

ORALS - invited

001

THE ROLE OF NON-CODING RNA IN HUMAN DEVELOPMENT

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It appears that we have fundamentally misunderstood the nature of genetic programming in humans and other multicellular organisms for the past 50 years because of the presumption, largely correct in prokaryotes but not in complex eukaryotes, that most genetic information is transacted by proteins, which form the main analog components of all cells. Humans have the same number of protein coding genes (19,500) as the nematode worm (~19,300), which has only 1,000 cells. Although only 1.2% of the human genome encodes proteins, the vast majority is actually transcribed in a developmentally regulated fashion, much of it on both strands. These transcripts include tens if not hundreds of thousands of small RNAs, including miRNAs, snoRNAs, piRNAs and other yet-to-be-discovered classes of regulatory RNAs, many of which are encoded in introns, and longer noncoding RNAs that exhibit dynamic expression patterns during germ cell and ES cell differentiation, gonadal development, muscle development, brain development, and macrophage and T-cell activation, to name a few. Many are dysregulated in disease, including neurological diseases and cancer. It is also now evident that most, if not all, complex genetic phenomena in the higher organisms are directed by RNA signaling pathways. Taken together, the data suggest that most of the human genome and those of other complex organisms, including transposon-derived sequences, is not junk nor evolving neutrally, but rather encodes a hitherto hidden layer of regulatory RNAs (many of which are species- or lineage-specific) that set the settings and direct the trajectories of differentiation and development via the control of chromatin architecture and epigenetic memory, promoter selection, splicing, RNA modification and editing, and mRNA stability and translation.

1. JS Mattick (2004) RNA regulation: a new genetics? *Nature Reviews Genetics* 5, 316.
2. JS Mattick (2005) The functional genomics of non-coding RNA. *Science* 309, 1527.
3. JS Mattick and IV Makunin (2006) Non-coding RNA. *Human Molecular Genetics* 15, R17.
4. A Aravin et al. (2006) A novel class of small RNAs bind to MILI protein in mouse testes. *Nature* doi:10.1038/nature04916.
5. A Girard et al. (2006) A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature* doi:10.1038/nature04917.

002

WHY MEN MAKE SPERM AND WOMEN MAKE OOCYTES: DISCOVERY OF THE MOLECULAR SIGNALS CONTROLLING GERM CELL FATE DURING EMBRYONIC DEVELOPMENT

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In mouse embryos, germ cells in developing ovaries enter meiosis and begin oogenesis around 13.5 dpc, whereas those in male gonads cease dividing until after birth, signaling a spermatogenic fate. It is widely believed that germ cells are intrinsically programmed to enter meiosis at a predetermined time, unless prevented from doing so by factors secreted by the male gonad. Instead, we now find that retinoic acid (RA) signaling controls the nexus between spermatogenesis and oogenesis.

We conducted an expression screen designed to identify genes expressed in a male- or female-specific manner during mouse gonadogenesis, and identified two genes encoding enzymes involved in RA metabolism. We detected abundant RA production in the adjacent mesonephroi of both sexes; the RA diffuses into the gonads in both sexes, persisting at high levels in the ovary, but is cleared from developing male gonads by the degradative enzyme Cyp26B1. By treatment of gonadal explants, we showed that either retinoic acid or an inhibitor of CYP26B1 induces XY germ cells to enter meiosis. Conversely, a retinoic acid receptor antagonist blocks entry of XX germ cells into meiosis. Meiotic markers were also induced in testes of *Cyp26b1* knockout mice. Together, our data suggest that retinoic acid, produced by the mesonephros, induces germ cells in the female gonad to enter meiosis but is prevented from doing so in the male gonad because of the actions of CYP26B1. Our data identify RA as a molecular trigger of meiosis in fetal gonads, a discovery that may be applicable to modulating human or animal fertility *in vivo* and production of functional gametes from germline stem cells *in vitro*.

A NOVEL APPROACH TO STUDY HUMAN PROSTATE DEVELOPMENT AND ITS DISEASES

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Men are not mice and rodent models have limited utility and relevance when studying human diseases. This is particularly true in the study of prostate disease, both benign and malignant, since these do not occur in mice. Yet mice are commonly used for this purpose and rodent models have provided controversial evidence for the early origins of adult prostate disease that is almost impossible to verify in humans. How do we develop model systems of human disease?

Our approach was to use the classical biological technique of tissue recombination together with stem cell technology to generate non-diseased human prostate tissue. We used rodent mesenchyme to establish reciprocal stromal-epithelial cell interactions with human ESC and directed their differentiation to fetal and mature human prostate glands, expressing PSA (prostate specific antigen), within 12 weeks; a process that takes 15 years or more in men. Glands derived from hESC of different genetic sex, first express fetal markers of prostate differentiation (eg Nkx3.1), followed by markers of its maturation (eg AR, p63 and PSA) and all the tissues are hormonally responsive.

This model provides new opportunities to study prostate disease. Firstly it provides normal tissue that is only available from young men aged 20-30yo; from this normal tissue, the process of disease initiation and progression can be studied, especially cancer. The mechanism of disease induction can be explored and verified by up or down regulating specific gene expression in the mesenchyme or stroma, eg using genetically modified mouse tissue or siRNA. Further, since fetal tissues are generated, we are able to study the early origins of adult disease, specifically the transgenerational effects of endocrine disruptors.

The conservation of stromal-epithelial signalling mechanisms between rodent and human species suggests this approach could extend to integumental, gastrointestinal and genital tissues, enabling the development of more relevant models of human diseases.

LIFE IN THE POUCH: WOMB WITH A VIEW

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Marsupials give birth to altricial young after a relatively short gestation period, but have a long and sophisticated lactation while the young develop, usually within a pouch. Their viviparous mode of reproduction thus minimises placentation in favour of lactation, effectively trading the umbilical cord for the teat. The special adaptations that marsupials have developed provide us with unique insights into the evolution of mammalian reproduction. Marsupials hold many mammalian reproductive “records”, for example they have the shortest known gestation but the longest embryonic diapause; the smallest neonate but the longest sperm. They have contributed to our knowledge of many mammalian reproductive events including embryonic diapause and development, birth behaviour, sex determination, sexual differentiation, lactation and seasonal breeding. Since marsupials have been genetically isolated from eutherian mammals for over 125 million years, sequencing of the genome of two marsupial species has made comparative genomics an exciting and important new area of investigation. This review will show how the study of marsupials has widened our understanding of mammalian reproduction and development, highlighting some of the mechanisms that are so fundamental that they are shared by all today's marsupial and eutherian mammals.

THE PIVOTAL ROLE OF IGF-II IN PLACENTAL INVASION, GROWTH AND FUNCTION

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The placenta has a myriad of functions including exchange of oxygen, nutrients and wastes between the maternal and fetal circulations. In early pregnancy, placental trophoblast cells invade and colonise the decidua and its vasculature to sequester a blood supply for the growing placenta. Impairments in this process have been implicated in pregnancy complications including IUGR, preeclampsia and pre-term birth. Our research is elucidating the pivotal role of IGF-II in placental invasion, differentiation and growth. IGF-II promotes trophoblast invasion while TGF β 1 inhibits it. We have discovered that IGF-II, under the influence of the low oxygen environment that characterises the first trimester, interacts with the IGF2R and the plasminogen activator system in 7-8 weeks human placental villous explants to promote trophoblast differentiation down the invasive pathway. In addition, treatment of the guinea pig with IGF-I or -II in early to mid pregnancy increases fetal weight near term. IGF-II improves the placental structural capacity for exchange near

term, while IGF-I reduces maternal fat deposition, presumably affecting substrate availability. Both IGFs sustainedly improve placental transport of glucose and amino acids. Hence, the type 1 and 2 IGF receptors are likely to mediate different IGF actions during early pregnancy. Gene ablation studies have shown that, later in gestation, IGF-II plays an important role in placental transport functions. Therefore, factors that reduce placental expression of IGF-II are likely to compromise placental exchange late in gestation when the demands of the fetus escalate. We have also discovered that repeated glucocorticoid treatment on days 104, 111 and 118 of pregnancy in the ewe significantly reduces placental IGF-II and IGF2R mRNA expression at day 145 just before term. This treatment also reduces fetal growth and therefore is likely to impair placental transport functions. Together these data demonstrate that IGF-II is a key player in placental development and function.

008

MACROPHAGE INHIBITORY CYTOKINE-1: ROLES IN TROPHOBLAST FUNCTION AND DECIDUAL PREPARATION

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Successful placentation is fundamental to the development of a healthy pregnancy and delivery of normal well grown baby. Understanding and manipulating placentation is therefore key to improving outcomes in various pregnancy disorders such as miscarriage, fetal growth restriction and pre-eclampsia. Over recent years, we have been exploring the roles of macrophage inhibitory cytokine-1 (MIC-1), a transforming growth factor- β superfamily member, in the regulation of placentation, decidualisation and subsequent pregnancy success. We have shown that MIC-1 is localized to the syncytiotrophoblast layer of the placenta and that MIC-1 production is down regulated in invasive extravillous trophoblast cells. Consistent with this, MIC-1 inhibits the activation of matrix metalloproteinases -2 and -9 in first trimester trophoblast and inhibits outgrowth from villous explants. These data suggest that MIC-1 may regulate trophoblast invasion/placentation. MIC-1 is also localized to the endometrium in both glandular and stromal cells with increasing immunostaining in secretory and decidualised tissues. In vitro, MIC-1 secretion by endometrial stromal cells increases during decidualisation and, in turn, MIC-1 facilitates the process of decidualisation. We have also undertaken a number of clinical studies of MIC-1 levels in maternal serum. In asymptomatic women who subsequently miscarry, first trimester MIC-1 levels are profoundly lower than in women with a subsequently normal pregnancy, consistent with MIC-1 having important roles in early pregnancy establishment. These data offer the potential for new clinical diagnostics and therapeutics. In summary, MIC-1 appears to have a number of potentially important functions in the early human placenta and decidua consistent with physiological roles in normal placentation. Whether these functions are key to successful pregnancy and the diagnostic utility of MIC-1 in early pregnancy remain key questions for our group.

009

INTERLEUKIN 11 AND LEUKEMIA INHIBITORY FACTOR: MECHANISMS AND INTERACTIONS IN IMPLANTATION AND PLACENTATION

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The successful implantation of the human embryo into a receptive endometrium leads to the formation of a functional placenta. Implantation failure results in infertility, while impaired implantation leads to inadequate placentation. Deficiencies in placental development can result in early abortion, or pre-eclampsia and intrauterine growth restriction. Currently there is no way of diagnosing endometrial infertility in women or of establishing whether the placenta is developing adequately. It is critically important to understand the molecular mechanisms of implantation because deficiencies in implantation have such serious consequences. Endometrial interleukin (IL)-11 and leukemia inhibitory factor (LIF) belong to the IL-6 family of cytokines and are two of very few molecules unequivocally required for embryo implantation and establishment of pregnancy in mice. In humans, IL-11 and LIF are produced by the endometrium and placenta in a spacial and temporal pattern suggestive of roles in uterine receptivity, endometrial stromal cell decidualization and trophoblast function. IL-11 advances decidualization of human endometrial stromal cells and LIF enhances endometrial stromal cell survival *in vitro*. However, roles for IL-11 in endometrial epithelial and trophoblast function are unknown, and for LIF, poorly understood. New evidence will be discussed in terms of the roles and interactions of IL-11 and LIF in uterine receptivity. A key issue in placental development is what controls trophoblast invasion during early placental development. Novel roles for IL-11 and LIF in trophoblast invasion will be presented. Studies that highlight the potential use of IL-11 and LIF as targets of infertility will also be discussed.

DETERMINANTS OF TISSUE AND LIGAND SELECTIVITY IN THE MINERALOCORTICOID RECEPTOR

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The mineralocorticoid receptor (MR) differs from the other steroid receptors in that it responds to two physiological ligands, aldosterone and cortisol (1). In epithelial tissues, aldosterone selectivity is determined by the activity of 11 β hydroxysteroid dehydrogenase type II. In other tissues, including the heart and regions of the CNS, cortisol is the primary ligand for the MR; in some tissues it may act as an antagonist. Clinical trials demonstrate a benefit of MR antagonists in the treatment of cardiac failure, however this benefit is compromised by hyperkalaemia. There is thus a need to search for tissue and ligand-specific determinants of MR activation.

Using a chimeric approach (2), we exploited the inability of the GR to bind aldosterone to identify the region of the MR ligand-binding domain (LBD) that confers aldosterone binding. We have narrowed this to a region of 25 amino acids, curiously the residues in this region that permit aldosterone binding do not contribute to the ligand-binding pocket.

Although the steroid receptors are modular, interactions may occur between domains. The N/C-interaction (3) is aldosterone-dependent but unexpectedly cortisol is an antagonist.

Nuclear receptor mediated transactivation is critically dependent on, and modulated by, co-regulatory molecules. A yeast-2-hybrid kidney cDNA library screen with the MR LBD has identified proteins which interact with one but not both MR ligands.

Further characterisation of these interactions may provide the basis of screens for the identification of "selective mineralocorticoid receptor modulators".

(1) Rogerson FM et al. *Journal of Biological Chemistry* 274:36305, 1999.

(2) Rogerson FM et al. *Molecular & Cellular Endocrinology* 200:45, 2003.

(3) Fuller PJ and Young MJ. *Hypertension* 46:1227, 2005.

ROLES OF ORPHAN RECEPTOR LRH-1 IN REPRODUCTION AND CANCER

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Liver Receptor Homologue-1 (LRH-1) is an orphan member of the nuclear receptor superfamily that belongs to the NR5A subgroup of receptors. Originally identified as a liver-specific factor that regulates expression of alphafetoprotein, LRH-1 has now been implicated in a variety of processes including cholesterol and bile acid synthesis, steroidogenesis and embryonic development. We have shown roles for LRH-1 in regulating aromatase expression in adipose tissue, testis and granulosa cells. LRH-1 also appears to mediate the over-expression of aromatase that occurs in adipose tissue of breast cancer patients, thereby providing the source of oestrogens for growth of postmenopausal ER+ tumours. In addition, LRH-1 directly stimulates proliferation of breast cancer epithelial cells by stimulating expression of G₁ cyclins. As such, it is an attractive target for drug development. As an orphan receptor, however, LRH-1 is constitutively active in the absence of ligand, and to date no antagonists have been identified. We are using complementary approaches to identify selective LRH-1 modulators. Firstly, by using phage display we have isolated small peptides that can inhibit LRH-1 activity by preventing its ability to interact with endogenous co-activators. Secondly, *in silico* approaches are being utilised to identify small "drug-like" molecules that either mimic the peptide antagonists by binding to the same site, or else occupy the classical ligand binding site of the receptor. These approaches may produce useful tools to dissect the functions of LRH-1 in endocrine tissues, and also have the potential to realise a new class of nuclear receptor modulators.

VITAMIN D-WNT PATHWAY INTERACTIONS IN SKELETAL AND NON-SKELETAL CELLS

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Active hormonal vitamin D, calcitriol, inhibits cell proliferation and induces differentiation and apoptosis in normal and tumor cells. Wnt signaling is involved in embryonic developmental and in adult tissue homeostasis, regulating cell fate specification, proliferation and differentiation. In addition, Wnt pathway dysregulation occurs in tumor cells. Secreted Wnt family glycoproteins act through Frizzled receptors to stimulate canonical β -catenin-mediated transcriptional responses. Evidence that human bone mass is strongly affected by mutations of LRP5, a Frizzled co-receptor, has led to investigation of interactions between Wnt and other bone regulatory pathways, including the vitamin D response

pathway. In addition, study of vitamin D-Wnt pathway interactions has also been stimulated by findings that vitamin D analogues promote differentiation of human colon carcinoma cells, in which β -catenin protein level or activity is often elevated. Calcitriol-bound vitamin D receptor (VDR) can directly inhibit the Wnt response by interaction with β -catenin, sequestering it away from the Wnt-responsive TCF transcription factor complex. As a result, activation of vitamin D responsive promoters is potentiated while transcriptional regulation of Wnt target genes is reduced. These divergent transcriptional effects are due to direct interaction between the β -catenin C-terminus and the VDR activation function-2 domain, with acetylation at the β -catenin C terminus differentially regulating the transcriptional responses¹. Calcitriol can also indirectly affect canonical Wnt pathway activity in osteoblasts by enhancing LRP5 gene transcription² and in colon carcinoma cells by stimulating expression of E-cadherin, which depletes nuclear β -catenin by promoting its accumulation at the plasma membrane³. The relative significance of these direct and indirect interaction mechanisms appears to be dependent at least in part upon cell type and the level of β -catenin present.

(1) Shah et al. 2006. *Molec Cell* 21: 799-809.

(2) Fretz et al. 2006. *Molec Endocrinology* e-published.

(3) Palmer et al. 2001. *J Cell Biol* 154: 369-387.

013

NUCLEAR RECEPTORS IN METABOLISM: THE SKI PHENOTYPES AND THE NORPHANS

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Nuclear Receptors (NR) are ligand-activated transcription factors that play key roles in growth, development and metabolism. The NR gene superfamily comprise the steroid receptors (estrogen, androgen, glucocorticoids amongst others), retinoid x-receptor (RXR) heterodimers (including thyroid, retinoic acid, and PPARs) and a large orphan receptor sub-family of receptors with no known ligands (NOR-1/Nurr77, COUPTF, LXR and many others). NRs expressed in skeletal muscle, fat and liver act as nutritional sensors to regulate metabolic target gene transcription to maintain energy homeostasis through direct effects on lipid, glucose and energy metabolism. As such, NRs involved in regulation of metabolism are primary targets for pharmacotherapeutic intervention to prevent and treat diabetes, cardiovascular disease and the metabolic syndrome. NRs being essential for normal growth and development interact with many other signalling pathways, including the transforming growth-factor- β (TGF- β) pathway, to amplify the diversity and complexity of their physiological control on growth. The Ski proto-oncogene, a negative regulator of the TGF- β signalling, recruits corepressors or prevents coactivator recruitment to the active transcriptional complex to determine the ultimate transcriptional outcome and the anti-proliferative potential. Ski *in vivo* and *in vitro* modulates skeletal muscle metabolism in part through an interaction and via regulation of NR-dependent effects on lipogenesis and metabolism. In this seminar, the functional role of several orphan nuclear receptors in skeletal muscle metabolism will be presented, in addition to the role of Ski crosstalk with the NR signalling pathway involved in development of the metabolic syndrome.

014

ASPECTS IN THE DIAGNOSIS AND MANAGEMENT OF PROLACTINOMA

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Human prolactin was isolated in the 1970's and it was soon recognised that hyperprolactinaemia resulted in a syndrome of amenorrhoea/galactorrhoea. Subsequently, it has been shown that hyperprolactinaemia may be the cause of secondary amenorrhoea in up to one third of cases. Prolactinomas are the commonest form of pituitary adenoma, and make up approximately 30% of all pituitary neoplasms. The basic principles of investigation involve excluding physiological and non-neoplastic causes of hyperprolactinaemia, pituitary neuroimaging and biochemical assessment of pituitary function. The recognised indications for treating hyperprolactinemia include hypogonadism (oligo-amenorrhoea in women, androgen deficiency in men), significant symptomatic galactorrhoea and tumour mass effect, particularly where visual pathways are compromised. Where hyperprolactinaemia is asymptomatic, no specific treatment other than periodic observation may be required. Once a prolactinoma is diagnosed, the usual first line of treatment is with a dopamine agonist. Currently, the dopamine 2 receptor specific agonist Cabergoline is the most widely used agent in clinical practice. Delivered once or twice weekly, it normalises prolactin levels in over 90% of subjects with pathological hyperprolactinaemia. The usual dose range is from 0.5 to 3mg per week, but higher doses may be used in resistant cases. Published data regarding macroadenoma shrinkage are uncontrolled but also demonstrate equal or superior efficacy compared to older studies of Bromocriptine, and occurs in approximately 80% of patients. Recent evidence suggests that over 60% of cases treated for 3-4 years with Cabergoline may enter a long term remission on cessation of the drug. Resolution of the adenoma on MRI is predictive of remission. Surgery is generally

reserved for a) dopamine-agonist resistant tumours, b) adverse effects of dopamine agonists or c) where it is desirable to obtain a histological diagnosis. Management of prolactinoma during pregnancy will be briefly discussed. Historically, Bromocriptine has been preferred although there is no evidence that Cabergoline is associated with an increased rate of foetal anomalies.

015

ACROMEGALY: NEW FRONTIERS IN MANAGEMENT

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Over the last few decades, there have been important advances in the fields of neuroendocrinology, cell biology, clinical chemistry, drug development, imaging, neurosurgery and radiotherapy, all of which have had a major impact in the management of acromegaly. The merits of growth hormone (GH) and insulin-like growth factor (IGF)-I measurements in the diagnosis and in the assessment of therapeutic outcomes of acromegaly have been intensively studied. The biochemical targets for treatment are a growth hormone of <2.5 ng/mL and a normal, age-adjusted insulin-like growth factor-1. Until 20 years ago, dopamine agonists were the only class of pharmaceutical agents available to control acromegaly. They have a limited adjunctive role, even with the development of second-generation selective agonists such as cabergoline. Surgery and radiotherapy were the mainstay of acromegaly management before the advent of the effective pharmacological therapies of the modern era: somatostatin analogues and pegvisomant, a growth hormone receptor antagonist. Somatostatin analogues achieve biochemical control in approximately 60% of patients. Pegvisomant, which is available in the USA and Europe and has just been registered in Australia, normalizes IGF-1 in nearly all patients but has no effect on tumour mass. Surgery is an appropriate first-line therapy for microadenomas as the chance of success is high. For large and/or invasive tumours where the prospect of surgical cure is remote, first-line therapy is somatostatin analogue treatment with debulking surgery having an adjunctive role to achieve tight control or to alleviate compression of the optic chiasm. Although acromegaly remains a challenging disease to manage, the expanding range of therapeutic options is likely to result in a better outcome for patients and offers the potential to tailor therapy based on a patient's individual requirements.

017

INSIGHTS INTO MONITORING THERAPY, AND CHALLENGES IN MANAGEMENT OF HYPOPITUITARY PATIENTS

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Pituitary hormone secretion is complex, dynamic and responsive to multiple external stimuli. This makes replication of normal hormone function in the hypopituitary patient very challenging. Evaluation of possible deficiencies of pituitary hormone secretion is the first challenge the clinician must face. The utility of different static and dynamic tests will be discussed. Replacement therapy with thyroid hormones and gonadal steroids are relatively straightforward, although the route of administration and dosage of sex hormones need to be optimized for each individual. More problematic is determination of the appropriate dosage and formulation for glucocorticoid replacement. We lack any effective method for measuring glucocorticoid action on target tissues, and measurements of circulating adrenal steroids are complicated by diurnal rhythms and variation in binding proteins. Possible approaches to monitoring of cortisol levels and action in ambulatory patients will be discussed. Even greater controversy surrounds the issues of Growth Hormone replacement in GH deficient adults, and androgen therapy in women with primary or secondary hypoadrenalism. Available evidence will be reviewed and balanced with practical aspects of cost and availability of appropriate therapeutic agents in Australia. Finally, some difficulties in management of diabetes insipidus will also be discussed. Patients with hypopituitarism have complex problems, and many remain symptomatic despite apparent "normalization" of their hormone concentrations. A flexible and individualized approach is important in achieving the best possible outcome for each patient.

NUTRACEUTICAL AND PHARMACEUTICAL EFFECTS ON UTERINE AND HORMONAL RESPONSES ASSOCIATED WITH EARLY PREGNANCY IN LACTATING DAIRY CATTLE

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A timed insemination program was used to investigate mechanisms through which polyunsaturated fatty acids (PUFA) and bovine somatotropin (bST) may increase fertility. Cows were assigned randomly to be inseminated (d 0) or not inseminated, and to receive 0 or 500 mg of bST (at d 0 and d 11) (i.e., C [cyclic], bST-C, P [pregnant], bST-P). Furthermore, a fish oil-enriched lipid supplement (FO; 1.9% of dietary DM initiated at 10 DIM) was evaluated in cyclic cows with (bST-FO; bST-C) and without (FO; C) bST. On d 17 (~94 d DIM) cows were slaughtered, uteri flushed and endometrial tissue collected. bST increased milk production, pregnancy rate (83% [5/6] > 40% [4/10]), conceptus length (45 > 34 cm) and interferon- τ in uterine luminal flushings (9.4 > 5.3 mg) with no effect on interferon- τ mRNA concentration in the conceptus. Feeding FO to cyclic cows increased proportions of eicosapentaenoic and docosahexaenoic acids while reducing the proportion of arachadonic acid in the endometrium. Cyclic cows fed FO had lower plasma insulin than control-fed cyclic cows, and FO altered plasma GH (bST-FO > bST-C) and IGF-I (bST-C > bST-FO) responses to bST. Endometrial IGF-I mRNA was reduced in pregnant cows. IGF-II mRNA was increased in the endometrium of P and bST-treated cows fed the control diet. Cows fed FO had increased concentrations of IGF-II mRNA when bST was not injected. IGFBP-2 mRNA was increased in bST-P cows, whereas bST decreased IGFBP-2 mRNA in all cyclic cows. FO decreased FGF-2 and increased progesterone receptor (PR) mRNAs. bST increased PR mRNA in endometrium of C but not in FO-fed or P cows. Concentrations of ER α mRNA and protein, and oxytocin receptor mRNA were decreased in P compared to C cows. Immunohistochemistry indicated that P and FO decreased ER α abundance in luminal epithelium. PGHS-2 protein was elevated in P cows and localized to the luminal epithelium. Both FO and bST treatments reduced staining intensity of PGHS-2 protein. In summary, pregnancy and bST altered endometrial gene expression. Cyclic cows responded differently to bST than pregnant cows. Feeding FO modulated responses in a manner that may favor maintenance of pregnancy.

IODINE SUFFICIENCY ACROSS AUSTRALIA: DO WE CURRENTLY MAKE THE GRADE?

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Over the past 7 years several localised, regional studies in South Eastern Australia and Tasmania have documented the re-emergence of mild to moderate iodine deficiency in adults and children. To provide a comprehensive snapshot of iodine nutrition throughout Australia we undertook a National Iodine Nutrition Study between mid 2003 and end 2004.

Design and Setting:

The survey was a cross-sectional study of 8 to 10 year old school children, randomly selected from government and non-government primary schools, in the 5 mainland Australian states of New South Wales, Victoria, South Australia, Western Australia and Queensland. The sample consisted of 1,709 students from 88 schools, comprising 881 boys and 828 girls. 1) Urinary iodine excretion levels (UIE) were determined and compared with WHO/ICCIDD criteria for the severity of iodine deficiency. 2) Thyroid volumes measured by ultrasound were compared with new international reference values (WHO/ICCIDD).

Results:

On a State basis, NSW and Victorian children are mildly iodine deficient with median UIE levels of 89 μ g/L and 73.5 μ g/L, respectively. South Australian children are borderline iodine deficient with a median UIE of 101 μ g/L. Both Queensland and Western Australian children are iodine sufficient with median UIE levels of 136.5 μ g/L and 142.5 μ g/L, respectively. Ongoing studies in NSW of iodine nutrition in pregnant women, and their offspring, confirm mild to moderate iodine deficiency is widespread throughout the State.

Conclusion:

The results of this study confirm the existence of inadequate iodine intake in the Australian population and call for the implementation of mandatory iodisation of all edible salt in Australia. In the interim, we recommend iodine supplementation be considered for pregnant women, those contemplating a pregnancy, and breastfeeding mothers.

SEARCHING FOR STEM CELLS IN THE KIDNEY

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The kidney, unlike the liver, never undergoes a structural repair or regenerative process in response to damage. Our understanding of kidney development suggests that nephron endowment is finalized prior to birth and that while they can hypertrophy, no new nephrons are 'born' after birth. However, recent data, particularly in the brain but also in other postnatal tissues including the heart and adipose, suggests that there may be stem cells present in adult organs previously regarded as non-proliferative during the postnatal period. We have taken several approaches to investigate the possibility of adult stem cells in the murine kidney. The first involved defining markers of renal progenitors by examining the expression profiling of the intermediate mesoderm as it commits to a renal fate. We have also thoroughly characterised the expression profile, multipotency and renal lineage potential of a putative stem cell population in the kidney, the Hoescht-effluxing side population. In this presentation, we will discuss the results of these studies and reflect on what implications these might have in the development of novel treatments for renal failure.

IDENTIFICATION OF MAMMARY STEM CELLS AND THEIR ROLE IN BREAST CANCER.

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The identity and purification of mammary stem cells (MaSCs) has proved elusive due to the lack of defined markers. However, we have recently isolated discrete populations of mouse mammary cells on the basis of cell-surface markers and identified a subpopulation (Lin-CD29hiCD24+) that is highly enriched for MaSCs as assayed by *in vivo* transplantation. Indeed, we demonstrated that a single cell, marked with a *lacZ* transgene, could reconstitute a complete mammary gland *in vivo*. The transplanted cell contributed to all three mammary epithelial lineages and extensive lobuloalveolar units were generated during pregnancy. Serial transplantation revealed that these cells have self-renewing capacity. These data establish that single cells within the Lin-CD29hiCD24+ population have the multipotent and self-renewing properties that define the MaSC. To further characterise the different mammary epithelial populations, we have investigated the expression of important prognostic markers of human breast cancer, including the estrogen receptor α (ER α), and progesterone receptor (PR), and provide evidence for differential expression amongst the various subsets. Finally, we show that the stem cell population was expanded in premalignant mammary tissue from MMTV-*wnt-1* but not MMTV-*neu* transgenic mice, indicating that stem cells are the likely tumour-initiating cells the *wnt-1* model of breast tumorigenesis.

GERMLINE STEM CELLS IN THE OVARY

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The notion of a fixed, non-renewable pool of oocytes in the ovary around birth (1) has been questioned (2,3,4). The presence of germline stem cells (GSC) giving rise to new oocytes in the adult ovary has been proposed (3,4) but challenged on theoretical and methodological grounds (5,6). Two reports provide new data that inform this debate (2,6). Byskov et al (6) injected 30-day old C57BL/6 mice with BrdU and could not find labeled oocytes in primordial or later stage follicles 8 days later. This argues against the presence of mitotically-active GSC in the mouse ovary, but does not exclude differentiated GSC arising from within the ovary or from external sources. We quantified all healthy follicles in C57BL/6 mouse ovaries between days 1 and 200 (n=6-10) using unbiased stereological methods (2). Mean numbers of healthy follicles fell 60% between days 1 and 7, primarily due to expulsion from the ovary. Although we saw no evidence for GSC, rare mitotic figures in unidentified cells were noted between days 1 and 12. From day 7 to 100 mean numbers of primordial or total follicles per ovary were not significantly depleted, but declined by day 200 to about 10% of day 1 levels. Our data supports the hypothesis of follicle renewal in postnatal and adult ovaries of C57BL/6 mice by an as yet unknown mechanism.

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023

ADULT STEM/PROGENITOR CELLS IN THE ENDOMETRIUM

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The human endometrium undergoes regeneration, differentiation and regression with each menstrual cycle, following parturition and in post-menopausal women taking estrogen replacement therapy. In other regenerative tissues, rare populations of adult stem cells have been identified. While it was postulated many years ago that endometrial stem/progenitor cells were responsible for endometrial regeneration, it was not until recently that we provided the first evidence for their existence in human and mouse endometrium, setting a new paradigm in uterine biology (1,2). We demonstrated that 0.22% of endometrial epithelial cells and 1.25% of stromal cells were clonogenic, each producing two morphologically distinct colony types (1). The large clones (colony forming units, CFU) of small, densely packed cells were rare (0.09% of epithelial and 0.02% of stromal), and the small clones comprised large, loosely arranged cells. Large epithelial and stromal CFU exhibited several adult stem cell properties; high proliferative potential and self-renewal. Large stromal CFU cultured in appropriate induction media also underwent multilineage differentiation into mesenchymal lineages; fat, smooth muscle, bone and cartilage. Bone marrow mesenchymal stem cell (MSC) phenotypic markers CD29, CD44, CD90, CD73, CD105, CD146 were expressed by large stromal CFU. Epithelial (2.1%) and stromal (0.2%) cells showed side population (SP) phenotype (stem cell property). Co-expression of CD146 and PDGF receptor- β produced a 10-fold purification of stromal cells with CFU and multilineage differentiation capacity. These cells were located around blood vessels. Our studies in mouse endometrium using the label retaining cell (LRC) technique identified the location of candidate endometrial epithelial stem/progenitor cells in the surface epithelium and MSC-like cells at the endometrial-myometrial junction (2). These rare epithelial and stromal LRC rapidly proliferated on estrogen-induced endometrial regeneration, despite lack of estrogen receptor- α expression. Our data suggest that rare populations of epithelial progenitors and MSC-like cells exist in human and mouse endometrium and may be responsible for initiating endometrial regeneration.

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024

NEW GENES THAT CONTROL REPRODUCTION IN THE HUMAN AND THEIR GENOTYPE-PHENOTYPES: EVIDENCE FROM HUMAN DISEASE MODELS

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In all mammalian species, gonadotropin-releasing hormone (GnRH) represents the key interface between the way that the CNS views the outside world and how it transmits signals internally to its reproductive endocrine milieu. Since it is the first and initiating hormone in a complex reproductive cascade that involves gonadotropin biosynthesis and secretion, gonadal steroidogenesis and germ cell maturation, and behaviour, it can be viewed as the "pilot light of reproduction".

Therefore, understanding the genes and signals that modulate the developmental fate and secretory actions of GnRH neurons in man remains a major question as defined in Science Magazine's 125 Outstanding Scientific Questions in 2005. To gain insight into this problem, we have used the human disease models of normosmic idiopathic hypogonadotropic hypogonadism (nIHH) and Kallmann Syndrome (KS) to elucidate the genes that control GnRH's secretion and action. Since patients with these conditions represent isolated defects in the secretion or action of GnRH, understanding their genetic basis has proven to be an important avenue of biologic insights into this problem.

This lecture will report on several new genes that are responsible for control of this key reproductive peptide in the human that we have identified using these human disease models, including GPR54, Metastin, FGFR1, and GnRHR. It will review new mutations in each and their genotype/phenotype correlations as well as useful clinical points for their counseling and management.

KISSPEPTIN ACTIVATION OF GNRH NEURONS TO INITIATE PUBERTY AND OVULATION.

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Evidence is accumulating for a critical role of kisspeptin signaling within the neural circuitry controlling fertility. Our studies have focussed upon understanding the cellular mechanisms through which kisspeptin controls the activity of the gonadotropin-releasing hormone (GnRH) neurons in mice.

Immunocytochemical approaches have demonstrated that a large population of kisspeptin neurons located in the rostral periventricular region of the hypothalamus develop just prior to puberty. Studies using a Cre-dependent Pseudorabies virus retrograde tracing technique in transgenic GnRH-Cre mice have shown that a sub-population of periventricular kisspeptin neurons project directly to GnRH neurons. Across development, GnRH neurons can be seen to receive kisspeptin-immunoreactive fibre appositions from postnatal day 25 onwards and electrophysiological studies in GnRH-GFP mice show that the percentage of GnRH neurons responding directly to kisspeptin increases across puberty. These studies suggest that periventricular kisspeptin neurons innervate and activate GnRH neurons directly to help bring about puberty.

Estrogen positive feedback initiates ovulation through a neural pathway that involves sexually dimorphic, estrogen receptor alpha (ER α)-expressing neurons that activate GnRH neurons. Our recent studies have shown that periventricular kisspeptin neurons in the mouse (1) are sexually differentiated with females having 10-fold great numbers of kisspeptin neurons compared with males, (2) express ER α , and (3) are activated by estrogen positive feedback using estrogen or ER α -selective agonists. Viral retrograde tracing shows that ER α -expressing kisspeptin neurons are primary afferents to GnRH neurons. Coupled with electrophysiological evidence for a massive and prolonged excitatory action of kisspeptin on GnRH neurons at proestrus, these data strongly suggest that periventricular kisspeptin neurons are a key component of the neural mechanism initiating ovulation.

THE BIOLOGY OF KISSPEPTINS AND GPR54

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In July 2005, Science magazine listed as one of its 125 greatest unanswered scientific questions "What controls puberty"? The power of genetics resulted in the discovery of the GPR54/Metastin system in the human by 2 groups in 2003 (DeRoux et al; Seminara et al). Since then, it has become clear that this system, previously completely overlooked by basic investigators attempting to determine the long-elusive "puberty gene", actually fulfills the criteria for a ligand receptor system that serves as a major gatekeeper of puberty and sexual maturation in several species including the human, mouse, and monkey. GPR54 receptors are located in the medial basal hypothalamus with >75% of GFP labelled GnRH neurons demonstrating their presence. GPR54 levels increase at puberty in both sexes in rodents and monkey. Their neuroanatomic localization in the arcuate nucleus and the AVPV, both important and known sites for the neuroendocrine control of GnRH secretion, position them well to be major regulators of GnRH secretion during puberty. GPR54 levels also appear to be sex steroid responsive although our understanding of the transcriptional control of GPR54 is still in its infancy. The precursor 145 aa peptide ligand for GPR54, Kisspeptin, is cleaved at a dibasic cleavage site and gives rise to an amidated 54 aa carboxy terminal fragment termed Metastin because of its previously described role in limited metastatic disease in several malignant cell lines. It appears that all biologic activity resides in the amidated carboxyterminal decapeptide sequence of Kisspeptin/Metastin although the circulating form of this precursor molecule is not yet clear. Metastin has been determined to be the most sensitive stimulator of GnRH-induced LH secretion yet discovered with levels as low as pM being able to stimulate GnRH secretion in rodents and primates. Metastin administration also causes GnRH antagonist-blockade of LH release (indicating the essential role of GnRH secretion and action in the ensuing LH release). Circulating levels of metastin are detectable in the human and increase 10,000 fold during pregnancy in a pattern that is quite different from hCG. This lecture will focus upon this system and its relationship with GnRH secretion.

KISSEPTIN AND GPR54: MOLECULAR CONDUITS FOR PUBERTY ONSET AND CENTRAL INTEGRATION OF ENERGY BALANCE AND REPRODUCTION

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Based on the observation that inactivation of the G protein-coupled receptor GPR54 is linked to hypogonadotropic hypogonadism in humans and mice, the essential role of this receptor and its putative ligands (kisspeptins, encoded by KiSS-1 gene) in the control of reproduction was first proposed in late 2003. Indeed, such a contention has now been fully substantiated by a number of genetic, molecular, physiologic and pharmacological studies. We will review herein the available evidence for the key role of KiSS-1/GPR54 system in the timing of puberty and signaling of energy balance and metabolic information onto the centers governing reproductive function. Concerning puberty onset, hypothalamic expression of KiSS-1 and GPR54 genes has been proven developmentally regulated, with maximum levels at puberty in rodents and primates. Moreover, functional studies have disclosed that enhanced KiSS-1 function (through increased kisspeptin tone and signaling efficiency) takes place at the time of puberty. Nonetheless, pubertal activation of KiSS-1 system appears to be exquisitely modulated, as excessive enhancement of KiSS-1 tone at puberty evokes the 'paradoxical' suppression of the gonadotropic axis. Regarding integration of energy status and reproduction, functional and expression analyses have demonstrated that situations of negative energy balance, linked to hypogonadotropism, decrease the hypothalamic expression of KiSS-1 gene, while exogenous kisspeptin is able to normalize acute gonadotropin responses and restore pubertal activation of the reproductive axis in undernutrition. Similar observations have been obtained in models of altered metabolism and gonadotropin secretion, such as experimental diabetes, where decreased KiSS-1 expression was associated to defective leptin levels, in line with recent data showing reduced KiSS-1 mRNA levels in ob/ob mice. Altogether, these data evidence that, among other essential roles in reproduction, the hypothalamic KiSS-1/GPR54 system operates as pivotal molecular conduit in the timing of puberty onset and for relaying metabolic information onto the centers governing the gonadotropic axis.

THE ROLE OF KISSEPTIN IN MEDIATING SEX STEROID FEEDBACK CONTROL OF GnRH

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The Kiss1 gene encodes a family of peptides called kisspeptins. These peptides are the endogenous ligands for the G protein-coupled receptor, GPR54, and play a vital role in the regulation of GnRH and in turn gonadotrophin secretion. In many species, centrally administered kisspeptin stimulates gonadotrophin secretion in a GnRH dependant manner. Moreover, virtually all GnRH neurons co-express GPR54. In the hypothalamus, the vast majority of kisspeptin producing cells (those expressing KiSS-1 mRNA) also express sex steroid receptors, particularly oestrogen receptor alpha. Thus, sex steroids are able to directly regulate the expression of KiSS-1 mRNA, implicating kisspeptin as a link between sex steroids and GnRH feedback. In the arcuate nucleus (Arc) of the rodent, sex steroids inhibit the expression of KiSS-1 mRNA, suggesting that the kisspeptin secreting neurons here are the conduit for the negative feedback regulation of GnRH/gonadotrophin secretion. However, in the anteroventral periventricular nucleus (AVPV), sex steroids induce the expression of KiSS-1 mRNA, implying that these kisspeptin neurons play a role in the positive feedback regulation of GnRH/gonadotrophin secretion. Thus, it is conceivable that kisspeptin neurons in the AVPV are central processors for generating the preovulatory luteinising hormone surge in the female.

LABORATORY MEASURES IN CLINICAL ENDOCRINE PRACTICE: BONE MARKERS

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Current investigations for patients with osteoporosis focus on assessment of bone mass with densitometry. However, clinicians commonly order laboratory tests to identify secondary causes of osteoporosis as well as monitor response to therapeutic interventions. The use of bone markers has been extensively studied and these markers reflect generalised skeletal remodelling. Bone markers may thus offer diagnostic utility, prognostic information and represent a useful tool for therapeutic monitoring. Bone markers are classified as either bone formation or bone resorption markers depending on which remodelling process they mainly represent. In most instances both remodelling processes are balanced and either bone marker will reflect the degree of bone remodelling activity. However, because bone resorption is shorter than formation, resorption markers respond faster to changes in remodelling than formation markers. A number of cases will be presented that highlight the current utility as well as limitations of bone markers in clinical practice. Specifically, a thorough understanding of preanalytical factors, analytical issues including lack of standardisation and postanalytical interpretation of results in the context of pathological skeletal disorders will be discussed. All of these issues can result in significant variation in results and an understanding is required when

interpreting individual bone marker responses. Bone markers have a limited role in diagnostic stratification, however could have a greater role in prognostic stratification and therapeutic monitoring which may result in routine clinical application. Recent evidence has indicated that pretreatment bone turnover may predict the reduction in nonspine fractures with alendronate therapy. Reduction in bone turnover occurs earlier than bone density changes following risedronate therapy and accounts for more than 50% of the predicted fracture reduction with treatment. Lastly bone markers may have a role in defining persistence with therapy and compliance with dosing recommendations.

031

MACROPROLACTIN

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Diagnosing hyperprolactinemia has always been confounded by the possible presence of macroprolactin which has been dubbed “false hyperprolactinemia”. If not properly investigated macroprolactin can lead to diagnostic confusion, invasive and unnecessary investigations. Prolactin can be present in the circulation as the monomer alone (molecular weight (MW) 23,000), or as a variable mixture of monomer, a polymeric complex of between 40 and 60,000 MW and the monomer bound to an immunoglobulin (MW 150-170,000). Both the monomer and the polymeric complex known as big prolactin have been reported to be biologically active. The immunoglobulin bound form is described as big big prolactin (macroprolactin), which has a long half life in the circulation and limited bioactivity. It is important to screen high prolactin levels for the presence of macroprolactin before commencing an investigation for an adenoma. Some prolactin assays do not measure macroprolactin and others show variable ability to determine its presence. Routine screening of high prolactin levels has to be done with an assay that detects macroprolactin and has been confirmed to be able to measure Prolactin in the presence of PEG. The immunoglobulin bound prolactin is precipitated with 12.5% PEG (w/v) and a recovery of 40% or less of the immunoreactive prolactin in the supernatant was indicative of the presence of macroprolactin. This also can be misleading and reporting only the PEG recovery can lead to some confusion. The presence of a microadenoma cannot be excluded, unless the recovered prolactin levels returned to the normal range after treatment. To add further confusion using gel exclusion chromatography we have found patients with big prolactin levels that would have appeared to be macroprolactinemic if only the PEG screening assay procedure was used. Many of the confusing patients may be being detected earlier now because of the increased awareness of macroprolactin in hyper prolactinemia. It is important to recognize the limitations of the screening assay as well as the ability of different prolactin assays to detect macroprolactinemia. It is concerning us that we are detecting an increasing number of patients with all three forms of prolactin in the circulation and a number of these may be incorrectly attributed to the presence of macroprolactinemia.

033

NUCLEAR RECEPTOR COREGULATORS – GETTING TO THE HEART OF HORMONE ACTION

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Discovery of the nuclear receptor coregulators, exemplified by SRC-1, has revolutionized our understanding of hormone action, and provided an opportunity to develop new diagnostic and prognostic markers as well as potential new therapeutics for a variety of human diseases. Numerous coregulators (comprising coactivators and corepressors) have been identified in the past few years, and the challenge is to define their molecular mechanisms of action, their functional role in a tissue- and disease-specific manner and translate these findings into meaningful clinical outcomes. In the past few years, as part of our studies to understand signaling pathways and identify novel targets in hormone-dependent cancers, we identified several novel nuclear receptor coregulators that bind a specific RNA coregulator (SRA, Steroid receptor RNA Activator) which is aberrantly expressed in human breast cancer (BCa). These SRA-binding coregulators contain distinct RNA-binding domains from two different structural families, and each modifies SRA-mediated coregulation of multiple nuclear receptor signaling pathways, including estrogen, androgen, glucocorticoid and thyroid. For example, SLIRP is a novel protein that corepresses the estrogen, thyroid and vitamin D nuclear receptor signaling pathways, interacts with multiple other coregulators, including SHARP, SKIP and NCoR, and augments the effects of tamoxifen. Remarkably, the majority of SLIRP resides in the mitochondrion, and it is most highly expressed in the energy-rich tissues (heart and skeletal muscle). Furthermore, SLIRP represses PPAR δ signaling suggesting an important role in energy homeostasis and metabolism in heart and skeletal muscle. Taken together, our studies provide insight into the important contribution of SRA-protein interactions to nuclear receptor transcription, support a key physiological role for each of these SRA-binding coregulators in a wide range of nuclear receptor signaling, especially estrogen signaling in BCa and suggest mechanisms by which aberrant expression could modulate anti-estrogen therapies. Furthermore, they illustrate the bifunctional nature of some coregulators, and for SLIRP, suggest key roles in both hormone-dependent cancer and energy homeostasis signaling pathways.

INTRACELLULAR STRESS, MITOCHONDRIAL DYSFUNCTION AND DIABETES COMPLICATIONS

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Intracellular stresses, such as oxidative stress and endoplasmic reticulum (ER) stress, are involved in the development of various diseases. In this lecture, our studies about the impacts of ER stress and reactive oxygen species derived from mitochondria (mtROS) on diabetes and its complications will be presented. The ER plays important functions essential to cell survival. Various conditions that interfere with ER function are called ER stress. Severe ER stress leads to apoptosis through induction of ER stress-associated apoptosis factor CHOP. The Akita mouse with a mutation (Cys96Tyr) in the insulin 2 gene develops diabetes with a reduced beta-cell mass. Overexpression of the mutant insulin in MIN6 cells induced CHOP expression and apoptosis. Targeted disruption of the CHOP reduced islet cell apoptosis and delayed the onset of diabetes in Akita mice. In addition, db/db mice displayed an increase of CHOP and other ER stress-related genes suggesting the involvement of ER stress in the progression of diabetes. The mtROS may play primary role in the development of diabetic complications. In MIN6 cells, hyperglycemia increased mtROS production, and the treatment of the beta-cells with H₂O₂ suppressed the first phase of glucose-induced insulin secretion. On the other hand, in hepatoma Huh7 cells, mtROS decreased tyrosine phosphorylation of IRS-1, an important insulin signal, via activation of ASK-1-JNK pathway. Therefore, mtROS could prevent insulin secretion and action, which may explain glucotoxicity in diabetes. To further study the role of mtROS in diabetic complications, we created a transgenic (eMnSOD-Tg) mouse that overexpresses MnSOD in endothelial cells. Expression of VEGF and fibronectin mRNAs in retinas was observed in STZ-induced diabetic WT mice, which was completely prevented in diabetic eMnSOD-Tg mice. In addition, the increase of 8-OHdG, a marker of oxidative stress was also suppressed in diabetic eMnSOD-Tg mice. In the relative hypoxia-induced in vivo retinopathy model, retinal flat-mount pictures showed typical central avascular areas in WT mice, which were reduced in eMnSOD-Tg mice. Therefore, normalizing hyperglycemia-induced mtROS could prevent diabetic complications in vivo. Our results indicate that intracellular stresses could be novel targets for prevention and treatment of diabetes and its complications.

NOVEL APPROACHES FOR THE TREATMENT OF DIABETES - WEARABLE ARTIFICIAL ENDOCRINE PANCREAS (AEP) AND MILD ELECTRIC AND THERMO GENERATOR (MET)

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Diabetic treatment is sometimes difficult in cases of insulin deficient status and of severe insulin resistance. To conquer these, our group has created a wearable artificial endocrine pancreas (AEP) and an instrument, named MET (mild electric current and thermo generator), which reduces insulin resistance in vivo by mild hyperthermia and electric current. AEP is a closed-loop system with glucose sensor, insulin infusion algorithm, and infusion pump to establish strict glycemic control. To establish the ideal insulin delivery route for AEP, we examined the effectiveness of portal and intraperitoneal insulin delivery routes. The closed-loop portal insulin delivery was feasible with regard to both insulin profiles and hepatic glucose handling in vivo. On the other hand, intraperitoneal route is less invasive and ~70% of infused-insulin could flow into portal vein, and achieved better glycemic control when compared with subcutaneous infusion. Therefore, the portal vein may be the most ideal insulin delivery route but intraperitoneal route could also be beneficial for glycemic control by AEP. Hyperthermia is known to reduce insulin resistance, at least in part through expression of HSP72. We recently found that combination of mild hyperthermia and weak electrical current could efficiently induce HSP72 in culture cells. Therefore, an instrument that can induce mild hyperthermia and weak electrical current in vivo, named MET, was created and applied for model mice of insulin resistance. Treatment of the high fat fed mice with MET twice a week for 8 weeks significantly reduced subcutaneous and visceral fat, reduced fasting insulin level, increased adiponectin level and improved glucose tolerance when compared with sham-treated mice. Fatty liver was also dramatically improved. The HSP72 induction was confirmed in tissues of MET treated mice, which paralleled with improved insulin signaling and increased insulin-stimulated GLUT4 translocation. Therefore, MET could be used for the treatment of patients with type 2 diabetes and/or metabolic syndrome.

INHALED INSULIN IN DIABETES TREATMENT

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The alveolar membrane is thin, permeable and has a large surface area. Unlike the gastrointestinal tract, it is relatively free of proteases. It therefore represents a suitable site for insulin delivery provided the hormone can be deposited in sufficient local concentration. Various techniques have been developed to make insulin particles of different dryness

and density to optimise its delivery by the inhaled route. Overall, absorption has been found to be reproducible with a rapid onset of action similar to the short acting insulin analogues now commonly in clinical use. However it seems to have a slightly longer duration of action which may stem from a gradual release of some pulmonary deposition of the inhaled insulin. In clinical trials now lasting up to 2-3 years, inhaled insulins have been shown to be non-inferior to subcutaneous insulin in both type 1 and type 2 diabetes and possibly associated with less hypoglycaemia. Its introduction is often associated with a transient cough. There is also a slight deterioration in lung function which is small and generally reversible after cessation of the inhaled insulin treatment. No other significant long term side effects have been demonstrated and one brand of inhaled insulin has already received regulatory approval for marketing in both Europe and U.S.A. Patients treated with inhaled insulin have shown good acceptance to the treatment and a better quality of life. Inhaled insulin is obviously more expensive than conventional products. Whether it can increase willingness to initiate insulin therapy and therefore reduce the morbidity of diabetes in the longer term is an important question yet to be answered.

037

INSULIN DELIVERY BY GENETIC ENGINEERING

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Type I diabetes is caused by the autoimmune destruction of the pancreatic beta cells. The only treatment for the disease is the injection of insulin to regulate blood glucose. Despite the best glucose-monitoring procedures the chronic complications of diabetes still develop: retinopathy, neuropathy, nephropathy and macrovascular complications. The only "cure" for diabetes is the transplantation of donor pancreatic tissue, but this is limited by lack of donors and the fact that patients must be immunosuppressed. Ultimately, other cures may come from xenotransplantation, generation of beta cells from human embryonic stem cells, or gene therapy by the creation of a surrogate beta cell. At the present time, xenotransplantation and stem cell therapy are both fraught with logistic, ethical and legal issues. My laboratory is investigating the use of somatic cell gene therapy as an alternate strategy for reversing diabetes. This strategy is based on the engineering of liver cells to synthesise, store and secrete insulin to glucose and other stimuli, thereby regulating patient blood glucose levels without the need for immunosuppression.

In collaboration with Prof. Tuch at Prince of Wales Hospital we have engineered a liver cell line, Huh7ins that responds in a regulated fashion to a glucose stimulus and corrects diabetes in an animal model. These cells are not destroyed by cytokines of the immune system that precipitate diabetes and this, or a similar cell line could possibly be used clinically following encapsulation and transplantation. An alternative strategy that we are also pursuing is the direct delivery of the insulin gene to the livers of diabetic animals. This procedure has resulted in normalisation of blood glucose levels for 500 days in streptozotocin-diabetic rats, storage of insulin in secretory granules and normal glucose tolerance. These studies give hope that gene therapy may be a treatment for Type I diabetes.

038

TESTOSTERONE DELIVERY - ROUTES OF ADMINISTRATION

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Since the first clinical use of testosterone in 1937 by frequent IM injection there has been a search for more convenient modes of delivery. Low oral bioavailability has led to almost exclusive use of parenteral preparations and the short half-life of testosterone means that it is best delivered by depot preparations.

Despite being available since the 1940's testosterone implants have been curiously neglected as a treatment modality until recent years. A single subcutaneous insertion of four 200 mg pellets maintains adequate testosterone levels in most men for 5 to 6 months. Side effects are infrequent and usually minor (bruising, bleeding, infection) although extrusion of one or more pellets occurs after about 10% of procedures. The major disadvantage of implants is the need for a minor surgical procedure by a trained operator.

Injectable testosterone esters provide reliable and adequate delivery of testosterone. Testosterone enanthate and combinations of esters approximate steady state levels when given weekly but for convenience are usually given second weekly, resulting in supraphysiological levels in the first few days and low levels towards the end of the second week. The recently availability of injectable testosterone undecanoate, which has been used at 12 weekly intervals, is likely to provide a significant advance in injection therapy.

Unlike synthetic 17-alkylated androgens which are potentially hepatotoxic oral testosterone undecanoate is safe. However multiple daily doses are needed and bioavailability is low and erratic. Transdermal preparations offer the same advantages of oral TU with better bioavailability.

The search for a satisfactory transdermal preparation has been hampered by the need for absorption of mg per day. Non-scrotal patches are large and absorption enhancers lead to a high incidence of skin irritation. Testosterone gel is better

tolerated than patches but care needs to be taken to avoid inadvertent transmission to female partners or children. Their short duration of action makes patches and gel particularly suitable for initiation of therapy and in situations where rapid withdrawal of therapy may be required. (e.g. men with treated prostate carcinoma). However they may not provide adequate testosterone delivery in severe androgen deficiency.

041

GENETIC CAUSES OF REPRODUCTIVE FAILURE IN THE MALE

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Infertility affects 1 in 25 Australian men. Of these, 40% present with primary spermatogenic failure (SgF) manifest as a combination of reduced sperm number, motility or structure/function. SgF is a heterogeneous group of disorders in which genetic causes are increasingly being recognized. A greater understanding of such genetic causes is not only essential in the diagnosis and potential treatment of men bearing the condition, but also in understanding the potential implications for children conceived through artificial reproductive technologies. Further, the identification of effective genetic barriers for male fertility represents a valuable tool for the development of novel male gamete based contraceptives.

Chromosomal disorders such as sex chromosome aneuploidies and autosomal translocations are seen in 13.7% of azoospermic and 4.6% of oligospermic men. Y chromosome microdeletions account for 3-5% of men with sperm densities of <5 million/ml, and depending on the region of the Y chromosome deleted present with a spectrum of histopathologies ranging from Sertoli cell only to hypospermatogenesis. More recently work from several groups has suggested that smaller deletions within the same region may or may not critically impair fertility depending upon as yet undefined modifiers on the Y chromosome (the Y haplogroup). Despite the development of many specific gene knockout mouse models with male infertility, the identification of critical single gene lesions in humans has proven to be very difficult. Those that have been identified include the cystic fibrosis transmembrane receptor (CFTR) gene which results in congenital absence of the vas deferens, several genes implicated in primary cilia dyskinesia and a group of genes involved in fibroblast growth factor receptor-1 signaling which are involved in Kallmann's syndrome. To date, however, only a single gene SCP3 has been unequivocally and mechanistically linked to human male SgF in the absence of somatic pathology.

042

ENDOMETRIAL PROPROTEIN CONVERTASE 6: A CRITICAL REGULATOR FOR EMBRYO IMPLANTATION

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Embryo implantation, during which the free-floating blastocyst attaches to and invades the uterus, is vital for mammalian embryo survival and development beyond the blastocyst stage. In women, implantation failure, resulting in embryonic death, is a major cause of early pregnancy loss and female infertility. Implantation failure also limits successful outcome of assisted reproduction (~70% of embryos transferred fail to implant). A better understanding of the molecular mechanisms of implantation is thus critically important in reproductive medicine. Successful implantation requires not only an implantation-competent blastocyst but also an appropriately prepared conducive endometrium. In a broader sense, endometrial preparation for implantation includes (i) differentiation of the endometrium into a receptive state so that at the expected time of implantation, the embryo will be able to attach and adhere to the luminal epithelium, and (ii) conversion of the endometrium into a competent condition so that appropriate tissue responses in the stroma and vasculature, will occur upon the attachment of the embryo, to allow properly controlled trophoblast invasion. We have established that proprotein convertase 5/6 (PC6), a serine protease of the proprotein convertase (PC) family, is a critical endometrial factor for implantation both in mice and primates. PCs control post-translational activation of a range of proteins with important functions (including HIV envelope proteins), and are regarded as "master switch" molecules and potential therapeutic targets (eg. for combating HIV infection). We propose that PC6 regulates endometrial function by activating a cohort of proteins of diverse functions essential for implantation. This presentation will discuss our current understanding of PC6 function in the endometrium and the potential implications of targeting PC6 for fertility control.

ENVIRONMENTAL INFLUENCES ON DNA METHYLATION IN EMBRYONIC CELLS: INVESTIGATING MECHANISMS AND PHENOTYPIC CONSEQUENCES.

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The epigenetic reprogramming in DNA methylation that occurs in the preimplantation embryo appears vulnerable to disruption when *in vitro* embryo production technologies are applied and may also be influenced by maternal nutrition *in vivo*. Thus we reasoned that blastocyst-derived, human embryonic stem cells isolated and cultured through a diverse range of protocols in different laboratories (hESC), may also be subject to epigenetic instability and variation (Allegrucci *et al.*, 2004. Lancet 364; 206-20), providing a novel model for the human embryo (Allegrucci *et al.*, 2005. Reproductive Toxicology 20; 353-367). In order to define the degree of epigenetic variation between independently-derived hESC lines we have employed Restriction Landmark Genome Scanning (RLGS) to examine the genome-wide methylation profiles of gene-rich CpG islands in hESC. Using *NotI/EcoRV/HinfI* digestion, our comparisons of hESC CpG islands to a normal human lymphocyte profile (comprising 2025 fragments for which a genomic *NotI/EcoRV* library was available) have revealed significant epigenetic variation between lines that cannot be accounted for by inherent genetic variability. Studies on the effect of a range of culture conditions revealed epigenetic instability over time in culture, with evidence of stable inheritance of changes occurring at lower passage number. The majority of loci which changed over time within a line were not in common between lines, suggesting that passage-associated changes are stochastic and unpredictable. In contrast, common methylation "hotspots" were identified as changing within the BG01 line when deviations from the standard protocol of culture on mouse embryonic fibroblast feeders with passage by manual dissection were applied. We are currently investigating the phenotypic and therapeutic consequences via examining the derivatives of human embryonic stem cells subjected to different environments expected to influence methyl cycle metabolism. Our data thus far suggest that further optimisation and standardization of hESC culture conditions is urgently required to ensure production of biosafe therapeutic products. Since the environmental factors implicated in altering DNA methylation in hESC cultures are in common with a range of human embryo culture media, hESCs might provide a novel model system to optimise culture conditions for assisted reproduction technologies.

In a complimentary approach to examine phenotypic consequences of methylation-relevant nutrients on programming the embryo *in vivo*, we have developed a sheep model. Maternally-applied methyl group deficiencies in diet during the periconceptual period are being examined for the effects on DNA methylation and subsequent conceptus development. Our rationale for these studies is that identification of key nutrients which can predispose early embryonic cells to programmed epigenetic change might uncover mechanisms pertinent to the developmental origins of adult disease. The latest results of these studies will be presented.

A MITOCHONDRIAL COMPONENT TO DEVELOPMENTAL PROGRAMMING

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Adult physiology is not simply dependent on the sequence of the nuclear genes we inherit. There is an increasing appreciation that very early environmental factors can determine development and adult phenotype¹. Recently, in experimental models it has been clearly demonstrated that environmental stress during pregnancy, particularly during the peri-conceptual period is a determinant of adult disease². Although these findings substantiate the concept of developmental programming – a process by which very early stress is proposed to have detrimental effects on offspring health. The molecular mechanisms of this phenomenon remain unclear. However, epigenetic changes to nuclear DNA (nDNA) have been implicated in this process³.

We have evidence that programmed deficits in mitochondrial function contribute to adult disorders in several diverse models of developmental programming. Mitochondria require both nDNA and mitochondrial DNA encoded transcripts to function⁴. Therefore persistent changes to either genome could cause abnormal mitochondria. Although we find that different maternal nutrient stresses result in similar deficits in mitochondrial function in adult offspring, namely abnormalities in the activity of mitochondrial electron transport complex iii, we have recently found that this deficit can arise from the environmental disruption of one of a number of distinct cellular processes. Oocytes and preimplantation embryos are particularly prone to environmentally induced changes to mitochondria whose effects can be mimicked *in vitro* and persist after the initial period of stress⁵. The cause and consequences of these changes will be discussed. Our work has particular relevance to assisted conception procedures and to embryo based stem cell derivation.

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METHYLCYTOSINE DEAMINATION BY DNA DEAMINASES AND EXPRESSION IN REPROGRAMMING TISSUES.

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Highly differentiated and specialised gametes undergo epigenetic remodeling essential to reconstitute the diploid nucleus of the zygote and restore totipotency. The remodelling involves DNA methylation and covalent modifications of core histones.

DNA methylation is important for epigenetic regulation of the genome. Through normal development it contributes to parental imprinting, X chromosome inactivation, silencing of transposable elements, and tissue-specific gene expression. It is also implicated in cancer and ageing. DNA methylation reprogramming occurs after fertilisation during early mammalian development and during germ-cell development. The loss of methylation without replication seen in these cases suggests an enzyme-catalysed reaction.

The loss of methylation from cytosine may occur directly but is energetically unfavourable. More likely is the deamination of methylcytosine to thymine followed by repair of the mismatch replacing methylcytosine with cytosine. The deamination of methylcytosine can also lead to mutations. With the discovery of cytosine deaminases and the otherwise paucity of methylcytosine directed activities, we are interested in determining whether these deaminases possessed any methylcytosine deaminase activity. We find that Aid and Apobec1 have robust deaminase activities against methylcytosine measured in both an *in vitro* assay and an *E. coli* based bioassay. These two deaminases are located in a cluster with other genes expressed in pluripotent tissues, and we find expression of these deaminases in oocytes, primordial germ cells and other pluripotent tissues. We are investigating the role of these deaminases in mice.

DNA demethylation is part of the epigenetic reprogramming that takes place in the early embryo and the establishment of the germ line. Understanding epigenetic events during these key phases of development will contribute to our knowledge of a process that is required for normal development and reproduction, and may prove useful in attempts to treat cancer, defer ageing, and reprogram somatic cells through nuclear transfer.