cross-linker, followed by extraction of the surface membrane proteins. Co-immunoprecitation of these cross-linked surface proteins from capacitated sperm has yielded a number of proteins associated with the chaperone HSP60. The characterisation of these proteins is the focus of ongoing research.

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ANALYSIS OF DNA DAMAGE INDUCED BY PRO-OXIDANT TREATMENT OF MAMMALIAN SPERMATOZOA *IN VITRO*

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Defects in the male genome produced as a consequence of oxidative insult have been associated with decreased fertility levels, an elevated incidence of childhood cancer and dominant genetic disease in the offspring (1). The objective of this study was to determine the relative susceptibility of sperm DNA of different mammalian species to oxidative injury. We applied a highly sensitive quantitative PCR assay (QPCR) to measure gene-specific DNA damage in nuclear and mitochondrial compartments of spermatozoa treated with H_2O_2. Human, murine and tammar wallaby (*Macropus eugenii*) spermatozoa were treated with H_2O_2 (0–5 mM) over a 1 h period. After DNA purification, DNA damage was assessed in a nuclear and a mitochondrial fragment of DNA by quantitative polymerase chain reaction assay (QPCR). DNA damage was detected as a decrease amplification of the target sequences. In murine and human spermatozoa, mitochondrial DNA exhibited greater sensitivity to oxidative damage than nuclear DNA. Doses ranging from 0.25–5 mM H_2O_2 induced DNA damage of up to 0.65 lesions/10 kb in the mouse, and 1.42 lesions /10 kb in the human. No significant effect on DNA damage was observed over this dose range in the nuclear DNA fragments investigated in these species. In contrast, tammar wallaby spermatozoa were susceptible to DNA damage at the 5 mM H_2O_2 dose in both nuclear (0.51 lesions/10 kb) and mitochondrial (0.55 lesions/10 kb) genomes. This study is the first to compare DNA damage in specific DNA sequences in spermatozoa of different mammalian species. Nuclear DNA of the metatherian species, the tammar wallaby, was more susceptible to oxidative damage than that of the eutherian species. A major difference between metatherian and eutherian spermatozoa is that, in general, the former possess protamines that are not stabilised by disulfide cross-linkage. These findings therefore suggest that sperm chromatin packaging affects the susceptibility of sperm DNA to oxidative damage.

(1) Sawyer and Aitken (2000) *Reprod. Med. Rev.* **8**, 107–126.

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THE EFFECTS OF VIAGRA ON SPERM FUNCTION AND EARLY EMBRYO DEVELOPMENT

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In an audit of UK fertility units, we have demonstrated that 42% prescribe Viagra to aid patient semen production. Viagra is a phosphodiesterase inhibitor (PDE5) and as non-specific PDEs have been shown to affect fertility, safety concerns have been raised. The aims of this study are to investigate the effects of Viagra on sperm function and early embryo cleavage. Human semen was incubated with and without Viagra (450 ng/mL sildenafil citrate, equivalent to plasma concentrations after 100 mg oral dose; Pfizer, UK). Aliquots were also prepared by a 90/45% density centrifugation gradient to separate good and poor subpopulations. All samples were analysed by computer assisted semen analysis (HTM-IVOS) up to 60 and 120 min. Prepared samples were also labelled with fluorescein isothiocyanate–peanut agglutinin to determine acrosome status. Male mice were gavaged with Viagra (equivalent dose/body wt) and mated with superovulating females. Twenty females were sacrificed 12 h later, their oviducts flushed and viable fertilized oocytes counted. Another 20 females were sacrificed 4 days after mating and their embryo numbers and cleavage stages determined. Viagra increased % progressive motility in semen ($n = 22$) by 38%, VAP by 21%, VSL by 21% and VCL by 16% at 60 min (all P values <0.001). These effects were sustained at 120 min. Sperm isolated from 90% ($n = 57$) and 45% ($n = 15$) fractions showed similar increases. Viagra also increased the proportion of acrosome reacted sperm in the 90% (+79%, $P < 0.001$) and 45% (+77%, $P < 0.001$) fractions. Further, Viagra caused a reduction in both the numbers of fertilised oocytes (–35%, $P < 0.001$) and those reaching blastocyst stage (–85%, $P < 0.001$). This study demonstrates that Viagra increases human sperm motility. However, Viagra induces human premature acrosome reactions and impairs mouse fertilisation and embryo cleavage. This study raises significant concerns for its use in assisted reproduction.

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EXPRESSION OF THE CHEMOKINE CXCL12 AND ITS RECEPTOR CXCR4 IN THE ACTIVATING MAMMALIAN OVARIAN FOLLICLE

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The mammalian ovary contains a finite number of oocytes that is determined during oogenesis in fetal life. As most of these oocytes are destined to undergo apoptosis, the initial primordial follicle population represents a valuable resource for the clinical manipulation of the female germ cell pool. However the events underlying the activation of the resting primordial follicle remain relatively poorly understood. A comparison was undertaken between whole 2-day and 7-day neonate mouse ovaries which represents ovaries with primordial follicles and primordial follicles / newly activated follicles respectively. The comparison took place at the level of gene expression utilizing cDNA microarray analysis. The mRNAs for the chemokine CXCL12 and its receptor CXCR4, were consistently shown to be up-regulated approximately 2-fold in 7-day tissue compared with 2-day tissue. Microarray results were confirmed for CXCL12 by real-time PCR analysis. CXCL12 and CXCR4 have been identified as essential for development of the haemopoietic, nervous and cardiovascular systems and have also been shown to be involved in the foetal migration of primordial germ cells to the gonads. These genes were selected for further analysis due to the known importance of other cytokine family members in primordial follicle activation. *In situ* hybridisation studies of CXCL12 revealed mRNA expression in oocytes of all stages, including primordial follicles, as well as epithelial, corpora luteal and stromal cells. In contrast CXCR4 mRNA expression appears to be restricted primarily to oocytes of all stages. This co-expression of ligand and receptor within the oocyte suggests an autocrine signaling mechanism. The age-dependent increase in CXCL12 and CXCR4 gene expression is likely to be a result of an increased oocyte cytoplasmic volume and/or the proportion of stromal cells present as follicles begin to activate in the neonate ovary. Examination of the timing of protein expression is currently underway to identify the role this chemokine signaling pathway plays in the initial activation of the mammalian primordial follicle.

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FGF9 STIMULATES OVARIAN PROGESTERONE PRODUCTION

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FGF9, a member of the fibroblast growth factor family (FGF), is known to be a male sex-determining factor involved in testicular cord formation (1). FGF9 knockout males are sex-reversed (2). However, nothing is known about FGF9's role in folliculogenesis because these mice die at birth (2). We previously reported the presence of FGF9 mRNA and protein in the immature rat ovary (3). In these studies we investigated: *(1)* the presence of FGF9 receptors (FGFR3) on granulosa cells (GC); and *(2)* the impact of FGF9 on GC progesterone production.

GC isolated from 21 day old diethylstilboestrol (DES)-treated rats were cultured for either 2 hours (RNA) or 2 days (progesterone) in McCoys 5C with FGF9 (0.1-50ng/ml) ± FSH (100ng/ml). Progesterone was measured in conditioned media by radioimmunoassay. RNA was extracted from the granulosa cells and reverse-transcribed for PCR. Specific primers for P450 side chain cleavage (SCC) amplified a 329 bp cDNA fragment. GAPDH was used for data normalisation. The FGF9 receptor FGFR3, was immunolocalised on formalin-fixed, paraffin-embedded sections of immature rat ovary.

FGFR3 protein was localised only to GC of the ovary. Progesterone production by cultured GC was significantly elevated by FGF9, consistent with the presence of FGFR3. Relative to a maximally stimulating dose of FSH, FGF9 increased progesterone production 10- fold. In preliminary studies, FGF9 increased the expression of P450 SCC mRNA by cultured GC revealing a mechanism by which FGF9 increases progesterone production. These data suggest a role for FGF9 not just in testicular formation, but in the regulation of ovarian steroidogenesis. *Supported by the NH&MRC of Australia (Regkey 241000 & 198705).*

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ANALYSIS OF OVARIAN MACROPHAGE POPULATIONS USING MACROPHAGE-SPECIFIC GREEN FLUORESCENT PROTEIN (GFP) TRANSGENIC MICE

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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 distinctive phenotypes and functions of ovarian macrophages such that many immunological mediators, such as cytokines and hormone receptors, are uniquely regulated within these cells across the oestrus cycle. In order to isolate macrophages from ovarian tissue by fluorescence activated cell sorting (FACS), we acquired transgenic mice (from DA Hume, Institute for Molecular Bioscience, University of Queensland) which express GFP exclusively in macrophages (1). In these mice GFP is expressed under direction of the *c-fms* gene promoter, which encodes the receptor for colony-stimulating factor-1 (CSF-1R), a major macrophage growth factor. Using flow cytometry we confirmed that 95% of peritoneal macrophages express GFP and 88% co-express GFP and the classical macrophage marker F4/80. The distribution of GFP+ macrophages in tissues was co-localized with macrophage markers F4/80 and major histocompatibility complex class II (MHCII) by immunohistochemistry using phycoerythrin (PE)-labelled antibodies. The liver, uterus and oviduct exhibited many GFP+ cells in characteristic macrophage distributions. Furthermore, GFP fluorescence was tightly co-localized with PE fluorescence of either F4/80 or MHCII, indicating that CSF-1R is expressed in the macrophages of these tissues. In contrast, macrophages in the ovary were positive for F4/80 and MHCII, but rarely expressed GFP. Thus unlike macrophages of other reproductive tissues, ovarian macrophages do not consistently express CSF-1R. In ovaries from gonadotrophin-primed immature females, GFP was not expressed in macrophages (F4/80+/MHCII+) surrounding follicles but was detected in macrophages within the regressing corpus luteum. Thus CSF-1R is a hormonally regulated gene, expressed only in specific subsets of ovarian macrophages suggesting that CSF-1 controls functional activities of ovarian macrophages at specific stages of the ovarian cycle.

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TGFβ1 DEFICIENT MICE EXHIBIT IMPAIRED FOLLICLE GROWTH AND LUTEAL MAINTENANCE

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Transforming Growth Factor β1 (TGFβ1) is essential for normal female reproduction. Mice with a targeted deletion in the TGFβ1 gene (TGFβ1–/–) have severely impaired fertility with pregnancy occurring in <25% of mated females. TGFβ1 is implicated in several aspects of ovarian function, including potentiation of granulosa cell proliferation and suppression of luteal cell apoptosis. Our initial observations indicate that estrous cycling is disrupted in TGFβ1–/– mice and that ovulation rate is reduced. To further investigate how impaired ovarian function contributes to the infertility of TGFβ1–/– mice, ovaries were isolated from TGFβ1+/+ and TGFβ1–/– littermates at proestrus and fixed and sectioned for examination of follicle morphology and growth. BrdU labelling was performed to detect granulosa cell proliferation and blood samples were obtained for analysis of gonadotrophins and ovarian steroid hormones. Histological examination showed that ovaries from TGFβ1–/– mice were smaller than those of TGFβ1+/+ mice, however large antral follicles were observed, indicating that TGFβ1 is not essential for granulosa cell proliferation. Compared to TGFβ1+/+ ovaries however, there were fewer antral follicles and only rare corpora lutea. Interestingly, in some cases there were large numbers of macrophages surrounding small follicles suggesting increased follicular atresia and/or altered macrophage activity in the TGFβ1–/– ovaries. Ovaries and serum were also isolated from females at d4 post-coital for assessment of corpora lutea morphology. TGFβ1–/– ovaries weighed less and had fewer corpora lutea than TGFβ1+/+ ovaries. TGFβ1–/– corpora lutea also contained increased numbers of apoptotic cells and infiltrating macrophages indicative of premature luteal regression. Circulating progesterone levels were reduced in TGFβ1–/– females, as was progesterone production per corpus luteum further indicating a functional defect in luteal maintenance. Cumulatively these observations show that TGFβ1 has essential roles in regulation of ovarian macrophage populations, in normal follicular development and in the generation, maintenance and steroidogenic function of corpora lutea.

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EFFECTS OF EXOGENOUS GONADOTROPHIN STIMULATION ON OVARIAN TISSUE GRAFTS IN THE MOUSE

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Ovarian tissue grafts commonly contain only limited numbers of follicles. The functional life span and ability to retrieve as many mature oocytes as possible from ovarian grafts is important when grafting is used to restore fertility. This study aimed to determine whether ovarian grafts responded to exogenous hormones in a similar manner to that of in situ ovaries. Ovaries of C57BlxCBA F1 mice were cut in half and grafted to one of three different graft sites in females of the same F1 line; bursal capsule (BC, n=12), kidney capsule (KC, n=6), subcutaneous tissue (SC, n=24). Three weeks after grafting, half of the graft recipients in each group were treated with 5IU PMSG followed by 5IU hCG 48 hours later. Oocytes were collected directly from the grafted ovaries 10 hours after the hCG injection and fertilized in vitro. Oocytes from the ovaries of superovulated normal mice (n=4) of the same hybrid strain were used as controls. Two-cell embryos were transferred to pseudopregnant recipients and collected at day 15 of gestation or the animals were allowed to go to term. Mature fertilisable MII oocytes were retrieved from stimulated grafts from all graft sites, however, the number (BC 9, KC 5, SC 2 oocytes per ovary) and proportion of two-cell embryos in each grafted group (BC 52%, KC 32%, SC 32%) was significantly ($P<0.05$) lower than in the in vivo matured control (16 oocytes, 85% two-cell). The fetal and placental weights of fetuses produced from graft-derived oocytes were not significantly different to the control group. Phenotypically normal pups were born in each of the graft and control groups. In conclusion, ovarian grafts treated with exogenous gonadotrophins produce significantly fewer mature oocytes and two cell embryos compared to in situ ovaries. *Work supported by ARC and NIH RFA.*

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CHARACTERISATION OF THE INFERTILITY EFFECT INDUCED BY A RECOMBINANT MURINE CYTOMEGALOVIRUS EXPRESSING MURINE ZONA PELLUCIDA 3

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A recombinant mouse virus, murine cytomegalovirus, that has been engineered to encode the fertility antigen murine zona pellucida 3 (mZP3), is being developed for fertility control in mice. A single inoculation of the recombinant virus induces complete infertility in female BALB/c mice which persists for the breeding life of the animal. The extent of this autoimmune response was unexpected especially as the incorporation of mZP3 appears to have immunologically attenuated the growth of the recombinant virus. The histological features of the infection are an initial depletion in tertiary follicles by 21 days post inoculation followed by a progressive depletion of primordial follicles, leading to an almost complete absence of follicles by 150 days post-infection. High titre, long lasting, zona pellucida-specific antibody is present in infertile BALB/c mice although infertility has not been linked with either a critical titre or a dominant immunoglobulin isotype. However, our evidence suggests that anti-ZP3 antibody plays a primary role in infertility since antibodies are detected *in vivo* bound to the zona pellucida of ovaries from recombinant virus-infected mice, and passively transferred antibody from infected animals induces infertility in the absence of recombinant virus. In addition, an experiment in which immunoglobulin-deficient mice remained fertile after inoculation with the recombinant virus indicates that antibody is crucial for the immunocontraceptive effect to occur. Other immune mechanisms are also being explored.

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OVARIAN PATHOLOGY IN MICE FOLLOWING IMMUNISATION WITH RECOMBINANT MURINE CYTOMEGALOVIRUS EXPRESSING MURINE ZONA PELLUCIDA 3

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Immunocontraception is a promising biological control for wild mice in Australia, having the potential to reduce the socioeconomic cost of plagues with minimal environmental impact. Inoculation of BALB/c mice with recombinant murine cytomegalovirus encoding murine zona pellucida antigen (mCMV-ZP3) confers total infertility characterised by depletion in ovarian tertiary follicles by Day 21 post inoculation followed by a progressive depletion in primordial follicles (1). The mechanisms underlying ovarian pathology are largely unknown but are likely to involve antibody mediated and cell mediated immune responses. The immune pathology may also be facilitated by acute responses involving antibody binding to ZP in growing follicles resulting in recruitment of inflammatory cells and oocyte destruction. The aim of this study was to investigate the effect of mCMV–ZP3 infection on leukocyte infiltration and expression of oocyte-derived signalling molecules in ovarian tissue. Fifteen BALB/c female mice were randomly allocated into three groups of 5 animals. Group one received an injection of PBS, group two and three received intraperitoneal inoculations of 2×10^4 p.f.u. of mCMV and mCMV-ZP3 respectively. Ovaries were retrieved at Day 7 post inoculation and one ovary from each mouse was sectioned for immunohistochemical analysis of resident leukocytes using mAb CD45 reactive with all leukocyte lineages. The other ovary was processed for real time quantitative RT-PCR analysis of growth and differentiation factor 9 (GDF-9) and connexin 43 (Cx43) expression. mCMV-ZP3 inoculation increased the abundance of ovarian leukocytes ($P = 0.08$), significantly increased expression of Cx43 mRNA ($p<0.05$), but did not alter GDF-9 mRNA expression. These results suggest that changes in expression of ovarian regulators due to ZP3 immunisation begins early after recombinant MCMV infection in mice, and implicates leukocyte infiltration in the mechanism leading to permanent ovarian failure. Further experiments are underway to investigate the dynamics of leukocyte trafficking and expression of oocyte-derived signals as the course of infection progresses. *This study is funded by the Cooperative Research Centre for Pest Animal Control.*

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EXPRESSION OF CHEMOKINES AND THEIR RECEPTORS AT THE HUMAN MATERNAL-EMBRYONIC INTERFACE

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Human embryo implantation is a complex process involving attachment of the developing blastocyst to the receptive endometrial epithelium, and subsequent trophoblast invasion through decidua. This is regulated by crosstalk between the maternal and embryonic cells, however little is known about the factors involved in enabling and directing trophoblast invasion. Chemokines are cytokines that regulate leukocyte chemotaxis via stimulation of adhesion molecules and cell migration. We have previously shown that two chemokines, fractalkine and MCP-3, are produced by endometrial epithelial and decidual cells, maximally around the time of implantation and early pregnancy (1, 2). We hypothesized that endometrially derived fractalkine and MCP-3 are important for the attachment/invasion of fetal trophoblast cells during implantation. To investigate this, expression of fractalkine, MCP-3 and their receptors (CX3CR1, CCR1, CCR2, CCR3 and CCR5) were assessed in cell types present at the maternal-embryonic interface. RNA, extracted from three trophoblast cell lines (JEG-3 and two trophoblast-choriocarcinoma hybrids), a human epithelial cell line (HES), primary endometrial epithelial cells, mid-secretory endometrium and placental tissue, was subjected to RT-PCR for the chemokines and receptors. Both chemokines were produced by endometrial and placental cells. Chemokine receptor expression was more variable, CX3CR1, CCR1, 2 and 3 were expressed by one or more of the trophoblast cells lines while CX3CR1, CCR1, 2 and 5 were expressed by endometrial cells. Marked differences in expression patterns in the different cell lines highlight the importance of studies to select those cell lines of most physiological relevance: in this case, one that most closely resembles early invasive trophoblasts. These data confirm that chemokines are produced by maternal and embryonic cells during implantation and the strong expression of their receptors on trophoblast cells supports a role for chemokines in embryo implantation. Further, these studies have characterized a number of trophoblast cells for future trophoblast migration and attachment assays.

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REGULATED EXPRESSION OF ADHESION AND ANTI-ADHESION MOLECULES IN MOUSE ENDOMETRIUM DURING EARLY PREGNANCY

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To implant and establish the connections that are vital for further development, the early embryo must attach to and then breech the barrier posed by the epithelium of the maternal tract. Expression of adhesion and anti-adhesion molecules in the luminal epithelium of the endometrium are thought to fluctuate in a temporal pattern to 'frame' the implantation site, with their expression regulated by endocrine and paracrine factors. Anti-adhesion molecules, such as members of the mucin family, provide a barrier to implantation in sites or at times unsuitable for embryo development. Expression of adhesion molecules, or specific integrins, are thought to aid in the adhesion of the embryo, allowing it to induce changes in the underlying tissue promoting embryo invasion and pregnancy. The aim of this study was to quantitate the expression of mRNA encoding the integrins αυ, α4 and β3 and MUC1 and MUC4 from Day 0 (oestrous) to Day 4 of pregnancy (implantation) using quantitative real time RT-PCR. Uterine tissues were collected at oestrous and at Days 1, 2, 3 and 4 of pregnancy (Day 1 corresponding to the presence of a vaginal plug), total RNA was extracted, DNAse treated, reverse transcribed into cDNA, and quantified by real-time PCR using SYBR Green chemistry. All specific primers were designed using GenBank sequences and data were normalised to β-actin mRNA expression. Expression of MUC1 and MUC4 mRNAs was dramatically reduced, with mean values 20-fold and 100-fold less than at oestrous respectively, by Day 4 of pregnancy. In contrast, expression of mRNAs encoding integrins αυ, α4 and β3 was detected throughout early pregnancy. These data demonstrate that adhesion and anti-adhesion molecules are differentially expressed in the murine uterus during early pregnancy and may be key mediators in embryo implantation, promoting attachment of the embryo to the luminal epithelium in an environment conducive to embryo growth and development. *Supported by a Clive & Vera Ramaciotti Project Grant to MJ Jasper.*

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AN INHIBITOR OF LEUKEMIA INHIBITORY FACTOR SIGNALLING BLOCKS EMBRYO IMPLANTATION IN THE MOUSE

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Embryo implantation is a critical step in the establishment of pregnancy. Endometrial leukemia inhibitory factor (LIF) is essential for embryo implantation in the mouse (1). Uterine LIF is expressed in the luminal epithelium on Day 3 of pregnancy (D3) (D0 = day of plug detection) and signals via activation of signal transducer and activator of transcription (Stat) 3 (2). We examined the effect of a novel LIF signalling inhibitor on the phosphorylation (p) of Stat3 during early pregnancy and on embryo implantation in the mouse. We injected LIF inhibitor into one uterine horn and PBS into the other uterine horn of the mouse at D3 and examined the effect on pStat3 immunostaining in the luminal epithelium between 30 and 360 min later. We found no immunoreactive pStat3 in luminal epithelium following treatment with LIF inhibitor at 60 and 90 min but variable staining at other time points. The PBS-treated uterine horn showed intense immunostaining at all times. LIF inhibitor (1mg/kg body weight per day) or PBS was administered to mice (a) subcutaneously, (b) intraperitoneally, at 8-hourly intervals for 3 days from D2, or (c) continuously into the peritoneal cavity via Alzet pumps from D2. No effect was seen on implantation at D6. When LIF antagonist (3.5mg/kg/day) or PBS were administered by Alzet pumps from D2 together with ip injections, 4-hourly from D3 for 36 h, there were no implantation sites in the uteri of treated mice ($n = 5$) while the control mice ($n = 4$) had 3.6 ± 0.5 sites ($P < 0.001$). Histologically, the uteri of the treated mice resembled non-pregnant uterus, while the control uterus resembled post-implantation uterus. The results demonstrate that treatment of mice during early pregnancy with a novel LIF inhibitor blocks LIF action in vivo and embryo implantation. This knowledge is important for development of novel contraceptives.

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PROGESTIN-INDUCED PROPROTEIN CONVERTASE 6 IS NECESSARY FOR DECIDUALISATION OF HUMAN ENDOMETRIAL STROMAL CELLS *IN VITRO*

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Decidualisation of human endometrium is an essential preparative event for successful establishment of pregnancy, and involves dramatic morphological and functional differentiation of the human endometrial stromal cells (ESCs). Proprotein convertase 6 (PC6) plays an important role in the processes of stromal cell decidualisation and embryo implantation in the mouse. PC6 is a member of the proprotein convertase family responsible for processing precursor proteins to their bioactive forms by selective proteolysis. In the present study we investigated the regulation of PC6 mRNA and protein expression in ESCs during decidualisation *in vitro*, and established a function for PC6 in decidualisation using morpholino antisense oligonucleotides (MOs). PC6 mRNA levels in ESCs during decidualisation were determined using quantitative real-time RT-PCR. 17β-oestradiol (E) plus medroxy-progesterone acetate (P) caused a significant increase in PC6 mRNA during decidualisation, whereas E alone did not increase PC6 mRNA expression. Consistent with the results of real-time PCR, much stronger PC6 immunostaining was observed in the cytoplasm of E plus P-treated ESCs (decidualised) compared to the E-treated ESCs (non-decidualised) on Day 12 of culture. This strong staining for PC6 was abolished by cotreatment with ZK 98299, a progesterone receptor antagonist. To investigate whether the induction of PC6 was necessary for decidualisation *in vitro*, MOs were used to block PC6 synthesis in cultured ESCs. PRL production, a typical marker for decidualisation, was significantly attenuated in decidualing ESCs following treatment with PC6 MOs in comparison to controls. These results suggest that PC6 plays a key role for decidualisation in human ESCs.

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SEMINAL PLASMA TGFβ ACTIVATES PRO-INFLAMMATORY CYTOKINE SYNTHESIS IN HUMAN CERVICAL EPITHELIAL CELLS

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Exposure to semen at intercourse in women elicits an inflammation-like response characterised by recruitment of inflammatory cells and expression of pro-inflammatory cytokines including GM-CSF, interleukin-6 (IL-6) and IL-8 (1). Studies in animal models have implicated TGFβ as the major active moiety in seminal plasma, and we have shown previously that TGFβ1 and TGFβ3 are present in high concentrations in human seminal plasma (>100 ng/mL), while TGFβ2 is less abundant. To investigate the physiological significance of each of the three TGFβ isoforms as pro-inflammatory agents in human seminal plasma, we have established *in vitro* model systems to measure human cervical cell cytokine synthesis. Primary cervical epithelial cells prepared from ectocervix of hysterectomy tissues or transformed Ect1 cells were incubated for 12 h with human recombinant TGFβ (isoforms 1, 2 or 3) or with seminal plasma in the presence or absence of isoform-specific TGFβ neutralising antibodies. Epithelial cell supernatants were recovered 24 h later and supernatants were analysed by commercial ELISA to quantify GM-CSF, IL-6 and IL-8 production. Each of the three TGFβ isoforms mimicked seminal plasma and were comparable in their capacity to stimulate >10-fold increases in both GM-CSF and IL-6 expression in a dose-responsive manner. In contrast, unlike seminal plasma none of the TGFβ isoforms induced IL-8 expression. Addition of neutralising antibodies to TFGβ1, TGFβ2 and TGFβ3 each effected >50% reduction in the ability of seminal plasma to induce GM-CSF and IL-6, but did not impair seminal plasma-stimulated IL-8 production. Together these data show that TGFβ1, TGFβ2 and TGFβ3 are major active constituents of seminal plasma, acting to elicit GM-CSF and IL-6 production in cervical epithelial cells. However, TGFβ does not fully account for the pro-inflammatory effects of human seminal plasma, and other active constituents remain to be identified.

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NOVEL IMMUNE MODULATION TO IMPROVE REPRODUCTIVE OUTCOMES IN PIGS

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Early embryonic mortality is a major factor limiting litter size and profitability in the pig industry. Pregnancy success requires an immunologically receptive reproductive tract, generated in response to exposure to immune modulating factors in the seminal plasma (SP). Both the short-term pro-inflammatory response of the endometrium to SP and to a novel immune-modulating product, mycobacterial cell wall extract (MCWE; 'Equimune', Bioniche Animal Health) and the reproductive outcomes from a large-scale farrowing trial are described. In the first experiment, 15 Large White gilts (24 weeks of age) were randomly allocated to three intrauterine treatments administered at onset of gonadotrophin-induced oestrus: *(1)* 80 mL PBS (control); *(2)* 80 mL SP; or *(3)* 80 mL PBS containing 500 µg MCWE. Gilts were slaughtered 32–34 h later and reproductive tracts retrieved. Luminal fluid leukocytes were assessed following fixation using DIFF-Quik stain, and indicated the proportion of lymphocytes increased with SP and MCWE treatment compared to the control group (14.0% and 17.0% *v.* 9.3%; $P < 0.05$), and the proportion of monocytes decreased (12.3% and 15.0% *v.* 25.0%; $P < 0.05$). In the second experiment, Large White or Large White/Landrace crossbred females ($n = 161$) were artificially inseminated twice, following standard industry practice, after detection of standing oestrus. Stratified for parity and breed, each was allocated to a treatment group: (a) Control: standard AI dose 1st and 2nd insemination; (b) 500 µg MCWE added to 2nd insemination; or (c) 500 µg MCWE included in the 1st and 2nd insemination. The overall pregnancy rate was 91%, with no significant effect of treatment on litter size, average piglet bodyweight at birth or litter variability. Although the use of MCWE at the time of AI did not improve reproductive outcome, significant potential remains in utilising its immune stimulating properties in 'priming' the reproductive tract, followed by mating at the next oestrus. This approach may improve gilt farrowing rates, thus increase overall herd productivity and efficiency.

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AMMONIUM AFFECTS MITOCHONDRIAL DISTRIBUTION AND FUNCTION IN MOUSE 2-CELL EMBRYOS

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Amino acids are key regulators of embryo function and are essential components in embryo culture media. Amino acids spontaneously breakdown and are metabolised by embryos resulting in ammonium build-up in the medium. While ammonium does not affect blastocyst development, the ability of these blastocysts to implant was reduced along with subsequent fetal growth rates. However, the mechanism for the inhibitory effect of ammonium is currently not known. It has been demonstrated in other tissues that mitochondrial bioenergetics can be disrupted by the presence of ammonium in the media which subsequently affects cellular viability. Therefore, the aim of this study was to examine the effects of ammonium on the mitochondria of mouse embryos cultured in the presence of ammonium. Mouse zygotes from superovulated females were cultured in medium G1.2 with or without 300 μM ammonium for 22 h at 37°C in $6\%CO_2 : 5\%O_2 : 89\%N_2$. *In vivo*-developed 2-cell embryos were flushed from the reproductive tract and assessed immediately. At the 2-cell stage mitochondrial distribution (Mitotracker) and membrane potential (JC-1) were assessed using confocal microscopy and images were quantitated using IP Lab software package. Differences between treatments were determined using ANOVA and Bonferroni's multiple comparison procedure. Culture of zygotes to the 2-cell stage in medium G1.2 did not affect mitochondrial distribution compared to *in vivo* controls. However, 2-cell embryos cultured with ammonium had a decrease in their mitochondrial nuclear : cortical ratio (97 ± 1 compared to 106 ± 1; $P < 0.05$) indicating that mitochondria were dispersing away from the nuclei. Culture with ammonium also significantly decreased the mitochondrial membrane potential (0.50 ± 0.01 mean pixel intensity ratio) compared to those cultured without ammonium (0.72 ± 0.3 mean pixel intensity ratio, $P < 0.001$). The data presented demonstrates that culture for only 24 h with ammonium disrupts both mitochondrial distribution and membrane potential and supports our hypothesis that mitochondria are an early target for the inhibitory action of ammonium.

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CALCIUM INVOLVEMENT IN GLUCOSE INDUCED GLUT3 EXPRESSION IN PREIMPLANTATION MOUSE EMBRYOS

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Despite their inability to utilise glucose for energy prior to compaction (E3), mouse embryos have a requirement for at least a brief glucose exposure to permit normal development. In the absence of this glucose pulse in vitro, we and others have found that embryos cleave to form morulae but fail to form blastocysts and subsequently degenerate. These embryos do not develop the capacity to utilise glucose preferentially and are unable to adapt to their nutrient environment and utilise alternate substrates (1). This inability to utilise glucose is due to failure to express GLUT3 at compaction (2). Brief glucose exposure prior to the 8-cell stage is sufficient to permit the embryo to undergo compaction, express GLUT3 and ultimately form a blastocyst, suggesting that glucose induces metabolic differentiation of the developing embryo. In this study we have explored the role of intracellular calcium in response to glucose given its central role in pancreatic glucose induced signalling events. Zygotes were cultured in the presence and absence of glucose and treated with either calcium mobilising agents, ethanol or ionomycin at 54 h post hCG or with the intracellular calcium chelator BAPTA-AM. Embryos were fixed and assayed for GLUT3 expression individually at 96 h post hCG using confocal immunofluorescence. Release of intracellular calcium by either ethanol or ionomycin, activated GLUT3 expression in a glucose like manner ($P < 0.01$) suggesting that calcium transients may be involved in glucose sensing. Moreover, buffering of calcium with the calcium chelator BAPTA-AM interfered with the ability of glucose to activate GLUT3 expression ($P < 0.05$), suggesting that glucose exposure does result in calcium transients that affect GLUT3 expression. It is unclear whether these calcium transients occur as a result of influx of extracellular calcium via voltage-gated ion channels or the release of calcium from intracellular stores via inositol triphosphate-gated calcium release channels in the endoplasmic reticulum.

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PAF INDUCED CHANGES IN INTRACELLULAR CA^{2+} AND MEMBRANE POTENTIAL IN THE 2-CELL MOUSE EMBRYO

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Platelet-activating factor (PAF) is an autocrine survival factor for the preimplantation embryo. PAF induces a transient increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in 2-cell embryos that is caused by the interdependent influx of external calcium and release of calcium from internal stores. A membrane current with L-type calcium channel properties is activated during PAF-induced calcium signalling. Since the L-type channel in many cell types is primarily voltage-gated we were interested to learn whether this was also the case in the 2-cell embryo. The present study investigated the relationship between the PAF-induced Ca^{2+} transient and changes in membrane potential (E_m) in the 2-cell embryo.

The perforated whole-cell patch-clamp technique was used to detect changes in Em and standard calcium imaging techniques were used to measure changes in $[Ca^{2+}]_i$ in 2-cell embryos from QS mice. Embryos were first loaded with Fluo-3 and then pretreated with PAF:acetylhydrolase to degrade the embryo derived PAF before patch clamping. Whole-cell perforated patch-clamping was performed by inclusion of 240mg/ml Nystatin in the pipette solution. Changes in E_m and $[Ca^{2+}]_i$ were recorded simultaneously after treatment of the embryo with PAF.

In 2-cell embryos PAF induced a change in E_m, consisting of an initial small depolarisation of 2.4 ± 0.2 mV (42 ± 4 sec after addition of PAF) followed by one or more transient hyperpolarisations of –8 ± 1 mV (100 ± 9 sec after addition of PAF). Transient increases in $[Ca^{2+}]_i$ paralleled the membrane hyperpolaristions and were initiated at 84 ± 8 sec after addition of PAF. These responses to PAF were seen in 58% of 2-cell embryos (n = 52). It is not yet clear whether these changes in E_m account for the activation of calcium influx through the L-type channel. The results show for the first time that the 2-cell embryo is an electrically active organism.

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DIFFERENTIAL EXPRESSION OF MONOCARBOXYLATE COTRANSPORTER PROTEINS IN PREIMPLANTATION EMBRYOS

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During preimplantation development mouse embryos demonstrate a switch in substrate preference. Pyruvate consumption, high during the first few cleavage stages, declines as the morula develops to a blastocyst, when glucose becomes the preferred substrate. Whilst pyruvate utilisation has been well characterised, changes in the function and expression of pyruvate transporters during this crucial period remain unclear. Pyruvate, lactate and other monocarboxylates are transported across mammalian cell membranes via a specific H^+-monocarboxylate cotransporter (MCT). Fourteen members of this family have been identified of which MCT1, MCT2 and MCT4 are well characterised. Although mRNA expression profiles are known during early mouse development (1,2), the specific roles of each protein isoform are unknown. In order to understand these, the expression pattern for each isoform and their cellular localisation during preimplantation development have been determined. Mouse embryos were freshly collected from superovulated Quackenbush mice at 24, 48, 72 and 96 h post-hCG and expression of MCT1, MCT2 and MCT4 analysed by confocal laser scanning immunohistochemistry. Our results confirm that all three MCT proteins are expressed in preimplantation embryos. Immunoreactivity for MCT1 and MCT2 appears diffuse throughout the cytoplasm of cleavage stage embryos. As development proceeds, MCT1 localised to the basolateral membranes of morulae and blastocysts, whilst stronger MCT2 expression was found on the apical trophectoderm as well as the inner cell mass. MCT4 immunoreactivity on the other hand is apparent at cell-cell contact sites in cleavage stage embryos and morulae, but it is not apparent in the blastocyst. The demonstration of different expression patterns for MCT1, MCT2 and MCT4 in mouse embryos implies specific functional roles for each in the critical regulation of H^+, pyruvate and lactate transport during preimplantation development.

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INSULIN RECEPTOR EXPRESSION 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, adult spleen, brain and tumours, where its expression has been associated with an undifferentiated state. IR-B is expressed primarily in classical insulin sensitive tissues such as adult liver and muscle. In contrast to other species, no evidence existed for IR expression prior to compaction in mice. However, both insulin and IGF-II have been shown to stimulate growth in cleaving embryos (Kaye 1997). We returned to this question using primers flanking exon 11 to detect IR-A and IR-B mRNA. Sequencing confirmed the identity of the amplified PCR products. IR-B mRNA was present in zygotes and all stages to blastocyst, IR-A mRNA was only detected in compacted morulae. Immunofluorescent confocal microsopy using a rabbit polyclonal antibody (against amino acids 128-205 of alpha-subunit) that recognizes both isoforms revealed nuclear IR localization in zygotes and cleavage stage embryos including morulae. The results demonstrate that mice like other species expresses IR throughout preimplantation development in contrast with earlier studies. Nuclear localisation of IR has been observed in hepatocytes and adipocytes and may be associated with transcriptional regulation. IR-A expression in compacted morulae may reflect the change in metabolism that occurs at this time and requires further study.

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DETERMINATION OF DIFFERENTIALLY DISPLAYED OXYGEN-SENSITIVE GENES IN BOVINE BLASTOCYSTS

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Oxygen-regulated gene expression in the bovine embryo contrasts markedly with that observed in the mouse. Under low (2%) oxygen moderate changes in gene expression are observed in the bovine blastocyst, compared with 3- to 4-fold increases in the mouse. We have determined that these moderate gene expression changes are most likely regulated by Hypoxia-Inducible Factor (HIF)-2 transcription factor activity in the bovine, in the absence of HIF1, although HIF2 target genes are largely unknown. The aim of this study was to screen, by differential display RT-PCR, for putative oxygen-regulated transcripts that might confer developmental competence in blastocysts cultured under varying oxygen atmospheres post compaction.

In vitro-produced bovine blastocysts were generated using standard protocols. Compact morulae were randomly allocated to treatments under either 2%, 7% or 20% oxygen for 72 h from Day 5. Blastocyst RNA was isolated using TriReagent and samples were reverse transcribed using Superscript II. cDNA was amplified using 10-mer primers in reactions containing ^{32}Pα-labelled dCTP. Resulting bands were detected by autoradiography, excised, purified and ligated into pGEMT vectors for transformation and sequencing. Seven clones were identified as having high homology with known sequences in GenBank. Real-time PCR was undertaken to confirm oxygen-regulation using Sybr green master mix.

Myotrophin mRNA was significantly increased following 2% oxygen culture, compared with 20% cultured blastocysts ($P < 0.01$), as was GLUT1 ($P < 0.01$). The expression of anaphase-promoting complex showed a significant association with oxygen, being higher in 2% cultured blastocysts ($P < 0.05$). Acetyl-coA-acetyltransferase I, chronic myelogenous leukemia tumor antigen (CML66), cyclin I, NADH dehydrogenase subunit 2 and ribonucleotide reductase M1, genes identified using differential display, were not altered by post compaction oxygen concentration.

This study has identified potentially HIF2-specific regulated genes, and supports the hypothesis that reduced oxygen concentrations post-compaction may influence bovine embryo development through oxygen-regulated changes in gene expression.

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SENSITIVITY OF BOVINE MORULAE AND BLASTOCYSTS TO HEAT SHOCK *IN VITRO*

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Thermotolerance of blastocysts and morulae is greater than that of zygotes. However, lower rates of pregnancy have been recorded when morulae rather than blastocysts were transferred. The aim of this study was to determine if morulae displayed lower thermotolerance than blastocysts in vitro. Embryos were produced from oocytes collected from abattoir-sourced ovaries. On Day 7 post fertilisation, embryos were classified as morulae or blasotcysts and subjected to either an increase in temperature from 39ºC to 41.5ºC over 1 h followed by a decrease to 39ºC over 2 h (HS) or maintained at 39ºC (NHS), using a water jacketed CO_2 incubator.The number of embryos progressing to expanded and hatched blastocysts was recorded after 48 h further culture at 39ºC. After arcsine transformation, the proportions progressing were submitted to a general linear model using adjusted sum of squares for tests of difference. Factors were embryonic stage, treatment and sire and the interaction terms of stage and sire with treatment. The Kruskall-Wallis test was also applied to the untransformed, non-parameteric data set. Non-parameteric , univariant analysis indicated non-significant effects of treatment (NHS median proportion progressing =72 %, HS = 64 %, $P = 0.12$) and of sire (NHS = 78 %, HS = 64 %, $P = 0.168$), while the effect of stage was highly significant (morulae = 35%, blastocysts = 79%, $P = 0.0000$). However, according to the general linear model, treatment and stage were significant factors ($F = 5.39$ and 38.3, respectively, and $P = 0.032$ and 0.000, respectively) and sire approached significance ($F = 4.09$, $P = 0.058$). Neither of the interaction terms was significant. It was concluded that embryos, which were morulae on Day 7, were less likely to progress to expanded or hatched blastocysts and that heat shock reduced developmental progression.

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FERTILISATION *IN VITRO* CAUSES PRECOCIOUS ACTIVATION OF TRANSCRIPTION FROM THE ZYGOTIC GENOME

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In vitro fertilisation (IVF) may have long-term adverse effects on progeny. Infants conceived by *in vitro* fertilisation are more likely to be born small for dates and, in a mouse model, culture *in vitro* caused changes in neurological performance of progeny. Changes in the pattern of gene expression in IVF embryos have been detected, and these may be one cause of the long-term effects. This study investigates the effect of IVF on the ontogeny of onset of expression from the embryonic genome in the mouse.

The expression of two markers for the onset of transcription (the transcription requiring complex (TRC) and hsp 70.1) was assessed in 2-cell embryos produced by IVF or fertilisation *in situ* (ISF). It was confirmed that the time from fertilisation to first cleavage was not different for IVF and ISF. Zygotes were cultured and at 1-hourly intervals those cleaved were 'picked off' (time 0 h after cleavage) and placed in groups of 10 in 10 µL of modified-HTF. The expression of the gene products was assayed at times after 'pick-off'.

The proportion of embryos expressing TRC increased with time after cleavage ($P < 0.001$). IVF embryos expressed it significantly earlier ($P < 0.01$) than ISF embryos. Some IVF embryos expressed TRC immediately after cleavage and this was never found for ISF embryos. All IVF embryos were TRC-positive by 2.5 h after cleavage, while this did not occur until 4.5 h post cleavage for ISF. Hsp70.1 transcripts were first detected in IVF embryos 2 h after cleavage but not until 6 h after cleavage in ISF embryos ($P < 0.01$).

The onset of transcription at the 2-cell stage is currently thought to reflect major reorganization of the nucleosomal structure of DNA. Evidence for precocious onset of transcription may indicate that this fundamentally import process is changed following IVF, and warrants further investigation.

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REPRODUCTIVE PERFORMANCE IN *CLOCK* MUTANT MICE

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The relationship between circadian rhythmicity and rodent reproductive cyclicity is well established, but the impact of disrupted clock gene function on reproduction has not been investigated. This study evaluated the reproductive performance of melatonin deficient and proficient mice carrying a mutation in the core circadian gene, *Clock*. In natural matings, melatonin deficient *Clock* mutant mice took 2 to 3 days longer to mate and to subsequently deliver pups than their control line. The melatonin proficient mutants (*Clock-MEL)* had a smaller, but still significant delay ($P < 0.05$). The *Clock* mutation resulted in smaller median litter sizes compared to the control lines (7 *v.* 8 pups, $P < 0.05$) while melatonin proficiency reversed this difference. Survival to weaning was 84% and 80% for the melatonin deficient and proficient *Clock* mutant lines respectively, compared to 94 to 96% for their control lines. When immature mice were subjected to a standard PMSG/HCG superovulation protocol, *Clock-MEL* mice had lowered fertility and significantly fewer ovulations than their control line although embryo development appeared to be only slightly affected (Table 1).

Table 1. Embryo development following superovulation in *Clock-MEL* mice

	Fertile matings	Embryos recovered at 96 h post HCG	Degenerated or unfertilised	2 cell to morulla	Blastocyst	Hatched blastocyst
WT-MEL	80% (12/15)	29 ± 4	10%	60%	29%	1%
Clock-MEL	53% (8/15)	19 ± 5	17%	48%	32%	3%

When kept in constant darkness, 7 of 15 *Clock-MEL* mice, became arrhythmic, but still became pregnant. The 7 mice that free ran for at least 14 days in constant darkness with a period of 27.1 h also became pregnant.

This study has shown that a mutation in the *Clock* gene that results in a protein incapable of initiating the transcription of target genes has significant, but subtle effects on reproductive performance. The capacity to produce melatonin or additional genes introduced along with the genes for the melatonin synthesising enzymes reduced the impact of the mutation further. It would appear that redundancy within the circadian timing system allows the reproductive cyclicity to persist in *Clock* mutant mice, albeit at a suboptimal level.

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STRESS APPLIED TO EWES AT DAY 2 AND 3 OF GESTATION INCREASES DAY 6 EMBRYO CELL COUNTS

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The effect of stress during pregnancy on fetal development has been demonstrated in studies such as the administration of glucocorticoids at 4 weeks of gestation which then 'programs' arterial blood pressure to be elevated during subsequent adult life (1). In the current experiment we have explored the effect of an acute stressful event applied to pregnant ewes at Day 2 and 3 of gestation.

The morphology and cell counts of Day 2 and 3 embryos were evaluated following surgical collection from 15 superovulated ewes. Seven of these ewes were subjected on Days 2 and 3 after insemination to a 15 min period of shearing (partial), isolation and confinement adjacent to a working dog. These stresses were intended to produce a short-term cortisol peak at the time embryos were at the 4–8 cell stage.

Embryos recovered from the 7 treated and 8 control ewes were graded according to IETS guidelines using a Nikon TE 300 inverted microscope. Cell counts were performed following staining of embryos with Hoechst 33342. Only embryos with >8 nuclei were included in the analysis to exclude embryos that were either unfertilised or died prior to the stressful events.

Subjective grading showed no difference in grading embryos as transferable (stage of compact morula or later with quality grade of 1 or 2) between embryos derived from stressed and non-stressed ewes (45/63 *v.* 45/64). However cell counts were dramatically different as stressed ewes produced embryos with higher cell counts (74.7 ± 32.0 *v.* 43.3 ± 22.9; $P < 0.001$ using one-way ANOVA).

As it is presumed that cell counts are directly related to embryo quality, our results suggest that stress may be manipulated to increase embryo cell numbers and thus embryo quality. The apparently beneficial effect of stress is surprising as it is commonly stated that minimising stress during commercial animal embryo transfer programs results in improved results. We look forward to the opportunity to follow the *in vivo* development of embryos derived from ewes stressed at Day 2 and 3 of gestation.

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NUCLEAR TRANSPORT OF GLI TRANSCRIPTION FACTORS DURING SPERMATOGENESIS

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Development is highly regulated by complex signalling cascades. One such pathway is the Hedgehog (Hh) signalling pathway which plays an essential role in spermatogenesis. The Gli family of zinc finger TFs, consisting of Gli1, Gli2 and Gli3, are mediators of the Hh signalling cascade. Gli1 is an activator of Hh target genes, whereas Gli2 and Gli3 can undergo proteolytic cleavage and function as both activators and repressors. Little is known regarding the nuclear import pathway of these TFs. In this study, the mRNA expression pattern of all Gli family members in the developing mouse testis was compiled by *in situ* hybridisation and shown to have unique expression patterns. In the adult mouse testis, Gli1 mRNA was detected in spermatogonia through to round spermatids whereas Gli2 was only found in spermatogonia and spermatocytes. Very low levels of Gli3 mRNA were detected in all ages and cell types. Since little is known regarding the import pathway for Gli1, expression vectors containing different fragments of the N-terminus of Gli1 were created and used to perform transfection experiments and generate vectors for bacterial GFP-fusion protein expression. Transfection experiments into African green monkey kidney Cos-7 cells, and the murine spermatogenic cell lines, Gc-1 and Gc-2 using 3 different constructs localised the NLS(s) required to target Gli1 to the nucleus in the zinc finger DNA-binding domain of Gli1. Preliminary results for *in vitro* binding of bacterially expressed Gli1 indicated no binding by importin β 1 or β3 but a weak interaction with the importin α/β heterodimer. This can be seen as the first step towards defining the nuclear import pathway for Gli1. The mechanisms by which Gli activity is modulated remain unanswered and the regulation of its nuclear entry may be an important means of doing so.

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INTERACTION BETWEEN BONE MORPHOGENETIC PROTEIN 4 AND RETINOID SIGNALLING IN MOUSE SPERMATOGENESIS

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Vitamin A (retinol, or ROL) is also essential for normal spermatogenesis in the rat and mouse. Vitamin A-deficient (VAD) rodents suffer various disorders including blindness and male infertility. The molecular mechanisms leading to infertility in vitamin A deficient rodents have never been fully elucidated. Following prolonged vitamin A withdrawal the only germ cells remaining in the VAD rodent testis are stem cell spermatogonia, type A1 spermatogonia, and a few preleptotene spermatocytes. Supplementing the diet of these animals with retinoic acid (RA) alleviates all symptoms of vitamin A deficiency, with the exception of sight and spermatogenesis. It is not until VAD animals are re-administered ROL through the diet, or RA is injected in repeated high doses directly into the testis, that normal spermatogenic function is restored. Here we report an interaction, in germ cells, between the Bone Morphogenetic Protein (BMP) 4 and retinoid signalling pathways that may help explain the molecular mechanics of vitamin A deficiency. We localised BMP4 gene expression to adult germ cells, in particular spermatogonia, at both the mRNA and protein level. We generated VAD mice and found that in the absence of retinoids *in vivo*, bmp4 gene expression was significantly upregulated in the testis. We also observed that the expression of bmp4 is downregulated by retinoid treatment in germ cells isolated from vitamin A sufficient mice. Expression of bmp4 mRNA in isolated spermatogonia was more sensitive to ROL rather than RA. Our results may reflect a direct requirement for ROL by germ cells for the resumption of spermatogenesis in VAD animals that involves the regulation of BMP4 expression. Furthermore our observations suggest that retinoid signalling in germ cells is different to that observed in somatic cells, and may provide insights into the role of retinoids in spermatogenesis.

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CHARACTERISATION OF PROSTAGLANDIN PRODUCTION IN THE NORMAL AND INFLAMED RAT TESTIS

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Prostaglandins E2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) play a role in Leydig cell function and in suppression of macrophage inflammatory functions. We predict that PGs also may play a role in interstitial fluid (IF) formation in the testis. Prostaglandin synthesis involves one of two distinct forms of cyclooxygenase (COX): constitutively expressed COX-1 and inducible COX-2. We recently demonstrated expression of both enzymes in macrophages, somatic and germ cells of the adult rat testis, and that COX-2 may the more important form in this organ. Adult male Sprague-Dawley rats were maintained on normal feed or 0.15% celebrex, a specific COX-2 inhibitor, for 5 weeks. Rats were subsequently treated with saline, or lipopolysaccharide (LPS; 0.1 mg/kg or 5 mg/kg), 6 h prior to collection of tissues. PGE_2 was measured by RIA in medium of cultured testis fragments and testicular cells from normal rats (± 10 µg/mL LPS, 24 h, 37ºC), and in testicular interstitial fluid. PGE_2 was constitutively produced by whole testis, Sertoli cells, Leydig cells and round spermatids, but not by resting macrophages or pachytene spermatocytes in culture. Stimulation with LPS upregulated PGE_2 in macrophage cultures, but not in other cells or whole testis. Normal PGE_2 levels in IF were 16–20 ng/mL; levels were not altered by low-dose LPS, but were reduced by high-dose LPS. Celebrex caused a reduction in IF PGE_2 levels in both the normal and low-dose groups, but not in the high-dose group. Celebrex elevated IF volume (25-50%) in all groups. Our experiments show cell-type specific regulation of PGE_2 production in the rat testis, and predict a role for COX-2 elicited PGs in the IF regulation and in post-meiotic cell function. Paradoxically, low-level inflammation does not alter testicular PGE_2 levels, as somatic and germ cells, which do not respond to LPS, appear to contribute most to the local levels of PGE_2.

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THE EFFECT OF TESTOSTERONE AND SEASON ON PRODYNORPHIN mRNA EXPRESSION IN THE PREOPTIC AREA-HYPOTHALAMUS OF THE RAM

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Progesterone stimulates prodynorphin mRNA expression in the hypothalamus of the ewe (1), but whether testosterone regulates dynorphin gene expression in the ram is unknown. In a previous study (2), we showed that both testosterone and season influence mRNA expression of another opioid, enkephalin, in the preoptic area and hypothalamus of rams. Using tissue from the same study, we tested the hypothesis that testosterone and/or season modulate prodynorphin mRNA expression in specific areas of the hypothalamus in the ram. Adult Romney Marsh rams were castrated either during the 'breeding' season or 'non-breeding' season and 1 week later received intramuscular injections of either peanut oil (vehicle) or testosterone propionate (8 mg/12 h for 7 days) (5/group). Blood samples taken every 10 min for 12 h were assayed for plasma LH and testosterone. Prodynorphin mRNA expression was quantified in hypothalamic sections by *in situ* hybridisation using a ^{35}S-labelled riboprobe and computer-aided image analysis. Plasma testosterone levels were higher in testosterone propionate-treated than oil-treated sheep. Mean plasma LH concentrations were reduced and the interpulse interval for LH pulses was greater in testosterone propionate-treated wethers compared to oil-treated wethers, with no change in LH pulse amplitude. Testosterone propionate treatment increased prodynorphin mRNA expression in the supraoptic nucleus and the bed nucleus of the stria terminalis, but only during the breeding season. Proenkephalin mRNA expression was also higher in the 'breeding' season than in the 'non-breeding' season in the caudal preoptic area and paraventricular nucleus. No differences were observed between treatments in five other regions of the hypothalamus. We conclude that testosterone and season regulate preproenkephalin mRNA levels in the preoptic area/hypothalamus in the ram in a region-specific manner.

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MOLECULAR BASIS OF OOCYTE–PARACRINE SIGNALLING THAT PROMOTES MOUSE GRANULOSA CELL PROLIFERATION

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Oocytes regulate follicle growth and development by secreting paracrine growth factors that act on granulosa cells (GC). We have recently determined that growth differentiation factor-9 (GDF-9) accounts for ~50% of the total mitogenic activity of oocytes, the remaining portion is as yet uncharacterised. This study was conducted to identify the receptor/signalling system utilised by oocytes to promote GC proliferation. We used an established oocyte-secreted mitogen bioassay, where denuded oocytes are co-cultured with primed-mouse mural GC. In this system, oocytes, GDF-9, TGF-b1 and activin-A all promoted GC DNA synthesis in a dose-dependent manner, but bone-morphogenetic protein-6 (BMP-6) and BMP-7 did not. The type-II receptor for GDF-9 is BMPRII and using real-time RT-PCR, cumulus cells (CC) and mural GC were found to express equivalent levels of BMPRII mRNA. We tested the capacity of the receptor ectodomain (ECD) to neutralise oocyte-stimulated mural GC proliferation. The BMPRII ECD antagonised both oocyte and GDF-9 bioactivity in a dose-dependent manner, completely abolishing activity of both mitogens at 1 ug/mL. The BMPRII ECD did not antagonise TGF-β and partially antagonised activin-A bioactivity, demonstrating its specificity. The TGFβR-II ECD, activin R-II ECD and activin R-IIB ECD all failed to neutralise oocyte- or GDF-9-stimulated GC DNA synthesis, whereas they did antagonise the activity of their respective ligands. The BMPRII ECD also completely antagonised oocyte-stimulated CC DNA synthesis. Using this oocyte-factor bioassay with mural GC transfected with Smad luciferase reporter constructs, we found that oocytes, GDF-9 and TGF-β (but not BMP-6) activated the Smad2/3 pathway. Consistent with this, oocytes and GDF-9 led to phosphorylation of GC Smad2 molecules as detected by Western blot. Conversely the Smad1/5/8 pathway was activated by BMP-6, but not by GDF-9, TGF-β nor surprisingly by oocytes. This study provides evidence that BMPRII is a key receptor for transmitting the paracrine actions of oocytes in GC. However, oocyte-secreted factors do not activate the BMP intracellular signalling pathway but rather the TGF-β/activin intracellular pathway.

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Withdrawn

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ACTIVIN A UPREGULATES ENDOMETRIAL METALLOPROTEASES: POTENTIAL MECHANISMS FOR PROMOTION OF DECIDUALISATION AND IMPLANTATION

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Activin and inhibin subunits are co-expressed by human endometrial epithelial and decidualised stromal cells. Activin A is a potent stimulator of decidualisation in vitro, but the mechanisms are unknown. Matrix metalloproteases (MMPs) are known to be important during decidualisation, as administration of a broad spectrum MMP inhibitor in the rat results in reduced decidualisation. Transforming Growth Factor(TGF)-βs are closely related to activins and inhibit MMP production in endometrial epithelial cells. We hypothesised that activins regulate MMP production during decidualisation and/or trophoblast invasion. Epithelial and stromal cells were isolated from human endometrium and treated for 24 h with activin, inhibin, activin/inhibin, and follistatin. Media were collected and subjected to gelatin and caesin zymography. In epithelial cells, activin A stimulated the expression of latent forms of MMPs-1, -2, -7 and -9, and increased formation of active forms of MMPs-2 and -7. Cotreatment with inhibin prevented this stimulation, whilst inhibin alone completely inhibited MMP production. Treatment with follistatin treatment reduced MMP levels. Similar regulation was seen in stromal cells for MMPs-1, -2 and -9. These data show that activin stimulates the production and activation of MMPs in both endometrial cells, and that inhibin is a potent inhibitor. It is interesting that activin is acting in an opposing manner to TGF-β, indicating that these two closely related proteins have divergent signalling pathways in endometrial cells. Further, this is the first demonstration of a role for inhibin in regulating MMPs and indeed for inhibin action in the endometrium. These findings are of potential importance in understanding regulation of MMPs in the peri-implantation endometrium. Activin is the predominant dimer produced by decidual and epithelial cells, where it may be promoting decidualisation though enhancing MMP production and activation. Furthermore, activin secretion by invasive cytotrophoblasts may stimulate focal decidual MMP production promoting their invasion during embryo implantation.

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CHEMOKINE PROFILING IN ENDOMETRIOSIS USING LASER CAPTURE MICRODISSECTION

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Endometriosis is an inflammatory condition with elevated leukocyte infiltrate; defined as the ectopic growth of endometrium-like tissue, characterised by epithelial glands, outside the uterus. Chemokines selectively regulate influx and activation of leukocyte subpopulations. Their non-immune functions during tissue remodelling and disease pathogenesis include up-regulation of adhesion molecules, stimulation of inflammatory mediators, angiogenesis, cell proliferation and motility (1). The aims of the present study were to compare the chemokine mRNA profiles expressed by the epithelial glands of: eutopic endometrium from patients with / without endometriosis. Tissue heterogeneity in the endometrium and in ectopic lesions hinders precise study of the contribution of cell-specific inflammatory responses. Laser Capture Microdissection (LCM) was therefore utilised. Frozen eutopic curettings and ectopic endometriosis lesions were sectioned, H&E stained and glandular epithelium laser captured. 327 captured glands yields approximately 27 ng of RNA from each endometrial sample. To obtain enough RNA for gene array analysis and verification studies, cellular mRNA was amplified. Two rounds of linear mRNA amplification provided a sufficient yield of >1.8 μg from 1 ng of RNA. RNA from 4 patients and 4 controls were pooled, amplified and probed on gene arrays to build a chemokine profile. We identified 45 chemokines / receptors that are specifically abundant in glandular epithelium. 39 were highly upregulated (2- to 60-fold) in women with endometriosis compared to controls. In contrast, only 2 genes were downregulated more than 2-fold in endometriosis patients. Most genes have not been previously studied for their roles in endometriosis. Verification studies are currently being carried out. This is a novel study providing evidence of a distinct profile of the chemokine/receptors collectively in women with endometriosis.

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REGULATION OF MOUSE CUMULUS EXPANSION BY OOCYTE-SECRETED GROWTH DIFFERENTIATION FACTOR-9 (GDF-9)

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Oocyte paracrine signalling is necessary for mouse cumulus cell expansion, an important preovulatory process. The oocyte-secreted factor growth differentiation factor-9 (GDF-9) signals through the bone morphogenetic protein receptor-II (BMPR-II) and is currently the primary candidate molecule for the cumulus expansion enabling factor (CEEF). The present study was conducted to determine whether in the mouse GDF-9 is the CEEF. Cumulus oocyte complexes (COC) were collected from eCG-primed mice and the oocyte was microsurgically removed to generate an oocytectomised complex (OOX). 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 recombinant mouse GDF-9 (score; mean +/– SEM: 2.7 +/– 0.1), recombinant TGF-β1 (score: 2.6 +/– 0.2) or co-culture with oocytes (score: 2.3 +/– 0.2). A GDF-9 neutralising antibody mAb-53, raised against hGDF-9, was effective in neutralising the response of OOX complexes to GDF-9 (score: 0.1 +/– 0.1), but had no significant effect on the expansion of OOX complexes co-cultured with oocytes (score: 2.3 +/– 0.2). Likewise, a TGF-β antagonist neutralised ($P < 0.05$) TGF-β-induced, but not oocyte-induced, expansion of OOX complexes. A soluble portion of the BMPR-II ectodomain, a known GDF-9 antagonist, failed to neutralise oocyte-induced cumulus expansion ($P > 0.05$) at the highest dose implying that BMPR-II is not a critical receptor involved in regulating cumulus expansion. Using real-time RT-PCR, hyaluronan synthase-2 (HAS2) mRNA expression by OOXs was upregulated 6- to 7-fold by oocytes and GDF-9. The GDF-9 neutralising antibody mAb-53, partially neutralised GDF-9-induced OOX HAS2 expression, but not oocyte-induced HAS2 expression. This study provides evidence that like TGF-β1, GDF-9 can enable FSH-induced cumulus expansion, however more importantly demonstrates that neither GDF-9 nor TGF-β1 alone account for the crucial oocyte-secreted factor regulating cumulus expansion in the mouse.

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OOCYTE-SECRETED FACTOR(S) REGULATE APOPTOSIS OF BOVINE CUMULUS CELLS

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Paracrine factors secreted by the oocyte affect cumulus cell proliferation and differentiation. These factors may also act in an anti-apoptotic manner, maintaining the low incidence of cellular apoptosis within cumulus cells. The purpose of this study was to determine whether the incidence of apoptosis within cumulus cells is regulated by oocyte-secreted factors (OSF). Bovine cumulus-oocyte complexes (COC) aspirated from abattoir-derived ovaries were randomly allocated to 3 treatments: *(1)* groups of 5 intact COC; *(2)* groups of 5 oocytectomised complexes (OOX), where oocytes were removed microsurgically; and *(3)* groups of 5 OOX co-cultured with 25 denuded oocytes (DO). To examine a dose effect of OSF, OOX were also cultured with increasing numbers of DO (OOX+5DO, OOX+25DO, OOX+50DO). In both experiments, complexes were cultured in their respective treatments for 24 h in 50 µl of oocyte maturation medium (+/– rFSH; 0.1 IU/mL). Apoptosis was assessed using TUNEL, with all nuclei counterstained with propidum iodide (PI). Nikon TE2000 laser confocal scanning microscopy was used to visualise and quantify the incidence of apoptosis (TUNEL/PI). The proportion of apoptotic cells was determined by image analysis. Oocyte removal lead to a significant increase in cumulus cell apoptosis (OOX, 67% apoptotic; COC, 15%; $P < 0.001$). However, the incidence of apoptosis in OOX was restored to COC levels when co-cultured with DO (18%; $P > 0.05$, COC *v.* OOX+DO). FSH prevented apoptosis in all treatment groups ($P < 0.05$) decreasing the incidence by 27% in OOX and by 12% in COC. Cumulus cell apoptosis within OOX (+/– FSH) was reduced in a dose dependent manner by treating with increasing numbers of DO. These results indicate for the first time that oocyte-secreted factor(s) regulate the incidence of apoptosis within cumulus cells.

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FOLLICLE STIMULATING-HORMONE (FSH) WITHDRAWAL INDUCES GERM CELL APOPTOSIS IN THE IMMATURE RAT TESTIS *IN VITRO*

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FSH is a key determinant of adult sperm output influencing both Sertoli and germ cell development. The aim of this study was to assess the impact of FSH action on Sertoli and germ cell proliferation and survival *in vitro*, and to identify FSH-regulated genes that may underpin these responses. Testis fragments from 17-day-old rats were cultured with recombinant human FSH for 2 or 24 h and then labelled with bromodeoxyuridine (BrdU) to identify proliferating cells. The testis fragments were then processed for analysis of cell numbers by stereology, BrdU incorporation by immunohistochemistry, and apoptosis by TUNEL. The TUNEL assay revealed that without FSH, spermatogonial apoptosis was induced to 195% and 179% ($P < 0.05$) compared to fragments with FSH after 2 and 24 h, respectively. No difference in apoptosis was observed in spermatocyte or Sertoli cell populations at these time points. No differences in Sertoli or germ cell proliferation were observed with or without FSH. To understand how FSH mediates spermatogonial apoptosis the response of 5 testicular genes of interest was examined. Expression of cyclin D2 (cell cycle, G_1-S), N-cadherin (N-Cad; adhesion molecule), Bax (pro-apoptotic), Bcl-w (anti-apoptotic), and stem cell factor (SCF; pro-apoptotic and other functions) was elevated to 151%, 348%, 209%, 258%, and 198%, respectively (all $P < 0.001$), in fragments cultured without FSH for 24 h, compared to fragments with FSH. No gene expression differences were observed at 2 h, except for SCF, which was elevated to 135% ($P < 0.01$). In conclusion, these studies have examined apoptosis and proliferation activities simultaneously in testis fragments *in vitro*, and demonstrated that FSH withdrawal induces both spermatogonial apoptosis and expression of testicular genes known to be involved in cell survival. This model will now be used to further investigate FSH-mediation of Sertoli and germ cell development.

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INTERLEUKIN-11 ENHANCES ENDOMETRIAL STROMAL CELL DECIDUALISATION VIA ACTIVATION AND INHIBITION OF TARGET GENES

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Differentiation of endometrial stromal cells into decidual cells is essential for successful embryo implantation. Interleukin (IL)-11 signalling is required for decidualisation in the mouse (1,2) and the expression pattern of IL-11 and its receptors during the menstrual cycle suggests a role for IL-11 in human decidualisation (3). Exogenous IL-11 has been shown to enhance hormone-induced decidualisation of human endometrial stromal cells in culture (4). This study aimed to determine the effects of IL-11 on downstream gene expression in endometrial stromal cells following 12 days of progesterone-induced decidualisation, and to examine the expression and functional significance of IL-11 target genes during this process. Stromal cells isolated from endometrial biopsies ($n = 6$) were decidualised with 17β-oestradiol and medroxyprogesterone acetate (EP) or EP with 100 ng/mL recombinant human IL-11. Medium was changed every 48 h, and total RNA extracted on Day 12 for gene expression analysis using custom-made 15K cDNA microarrays. Quantitative real-time RT-PCR was performed on the same samples to confirm gene expression levels. In subsequent experiments ($n = 2$), cells were cytocentrifuged onto glass slides for immunocytochemistry using specific antibodies. Microarray analysis revealed 16 upregulated and 11 downregulated cDNAs in EP + IL-11 compared to EP treated cells. Among these were IL-1β (6.1-fold upregulated) and insulin-like growth factor binding protein (IGFBP)-5 (3.6-fold downregulated). Using real-time RT-PCR, IL-11 was confirmed to increase IL-1β (fold change 1.3–107.1) and decrease IGFBP-5 (fold change 2.8–469.0) transcript abundance in 6 patients. Immunolocalisation of IL-1β in EP and EP + IL-11 treated cells revealed more intense vesicular cytoplasmic staining with IL-11 treatment, while staining intensity for IGFBP-5 was not affected. Interactions between IL-11 and its downstream targets IL-1β and IGFBP-5 are likely to have functional importance in early pregnancy, and may provide novel targets for the manipulation of human fertility.

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DNMT3L: A COORDINATOR OF EPIGENETIC MODIFICATIONS DURING SPERMATOGENESIS

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Spermatogenesis is a process with unique epigenetic requirements. The differentiation from diploid spermatogonia to haploid spermatozoa requires regulation of genomic imprint establishment, stage specific gene expression, meiotic division, and the histone-protamine transition. The methyltransferase regulator, Dnmt3L, is expressed during gametogenesis and is necessary for establishment of maternal methylation imprints in the oocyte. Targeted disruption of Dnmt3L does not appear to affect oogenesis, as mature oocytes are generated, however resultant heterozygous progeny die mid gestation due to biallelic expression of imprinted genes. Dnmt3L–/– males however show spermatogenic arrest. We found that this arrest occurs during prophase I of meiosis, with spermatocytes lost by both apoptosis and germ cell sloughing. A progressive degeneration ensues, resulting in a Sertoli cell phenotype. Electron microscopy of meiotic spermatocytes revealed that homologous chromosomes fail to align and form synaptonemal complexes. Furthermore, Dnmt3L–/– spermatocytes show abnormal methylation on paternally imprinted genes and abnormal global retention of histone acetylation, implicating Dnmt3L in histone deacetylase recruitment. Thus, during spermatogenesis, Dnmt3L is crucial for two distinct epigenetic modifications; imprint establishment and global histone deacetylation prior to homologous chromosome alignment. The latter defect is likely to affect the alignment of homologous chromosomes and trigger the pachytene checkpoint leading to spermatocyte death. Since Dnmt3L has no DNA methlytransferase or HDAC activity itself, we propose that Dnmt3L is essential for the coordination of epigenetic layers, at least during spermatogenesis.

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DEVELOPMENT OF A LABEL RETAINING CELL METHOD TO IDENTIFY STEM CELLS IN MOUSE ENDOMETRIUM

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Epithelial and stromal stem/progenitor cells have been demonstrated in human endometrium by their clonogenic activity (1). To understand the regulation and growth kinetics of endometrial stem cells (SCs), an animal model is being established using the label retaining cell (LRC) approach. Tissue SCs are quiescent and will retain a DNA synthesis label (BrdU), while the label is diluted out in more mature dividing cells during a chase period. Those cells retaining the label over long period of time are LRCs and have been identified as SCs (2). An optimum time to initially label the majority of the cells is critical. We hypothesised that during mouse endometrial development, there is a growth window for maximum labelling of epithelial and stromal cells. The aim of this study was to determine the optimum age and length of time for maximum endometrial BrdU labelling. Two stages of endometrial growth were exploited: *(1)* postnatal growth before gland development (3 days old – P3); and *(2)* oestrogen induced growth in ovariectomised prepubertal mice (4–5 weeks old). C57/CBA P3 mice received subcutaneous BrdU injections twice daily for 3 consecutive days. Four to five-week-old ovariectomised mice received BrdU filled mini-osmotic pumps implanted subcutaneously for 7 and 14 days, with daily oestrogen injections. Mice were killed 4 h after the last treatment, uteri collected, and BrdU labelled cells were detected by immunohistochemistry. Results are shown in the table below:

Age (days)	P3	(OVX, prepubertal) 28	(OVX, prepubertal) 35
Total dose (μg/g)	306	277	277
Length of labelling (days)	3	7	14
Epithelial BrdU cells (%)	85.70 ± 2.27 (n = 3)	59.65 ± 0.70 (n = 4)	66.35 ± 2.64 (n = 2)
Stromal BrdU cells (%)	78.43 ± 2.98 (n = 3)	70.52 ± 1.05 (n = 4)	59.44 ± 4.80 (n = 2)

The data shows that maximum labelling for epithelial and stromal cells was achieved for P3 mice, possibly due to greater endometrial growth of the Müllerian duct compared to oestrogen induced endometrial growth following ovariectomy. Chase experiments are currently in progress on P3 labelled mice to identify the location of LRCs in mouse endometrial glands and stroma.

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

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SNTs (Suc-1 associated Neurotrophic Factor Targets) are FGF signalling adaptors and crucial activators of the MAP/PI3 kinase pathways via FGFRs. Screening a rat testis library identified snt-2 as a potential ODF component. ODFs are a major constituent of the sperm tail that we hypothesise play an active role in motility. Using western blot analysis I have localised Fgfr-1 to the sperm tail. As such, I propose that Fgf signalling through snt-2 is involved in sperm tail development/function. To test this hypothesis, I created transgenic mice carrying a dominant-negative variant of Fgfr-1 driven by the protamine 1 promoter (haploid specific). Breeding experiments confirmed males were fertile, although one line showed a tendency towards reduced pup numbers. This effect was strengthened by Daily Sperm Production (DSP), showing significantly reduced DSP (30%↓) compared to wt mice. Transgene expression levels were expressed up to 70 times above native mRNA levels in wt mice; however there was a concurrent up-regulation of the native receptor in transgenic mice. Cumulatively this resulted in only a 6x over-expression in transgene: native mRNA, and illustrated the presence of a feedback mechanism controlling Fgfr-1 expression. To increase transgene expression, I crossed independent lines (double heterozygous, DH) males. Breeding experiments showed males from 1 cross were significantly subfertile (2 *v.* 10 in wt mice) . DSPs were further reduced, (41%↓) compared to wt mice. Collectively this data shows Fgfr-1 signalling is required for quantitatively normal spermiogenesis, but is also likely to have a post testicular role in sperm function. I hypothesise this is mediated via activation/regulation of motility through the MAP/PI3 kinase pathways. Further, these mouse models provide compelling evidence that infertility in Kallmann's Syndrome patients is composed of both hypothalamic and testicular components. These mice will provide valuable insights into the signal transduction mechanisms controlling sperm function and avenues for contraceptive development.

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NATURAL KILLER CELL ACTIVITY IN THE ADULT RAT TESTIS

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Immune privilege of the testis is due to an inability to recognise and respond to antigens within the testis, and implies that innate immunity against tumours and infections, such as that afforded by natural killer (NK) cells, may be important. Our previous studies established the existence of cells that express NK markers in the rat testis, but the functional activity of these cells has not been assessed previously. Testicular interstitial cells were collected from adult male Sprague-Dawley rats by mechanical dissociation, and macrophages were removed by adherence depletion. These interstitial cell preparations contained on average 3% cells expressing the NK cell marker, CD161. The interstitial cells (effector cells) were labelled with 5-(6)-carboxy-fluorescein succinimydyl ester (CFSE), and cultured at various effector : target cell ratios with YAC-1 tumour cells (target cells) overnight. The combined cells were labelled with 7-amino actinomycin D (7-AAD), which stains the nuclei of cells undergoing apoptosis. Cells were fixed with paraformaldehyde and analysed by flow cytometry. The proportion of target (CSFE negative) cells expressing 7-AAD staining (mean ± SD, $n = 4$ experiments) increased as the effector : target cell ratio was increased: 3.5 ± 3.6% (no. effector cells; background), 24 ± 7.0% (effector : target cell ratio, 2 : 1), 25.8 ± 11.7% (7 : 1), 41.8 ± 17.0% (20 : 1), and 71.7 ± 12.7% (60 : 1). These data indicate that functional NK cells are present in the rat testicular interstitial cell preparations, and may contribute to innate immune protection in the rat testis.

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IDENTIFICATION OF ELEVATED LEVELS OF APOPTOSIS AMONG T-CELLS ISOLATED FROM THE RAT TESTIS

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Protection of the developing gametes from attack by the immune system is essential for reproductive success. Autoimmune infertility represents a failure of this protection. Specific T-cell apoptosis is the main mechanism for control of antigen-specific immune responses. Studies were undertaken to investigate this regulatory process in adult rat testes. Flow cytometry was employed in conjunction with annexin-V/propidium iodide dual staining to identify apoptotic cells concurrent with CD3 staining to identify T-cells. CD3-positive cells isolated from the testicular interstitial tissue were shown to be 34.12 ± 3.0% apoptotic (mean ± s.e.m., $n = 3$) at collection. This was consistently greater than the numbers of apoptotic CD3-positive cells isolated from lymph nodes (4.04 ± 1.95%, $n = 2$), spleen (16.77 ± 4.73%, $n = 4$) and peripheral blood (9.64 ± 1.44%, $n = 2$). These results also were confirmed by using T-cells purified with MACS microbeads against the pan T-cell marker OX52 to improve sample purity: 40% of isolated testicular T-cells and 3% lymph node T-cells were found to be undergoing apoptosis. The level of apoptosis among T-cells isolated from another non-lymphoid organ, the liver, was only 6%. It is hypothesised that the immunosuppressive milieu of the testis induces an increased level of apoptotic deletion among T-cells that gain entry into the testis and potentially threaten gamete viability. Further studies of the mechanism responsible for this elevated level of T-cell apoptosis in the testis will significantly enhance our knowledge of how testicular immune tolerance is maintained.

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SPERM TRAINS AND MATING BEHAVIOUR IN WOOD MICE OF THE GENUS APODEMUS

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There is evidence of possible correlation between mating behaviour, sperm morphology and sperm behaviour in the female reproductive tract prior to fertilization. As shown in *A. sylvaticus* (1), cooperation between spermatozoa of an individual confers a significant advantage for fertilization, where inter-male sperm competition is intense. In our study we aimed to focus on three other species of rodent genus *Apodemus; A. flavicolis, microps, agrarius* versus *A. sylvaticus*, whose either promiscuous or monogamous mating behaviour has already been demonstrated (Stopka *et al*., unpublished). We placed spermatozoa from cauda epididymis of adult males of selected species into mouse *in vitro* fertilisation medium, and compared sperm behaviour of individual species. Surprisingly, despite monogamous or polygamous mating behaviour, sperm of all the above species rapidly aggregated into motile trains, as in *A. sylvaticus*, consisting of hundreds of cells. However, size and cell–cell coupling differed between each species. This may point to another aspect besides mating behaviour that may be involved in building up a mechanism for successful sperm delivery to the egg. These unique and organised aggregations can significantly increase sperm progressive motility. A detailed study of the main cytoskeletal proteins, using immunofluorescent methods, together with confocal microscopy, shows that in sperm-sperm binding especially protein actin plays an important role. According to *in vitro* experiments, we speculate that the viscous environment of the female reproductive tract may have played a crucial role in the evolution of sperm behaviour. Disintegration of motile sperm trains was associated with the majority of spermatozoa undergoing a premature acrosome reaction. However, the mechanism that triggers it remains to be determined. To conclude, it is likely that a myriad of changes in social behaviour of particular species were not accompanied by changes in sperm behaviour, but instead they retained original ancestral tactics typical for the genus *Apodemus*.

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PROBIOTIC LACTOBACILLUS IN SEMINAL PLASMA

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Commensal bacteria of the *Lactobacillus* genus are implicated in beneficial 'probiotic' roles in the gut and other mucosal tissues. Their presence reduces the incidence of pathogenic infections, both passively and via production of antimicrobial substances, and through Toll-like receptor-mediated activation of cytokine expression in host tissues. Lactobacilli are present in the female reproductive tract but have not been examined in the male. This study aimed to investigate, by selective culture techniques and real-time quantitative PCR, the prevalence in boar seminal plasma of Lactobacilli compared with other pathogenic bacteria. Using acidified Rogosa Agar, Lactobacilli were cultured from 3/3 fresh semen samples and were found to be most prevalent in the first fraction of the ejaculate. For PCR, DNA was extracted from reference bacterial cultures and archived seminal plasma samples from 40 healthy boars. Bacterial species-specific primers targeting *Lactobacillus* 16s and 16s-23s rDNA sequences, and *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus*-specific Sau3AI, oprL, and 16s rDNA genes respectively, were used in real-time PCR assays employing SYBRgreen (Applied Biosystems) technology. Lactobacilli were detected in 22/40 (55%) of seminal plasma samples, while pathogenic bacteria were detected in <10% of samples (*Staphylococcus aureus*, 1/40; *Pseudomonas aeruginosa*, 2/40; and *Bacillus*, 3/40). The *Lactobacillus* content of individual boars ranged from 1.5 to 15×10^6 cells/mL, and within boars, content varied within 30% of the mean value in successive samples over a 6-month period. We conclude that Lactobacilli are present in abundance in boar seminal plasma compared to other potentially pathogenic bacteria. These bacteria may protect the male tract from pathogen infection, and after ejaculation, may influence the female immune response to male antigens. Ongoing studies will investigate whether Lactobacilli abundance in seminal plasma correlates with boar fertility, and examine the potential value of improving reproductive performance in pigs and other species by administration of probiotic agents.

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HOW THE ELEPHANT GOT ITS EPIDIDYMIS

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An idea has been promoted for more than 30 years, that the elephant does not have an epididymis (1,2), that each derivative of the mesonephric (Wolffian) ducts only develops into a ductus deferens. Whilst the location of some of the duct provides some support for this idea, it is considered that the interpretation is misleading in implying that the testes only have ejaculatory ducts that don't function like the epididymides of other mammals. Modern interpretation indicates that all mammals have epididymides, but some species exhibit adaptations of the epididymides that are determined by the structure and general physiology of the species, and the reproductive strategy of the males to achieve paternity. The modern interpretation of epididymal evolution is based on comparative cytological studies of duct differentiation, the functions of the ducts in regulating their milieu and effecting post-testicular sperm development and storage, and the biological significance of the epididymis in natural selection. It has been shown that most of the more than 50 m length of the elephant's ductuli epididymides do lie on or close to the testis. However, more importantly, it has been shown that mature elephants have epididymides that are structurally differentiated into an initial segment (a feature that is unique to the mammalian epididymis) and 'middle' segments where sperm mature, and a terminal segment that is adapted for sperm storage both in structure and in the regulation of the luminal milieu. Each ductus epididymidis is connected to the urethra by a short ductus deferens.

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EFFECTS OF ANIMAL PAIRING ON MARMOSET SPERM COLLECTED BY PENILE VIBRATORY STIMULATION

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The marmoset monkey (*Callithrix jacchus*) is an important model species for the development of reproductive technologies in humans and endangered primates. Obtaining sufficient sperm of high quality is a limiting factor in implementing marmoset IVF. A method for the collection of marmoset semen, using penile vibratory stimulation (PVS), has recently been described (1). Due to the high rate of copulation in marmosets, the pairing of males with females may affect semen collection. The aim of the present study was to determine whether the quantity and motility of sperm collected by PVS is affected by animal pairing. A total of 10 adult male marmosets were used, of which 3 were paired with another adult or juvenile male, and 7 were paired with an adult female. Semen was collected from each male on up to 5 separate occassions in sterile glass tubes. PVS involved the application of successive sequences of increasing vibration to the penis using a FertiCare personal vibrator. Immediately following collection, pre-warmed Hepes-buffered TALP medium (200 µl) was added to the ejaculate. Sperm suspensions were evaluated for total sperm count and sperm motility. Ejaculates were obtained from male-paired males on every attempt (12 of 12), whereas 5 of 23 attempts failed to yield an ejaculate from female-paired males. The number of sequences of stimulation needed to obtain an ejaculate differed between males but was unaffected by animal pairing. The ejaculates collected from female-paired males had lower total numbers of sperm ($3.9 \pm 1.4 \times 10^6$ *v.* $10.1 \pm 2.2 \times 10^6$; $P < 0.05$) with a lower percentage motile ($35 \pm 9\%$ *v.* $85 \pm 13\%$; $P<0.01$), compared with those from male-paired males. We conclude that housing males separately from females increases the quantity and motility of sperm collected by PVS.

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THE EFFECTS OF PROGESTERONE ON ENDOMETRIAL ANGIOGENESIS IN PREGNANT AND OVARIECTOMISED MICE

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In mice, early pregnancy is associated with an increase in endometrial angiogenesis in preparation for the implanting embryo. The aims of this study were to quantify endometrial angiogenesis in pregnant mice and to investigate the role of progesterone in promoting endothelial cell (EC) proliferation in ovariectomised mice; we hypothesised that EC proliferation would increase with increasing plasma progesterone concentrations in pregnant mice and that progesterone would stimulate EC proliferation in ovariectomised mice, but only following oestrogen priming. Uterine tissue from CBA x C57 mice was collected on Days 1–4 of pregnancy (n = 4–5/day) when circulating progesterone concentrations are increasing but before implantation occurs. Prior to dissection, mice were injected with BrdU enabling proliferating EC to be quantified and localised within blood vessels by CD31/BrdU double staining immunohistochemistry. There was a significant increase in proliferating EC (Kruskal-Wallis statistic (KW) = 17.1, P = 0.002) on Day 3 of pregnancy (Days 1 and 2, no proliferation; Day 3,126.6 ± 45.6 proliferating EC/mm^2 (mean ± s.e.)), when plasma progesterone also began to increase (as measured by radioimmunoassay). To determine if the EC proliferation was due to progesterone, a second experiment was performed on ovariectomised mice. One group of mice (n = 6) were treated with a single injection of 100 ng of estradiol on day eight after 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 following BrdU injection on Day 13 following ovariectomy. Unexpectedly, mice treated with progesterone only had the highest amount of EC proliferation (114.7 ± 30.9 proliferating EC/mm^2); oestrogen priming was not required and actually significantly reduced progesterone induced EC proliferation (44.8 ± 15.5 proliferating EC/mm^2; KW = 13.8, P = 0.008). We are currently investigating the interaction between progesterone and VEGF using immunohistochemistry and inhibition studies.

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MATRIX METALLOPROTEINASES IN THE MOUSE MODEL OF MENSTRUATION: EFFECT OF DOXYCYCLINE INHIBITION

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Strong correlative evidence supports a role for matrix metalloproteinases (MMP) in the tissue breakdown at menstruation. Because menstruation occurs only in women and a few old-world primates, it has not been possible to examine the functional significance of potentially key mediators of this process. To this end, we developed a mouse model for menstruation (1), in which ovariectomised mice are subjected to a decidualising stimulus: injection of oil into the uterine lumen following appropriate hormone-priming. During the 24 h following withdrawal of progesterone (P), the decidualised tissue progressively breaks down, in a manner that morphologically resembles that of human endometrium at menstruation. The aims of the present study were to examine the pattern of MMP expression during the time from progesterone withdrawal until complete tissue breakdown, and to determine whether administration of doxycycline, (a known MMP modulator), 3 h prior to P withdrawal, affected the expression or activity of the MMPs or restrained the tissue destruction. MMP-3 was present at foci in the decidual zone: these were initially associated with the restructuring at decidualisation and subsequently with the tissue destruction. MMP-7 was detected both in epithelium and in leukocytes, predominantly neutrophils. These were first apparent in the basal zone during the earliest stages of tissue instability, and dramatically increased in numbers as breakdown progressed. MMP-9 was found only in leukocytes, predominantly neutrophils and some macrophages, with greatly increased numbers with time. Zymography revealed a dramatic increase in both latent and active MMP-9 as tissue breakdown proceeded. Doxycycline reduced immunoreactive MMP-3 but not MMP-7 or MMP-9 in the tissue, and also decreased gelatinase activity. However, no apparent effect on tissue breakdown was observed. Further studies with a more potent MMP inhibitor are required to fully establish the importance of MMPs in these processes.

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HUMAN ENDOMETRIAL CYCLE STAGES CAN BE DETERMINED BY GLOBAL GENE EXPRESSION PROFILING

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Endometrium is a dynamic tissue which undergoes cyclic changes each month, under the overall control of oestrogen and progesterone. The aims of this study were to investigate the changing global gene expression profile of human endometrium during the menstrual cycle using microarray technology and to determine the correlation between histopathological evaluation and molecular profile of the samples. Curettings of endometrium were collected from 43 cycling women and immediately snap frozen. The menstrual cycle was divided into seven stages by histological evaluation. Standard two-color cDNA microarrays were performed on the 43 samples against a common reference, using a 10.5 K cDNA glass slide microarray. Expressed genes were identified using a Scanarray 5000 UV laser scanner. Quantarray software was used to quantify the relative gene expression values. Normalisation and visualisation of the gene expression changes were performed using the GeneSpring software package. Hierarchical clustering of all 43 samples was performed, based on the expression profile of 571 genes, which were identified as differentially expressed by parametric ANOVA with Benjamini-Hochberg correction. The 43 samples were sorted into nine groups which all agreed with histopathology by either being in the same group or an adjacent group apart from four samples. For further analysis, the four outliers were removed, one group was excluded due to lack of replicates and two groups were merged to get the final molecular classification of the cycle. The statistical analysis was repeated and 1452 genes were identified as differentially expressed at $P \leq 0.05$. The data were also independently analysed by a CSIRO algorithm called GeneRave and the results from both methods were comparable. mRNA expression profiles of the genes TGF a (Hs.170009), NCR3 (Hs.509513) and FUT4 (Hs.390420) were verified using real-time PCR. We have shown for the first time that endometrial cycle stage prediction is possible based on global gene expression profile.

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LEUKOCYTE MATRIX METALLOPROTEINASE EXPRESSION IS REGULATED IN RESPONSE TO SELECTED CHEMOKINES: IMPLICATIONS FOR BREAK-THROUGH BLEEDING

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Break-through bleeding is a problem common to many women using long-acting progestin-only contraceptives such as Implanon, and is the main reason many women discontinue using them. Our previous studies (1) identified a number of chemokines (chemoattractive cytokines) that are highly expressed during the menstrual cycle with the capacity to selectively attract the leukocyte subsets present in each phase. Leukocytes produce factors thought to be critical for endometrial breakdown and remodelling, including matrix metalloproteinases (MMPs). We hypothesised that progestins act on endometrial epithelial cells to activate chemokine expression. The chemokines attract leukocytes into the tissue and stimulate their production of MMPs, contributing to tissue breakdown and break-through bleeding. The present study investigated the role of appropriately selected chemokines on leukocyte MMP production. The eosinophilic cell line EOL-1 and the mast cell line HMC1 were treated with four chemokines (fractalkine, HCC-1, MCP-3 and IL-8) at a range of concentrations. MMP production and activation were analysed by gelatin and casein zymography of culture medium. No response was observed from the mast cells under any of the treatments. However, there was a significant increase in latent MMP-9 production by eosinophils in response to increasing concentrations of IL-8 and MCP-3. Results show a greater than 2-fold increase in the amount of latent MMP-9 in response to IL-8 or MCP-3 (300 ng/mL and 80 ng/mL respectively) compared to non-treated controls. These chemokines had no significant effect on levels of active MMP-9 or latent and active forms of MMP-2. HCC-1 and fractalkine had little effect on either MMP-9 or MMP-2 production by these cells. These data support the hypothesis that MMP production by leukocytes is regulated, at least in part, by selective chemokines expressed during menstruation. Future studies will expand the leukocyte subtypes tested and investigate the role of synthetic progestins in chemokine expression from endometrial epithelial cells.

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IDENTIFYING MARKERS FOR STROMAL STEM/PROGENITOR CELLS IN HUMAN ENDOMETRIUM

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The endometrium is divided into upper functionalis, which rapidly grows then differentiates before being shed, and lower basalis, from which cyclical regeneration begins. A small proportion of endometrial stromal cells have been identified with clonogenic activity, a functional property of stem cells (1). We hypothesised that stromal stem/progenitor cells expressing known stem cell markers reside in the basalis. The aims of this study were to: *(1)* investigate the clonogenic activity of human endometrial stromal cell populations enriched and depleted for known stem cell markers, and *(2)* identify a marker that will differentiate basalis from functionalis stroma. Endometrial tissue acquired from 23 ovulating women undergoing hysterectomy was digested with collagenase to produce single cell suspensions. Leukocytes and epithelial cells were removed, and stromal cells analysed by flow cytometry, FACS sorted into enriched and depleted populations, and cultured for clonal analysis as described (1). Markers analysed included stem cell markers, STRO-1, CD133, CD45 and CD34, and an endometrial stromal cell marker, CD90 (2).

Immunohistochemical analysis of CD90 was performed on full thickness human endometrial tissue. $CD45^-$ endometrial stromal cell populations contained 2.13 ± 0.65% (n = 13) STRO-1^+, and 5.43 ± 1.42% (n = 16) $CD133^+$ cells. Stromal cell populations enriched (0.65 ± 0.42%) and depleted (0.95 ± 0.58%) for STRO-1 showed no significant difference (P = 0.19, n = 5) for clonogenic activity. Surprisingly, clonogenicity of $CD133^+$ stromal cells (0.74 ± 0.56%) was lower than $CD133^-$ (3.89 ± 1.35%) cells (P = 0.03, n = 6). Immunohistochemical staining showed strong CD90 staining in the functionalis, with lighter staining in the basalis. These observations were confirmed by flow cytometric analysis which identified two distinct populations (n = 9), $CD90^{low}$ (19.55 ± 4.35%) and $CD90^{hi}$ (74.71 ± 5.20%). Clonogenic analysis of these two populations is underway. Interestingly, dual-colour flow cytometry showed the $CD133^+$ cells to be $CD90^{low}$ (n = 7). Further analysis suggests that the $CD90^{low}CD133^+$ population are $CD45^-CD34^+$, suggesting endothelial progenitor cells. This study identified CD90 as a marker that distinguishes basalis and functionalis stroma, and demonstrated that STRO-1 and CD133 are not functional markers for clonogenic endometrial stromal stem/progenitor cells.

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ESTROGEN AND PROGESTERONE RECEPTOR EXPRESSION IS SIGNIFICANTLY REDUCED IN CULTURED MYOMETRIAL AND FIBROID SMOOTH MUSCLE CELLS

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Fibroids are benign neoplasms of the smooth muscle cells of the uterus. Cultured myometrial and fibroid smooth muscle cells (MSMC & FSMC) have been widely used as a model for the study of fibroid growth. However, there is ongoing controversy regarding expression levels of estrogen and progesterone receptors (ER and PR) in cultured cells vs tissue. The aim of the present study was to measure levels of mRNA for ERα and PR in myometrium and fibroid, and in cultured MSMC & FSMC. Myometrium and fibroids were collected from hysterectomy specimens (n = 8). Part of the tissue was snap frozen and the rest was used to isolate SMC, which were cultured for 3 passages and collected for RNA at P0 (2 weeks in culture) and P3 (5 weeks in culture). ERα and PR levels were quantified using real-time PCR and normalized using 18S rRNA as an internal control. Both ERα and PR were detected in all samples. Large variability in receptor levels between different isolates was detected. Surprisingly, despite large differences between the means, none of comparisons of tissue *v.* P0 cells were significant by non-parametric tests. However, there was a statistically significant reduction in both ERα and PR expression between whole tissue and isolated cells at P3 (Table 1).

Table 1. Mean ± s.e.m. ERAα or PR *v.* 18S rRNA

	Myo	Fib	MSMC P0	FSMC P0	MSMC P3	FSMC P3
ER α ($\times 10^{-3}$)	4.82 ± 0.97	7.18 ± 1.57	0.88 ± 0.68	0.21 ± 0.03	0.15 ± 0.03*	0.10 ± 0.03**
PR ($\times 10^{-3}$)	93.36 ± 24.28	67.77 ± 17.86	10.36 ± 2.19	18.62 ± 6.79	3.99 ± 1.04*	2.42 ± 0.61*

*P < 0.01; **P < 0.001, compared to matching tissue.

This is the first study to provide objective data to support a significant decline in ERα and PR expression in cultured MSMC and FSMC. Despite this decline, detectable levels of ERα and PR mRNA were present at both P0 and P3, potentially explaining why some published studies have been able to demonstrate *in vitro* response to steroids in these cells.

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MACROPHAGE INHIBITORY CYTOKINE-1 AT THE MATERNAL–FETAL INTERFACE IN EARLY HUMAN PREGNANCY

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Macrophage inhibitory cytokine-1 (MIC-1) is a transforming growth factor-β (TGF-β) superfamily member, first isolated from activated macrophages and subsequently localised in the human placenta. We previously reported that decreased circulating levels in very early pregnancy are associated with subsequent miscarriage. We undertook these current *in vitro* studies to investigate possible roles for MIC-1 in early pregnancy: *(1)* regulation of placental matrix metalloproteinase-2 and -9 (MMP-2 and -9); *(2)* effect on placental apoptosis; and *(3)* regulation of endometrial stromal cell decidualisation. *(1)* First trimester placental explant cultures were treated with 100–200 ng/mL MIC-1 ± 1/1000 (v/v) anti-MIC-1 antibody. MMP-2 and -9 were measured by gelatin zymography. MMP activation via the plasminogen activation pathway was examined by measuring mRNA expression for urokinase plasminogen activator and its receptor (uPA, uPAR) and type-1 plasminogen activation inhibitor (PAI-1). *(2)* In first trimester trophoblast explants, apoptosis was induced *in vitro* with tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) ± 200 ng/mL MIC-1. The pro-apoptosis factor caspase-3 was localised by immunohistochemistry. *(3)* Using an established model of oestrogen and progesterone induced endometrial stromal cell decidualisation, MIC-1 production was measured and correlated with morphological changes. Cultures were also treated with 20 ng/mL MIC-1. MIC-1 treatment inhibited activation of both MMP-2 and MMP-9 while treatment with anti-MIC-1 antibody blocked the inhibition. uPA, uPAR and PAI-1 mRNA did not change with either treatment. MIC-1 treatment mitigated TNF-α/IFN-γ induced trophoblast apoptosis. MIC-1 production increased during induced decidualisation and MIC-1 treatment facilitates further decidualisation in this model. MIC-1 appears to have a number of potentially important functions in the human placenta and decidua consistent with physiological roles in normal placentation. Whether these functions are key to successful pregnancy remains to be studied.

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ROLE OF HOMEOBOX GENE *HLX* EXPRESSION IN NORMAL PLACENTAL DEVELOPMEMT

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In a screen for homeobox genes in the human placenta, we cloned and characterised *HLX1* (also known as *HB24*) (1). Furthermore, we provided evidence that *HLX1* may be a regulator of human placental develolpment (2). We have since shown that the mouse homologue of *HLX1*, called *Hlx*, is expressed in the murine placenta. *In situ* mRNA hybridisation studies and antibody localisation of *Hlx* revealed expression in the labyrinth layer (LL), secondary giant cells (GC) and in the spongiotrophoblast layer (STL) (3). The STL is required for structural support of the placenta. Targeted gene mutation of *Hlx* resulted in embryonic defects in the developing gut and the liver (4) but the effects on placental development were not investigated. Histological preparations of placental tissues collected from Days 10.5, 13.5 and 19.5 from *Hlx* mutant mice were investigated for morphological changes. Our preliminary observations reveal that by haematoxylin and eosin staining the STL of the mutant murine placenta is severely disrupted but the overlying GC layer appears to be unaffected. Endogenous alkaline phosphatase staining of the LL further confirmed that the highly vascularised LL where fetal- maternal exchange occurs, is disorganised and expands into the region normally occupied by the STL. These observations indicate that *Hlx* is essential for normal placental development.

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DECREASED EXPRESSION OF OESTROGEN RECEPTOR β IN THE REPRODUCTIVE TRACT OF PREGNANT RELAXIN-DEFICIENT (*Rlx*$^{-/-}$) MICE

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The peptide hormone relaxin (RLX) is reported to directly affect uterine oestrogen receptors (ERs) in the rat (1). Treatment of immature ovariectomised rats with porcine RLX causes a decrease in uterine ERβ mRNA levels within 6 h. However, RLX has no effect on ERα expression. As both ERβ1 and ERβ2 inhibit ERα-mediated transcriptional activity, this RLX-induced downregulation in ERβ could be a prerequisite for oestrogen to exert its effects on target tissues. The aim of the current study was to use relaxin-deficient (*Rlx*–/–) pregnant mice to investigate if relaxin deficiency results in alterations in either ERβ or ERα mRNA expression in reproductive tissues. Cervix and vagina tissues were obtained from adult C57/Blk6J wild-type mice at five stages of gestation (Days 7.5, 10.5, 14.5, 17.5, 18.5 pc) and *Rlx*–/– littermates on Days 7.5, 14.5 and 18.5 pc. Q-PCR with TaqMan probes in the Opticon 2 thermal cycler (MJ Research, GeneWorks) was used to quantify ERα and ERβ gene expression. ERα mRNA levels were significantly ($P < 0.05$; ANOVA) increased in the cervix/vagina on Days 17.5 and 18.5 pc in *Rlx*+/+ mice. The increase in ERα in *Rlx*+/+ mice was negatively correlated with a significant decrease in ERβ expression from Day 14.5 pc. In contrast, there was no decrease in ERβ gene expression in the cervix/vagina of *Rlx*–/– mice; ERβ mRNA levels were significantly ($P < 0.05$) higher compared to *Rlx*+/+ mice on Days 14.5 or 18.5 pc. However, there was no corresponding reduction in ERα expression in the cervix/vagina of the *Rlx*–/– mice, so that ERα mRNA levels were still elevated at term despite the maintenance of high ERβ expression. In summary, these data show changes in ERβ expression in the cervix/vagina of relaxin-deficient mice, which may subsequently affect ERα-mediated transcriptional activity.

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INUSLIN-LIKE GROWTH FACTOR TREATMENT OF PREGNANT GUINEA PIGS DURING EARLY PREGNANCY PROMOTES FETAL GROWTH

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Insulin-like growth factor (IGF)-II is an important regulator of growth in many tissues and is abundantly expressed in the placenta during pregnancy. Gene ablation studies performed in mice have shown that IGF-II deficiency results in both impaired fetal and placental growth, whereas deficiency in IGF-I reduces fetal growth only. Conversely, maternal IGF supplementation in early pregnancy in the guinea pig increases placental and fetal size by mid pregnancy. This study aimed to determine whether these anabolic effects persist into late pregnancy after cessation of treatment. On Day 20 of pregnancy, mothers were anaesthetised and a mini osmotic pump was implanted subcutaneously, to deliver 1mg/kg/day IGF-I (n = 7), IGF-II (n = 9) or vehicle (n = 7) for 17 days. Guinea pigs were killed on Day 62 of pregnancy (term ~67 days). Fetal and placental weights, and maternal and fetal body composition, were measured. Total litter size was unaffected by IGF treatment; however, IGF-II increased the number of viable fetuses by 26% (P = 0.01). After adjusting for the number of viable pups per litter, maternal IGF treatment increased fetal growth by increasing abdominal circumference, crown-rump length and fetal weight (fetal weight: IGF-I 79+/–2.5 g; IGF-II 78+/–2.6 g; vs vehicle 68+/–2.5 g, P = 0.02). IGF treatment did not alter absolute or relative fetal organ weights. IGF-I reduced placental weight by 9% and IGF-II increased it by 9%, but not significantly. IGF-I increased the fetal weight : placental weight ratio (19+/–0.9 vs 15+/–0.9, respectively P = 0.043). IGF treatment did not affect maternal weight gain during pregnancy nor net carcass weight; however, IGF-I reduced maternal lung and adipose tissue weights. In conclusion, maternal IGF-II treatment during early pregnancy improved fetal growth into late gestation, possibly by modulating placental efficiency. As poor placental development is implicated in fetal growth restriction, increasing maternal IGF abundance in early to mid pregnancy may be a potential therapeutic approach to placental insufficiency.

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SEMEN EXPOSURE IN EARLY PREGNANCY IMPACTS FETAL AND NEONATAL GROWTH

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Optimal uterine receptivity is critically important to embryo implantation and subsequent development of the fetus and placenta. Modulators of the uterine cytokine and immune environment are present in seminal plasma. To determine the extent to which seminal plasma programs uterine receptivity and subsequent fetal growth, an embryo transfer model was developed. Embryos were transferred to female recipients prepared by mating with either vasectomised (vas) males or vasectomised males from which seminal vesicles, the major source of seminal plasma, were removed (vas+svx). Placental and fetal parameters were measured at Day 18 of pregnancy, and an additional cohort of embryo recipients progressed to birth to allow growth trajectories and body composition measurement in progeny. At Day 18 of pregnancy, the number of viable implantation sites was unaffected but fetal weight was reduced by 4% (P = 0.05) when females were mated to vas+svx males compared with control pregnancies. Placental weight was not affected. Histological analysis of Day 18 placentas showed decreases in the total mid-sagittal cross sectional area and that of labyrinthine (nutrient exchange) tissue of placentas derived from vas+svx mated females. In term experiments, neonates from matings with vas+svx males were 12% smaller 24 h after birth (P < 0.001) and 14% smaller at 8 days (P < 0.001). Mice derived from vas+svx matings exhibited lower weights compared to those from vas matings until 14 weeks of age. The data demonstrates a critical role for semen in preparing the pre-implantation uterine environment so as to optimise subsequent growth of the fetus and neonate. The effects of seminal plasma are likely to be mediated through the maternal immune response and cytokine expression which influence placental morphogenesis and nutrient transfer function. Our findings have relevance to assisted reproduction programs, where pregnancies are routinely initiated in the absence of female tract exposure to semen.

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CALMODULIN-DEPENDENT NUCLEAR IMPORT PATHWAY OF THE TESTIS-DETERMINING FACTOR SRY

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Modulation of the nuclear entry of transcription factors (TFs) and chromatin components is a means by which eukaryotic cells can regulate gene expression in response to extracellular signals and the cell cycle during differentiation and development. TFs and chromatin components access the nucleus through nuclear localisation sequences (NLSs), which mediate interaction with components of the cellular nuclear import machinery, such as members of the importin superfamily. The Ca^{2+}-binding protein calmodulin (CaM) has previously been shown to bind at or near NLSs in several nuclear-localising proteins that have important roles in testis development including the Y chromosome-encoded HMG-domain-carrying chromatin remodelling factor SRY, and related factor SOX9, both of which are key regulators of gonadal development. SRY function in the nucleus of somatic cells of the fetal gonad, in particular, is essential for development of a testis in males. Here we present new findings implicating a role for CaM in modulating SRY nuclear accumulation, whereby treatment of transfected cells with CaM antagonists significantly reduces nuclear accumulation of green fluorescent protein (GFP)-fusion proteins encoding either full length SRY or the SRY HMG domain alone. An *in vitro* nuclear transport assay using bacterially expressed fluorescent proteins showed similar results, with native gel electrophoresis/fluorimaging and fluorescence polarisation assays, indicating direct binding of CaM to the SRY HMG domain in Ca^{2+}-dependent fashion. Since clinical mutations resulting in sex reversal occur within SRY's CaM-binding NLS, these results may shed new insight into CaM-dependent pathways of nuclear protein import, and how this may relate to testis development.

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TARGETS OF THE ACTION OF NUCLEAR TRANSPORT FACTORS IN SPERMATOGENESIS

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During spermatogenesis, precise and orderly switches in gene expression are required. The movement of transcription factors (TFs) and nuclear proteins into and out of the nucleus is highly regulated, thus determining the extent and timing of gene expression. Most nuclear transport events are mediated by members of the importin superfamily that specifically recognise their cargoes and facilitate the passage of receptor-substrate complexes through the nuclear pore complex (NPC) which is made up of nucleoporin proteins. Eight human importin α isoforms are known that function in heterodimeric form with importin β whilst there are 20 members of the importin β family, which mediate the nuclear import or export of a very diverse set of protein or RNA cargoes. Understanding of importin and TF/chromatin component interaction during spermatogenesis should identify potential developmental switches, critical steps in the spermatogenic process. We are interested in the expression of different importins during spermatogenesis, and their specific nuclear import/export substrates as candidates in developmental switches. Preliminary analysis has shown that the nuclear import factors importin β1 and 3 are not only expressed in germ cells, but also alter their cellular distribution during maturation. In a yeast two-hybrid screen, using truncated importin β3 as bait and a library made from adult mouse testis, we identified a transcriptional repressor gene involved in cell cycle regulation, and an enzyme of the purine nucleotide biosynthesis pathway as candidate binding partners. Further studies will focus on elucidating the biological significance of these interactions in spermatogenesis.

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THE ROLE OF PP60C-SRC TYROSINE KINASE IN SPERM CAPACITATION

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A protein kinase A-dependent tyrosine phosphorylation pathway in mammalian spermatozoa has been demonstrated to exist, and is unique to this cell type. As PKA is incapable of directly phosphorylating substrates on tyrosine residues, much research has focused on the identification of an intermediary tyrosine kinase which can be activated by serine/threonine phosphorylation via PKA. Inhibitory studies using genistein, tryphostin, erbstatin and herbimycin A, have demonstrated that the src family of kinases may be responsible for the tyrosine phosphorylation events seen during capacitation (1). Although one src family member, c-yes, has been implicated as the kinase responsible for these events (2), this has since been disputed (3). Another src family member, pp60c-src, can be activated by phosphorylation on Ser-17 by cAMP-dependent protein kinase and Ser-12 by calcium-phospholipid dependent protein kinase C (4), and may be the intermediary tyrosine kinase of interest. Western blot analysis demonstrated the presence of pp60c-src in rat sperm samples isolated from the caput and cauda epididymis. Furthermore, co-immunoprecipitation studies revealed a number of pp60c-src-associated proteins including outer dense fibre 2 (ODF2) and A-kinase anchoring protein 4 (AKAP4). Interestingly, both of these proteins become phosphorylated during capacitation of mouse sperm (data not shown) and AKAP4 is tyrosine phosphorylated in capacitated human sperm (5). These data implicate pp60c-src kinase activity in the phosphorylation of a number of sperm midpiece proteins, which may regulate hyperactivation during capacitation. Further research focusing on the activity of pp60c-src in non-capacitated and capacitated sperm will be conducted.

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BIOACTIVE ACTIVIN AND ITS AFFECTS ON MURINE TESTIS DEVELOPMENT

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Activin is a member of the TGF β (Transforming Growth Factor β) superfamily of ligands which influence many aspects of male germ cell development. Formed by the linkage of two common β subunits, activin A (a βA:βA dimer) has been reported to *(1)* cause apoptosis of primodial germ cells, *(2)* inhibit the cellular transition of gonocytes into undifferentiated spermatogonia, and *(3)* enhance FSH-mediated stimulation of Sertoli cell proliferation in the newborn rat testis. Although closely related, activin βA and activin βB (a βB:βB dimer) differ in that activin βB is less bioactive. In this study we examined the role of activin during the first wave of spermatogenesis using knockout ($Inhba^{-/-}$) and transgenic ($Inhba^{BK/BK}$; two copies of the *βB* subunit gene coding sequence replace the *βA* coding sequence) mouse models with reduced levels of bioactive activin. Absolute gonocyte and Sertoli cell numbers were significantly elevated in the absence of activin A in newborn $Inhba^{-/-}$ testes compared to wild type, as determined by optical disector analysis. As the $Inhba^{-/-}$ mice die at birth, we next studied the BK/BK mice to examine postnatal effects of reduced activin bioactivity. Surprisingly, both body weight and testis weight were lower in the BK/BK compared to wild type mice at Day 7 and 14, but testis growth in proportion to body weight was significantly reduced between 7 and 14 days. At 2 weeks of age, the BK/BK animal displays a significant reduction in Sertoli cells and specific subpopulations of germ cells, the latter of which was evident only in heterozygote animals. Examination of these two models has identified that lower levels of bioactive activin affect Sertoli cells and germ cells at different stages of testicular development. Our ongoing studies involving RNA analyses of various candidate target genes will facilitate a greater understanding of the molecular basis for these observations.

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INFERTILITY IN MICE WITH NULL MUTATION OF THE EGR-1 TRANSCRIPTION FACTOR

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Female infertility has been reported in two lines of mice with mutation of the *Egr-1* gene. One underlying cause of this defect is deficient LH production by pituitary gonadotropes. However, Egr-1 is also acutely regulated by both FSH and LH in ovarian granulosa cells (1). A role for this transcription factor in regulating gonadotrophin responsive target genes and ovarian function is hypothesised. Indeed the LH-receptor is a proposed target of Egr-1 regulation, but this has not been investigated in detail *in vivo* and is difficult to reconcile with the pattern of Egr-1 expression.

In this study, the role of Egr-1 within the ovarian follicle was investigated using exogenous gonadotropin replacement in *Egr-1*$^{-/-}$ mice . Adult *Egr-1*$^{-/-}$ female mice superovulated by sequential PMSG and hCG stimulation and mated with proven male breeders failed to produced offspring while 90% of heterozygous females got pregnant and produced litters (7.4 ± 2.9 pups per litter) within 22 days of stimulation. Recovery of oocytes from oviducts of immature superovulated mice revealed a reduced ovulation rate in null females (6.3 ± 3.8 oocytes) compared to their heterozygous (18.0 ± 6.5) and WT (17.8 ± 6.8) littermates. Gross morphology and histology of exogenously stimulated ovaries were indistinguishable from their heterozygous or WT counterparts. Surprisingly, no alteration was detectable in the mRNA expression of previously reported direct Egr-1 responsive genes, namely LH-receptor and membrane prostaglandin E synthase (mPGES). Nor were mRNA for two critical ovulatory genes with putative Egr-1 response elements, ADAMTS-1 or versican V1 altered. Temporal and spatial expression of genes involved in ovarian steroidogenesis, P450scc and Cyp17 and LH-receptor, were indistinguishable from normal littermates during exogenously controled follicular development.

Combined observations of acute Egr-1 induction by gonadotropins, reduced ovulation and complete infertility suggest an important role for Egr-1 in ovarian function. However, genes identified as targets of Egr-1 regulation in other studies proved to be Egr-1 independent in this model.

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REPRODUCTIVE CONSEQUENCES OF CIRCADIAN DYSFUNCTION: FERTILITY IN THE *BMAL1* NULL MOUSE

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Circadian rhythms are generated by a suite of genes called clock genes that are expressed in the brain and also in many peripheral tissues. In the peripheral tissues, these genes assist in regulating the expression of many genes involved in cell growth, angiogenesis and development. *Bmal1* is a critical gene involved in circadian rhythm generation. Here we report on the fertility and fecundity of *Bmal1* knockout mice (*Bmal1*$^{-/-}$). Male *Bmal1*$^{-/-}$ mice have impaired fertility compared to controls [(litters produced/number of animals) wild type (5/5), CBA controls (5/5), *Bmal1*$^{-/-}$ (1/15)]. Fifty percent of male *Bmal1*$^{-/-}$ mice had defective caudal sperm, showing sperm that was both non-motile and malformed. Seminal vesicle weight was significantly reduced in the *Bmal1*$^{-/-}$ mice (50% reduction) in males at both 4 and 5.5 months old. Female *Bmal1*$^{-/-}$ mice had irregular oestrus cycles and failed to maintain a pregnancy to term following natural mating [(litters produced/number of animals) wild type (5/5) CBA controls (5/5) *Bmal1*$^{-/-}$ (0/5)]. When embryos were flushed from the uterus 4 days after natural mating, there was a reduced number of released oocytes and a reduced development to blastocysts in the *Bmal1*$^{-/-}$ female mice. Following a standard PMSG/HCG super ovulation protocol, *Bmal1*$^{-/-}$ mice showed both a reduction in ovulation rate as well as a slowed progression of embryos to blastocyst stage (Table 1).

Table 1. Embryo development following superovulation of *Bmal1*$^{-/-}$, *Bmal1*$^{+/-}$ and *Bmal1*$^{+/+}$ mice

	Fertile matings (%)	Embryos recovered	Degenerating/unfertilised (%)	2 cell-morula (%)	Blastocyst (%)	Hatching blastocyst (%)
Bmal1$^{+/+}$	50	31 ± 5	9	32	58	1
Bmal1$^{+/-}$	94	33 ± 4	13	36	46	5
Bmal1$^{-/-}$	60	20 ± 5	2	64	31	3

These results suggest that disruption of a key clock gene has detrimental consequences on fertility in the mouse. Further, this reduction in fertility appears to be acting at multiple levels. Continued investigation into the importance of rhythm genes in reproductive function is required.

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A NOVEL SCAVENGER RECEPTOR DOMAIN CONTAINING GENES DIFFERENTIALLY EXPRESSED IN THE EMBRYONIC MOUSE TESTIS

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Sex in mammals is determined by the constitution of the sex chromosomes: two X-chromosomes results in the development of ovaries and a female phenotype, while an X and a Y chromosome result in testis development and a male phenotype. The *SRY* gene on the Y chromosome has been identified as the genetic switch, which initiates testis formation. The importance of *SRY* and the related *SOX9* gene in sex determination has been well characterised in humans and mouse. Mutations in *SRY* and *SOX9* account for approximately 20% of sex reversed XY-female patients suggesting that other genes are required for normal testis development. The mouse model was used to identify and characterise differentially expressed genes in the embryonic testis/ovary that may represent new sex determining candidates.

A suppression subtraction hybridisation screen was conducted on testes/ovaries mouse cDNA between 12.0-12.5 *dpc*. Differential clones were sequenced, bioinformatic analysis was performed and expression patterns verified by whole mount *in situ* hybridisation (WISH) on 12.5 *dpc* mouse partial embryos.

We identified a novel gene (B7) containing a scavenger receptor domain of unknown function. B7 was expressed in the gonads of both sexes from 11.5-12.0 *dpc*. From 12.5 -13.5 *dpc*, B7 expression was present in the testis but not in the ovary. We termed B7 Testis Expressed Scavenger Receptor (Tesr). Expression of *Tesr* was also seen in the mouse brain, eye, head, heart, neural arch and cartilage primordium. Furthermore, *Tesr* expression was analysed in the chicken embryo. Low expression was seen in the male gonads and mesonephric tubules from day 3.5-7.5. *Tesr* was expressed in chicken embryo's heart, notochord, neural tube, dorsal aorta and the somites at day 3.5 and 4.5.

Tesr may play a role in phagocytosis of apoptotic cells and may be involved in scavenger, adhesion, defence functions and immune responses. Another possible role for *Tesr* in the developing testis may be cholesterol delivery for steroidogenesis.

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DIFFERENTIAL EXPRESSION OF THE RELAXIN RECEPTOR (LGR7) IN THE MAMMARY APPARATUS OF THE LACTATING TAMMAR WALLABY (*MACROPUS EUGENII*)

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Growth and development of the mammary apparatus (nipple and mammary gland) are important aspects of lactation. Macropodid marsupials can suckle young of two different ages simultaneously, a phenomenon known as asynchronous lactation. As a result, the type of milk produced and the structure of the two mammary glands supporting young of different ages vary considerably. A role for the peptide hormone relaxin in lactation has been demonstrated in relaxin receptor (LGR7)-deficient mice, which fail to deliver milk to their offspring due to impaired nipple development (1). This study investigated the distribution of LGR7 in the different mammary glands and nipples during asynchronous lactation in the tammar wallaby. The specific aim was to determine if the age of the pouch young influences LGR7 gene expression. Tissues were collected from the mammary apparatus sustaining the neonate and an older pouch young in the same mother, between Days 0 and 21 of lactation (n = 5/stage). A partial sequence (250-bp) of the tammar LGR7 was first obtained from a region close to the N-terminus of the soluble ectodomain, with 82% amino acid homology compared to the human LGR7 sequence. LGR7 gene expression was then measured by quantitative-PCR, using a TaqMan probe in the Opticon 2 thermal cycler (MJ Research, GeneWorks). Expression of LGR7 was upregulated in both the nipple and mammary gland supporting the neonate between 5 and 11 days after birth. There was no difference in LGR7 expression between these two tissues. However, LGR7 mRNA concentrations were significantly ($P < 0.05$: paired t-test) higher in the mammary apparatus supporting the neonate compared with that of the older young. These data suggest that a local stimulus, such as the continuous sucking by the neonate, may influence LGR7 expression in the mammary apparatus.

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EFFECTS OF PROSTAGLANDINS ON SOCS EXPRESSION IN T-47D BREAST CANCER CELLS

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There is increasing evidence to suggest that prostaglandins can upregulate suppressor-of-cytokine-signalling (SOCS) expression and so modify cellular responses to cytokines. Here we examined this possibility in two breast cancer cell lines. Initially we characterised prostaglandin receptor expression by reverse transcription-PCR, and found that T-47D cells express EP2, EP3 and EP4 receptors but not FP or EP1 receptors whereas MCF-7 cells expressed EP1 and EP4 receptors. A range of prostaglandin agonists were then used to elucidate whether prostaglandins affect SOCS expression and the receptor subtypes involved. SOCS 1-3 and CIS expression were measured by Real-Time PCR. In MCF-7 cells, PGE2 caused only minor increases in SOCS1 and SOCS3 expression. However in T-47D cells, PGE2 strongly induced SOCS3 expression with 2- and 5-fold increases in mRNA at 30 and 60 min respectively, returning to baseline at 120 min. SOCS1 was also upregulated at 30 and 60 min (3- and 5-fold respectively) and remained elevated (6-fold) at 120 min. In contrast, CIS and SOCS2 were not induced. Cloprostenol, Butaprost, Latanoprost and Sulprostone had no effect on SOCS expression, suggesting that the PGE2 response is mediated via the EP4 receptor. The induction of SOCS expression by PGE2 was not due to increased STAT3- or STAT5-tyrosine phosphorylation, and indeed we observed a decrease in STAT5 tyrosine phosphorylation 10 min and 1 h after PGE2, as determined by ip/western blotting. In summary, we propose that prostaglandin-induced SOCS1 and 3 expression in T-47Ds could constitute a means whereby cellular resistance to PRL is induced. *Supported by the ARC.*

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EXPRESSION OF HTRA1, 2 AND 3 IN HUMAN ENDOMETRIAL CANCER

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The mammalian HtrA family consists of serine proteases with distinct domains homologous to the bacterial high temperature requirement factor (HtrA). Three human HtrA members have been reported: HtrA1 (PRSS11 or L56), HtrA2 (OMI) and HtrA3 (PRSP). The function of HtrA1 is not well characterised, but it has been shown to be downregulated in malignant tissues (1–3) indicating that the downregulation of HtrA1 is associated with cancer progression. HtrA2 regulates apoptosis by interacting with X-linked inhibitors of apoptosis (XIAP) thus preventing the caspase-inhibitory function of XIAP (4). The function of newly identified HtrA3 is not known, however it shares a high degree of sequence and domain homologies with HtrA1 and may therefore share a functional similarity with HtrA1 (5). Endometrial cancer (EC) is a prevalent gynaecological cancer, commonly affecting women after menopause. In this study we examined the expression of HtrA1, 2 and 3 in EC. Reverse transcriptase-PCR (semi-quantitative) analysis showed decreased mRNA expression of both HtrA1 and HtrA3, but no significant change for HtrA2, in EC tissue samples compared to normal endometrium. We then determined the protein level of expression and the cellular localisation of all three HtrA members in EC progression using immunohistochemistry. HtrA1 and HtrA3 showed a similar pattern of expression and both decreased dramatically with the progression of cancer from grade 1 through to 3. Surprisingly, HtrA2 protein expression was also decreased with cancer progression, but the decline was not as dramatic as that for HtrA1 and HtrA3. Interestingly, considerably less staining was observed for all three HtrA proteins in grade 3 cancer tissues. These data suggest that decreased expression of HtrA proteins, particularly HtrA1 and HtrA3, is associated with the progression of endometrial cancer.

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INCREASED PERINATAL MORTALITY FOLLOWING RESTRICTION OF PLACENTAL AND FETAL GROWTH

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Intrauterine growth restriction and subsequent low birth weight in humans are associated with increased perinatal mortality and morbidity. Impaired placental function is a major cause of IUGR in humans, but its impact on perinatal survival has not been clearly defined. We have therefore investigated the effect of restriction of placental and fetal growth on perinatal survival and behaviour in the neonatal lamb. Placental growth was surgically restricted (PR) by removal of the majority of endometrial implantation sites prior to pregnancy, leaving either 6 to 7 (moderate PR) or 3 to 4 (severe PR) visible caruncles in each uterine horn, and ewes were mated following at least 10 weeks recovery. Perinatal outcomes (stillbirths and neonatal death before 3 days of age) were recorded in a cohort of 48 control (30 singleton, 18 twin) and 28 moderate PR (14 singleton, 14 twin), and 21 severe PR (11 singleton, 10 twin) lambs, and effects of PR or twinning were evaluated by Chi-square analysis. Rates of stillbirth ($P = 0.006$) and total perinatal deaths ($P < 0.001$) were higher in severe PR pregnancies than in control or moderate PR lambs, overall (see Table).

		Control	Moderate PR	Severe PR
Singletons	Stillborn	1/30	0/14	2/11
	Neonatal death	2/30	1/14	1/11
Twins	Stillborn	4/18	1/14	7/10
	Neonatal death	3/18	2/14	1/10

Similarly, severe PR increased stillbirths and perinatal deaths in twins alone ($P = 0.003$ and $P = 0.015$ respectively), but the effects of PR were not significant in singletons ($P = 0.10$, $P = 0.26$ respectively). Twinning increased stillbirths and perinatal deaths overall ($P = 0.002$, $P = 0.001$) and in control lambs alone ($P = 0.038$, $P = 0.017$). Restricted fetal growth due to twinning or severe surgical restriction of placental growth thus decreases perinatal survival, due to increased stillbirths. We will further investigate the characteristics of neonatal morbidity following PR by recording neonatal behaviour, including time taken to stand and suckle, in a subsequent cohort of control and PR lambs.

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NUCLEAR FACTOR κB DOWNREGULATION IN HUMAN T-CELLS IS ESSENTIAL FOR THE MAINTENANCE OF THE CYTOKINE PROFILE REQUIRED FOR PREGNANCY SUCCESS

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Human pregnancy is associated with a shift away from Th-1 type and a bias towards Th-2 type immune responses. The molecular mechanisms that regulate this are unknown. We assessed the expression and activity of Nuclear Factor (NF)-κB, a transcription factor that plays a central role in regulating immune responses. Nuclear and cytoplasmic fractions were prepared from isolated T-cells from non-pregnant and pregnant females and subjected to Western blotting to assess NF-κB and its' inhibitors IκBα and β expression. NF-κB activity in nuclear extracts was determined by Electrophoretic Mobility Shift Assays. Isolated T-cells were pre-incubated with/without the NF-κB translocation inhibitor SN50 and subsequently stimulated with PMA/ionomycin in the presence of the protein transport inhibitor Brefeldin A. Cytokine production was determined using flow cytometry.

Our results demonstrated high levels of immunoreactive NF-κB (p65) in all nuclear fractions of T-cells from non-pregnant females. In contrast, low levels of p65 were detected in nuclear fractions of T-cells from pregnant females. Levels of IκBα and β were also higher in cytoplasmic fractions of T-cells from non-pregnant than from pregnant females. The reduction in p65 levels in pregnancy was consistent with reduced levels of active NF-κB in T-cells from pregnant relative to non-pregnant females. Stimulation of T-cells from non-pregnant females with PMA/ionomycin resulted in IκBα degradation, p65 translocation and subsequent production of Th-1 cytokines IFN-γ and IL-2. In contrast, PMA stimulation had no effect on NF-κB activity in T-cells from pregnant females and a reduced effect on IFN-γ and IL-2 production. In the presence of SN50, IFN-γ and IL-2 production by T-cells from non-pregnant females was attenuated demonstrating a specific role for NF-κB in the production of these Th-1 cytokines. We can therefore conclude that, specific down-regulation of NF-κB in T-cells in pregnancy is an essential requirement for maintaining the cytokine profile necessary for pregnancy success.

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DIFFERENTIAL EXPRESSION OF PLASMINOGEN ACTIVATION CASCADE COMPONENTS IN HUMAN PRETERM DELIVERY WITH AND WITHOUT PRETERM PREMATURE RUPTURE OF THE FETAL MEMBRANES

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Previous studies from our laboratory have shown that plasminogen activation cascade (PAC) components are involved in the rupture of fetal membranes at term. The aim of this study was to test the levels of 4 key components of the PAC in fetal membranes collected from preterm deliveries (PTD) with and without preterm premature rupture of the fetal membranes (PPROM). In contrast to previous studies of the PAC in fetal membranes which have focused on labour at term, this study has examined PTD with PPROM (n = 10) and PTD without PPROM (n = 10).

Using immunohistochemistry, immunoblotting and zymography, we examined the expression and activity levels of key components of the PAC; plasminogen, PAI-2, uPA and uPAR for expression and plasminogen, plasmin and uPA for activity. The data presented here show significant changes in the expression and activity of PAC components in PTD + and – PPROM samples. When compared to term labour control samples both PTD + and – PPROM show increased expression and activity of PAC components. When PTD + and – PPROM are compared to each other differences in expression and activity can be seen. uPA expression and activity increases in PTD – PPROM, relative to term delivery samples, and increases further in PTD + PPROM samples. Immunohistochemical analysis reveals the uPAR is expressed at very low levels in the amnion epithelium, basement membrane and mesenchyme/intermediate zone of PTD + PPROM but is highly expressed in PTD – PPROM.

These data demonstrate differential expression of the PAC in PTD + PPROM cases compared with PTD – PPROM cases, which suggests different aetiologies and/or mechanistic pathways for the two types of PTD. These differences may be of importance in defining optimal clinical management of the two situations.

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LABOUR-ASSOCIATED CHANGES IN HUMAN FETAL MEMBRANES AND PLACENTAL ADRENOMEDULLIN MRNA EXPRESSION

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We have recently shown that total adrenomedullin (AdM) concentrations are elevated in choriodecidual and amniotic tissues, but not in placental, in response to human labour at term and pre-term (1). Therefore, the present study was designed to determine whether AdM mRNA expression was increased with labour in term and pre-term samples by using Northern Blot Analysis. Placentas were collected either at elective caesarean section (not-in-labour, NIL), after normal vaginal deliveries and at caesarean section during labour (in-labour, IL) from women with singleton pregnancies at term (>36 weeks gestation, 39.2 ± 0.2) or pre-term (between 24 to 36 weeks gestation, 32 ± 0.4), but with otherwise uneventful antenatal courses.

There were significant labour-induced increases in AdM mRNA expressions in amnion and choriodecidua in both groups, pre-term and term.

Relative abundance of AdM mRNA expressed as a ratio to 18S rRNA (mean ± s.e.m.)

	Pre-term NIL	Pre-term IL	Term NIL	Term IL
Amnion	1.4±0.15, n = 13	2.4±0.9*, n = 10	3.4±0.7, n = 11	10.1±1.3*, n = 10
Choriodecidua	5 ±0.5, n = 10	13.1±1.2*,n = 15	7.5±0.7, n = 15	14.1±1.1*, n = 10
Placenta	13.7±1.9, n = 10	19 ± 2*, n = 10	13 ± 1.9, n = 10	14.1 ± 1.5, n = 11

*Significantly increased compared with corresponding NIL group (ANOVA).

No difference was found in those who had vaginal deliveries with those who had caesarean sections after the commencement of labour at pre-term. Amongst term samples in labour all subjects were delivered vaginally uneventfully, except two cases where forceps were used in the third stage of labour.

AdM mRNA levels are increased in association with both term and pre-term labour, both in amnion and choriodecidua. We postulate that increased production of AdM by fetal membranes in association with labour may be involved in fetal and/or maternal adaptations to labour. For example, AdM may compensate for local-acting vasoconstrictor substances that are increased during labour, or it may act on the fetal lung in preparation for extra-uterine life.

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