

## ABSTRACTS - ORALS

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### FOUNDATIONS OF FERTILITY: SIGNALING PATHWAYS REGULATING FETAL GERM CELL DEVELOPMENT

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Germ cells respond to molecular cues that regulate sex-specific development, and the decision to withdraw from the cell cycle, in the fetal gonads. In fetal ovaries, germ cells enter meiosis whereas in fetal testes, germ cells arrest mitotically. We report here three signaling systems that contribute to the control of fetal germ cell fate *in vitro* and *in vivo*.

1. Germ cells in a female mouse fetal gonad are triggered to enter meiosis by the potent signaling molecule retinoic acid (RA). RA induces germ cells to express *Stra8*, which is essential for initiation of meiosis. In the developing testis, germ cells avoid entering meiosis because RA is actively degraded by a cytochrome P450 enzyme, CYP26B1.

2. FGF9 acts directly on testicular germ cells to prevent up-regulation of *Stra8* and hence their entry into meiosis. Thus, *Stra8* expression, and hence meiosis, is regulated both positively and negatively by RA and FGF9 respectively.

3. The TGF $\beta$  morphogen Nodal and its co-receptor Cripto regulates the balance between continued germ cell proliferation and cell fate commitment in the developing testis. Compromised Nodal signaling in male germ cells led to reduced pluripotency, premature differentiation, and fewer adult spermatogonial stem cells. Conversely, human testicular germ cell tumours showed aberrant activation of NODAL and CRIPTO proportional to tumour severity and number of malignant cells. Thus, Nodal signaling in the male germline provides the molecular control mechanism that regulates cell potency, and dysregulation of this pathway determines tumorigenic potential.

Our results support the hypothesis that testicular germ cell tumours and some forms of infertility have their origins in dysregulation of pathways controlling germ cell behaviour in the embryo.

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### RODENT PREIMPLANTATION MATERNAL LOW PROTEIN DIET MODEL AND ADULT CARDIOVASCULAR DISEASE

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The composition of maternal diet around the time of conception in several animal models has been shown to influence the plasticity of the developmental programme leading to altered postnatal phenotype. The developmental consequences can affect growth, physiological and metabolic parameters and often associate with onset of adult disease. These environmental effects mediated during the periconceptual period also have implications for human assisted conception treatment and the use of reproductive biotechnologies used in domestic animal species. We have investigated such developmental programming mechanisms in rodent models in particular with a maternal low protein diet administered for the period of preimplantation development after mating, with normal control nutrition provided for the remainder of gestation and postnatally. This protein restriction model has revealed cardiovascular dysfunction, notably relative hypertension and associated disturbance in arterial vessel relaxation potential, behavioural abnormalities, metabolic effects and altered growth in offspring, with evidence of gender-specific sensitivities. Analysis of preimplantation embryos derived from diet-challenged mothers indicate programming of developmental plasticity has occurred by the blastocyst stage. Our data indicate that the extra-embryonic lineages associated with both the development and function of the visceral yolk sac and trophoblast are altered by the diet challenge. These changes are suggestive of compensatory mechanisms to increase the efficiency of nutrient retrieval likely to protect foetal growth and stabilise competitive fitness in the next generation. In addition, use of embryonic stem cell lines derived from blastocysts following maternal diet treatments provide further evidence for developmental plasticity. Activation of embryo responses and resulting changes in foetal development appear to involve physiological and epigenetic components and are mediated through initial 'sensing' by the early embryo of the maternal nutrient environment. Collectively, our rodent models provide evidence to support the concept that the developmental origins of adult disorders can be traced back to maternal-embryonic interactions prior to implantation. Funded by NICHD, USA; BBSRC, UK, MRC, UK and Gerald Kerkut Trust, UK.

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### ONE-CARBON METABOLISM AND EPIGENETIC PROGRAMMING IN THE OOCYTE AND PRE-IMPLANTATION EMBRYO

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Long-term programming effects of specific dietary B-vitamins (e.g. vitamin B12, folate) and sulphur amino acids (e.g. methionine) on key epigenetic processes during gametogenesis and pre-implantation development have been the focus of research endeavours at Nottingham in recent years. In the sheep we have demonstrated that physiologically relevant reductions in the dietary supply of these one-carbon related metabolites to intending mothers can lead to epigenetic modifications to DNA methylation in progeny associated with hypertensive adult offspring that are also insulin resistant; effects most pronounced in male offspring [Sinclair et al., 2007]. Parallel studies in the rat reveal common phenotypic effects which are also male specific [Maloney et al., 2011]. These studies set a precedent for the long-term programming effects of specific micronutrients during gametogenesis and pre-implantation development. Current studies are seeking to develop our understanding of how disturbances to one-carbon metabolism can lead to epigenetic dysregulation of DNA methylation in germ cells. To that end we have been developing mathematical models of these cycles in the ovary, which have served to direct current *in vitro* experiments with oocytes and somatic cells from the ovarian follicle. Long-term follow-up studies in sheep offspring also point to endoplasmic reticulum stress as a putative mechanism underlying insulin resistance, and the epigenetic regulation of selected genes involved in this process is currently under investigation. These emerging data together with current concepts and thoughts on future directions will be discussed.

(1) Sinclair et al. 2007. PNAS 104: 19352

(2) Maloney et al., 2011. J. Nutr. 141: 95-100

## MATERNAL OBESITY AND THE EARLY ORIGINS OF CHILDHOOD OBESITY: WEIGHING UP THE BENEFITS AND COSTS OF MATERNAL WEIGHT LOSS IN THE PERICONCEPTIONAL PERIOD FOR THE OFFSPRING

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As the prevalence of obesity has increased in the developed world, more women are entering pregnancy with a high Body Mass Index (BMI) in the overweight (BMI>25 kg/m<sup>2</sup>) or obese (BMI>30kg/m<sup>2</sup>) range. Population and clinical studies have shown maternal obesity results in fertility problems and suboptimal outcomes for the mother and her fetus during and after pregnancy. It is also the case that maternal obesity is associated with an increase in BMI in her offspring – as an infant, during childhood and into later life. There is a need to understand the separate or interdependent contributions of maternal pre pregnancy BMI, gestational weight gain, glycaemic control and macronutrient intake on the metabolic outcomes for the offspring. Experimental studies highlight that there may be separate influences of maternal obesity during the periconceptional period and late gestation on the adiposity of the offspring. Recent studies in our laboratory<sup>1</sup> have shown that while a period of dietary restriction in obese mothers may ablate the programming of obesity, it is associated with an activation of the stress axis in the offspring. Thus maternal obesity may result in epigenetic changes which predict the need for efficient fat storage in postnatal life while maternal weight loss may lead to epigenetic changes which predict later adversity. Thus development of dietary interventions for obese mothers during the periconceptional period requires a greater evidence base which allows the effective weighing up of the metabolic benefits and costs for the offspring.

1. Zhang S, Rattanatray L, MacLaughlin SM, Cropley JE, Suter CM, Molloy L, Kleemann D, Walker SK, Muhlhausler BS, Morrison JL, McMillen IC. (2010) Periconceptional undernutrition in normal and overweight ewes leads to increased adrenal growth and epigenetic changes in adrenal IGF2/H19 gene in offspring. *FASEB J.* 4: 2772-82.

## DIET INDUCED PATERNAL OBESITY EPIGENETICALLY MODIFIES SPERM AND IMPAIRS THE REPRODUCTIVE HEALTH OF TWO SUBSEQUENT GENERATIONS IN A MOUSE MODEL.

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Obesity and related conditions such as type 2 diabetes and sub-fertility are increasingly prevalent. Although the paternal genomic contribution to the next generation is apparent, a dearth of information surrounds the non-genetic components of sperm and its impact on the health of subsequent offspring.

We have previously established that male obesity increases DNA damage and reactive oxygen species (ROS) levels in sperm, concomitant with decreased fertility indicated by reduced sperm binding, fertilisation, implantation and early embryo development.

Here, we demonstrate that paternal exposure of C57Bl6 mice to a high fat diet (HFD) induces obesity without diabetes and diminishes the reproductive viability of male and female first generation (F<sub>1</sub>) offspring. F<sub>1</sub> males exhibited perturbed sperm parameters of reduced motility (-13.8%  $P=0.04$ ), sperm binding (-38.9%  $P=0.0007$ ), fertilisation (-33.3%  $P=0.009$ ) and increased ROS (+18.0%  $P=0.03$ ). F<sub>1</sub> females oocytes had impaired meiotic progression (GV to MII; -24.6%  $P=0.04$ ) and mitochondrial function (mitochondrial membrane potential; MMP).

Strikingly, sub-fertility was further transmitted to both sexes of the second generation (F<sub>2</sub>) through both F<sub>1</sub> parental lines. F<sub>2</sub> males from both F<sub>1</sub> parental lineages presented with sperm motility defects and elevated ROS, with F<sub>2</sub> males from F<sub>1</sub> females also having smaller testes (-13.2%  $P=0.002$ ), seminal vesicles (-12.9%  $P=0.05$ ) and reduced peripheral testosterone (-26.7%  $P=0.05$ ). Conversely only F<sub>2</sub> females sired by F<sub>1</sub> males had oocyte defects of increased ROS (+16.3%  $P=0.03$ ) and aberrant mitochondrial function (MMP). The compromised F<sub>2</sub> gametes imply that the reproductive health of the third generation may also be jeopardised.

Founder male obesity reduced global methylation of DNA extracted from both whole testes (-27.0%  $P=0.004$ ) and enriched spermatid populations (-24.9%  $P=0.00002$ ). This study provides the first evidence that paternal HFD induced obesity initiates alterations to the epigenome of sperm possibly inducing the observed intergenerational transmission of impaired reproductive function.

## MATERNAL INSULIN RESISTANCE CAUSES OXIDATIVE STRESS AND MITOCHONDRIAL DYSFUNCTION IN OOCYTES

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Insulin resistance (IR) and hyperinsulinemia compromise fertility in females. However, the precise underlying mechanism remains unclear. By establishing IR in a mouse model, we have investigated how IR impairs oocyte quality and early embryonic arrest. Our data demonstrated that an imbalance in oxidative products such as high reactive oxygen species, and decreased antioxidative products, such as GSH and GSH/GSSG, resulted in oxidative stress (OS), impairing mitochondrial function in GV and MII oocytes of insulin-resistant mice. These dysfunctional mitochondria displayed a decrease in the ATP content and mitochondrial DNA (mtDNA) copy number in MII oocytes. However, GV oocytes were characterized by high ATP content concomitant with increased clustered mitochondrial distribution and high inner membrane potential, indicating high mitochondrial oxidative phosphorylation. Low demand and high supply of ATP contributed to OS in GV oocytes of insulin-resistant mice. Notably, GV oocytes from insulin-resistant mice showed early stage apoptosis, which affected oocyte maturation and was associated with reduced retrieved MII oocytes and poor quality oocytes. Dysfunctional mitochondria and OS contributed to abnormal spindles and misaligned chromosomes. Together, our data show that IR contributes to OS, which, in turn, impairs mitochondrial function in mouse oocytes. This may impair the accurate transmission of mtDNA from one generation to the next. Therefore, our results suggest that OS and mitochondrial dysfunction are responsible for poor oocyte quality of insulin-resistant mice, and may provide novel targets to improve low fertility in females with IR.

## REGULATION OF EXIT FROM MEIOTIC PROPHASE

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It is important that the exit from the first meiotic prophase into the division phase be precisely timed. Failure to coordinate it with appropriate resolution of recombination events can lead to failure of homologs to separate (nondisjunction), with ensuing gamete aneuploidy and pregnancy loss. Competency for meiotic prophase exit arises after assembly of the synaptonemal complex (SC). Both unbiased mutagenesis and biased “candidate protein” approaches have identified crucial regulatory steps. A mutation in the mouse *Eif4g3* gene, encoding eukaryotic translation initiation factor 4 gamma, 3, causes male infertility, with failure in efficient translation of HSPA2, an SC-localized chaperone protein for CDC2A kinase, in spite of the presence of *Hspa2* transcript. The HSPA2 protein is known to be required for desynapsis, including disassembly of the central element of the SC, but the precise kinases bringing about desynapsis have not been identified. We have found that activity of polo-like kinase 1 (PLK1) is essential for disassembly of central element components of the synaptonemal complex. Moreover, SC central element proteins undergo PLK1-mediated phosphorylation during exit from meiotic prophase. Together, these results implicate coordinated activities of a translation factor, a chaperone protein and a kinase; and suggest a regulatory pathway whereby SC-associated proteins lead to kinase activation that promotes timely exit from prophase and ensures accurate chromosome segregation. Supported by NIH HD33816.

## CDH1/FZR1 IN THE MAINTENANCE OF PROPHASE ARREST AND PREVENTION OF ANEUPLOIDY

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Cdh1 (a.k.a. Fzr1) and Cdc20 are two well-established activators of the Anaphase-Promoting Complex/Cyclosome (APC/C). In the mitotic cell cycle division the APC/C has the essential task of getting a cell to complete M-phase properly and ensuring that S-phase does not start precociously. It is an E3 ubiquitin ligase, and its ubiquitinated substrates are thereby earmarked for immediate proteolysis through the 26S proteasome. Targeting distinct substrates at specific times in the cell cycle is key in understanding APC/C function, and in part this achieved by whether the E3 ligase is bound to Cdh1 or Cdc20.

Mammalian oocytes have distinct stops and starts to their meiotic divisions: a protracted prophase I arrest, hormonal triggered meiotic resumption, re-arrest at metaphase II, and a sperm-triggered completion of meiosis II at fertilization. Over recent years, work from our lab and those of others have helped establish that APC/C activity is essential in many aspects of these meiotic transitions.

Of particular note is the role played by Cdh1 in controlling APC/C function in oocytes. Using antisense knockdown, and latterly an oocyte-specific knockout (loxP/ZP3-Cre), we find that APC/C<sup>Cdh1</sup> activity is essential for the maintenance of prophase I arrest by preventing accumulation of its substrate cyclin B1, the regulatory subunit of CDK1. Using both antisense knockdown and knockout approaches, we also demonstrate that Cdh1 plays an important function in meiosis I, where it helps time the metaphase-anaphase transition and so prevents the oocytes from mis-segregating homologs.

The involvement of Cdh1 both in the process of the timing of prophase I arrest and the timing of homolog segregation in meiosis I make it an important regulator of female meiosis.

## MECHANISMS REGULATING ACCURATE CHROMOSOME SEGREGATION DURING MOUSE OOCYTE MEIOSIS

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To understand the molecular pathways regulating chromosome segregation during oocyte meiosis, by using overexpression and RNA interference (RNAi) approaches, we analyzed the role of Bub3 in mouse oocyte meiosis. Our data showed that overexpressed Bub3 inhibited meiotic metaphase-anaphase transition by preventing homologous chromosome and sister chromatid segregation in meiosis I and II, respectively. Misaligned chromosomes, abnormal polar body and double polar bodies were observed in Bub3 knock-down oocytes, causing aneuploidy. Furthermore, through cold treatment combined with Bub3 overexpression, we found that Bub3 was involved in the attachment of microtubules to kinetochores. We propose that as a member of SAC, Bub3 is required for regulation of both meiosis I and II, and is potentially involved in kinetochore-microtubule attachment in mammalian oocytes.

Two-step releases of cohesion between sister chromatids ensure that homologous chromosomes separate in meiosis I and sister chromatids separate in meiosis II, generating haploid gametes. We found that Sgo1 overexpression kept homologous chromosomes and sister chromatids not to separate in meiosis I and meiosis II, respectively, while the Sgo1 RNAi promoted premature separation of sister chromatids before meiosis II. Our results reveal that prevention of premature separation of sister chromatids in meiosis I requires the retention of centromeric Sgo1, while normal separation of sister chromatids in meiosis II requires loss of centromeric Sgo1.

## CHROMOSOME SEGREGATION IN MAMMALIAN OOCYTES

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Errors in chromosome segregation during cell division lead to erroneous DNA content of daughter cells, known as aneuploidy. Whereas aneuploidies are rare in somatic cells, errors are relatively common in mammalian oocytes, both in meiosis-I and meiosis-II, and are a major cause of embryo loss. Chromosome segregation in most cell types occurs as a result of poleward chromosome motion (anaphase-A), and separation of spindle poles (anaphase-B). Despite significant advances in many cell types, next to nothing is known about the mechanisms by which spindle microtubules drive chromosome segregation in mammalian oocyte meiosis. Moreover, the mechanisms which oocytes employ to attempt to prevent aneuploidy, and how these mechanisms go awry in oocytes from older mothers are poorly defined. In this talk I will discuss some of our recent work on chromosome segregation in mammalian oocytes, including recent analyses of the anaphase mechanism in mouse eggs. Live imaging reveals that spindles elongate

## METABOLIC REGULATION OF THE REPRODUCTIVE AXIS

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As a general rule, hypothalamic neuropeptides that stimulate food intake act to inhibit the reproductive axis. Thus, our studies in sheep indicate that neuropeptide Y acts to stimulate feeding but inhibits the reproductive axis. Melanocortins, such as melanocyte stimulating hormone-  $\alpha$ ,  $\beta$  and  $\gamma$ , are products of the pro-opiomelanocortin (POMC) gene, produced in cells of the arcuate nucleus and act to inhibit feeding. Melanocortins also stimulate the reproductive axis. In lean hypogonadotropic ovariectomised ewes, POMC gene expression is reduced, but intracerebroventricular (icv) infusion of leptin increases expression of this gene and peptide levels as well as restoring pulsatile luteinising hormone (LH) secretion. Icv infusion of a melanocortin receptor agonist also increases LH secretion in lean animals, suggesting that the melanocortin system may be the means by which the reproductive axis is regulated by changing metabolic state. Melanocortin cells have reciprocal communication with kisspeptin cells of the arcuate nucleus, so effects of these two systems on the gonadotropin releasing hormone (GnRH) cells may be integrated.

Gonadotropin inhibitory hormone (GnIH) is produced by cells of the dorsomedial hypothalamic nucleus/paraventricular nucleus. These cells project to GnRH cells and appetite regulating cells as well as to the neurosecretory zone of the median eminence. In sheep at least, GnIH acts to inhibit both GnRH cells and pituitary gonadotropes. The peptide also has a potent effect to stimulate food intake. GnIH gene expression is reduced in the periovulatory period, consistent with data from other species showing a reduction in appetite at the time of ovulation. Thus, GnIH signals to inhibit reproduction and stimulate food intake.

## GNRH PULSE GENERATION BY HYPOTHALAMIC KISSPEPTIN/NEUROKININ B/DYNORPHIN NEURONS

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The gonadotropin-releasing hormone (GnRH) pulse generator is thought to play pivotal roles in reproduction by integrating a wide variety of inputs that convey information about the internal and external environment. Although previous studies indicated that the pulse generator is located in the hypothalamus, the precise neural identity had remained unknown for a long period. However, recent evidence suggests that a group of kisspeptin neurons in the arcuate nucleus (ARC) is a likely candidate for the pulse generator. Our laboratory has been studying this issue in the goat model. By recording multiple unit activity (MUA) in close proximity of ARC kisspeptin neurons, we found that rhythmic bursts of MUA (MUA volleys) occur with a constant interval and each MUA volley is invariably accompanied by a luteinizing hormone (LH) pulse in gonadectomized male and female goats. Treatments of the goats with estrogen and progesterone decreased the frequency of the rhythmic bursts, possibly reflecting the negative feedback action of the steroids. Histochemical studies revealed that neurokinin B (NKB) and dynorphin (Dyn) are contained in most of ARC kisspeptin neurons, and suggested that kisspeptin/NKB/Dyn neurons form a neural network in the ARC. Then, physiological roles of NKB and Dyn were examined in the MUA system. Intracerebroventricular injection of NKB immediately induced the MUA volley, whereas Dyn inhibited it. On the other hand, an NKB receptor antagonist suppressed the occurrence of MUA volley, whereas a Dyn receptor antagonist accelerated it. Taken together, we propose that the intrinsic source of the GnRH pulse generator is the group of ARC kisspeptin/NKB/Dyn neurons, in which rhythmic activity is generated by a reciprocal action of NKB (stimulatory) and Dyn (inhibitory). Kisspeptin would play a role in transmitting the rhythmic signal to GnRH neurons to generate pulsatile GnRH release.

## THE DESIGN OF A KISSPEPTIN-10 ANALOGUE WITH GREATER *IN VIVO* BIOACTIVITY THAN KISSPEPTIN-10.

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The kisspeptins are neuropeptides critical to the activity of the hypothalamo-pituitary-gonadal (HPG) axis. The smallest endogenous kisspeptin, kisspeptin-10 (KP-10), binds to the receptor KISS1R with a similar affinity to the full-length peptide, kisspeptin-54 (KP-54), but is less effective *in vivo*, possibly because of increased enzymatic breakdown or clearance. The kisspeptin system may have therapeutic potential in the treatment of reproductive disease. Rational modification of the structure of KP-10 resulted in KP-10 analogues with varied binding affinities for the KISS1R. Those analogues that bound with relatively high affinity to KISS1R were tested for ability to stimulate ERK1/2 phosphorylation *in vitro* and for their ability to stimulate the HPG axis *in vivo*. The analogue [dY](1)KP-10, bound to KISS1R with lower affinity to KP-10 but exhibited similar bioactivity *in vitro*. *In vivo*, peripheral administration of [dY](1)KP-10 increased plasma LH and testosterone more potently than KP-10 itself in mice. At 60 min postinjection, 0.15 nmol [dY](1)KP-10 significantly increased total testosterone levels in mice whereas the same dose of KP-10 had no significant effect. Long-lasting KP-10 analogues such as [dY](1)KP-10 may have therapeutic potential in reproductive disease.

## EFFECTS OF PRE-NATAL STRESS AND POST-NATAL PAIN ON THE STEROIDOGENIC CAPACITY OF YOUNG PIG GONADS

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We have shown that prenatal social stress and tail amputation early in life reduces testosterone concentrations and testis size, respectively, in young male pigs (Hoeks et al. 2010). This work has now been extended to better understand how these treatments affect male and female gonad steroidogenesis. Eight pregnant sows were stressed by mixing with unfamiliar older sows for 2 1-week periods during mid-pregnancy while 8 control pregnant sows were not mixed. On day 3 after birth, half the piglets from each litter had their tails amputated while the other piglets were left intact. Gonad tissue and plasma was obtained from 1 tail-amputated and 1 intact piglet of each sex from each litter at slaughter on 40.44±0.27 days of age. Oestradiol concentrations were determined by radioimmunoassay. Ovaries were preserved in Bouin's solution, sectioned and stained with haematoxylin and eosin to count numbers and developmental stages of ovarian follicles. Testes were snap frozen and expression of 17 $\alpha$ -hydroxylase determined by qPCR. Data were analysed in a 2x2 analysis of variance, fitting age at slaughter as a covariate. Oestradiol concentrations were lower in prenatally stressed male (22.88 versus 38.70 pg/ml, s.e.d.= 6.06;  $P=0.015$ ), but not female pigs and in both male (25.61 versus 34.92 pg/ml, s.e.d.= 6.05;  $P=0.021$ ) and female (4.76 versus 6.88 pg/ml, s.e.d.= 1.47;  $P=0.030$ ) tail-amputated compared with intact pigs, respectively. Prenatally stressed female pigs had fewer primordial ovarian follicles (-4.32 versus -3.96 log/ $\mu\text{m}^2$ , s.e.d.=0.14;  $P= 0.027$ ). There were no other treatment effects on ovarian follicle populations. There was a significant interaction between the effects of pre-natal stress and post natal pain on 17 $\alpha$ -hydroxylase expression, such that amputation increased expression in pigs born to control sows, but reduced expression in animals born to stressed sows. These results confirm the sensitivity of the developing reproductive axis to both pre- and early post-natal stress.

(1) Hoeks C, Rutherford KMD, Hogg CO, Duncan C. & Ashworth CJ 2010 Pre-natal stress and post-natal pain affect testis development in young pigs. Proc SRF Annual Meeting, Nottingham. P66

## A TRANSCRIPTOMIC EXAMINATION OF SEXUAL DIFFERENTIATION IN ZEBRAFISH

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The zebrafish (*Danio rerio*) is a popular vertebrate model. Although many studies investigating zebrafish developmental biology have been conducted, molecular pathways underpinning sexual development in zebrafish are poorly understood. At present, neither sex chromosomes nor sex markers have been identified. It is likely, however, that genes do play important roles in zebrafish sex determination. Apart from genetic factors, environmental factors can also influence sex ratios. While little is known of the molecular genetics of sexual development in zebrafish, the importance of sex hormones, such as estradiol and testosterone, in gonadal differentiation and the maintenance of sexual phenotype and reproductive potential in the differentiated gonad of zebrafish is well established. In fact, manipulation of the sexual phenotype of fishes using sex steroids is a routine procedure utilised for the production of monosex populations for aquaculture. In this study, we take advantage of hormonal manipulation of the sex of developing zebrafish embryos to unravel the unknown genetic pathways that underlie sex determination. Consistent with published reports, we achieved full masculinisation of the zebrafish with methyltestosterone. However, ethinylestradiol treatment suppressed gonad development in presumptive genetic males at low doses and prevented gametogenesis in both sexes at higher doses. While previous studies have focused on investigating the genes involved in sex hormone induced-sex reversal in the zebrafish gonad through candidate gene and microarray approaches, next generation sequencing technologies are used in this study to identify genes mediated by sex hormones in both the brain and gonad of zebrafish. Among the genes whose expression patterns have been altered through sex hormone exposure, we expect to find major players involved in the regulation of sex differentiation via the hypothalamus-pituitary-gonad axis (e.g., Cytochrome p450 aromatase B and Gonadotrophin-releasing hormone). We anticipate these data will help elucidate the roles of sex hormones in teleost sex differentiation with potential benefits for the control of sex in aquaculture.

## MOLECULAR DETERMINANTS OF BLASTOCYST COMPETENCY FOR IMPLANTATION

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Early pregnancy loss in humans, which often occurs due to defects that occur before, during or immediately after implantation, is a worldwide social and economic concern. For successful implantation to occur in the receptive uterus, the blastocyst must also attain implantation competency. The first evidence that the state of activity of the blastocyst determines the "window" of implantation in the receptive uterus was derived from reciprocal blastocyst transfer experiments in a delayed implantation mouse model. This model is a powerful approach to define the molecular signaling components that direct blastocyst activation or dormancy. Nearly 100 mammals in seven different orders undergo delayed implantation, but the underlying mechanism remains largely unknown. There is evidence that catecholestrogens produced from primary estrogens in the uterus activate blastocysts. Another lipid signaling molecule that targets blastocysts is an endocannabinoid anandamide, which activates G-protein coupled cannabinoid receptors CB1 and CB2. Expression of CB1 in the Tr, and uterine synthesis of anandamide, suggest that endocannabinoid signaling is critical to implantation in mice. Levels of uterine anandamide and blastocyst CB1 are coordinately downregulated with the attainment of uterine receptivity and blastocyst activation, respectively, in contrast to their elevated levels in the nonreceptive uterus and dormant blastocysts. Anandamide regulates blastocyst function by differentially modulating MAPK signaling and Ca<sup>2+</sup> channel activity via CB1. Using delayed implantation model, a global gene expression study showed that these two different physiological states of the blastocyst are molecularly distinguishable. The main functional categories of altered genes include cell cycle, cell signaling and energy metabolic pathways. This study also showed an upregulated expression of heparin-binding EGF-like growth factor (HB-EGF) in activated blastocysts and is complementary to earlier reports of upregulated expression of its receptor ErbB1 and ErbB4 in similar blastocysts. Recently, we demonstrated that silencing of Wnt-beta-catenin signaling in mice does not adversely affect the development of preimplantation embryos to blastocysts and uterine preparation for receptivity, but, remarkably, blocks blastocyst competency to implantation. A coordinated activation of canonical Wnt-beta-catenin signaling with Cox-2-PPAR $\delta$  signaling pathway ensures blastocyst competency to implantation. These findings constitute novel evidence that Wnt signaling is at least one pathway that determines

blastocyst competency for implantation. More insight into the molecular basis of blastocyst competency for implantation might help to improve pregnancy rates in human IVF programs.

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## SEX AND THE SINGLE FOLLICLE: MECHANISMS INVOLVED IN SELECTION OF ONE OR MULTIPLE DOMINANT FOLLICLES IN CATTLE

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Selection of a single dominant follicle in cattle involves a sequence of endocrine, intercellular, and intracellular changes that produces a single follicle that continues to grow, whereas the non-selected follicles cease growth and eventually undergo atresia. Technical breakthroughs have helped to unravel the pattern of changes during selection but have not yet definitely established the role of each specific change in the follicle selection process. In Holstein heifers morphologically distinguishable selection of a single dominant follicle occurs when the follicle reaches ~8.5 mm in diameter. The circulating FSH concentrations, on average, reach a nadir near this time, although individual cows show surprising variation in the magnitude and pattern of the changes in FSH that accompany follicle selection. Blockade of LH pulses by treatment with the GnRH receptor antagonist, Acyline, does not inhibit follicle growth before 8 mm but follicles do not proceed past the point of follicle selection in Acyline-treated animals. This effect is consistent with the dramatic increase in expression of LH receptors in granulosa cells near the time of follicle selection and suggests that a shift from FSH-dependence to LH-dependence occurs in the dominant follicle. Surprisingly, Acyline-treated animals do not have the characteristic increase in LH receptor expression in granulosa cells indicating a potential role for LH pulse in induction of LH receptors in the dominant follicle. A myriad of other gene expression changes have been reported in granulosa cells near the time of follicle selection including changes that would: increase estradiol production, inhibit apoptosis, change intrafollicular paracrine regulators, change the extracellular matrix, and alter metabolism and cell proliferation. The precise roles of these molecular and cellular changes in follicle selection are still being investigated. In dairy cows, high milk production is associated with a dramatic increase in frequency of double ovulation and dizygotic twinning. An increase in circulating FSH during the 24 h prior to follicle selection is associated with selection of multiple, compared to single, dominant follicles in lactating dairy cows. Interestingly, carriers of gene that results in high ovulation in cattle also demonstrate a distinct change in the pattern of circulating FSH near follicle selection. Although there are no difference in circulating FSH prior to follicle selection, the characteristic decline in FSH associated with follicle selection does not occur in carriers of the multiple-ovulating genotype and this may underlie the selection of multiple ovulatory follicles in this genotype. The physiology producing this change in FSH (follicular or pituitary) and the intrafollicular and molecular changes associated with this genotype have not yet been defined. Thus, a dynamic physiological model of follicle selection continues to emerge, although many key features are not yet defined.

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## HIERARCHICAL OVARIAN FOLLICULAR DEVELOPMENT AND OVULATION-RATE

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Peters et al (1975) reported that ovarian follicles, in mice, grow sequentially, and grow without rest until they die or ovulate. Subsequent studies in humans, sheep and cattle indicated that at any moment in time few, if any, antral follicles share an identical endocrine microenvironment or somatic cell composition thereby supporting the notion that follicular growth is hierarchical. More recently, from studies in sheep with genetic mutations that result in ovulation-rates of between 5 and 14, instead of the usual 1 to 3 in wild-types, or in animals subjected to a super-ovulation treatment, it is also evident that the hierarchical pattern of follicular development, as assessed by somatic cell composition and gonadotrophin-responsiveness of the granulosa cells in vitro, is retained. The question therefore arises as to whether oocytes in multiple ovulation-rate phenotypes or after exogenous gonadotrophin treatments share similar functional characteristics. In sheep, the growth factors, bone morphogenetic protein 15 (BMP15) and growth differentiating factor 9 (GDF9), are expressed exclusively by oocytes and are essential for normal follicular development and determining the ovulation-rate phenotype. In individual ovine oocytes, the expression levels of BMP15 and GDF9 mRNA are consistently 1:1 and highly correlated ( $R^2 > 0.90$ ), irrespective of follicular health, stage of antral follicular development and ovulation-rate phenotype. However, in the presumptive preovulatory follicles of sheep, differing in ovulation-rate between 1 and 14, the levels of BMP15 and GDF9 mRNA were highly variable. These findings suggest that oocytes in multiple preovulatory follicles are not functionally similar, at any moment in time, and that the levels of BMP15 and GDF9 mRNA are not reliable predictors of oocyte quality or likely pregnancy outcome. Thus, significant challenges remain with respect to improving the efficiencies of generating high yields of oocytes for embryo production.

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## FACTORS AND MICROENVIRONMENTS REGULATING CL FUNCTION - HYPOXIA IN LUTEAL PHYSIOLOGY

**K. OKUDA**

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Luteolysis is characterized by a decrease in progesterone (P4) production (functional luteolysis), followed by a decrease in luteal size (structural luteolysis), during which cells of the corpus luteum (CL) undergo apoptosis. We have provided evidences for the importance of low oxygen conditions (hypoxia) in luteolysis in cattle. Hypoxia inhibited P4 synthesis [1] and induced luteal cell death [2], suggesting that oxygen deficiency is one of the major factors contributing to both functional and structural luteolysis in cattle. Hypoxia induces activation of a transcription factor, hypoxia-inducible factor 1 (HIF1), which is recognized to stimulate angiogenesis via transcriptional regulation of angiogenic factors, such as vascular endothelial growth factor (VEGF). HIF1 is an obligatory heterodimeric protein composed of two members of the basic-helix-loop-helix (bHLH)-containing PER-ARNT-SIM (PAS) domain family, HIF1A (alpha subunit) and aryl hydrocarbon receptor nuclear translocator (ARNT) [3]. ARNT expression is not regulated by hypoxia, whereas HIF1A is stabilized only under hypoxic conditions [3]. Since CL develops with a rapid angiogenesis after ovulation, we investigated the expression of these factors in the CL throughout the estrous cycle to clarify whether hypoxia-induced angiogenesis occurs during CL development. We recently obtained the following findings [4]: mRNA expression of HIF1A was down-regulated at the regressed stage, while protein expression of HIF1A was highest at the early luteal stage and decreased at the latter stages. VEGF expression was highest at the developing luteal stage in both mRNA and protein levels. In addition, hypoxia increased the amounts of HIF1A protein, VEGF mRNA and protein in cultured bovine luteal cells, suggesting that hypoxia formed after a follicle rupture is important for the formation of luteal vasculature

in cattle. Based on the overall findings and our recent reports, we conclude that hypoxia is an important condition for both luteal formation and regression in cattle.

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- (3) Semenza GL. *Physiology (Bethesda)* 24, 97-106 (2009)
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## STEROIDAL CONTROL OF SERTOLI CELL AND SPERMATOGENIC DEVELOPMENT

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Steroids are vital for spermatogenesis, yet defining precise steroid-regulated pathways in the testis remains a challenge. Our research established several mouse models to dissect specific hormone actions during postnatal spermatogenic development. Steroid-deficient hypogonadal (*hpg*) mice provided a versatile model to identify specific hormone contributions during postnatal Sertoli and germ cell development. Restoration of steroidogenic and transgenic (Tg) FSH activity in *hpg* mice identified androgen- and FSH-responsive germ cell populations and Sertoli cell factors. Estradiol (E2) also stimulated Sertoli cell function and spermatogenesis in *hpg* testes. However, postnatal aromatase activity is not required to achieve normal Sertoli cell (SC) numbers, and E2-treated androgen receptor (AR)-deficient males showed that E2- and FSH-induced spermatogenesis and SC proliferation requires a functional AR. Sertoli-specific loss of AR function by Cre-loxP-mediated disruption of the AR DNA-binding domain (DBD) produced infertile SC<sup>[AR-DBD]</sup> males exhibiting limited postmeiotic germ cell development. Sertoli cell-specific AR-regulated transcripts (*Rhox5*, *Eppin*) were decreased in postnatal (5-10 day old) SC<sup>[AR-DBD]</sup> vs WT testes. However, *Rhox5* expression declined, whereas *Eppin* expression increased, in adult SC<sup>[AR-DBD]</sup> testes containing normal testosterone levels, revealing differential temporal control for distinct AR-regulated transcripts. Sertoli cell AR-mediated actions in early postnatal development were identified in mice expressing Tg-hAR under the Sertoli-specific *Abp* promoter, denoted SC<sup>[TgAR]</sup>. Premature Sertoli cell-specific AR expression elevated known androgen-regulated genes (*Rhox5*, *Eppin*, *Cldn11*) in 5 do SC<sup>[TgAR]</sup> vs WT testes, whereas postnatal *Amh* expression, reported to be downregulated by AR, was independent of SC<sup>[TgAR]</sup> actions. In addition, our SC<sup>[TgAR]</sup> model showed that promiscuous AR-regulated expression of important Sertoli cell transcripts had no impact on early postnatal mitotic (*ckit*) and meiotic (*Stra8*, *Piwil2*, *Sycp1*) germ cell markers, but elevated transcripts (*Dmcl1*, *Spo11*) crucial for pachytene meiotic development. These loss- and gain-of-function approaches provide unique opportunity to differentiate AR-regulated genes and pathways during Sertoli and germ cell maturation.

## REGULATION OF SPERMATOGENESIS BY SMALL NON-CODING RNAs

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The discovery of abundant small non-coding RNA (sncRNA) populations in the transcriptome of the cell has changed our view on the makeup of our genome and the complexity of the regulatory networks imposed by these sncRNAs in every aspect of cellular functions. Testicular cells express almost all sncRNAs species identified to date and some of the sncRNAs are exclusively or preferentially expressed in the testis (e.g. piRNAs). Using the next generation sequencing technology, we have determined the sncRNA transcriptome of spermatogenic cells in the adult testes. In addition to known sncRNA species (e.g. miRNAs, piRNAs, snoRNAs, tRNAs, rRNAs), we also identified novel endogenous-siRNAs (endo-siRNAs) and mitochondrial genome-encoded small RNAs (misRNAs). The biogenesis of endo-siRNAs and misRNAs may share pathways common to the production of miRNAs, but also involves yet-to-be-defined mechanisms.

miRNAs are believed to act as translational suppressor by binding the 3'UTRs of mRNAs followed by recruiting RNA-induced silencing complex (RISC). However, increasing lines of evidence have shown that miRNAs can also promote translation. Using spermatogenesis as a model, we found that miRNAs could either suppress or promote translation depending upon the subcellular compartmentalization and the effector complexes that miRNAs recruited. In spermatocytes and spermatids, MIWI is a housekeeping member of the miRNA effector complexes. Proteomic analyses reveal that the MIWI-miRNA complexes contain RNA-binding proteins that are involved in classical mRNA decay or translation-suppressive/promoting pathways.

Conditional inactivation of *Dicer* or *Drosha* in each every spermatogenic cell type revealed that neither *Dicer* nor *Drosha* is required for the spermatogonia stem cell renewal and differentiation in adult testes. But they are essential for the meiotic and haploid phases of spermatogenesis. Slight differences in the phenotypes between *Dicer* and *Drosha* knockout testes suggest that endo-siRNAs may play a role. Indeed, we have identified numerous endo-siRNAs expressed mainly by spermatogenic cells in the adult testes.

Our data start to shed light on the role of sncRNAs in the regulation of spermatogenesis.

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## SPERM STEM CELL BEHAVIORS IN MOUSE TESTIS

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In mammalian testis, numerous sperms are produced persistently for a prolonged period, which plays an essential role for the continuity of life. It is generally considered that stem cells that are both capable of self-renewal and differentiation supports the continuity of spermatogenesis. Stem cells are also considered to be crucial not only for steady state spermatogenesis but also for regeneration after damage or transplantation. However, it is still largely to be elucidated what is the cellular identity and characters of the stem cells and how they behave in the testis.

We have been challenging this issue by means of live imaging and pulse labeling, which allow investigating the cellular behavior in undisturbed testis that undergoes steady-state spermatogenesis. These have shown us, slowly but steadily, how the stem cell functions are achieved. It has been suggested that, rather than a very limited number of defined stem cells, an extended population of undifferentiated cells with different degree of self-renewing and differentiating probabilities compose the functional stem cell compartment. Interestingly, it is largely influenced by the tissue situation (steady-state, regenerating after damage, or post-transplantation) how much the cell population is recruited to the active population with the stem cell functionality. These findings may expand our view regarding the definition of stem cells in the mouse spermatogenesis.

## DEVELOPMENT OF NOVEL CLONING AND ART METHODS FOR ANIMAL PRODUCTION AND SPECIES PRESERVATION

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We previously reported that treating cloned embryos with Trichostatin A (TSA), an inhibitor of histone deacetylase (HDACi) that also remarkably reduces DNA methylation, can increase the efficiency of hybrid mouse cloning up to 5-fold. Recently, we found that Scriptaid, another HDACi, allowed us to generate cloned mice from even inbred strains, such as C57BL/6 or C3H. On the other hand, ES cell can be established from somatic cell via nuclear transfer (ntES cell), and the establish rate is ten-fold higher than production rate of cloned mice. ntES cells were identical to the ES cells derived by fertilization in terms of their global gene expression and their differentiation potential, and healthy cloned mice are generated from ntES cell nuclei via serial nuclear transfer with relatively high success rate. By combining the cloning and the ntES cell techniques, this approach could be applied to fertility treatments using somatic cells instead of gametes. For example, we could obtain offspring from very old (nearly 3 years old) or infertile mutant mice using this combination technique. Surprisingly, cloned mice now can be generated from even frozen dead bodies or freeze-drying cells, suggesting that extinct animals, such as the mammoth, can be possibly resurrected by this technology if non-damaged nuclei are retrieved from the permafrost. Thus, although there remain many cloning issues, it has the emerging potential to become a powerful new research tool with broad based applications in the study of basic biology, especially for preserving mouse strain without germ cell. In this talk, I will describe this unique technique and the possible applications.

## PIONEERING THERAPIES TO PRESERVE OR PREVENT SPERM FUNCTION AND TO PROTECT AGAINST SEXUALLY TRANSMITTED DISEASES (STDs)

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There have been no radically new forms of contraception since the pill was introduced 1960 and even this technology was based on research conducted in the 1950s utilizing biochemical advances that can be traced back to the 1920s. Whatever new forms of fertility regulation we introduce for the future they should exploit the massive advances that have been made in our understanding of the reproductive system over the last half-century and be tailored to the needs of the 21<sup>st</sup> century. In this context, there is an urgent need to develop novel, safe, effective, dual-purpose contraceptive agents that combine the prevention of pregnancy with protection against sexually transmitted diseases (STDs). To achieve this aim we have researched a class of a topical contraceptive agent that can selectively and instantaneously immobilize millions of spermatozoa, while suppressing the infectivity of any pathogenic microbes, such as Chlamydia, that might also be present in the ejaculate. This approach is based on the ability of small molecular mass organic compounds to selectively and covalently adduct key proteins involved in the regulation of sperm movement, while modifying key surface proteins that control the infectivity of Chlamydia. We have also successfully developed strategies for the design of latent 'spermostatic' formulations that only become activated on contact with seminal plasma. Such developments pave the way towards a radical rethink of the topical contraception approach, since they enable delivery of the contraceptive agent to be temporally dissociated from the coital act. The further development and refinement of this pharmaceutical approach should permit a radical rethink in the design of safe, effective topical contraceptive methods that protect the user against both fertility and the spread of STDs.

## REGULATION OF IMPLANTATION AT THE FETAL-MATERNAL INTERFACE. OPPORTUNITIES FOR NEW CONTRACEPTIVES. Â

E. Dimitriadis

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Despite huge increases in access to contraceptives globally over 80 million women have unintended or unwanted pregnancies annually. Remarkably, there have been no new methods of contraception developed in the last 50 years. The World Health Organisation has recognised an urgent need to develop novel contraceptive options. We have utilized unique long-acting cytokine inhibitors in an anti-implantation contraceptive strategy. This is an entirely new approach that pharmacologically targets the uterus maintaining it in a non-receptive state so that embryo implantation is totally prevented. Studies in mice have demonstrated that an inhibitor to the interleukin 6-type cytokine, leukemia inhibitory factor (LIF), conjugated to polyethylene glycol (PEG) totally prevents embryo implantation via intraperitoneal injection or vaginal gel delivery without embryo toxic effects. In addition non-uterine target effects in bone and central nervous system are abolished via vaginal gel delivery in mice suggesting it is a preferable and suitable delivery option in women. In support, in women vaginally administered drugs preferentially localise to the uterus suggesting that vaginal administration of a compound is a suitable delivery method for an anti-implantation contraceptive. This is the first study to demonstrate the contraceptive efficacy of a PEGylated compound delivered vaginally. Contraceptive trials in non-human primates are currently underway. If effective, this will offer opportunities for new, non-hormonal contraceptives for women.

## OOCYTES UNDER ARREST: A “WHODUNIT”

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The progression of meiosis in female mammals is subject to start and stop signals driven by both intrinsic and extrinsic signals. In 1935 it was discovered that signals from ovarian somatic cells arrested meiosis at prophase I until the LH surge. Since then the identity of these signals has been a mystery with false leads, important clues, and a wide cast of suspects. Now the exciting interactions of characters, cell types and molecular signals, are coming together to shape an intriguing resolution of the “Whodunit.”

Sustaining elevated levels of cAMP in oocytes is essential for maintaining meiotic arrest in antral follicles. Activity of an oocyte-specific phosphodiesterase, PDE3A, degrades cAMP to initiate GVB. cGMP inhibits PDE3A activity, is produced in cumulus cells, and is transferred to oocytes where it acts to inhibit PDE3A, thus preventing reduced cAMP levels.

What regulates cGMP? Natriuretic peptide type C (NPPC) is expressed by mural granulosa cells and its cognate receptor natriuretic peptide receptor 2 (NPR2), a guanylyl cyclase, by cumulus cells. Treatment of isolated COCs with NPPC increases levels of cGMP in both cumulus cells and oocytes, of cAMP in oocytes, and in inhibition of GVB. Moreover, precocious resumption of meiosis occurs in Graafian follicles in loss-of-function mutants of either *Nppc* or *Npr2* demonstrating the crucial function of NPPC and NPR2 in maintaining arrest. Expression of *Npr2* mRNA, NPR2 function, and ability of NPPC to maintain meiotic arrest are regulated by oocyte-derived paracrine factors and estradiol.

The jury is in. They conclude that there is a conspiracy of complex interactions of ovarian follicular cell types (mural and cumulus granulosa cells and the oocyte), ligands (NPPC, oocyte-derived paracrine factors, and estradiol), and key NPR2 receptors on cumulus cells, all enable cumulus cell production of cGMP, which, when transferred to oocytes, is guilty of preventing meiotic resumption.

## THE ROLE OF MICRORNAS ON THE EPIGENETIC REGULATION OF FERTILIZED AND CLONED EMBRYO DEVELOPMENT

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Cloning or somatic cell nuclear transfer (SCNT) using adult somatic cell to derive cloned embryos is a promising new technology with potential applications in both agriculture and regenerative medicine. Mammalian embryos derived by nuclear transfer are capable of development to the blastocyst stage with a relatively high efficiency of 30–50%. However, in full-time development, usually only 2% of NT embryos can result in live births due to abnormalities in placenta formation. In SCNT embryos, the donor cell nucleus is epigenetically reprogrammed by oocyte cytoplasm during development. Incomplete reprogramming of the donor cell genome is considered a major reason for low cloning efficiency. Aberrant epigenetic modifications include DNA methylation, histone modification and X-chromosome-inactivation. Due to a lack of basic knowledge regarding the embryos following nuclear transfer, the success rate of cloning is low. Therefore, elucidation of the molecular mechanism of SCNT embryo development will be of great value for further research.

microRNAs (microRNA) are single-strand RNA molecules of about 19–23 nucleotides in length, which regulate gene expression by imperfect base pairing with target mRNA, subsequently guiding mRNA cleavage or translational repression. Since the first discovery and functional annotation in 1993 of the small RNA, *lin-4* and *let-7*, which are involved in developmental timing and gene regulation during *C. elegans* larval development, microRNAs have received scientific attention. Now hundreds of microRNAs have been identified in various multicellular organisms, and many microRNAs have been shown to be evolutionarily conserved. The roles proposed for this novel class of tiny RNA molecules are diverse. They are likely to be involved in developmental timing, differentiation, cell proliferation, signaling pathways, apoptosis, metabolism, heterochromatin formation, genome rearrangement, brain development and carcinogenesis.

Currently (2006- present) we are working to determine the role of microRNAs on the epigenetic regulation of fertilized and cloned embryo development. The general hypothesis of our research is that genetic and epigenetic factors regulate the development of preimplantation mammalian embryos, and aberrant modulations in cloned embryos are causes of abnormal development and low success rate of cloned embryos.

## PRODUCTION OF OFFSPRING FROM GERM LINE STEM CELLS IN NEW BORN OVARY

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Our recent study showed that a neonatal mouse female germline stem cell (FGSC) line with normal karyotype has been established by immunomagnetic isolation and culture for more than 15 months. The FGSCs from adult mice were isolated and cultured for more than 6 months. These FGSCs were infected with GFP virus and transplanted into ovaries of infertile mice. Transplanted cells underwent oogenesis and the mice produced offspring that had the *GFP* transgene. For further study, we have tested 3 proteins with the aim of improving the efficiency of FGSC purification. Immunofluorescence assays and magnetic sorting were performed using short-type PB-cadherin (*Stpb-c*), CD9, and interferon-inducible transmembrane protein 3 (*Iftm3*, *Fragilis*), all of which are expressed in germ cells. Although all 3 proteins were expressed in FGSCs, CD9 was unsuitable because of its lack of germline specificity, and *Stpb-c* was also unsuitable because of the unavailability of an appropriate primary antibody. The efficiency of FGSC purification was remarkably enhanced using the germline-specific protein *Fragilis*, compared with that using MVH. Next, we demonstrate the successful generation of transgenic or gene knock-down mice using FGSCs. FGSCs from ovaries of 5-day-old and adult mice were isolated and either infected with recombinant viruses carrying green fluorescent protein, Oocyte-G1 or the mouse dynein axonemal intermediate chain 2 gene, or transfected with the Oocyte-G1 specific shRNA expression vector, and then transplanted into infertile mice. Transplanted cells in the ovaries underwent oogenesis and produced heterozygous offspring after mating with wild-type male mice. The offspring were genetically characterized and the biological functions of the transferred or knock-down genes were investigated. Efficiency of genetransfer or gene knock-down was 29%–37% and it took 2 months to produce transgenic offspring. Gene manipulation of FGSCs is a rapid and efficient method of animal transgenesis and may serve as a powerful tool for biomedical science and biotechnology.

## PLURIPOTENCY AND THERAPEUTIC POTENTIAL OF INDUCED PLURIPOTENT STEM CELLS

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Ectopic expression of four transcription factors including Oct4, Sox2, Klf4 and c-Myc in differentiated fibroblast cells could reset the cell fate of fibroblast cells to pluripotent state (Takahashi and Yamanaka, 2006). Subsequently, full pluripotency of these so-called induced pluripotent stem cells (iPSCs) has been demonstrated as viable mice could be generated autonomously from iPSCs through tetraploid blastocyst complementation (Kang et al., 2009; Zhao et al., 2009). Moreover, the generation of human and patient-specific iPSCs has raised the possibility of utilizing iPSCs clinically. In this talk, I will briefly summarize the recent progress in our laboratory in understanding the pluripotency of iPSCs. Furthermore, I will further introduce the advances that we achieved recently in understanding the molecular mechanism of defined transcription factors mediated somatic cell reprogramming. Moreover, I will further discuss the potential of using patient-specific iPSCs for disease modeling and therapeutic purpose.

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## KNOWLEDGE FROM MODEL; GERMLINE- OR NONGERMLINE-DERIVED, STEM CELL-LIKE CELLS AND THEIR APPLICATION FOR DERIVING TISSUE-SPECIFIC STEM CELLS

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Biomodelling that employs various species and different anatomical levels have been developed for establishing innovative biotechnologies and therapeutic technologies. Especially in stem cell research, employing of various animal models greatly contribute to overcoming technical limitation of current stem cell engineering. The major interest of our research group is developing innovative technologies for therapy-friendly establishment of stem cells, which does neither employ embryonic tissue nor undertake gene target. So, we have continuously made our effort on establishing stem cells from non-germline, somatic tissue, and in particular, we are attempting to manipulate cell niche for deriving immune-specific stem cells without undertaking genetic manipulation. As results, we have succeeded to transform non-germline somatic cells retrieved from ovarian stoma tissue into embryonic stem cell-like, colony-forming cells in mice. Total two lines were established by co-culturing of ovarian stoma cells with fibroblasts. They have a typical embryonic stem cell (ESC)-like activity such as the reactivity to stem cell-specific markers, expression of stemness-related genes, in vitro-differentiation into embryoid bodies expressing of germline-specific genes and in vivo-differentiation into teratomas. However, there was a considerable difference in methylation status of imprinted genes and SNP profile compared with referenced stem cells. Those factors might affect their capacity to induce germline transmission and cellular activity as stem cells. A series of experiments is undertaken to find key factors yielding such difference using various tools of biomodelling. Concomitantly, we have attempted to establish stem cell lines from other tissue using these cell lines established.

## PLURIPOTENT STEM CELLS AND THE POTENTIAL APPLICATION IN REGENERATIVE MEDICINE

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Stem Cell, with its property of unlimited proliferation and fully developmental potential, has become important cell resource in regenerative medicine research. For most researches focus on embryonic stem cells, the ethical issue has been an obstacle for its application. Induced pluripotent stem (iPS) technique is not limited by availability of donor cells or ethical objections, so it has been the hotspot of stem cell research and expected to have broad applications in regenerative medicine since it was invented in 2006. Up to now, ES cells and iPSCs have been successfully derived from mice, human, monkeys, rats and pigs and have been differentiated into many tissue specific cells. Recently, several progresses in stem cell research field have been achieved: Using the tetraploid complementation method, Zhao et al successfully produced viable mice from iPSCs, proven that fully reprogrammed iPSCs possessed similar pluripotency as ES cells; microarray and high-throughput sequencing data revealed that mouse ES and iPSC cells with tetraploid complementation ability (4n-iPSC cells) shared similar expression patterns, but were distinguishable from tetraploid complementation incompetent iPSCs in the Dlk1-Dio3 imprinting region; we also demonstrated that this marker region was also positively correlated with the pluripotent levels of 3F-iPSCs, indicating the feasibility of using this region as a marker to select full-pluripotent 3F-iPSCs; by comparing the development of embryonic and adult mice derived from iPSCs, we found it exhibited similar developmental features as ES cells, yet were prone to tumorigenesis.. These achievements will greatly promote the application of stem cell in both basic research and regenerative medicine.

## MOLECULAR MECHANISMS OF OVULATION

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Ovulation, triggered by the preovulatory gonadotropin surge, is a complex and multifaceted process that culminates in follicular rupture and oocyte release after around 36h in the human. The major events in the ovulatory process are breakdown of the ECM of the follicular wall and increases in flow and vascular permeability of the follicle. The consequential progressive decrease of the tensile strength of the exterior follicular wall and a maintained positive intrafollicular pressure will ultimately lead to rupture of the exterior follicular wall and oocyte release. Several intraovarian mediator pathways are orchestrating this ovulatory process with participation of theca cells, granulosa cells, the oocyte and invading leukocytes.

Important transcriptional regulators are CEPBb, RUNX1 and the progesterone receptor. One result is increased follicular production of eicosanoids, with several subtypes of leukotrienes and prostaglandins, being involved in promoting ECM degradation and increased vascular permeability. Aquaporin 2 and 3 are highly upregulated in the human follicle during ovulation, and may be the major permeability-enhancing factors that ensure an expansion of the follicular antrum during ovulation. Other ovulation-associated angiogenic factors are nitric oxide, VEGF and angiotensin II. The EGF-like growth factors, amphiregulin and epiregulin induces ovulation promoting changes in the cumulus cells. The major ovulation-associated proteases are the MMPs and the ADAMTS. In the human ovulatory follicle some specific MMPs and TIMP-1 are upregulated in a time- and site-specific fashion, suggesting a role in ECM breakdown. A massive follicular invasion of leukocytes, especially macrophages and neutrophils, takes place during ovulation. Certain cytokines and chemokines attract these immune cells into the follicle to activate them, as major contributors to ECM remodelling during ovulation and luteinisation.

In conclusion, the ovulatory process is tightly coordinated by several mediator pathways. Further studies of molecular mechanisms of mammalian ovulation should include both experimental animal models and human tissue. This research should then be directed to solve clinical problems including anovulation and also to explore new strategies for ovulation inhibition in contraception.

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### NOVEL PATHWAYS LINKING ADIPOSITY AND DYSFUNCTION OF OVULATION AND OOCYTES

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Obesity in women is associated with anovulation and reduced conception rates. Utilizing dietary and genetic mouse models, we found that obese female mice also exhibit reduced ovulation, impaired fertilization and delayed blastocyst development, which is associated with lipotoxicity responses in the ovary. Specifically, the cumulus-oocyte complexes of obese mice contain excess lipid, exhibit endoplasmic reticulum (ER) stress, and have increased apoptosis. To identify mechanisms by which lipotoxicity impacts ovulation and oocyte quality we utilized an in vitro system and found that high levels of saturated fatty acid induced multiple ER stress pathways in the cumulus-oocyte complex, impaired cumulus protein secretion and altered mitochondrial activity in oocytes. The induction of ER stress in the cumulus-oocyte complex impaired normal oocyte maturation, fertilization and blastocyst development.

This lipotoxicity mechanism likely contributes to the reduced fertility that affects obese women. Supporting this we found that ovarian follicle fluid from obese women contains high levels of lipid and inflammatory cytokines, and granulosa cells express ER stress markers. To understand the impact this may have on peri-conception oocyte maturation, mouse oocytes were used as substitutes for human oocytes and were stimulated to mature in vitro in the presence of lipid-rich follicle fluid from obese women or lipid-poor follicle fluid from non-obese women. Oocytes exposed to obese follicle fluid during their maturation had increased oocyte lipid content, induction of ER stress and dramatically impaired nuclear maturation. Thus the follicular environment of obese women is markedly different from that of moderate weight women and maturation within this environment is detrimental to oocytes.

Overall we have elucidated cellular mechanisms by which obesity/ insulin resistance/ dyslipidemia disrupt oocyte developmental competence. Investigation of these critical regulatory pathways in ovarian cells of women with obesity and PCOS provides insight into how the altered follicular environment in these women contributes to their infertility.

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### THE DIVERSE ROLES OF NEUROTROPINS IN OVARIAN FUNCTION

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The neurotrophins are now recognised to have a diverse range of functions within the ovary, from early development and follicle formation, through initiation of follicle growth to ovulation and determination of oocyte developmental competence. In addition to nerve growth factor, the family members brain derived neurotrophic factor (BDNF), neurotrophin 3 and neurotrophin 4/5 (NT4/5) play roles acting through their selective tyrosine kinase receptors (Trks A, B and C) as well as the common p75 receptor. NT4/5 and BDNF are expressed in the developing ovary of both mouse and human, predominantly in the somatic cells that will become granulosa cells. Expression increases towards follicle formation, and they may be involved in germ cell survival in a pathway involving germ cell-derived activin. Species differences exist, with NT4/5 predominant in the mouse, but BDNF in the human.

Understanding the roles of Trk receptors is complicated by varying phenotypes in different models. The truncated TrkB receptor is expressed by immature oocytes, and the full-length form is expressed in response to the ovulatory LH surge, and is necessary for oocyte survival. BDNF promotes meiotic progression and developmental competence in both mouse and bovine. The mature human follicle expresses several NTs and their receptors, particularly in the cumulus and oocyte allowing for comparable development-promoting effects, and BDNF expression in cumulus correlates with normal fertilisation and early embryo development. However in immature human oocytes, BDNF has a negative effect on embryo cleavage. This may reflect changing Trk receptor expression with maturation. In conclusion, neurotrophins have a expanding repertoire of functions at all stages of oocyte development, and there is the potential for therapeutic use in assisted conception in both human and domestic species.

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### EMBRYO YIELD AND QUALITY AFTER NON-HCG PRIMED IVM AND CONVENTIONAL ICSI IN PCO PATIENTS

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Introduction: In vitro maturation (IVM) of oocytes is more patient-friendly than hormone driven ART, but less efficient, partly because of an inferior embryo developmental potential. We performed a within-patient analysis to compare the embryo yield of non-hCG-primed IVM and conventional ICSI treatment.

Material and Methods: PCO patients undergoing IVM after previous unsuccessful conventional ICSI (group 1, n=9) or conventional ICSI after previous unsuccessful IVM (group 2, n=10).

Baseline parameters (age, BMI, AMH) did not differ between both groups.

In group 1, cumulus oocytes complexes (COCs) were retrieved and cultured in IVM culture media (Origio) for 38h, followed by ICSI and embryo culture.

Group 2 patients underwent a gonadotropin-stimulated/GnRH-antagonist cycle with ICSI after  $\geq 1$  unsuccessful IVM cycle. COC yield, maturation and fertilisation rate, embryo yield (number of embryos suitable for transfer or cryopreservation) and quality were evaluated.

Results: In group 1, IVM yielded on average 12.0 COC, compared to 21.0 COC in preceding conventional ICSI cycles ( $p < 0.01$ ). The embryo yield per IVM cycle was significantly lower compared to previous ICSI cycles (2.4 vs. 7.4 embryos,  $p < 0.05$ ). However, the percentage of top quality embryos per fertilised oocyte was similar in both groups (33.6% (IVM) vs. 25.2% (ICSI),  $p = 0.47$ ).

In group 2, 11.3 COC were obtained on average after conventional ICSI, compared to 15.2 in preceding IVM cycles ( $p = 0.24$ ). The embryo yield per ICSI cycle was higher (6.3 ICSI vs. 4.1 IVM embryos,  $p = 0.09$ ). The percentage of top quality embryos was significantly higher after conventional ICSI (67.4% vs. 24.1%,  $p < 0.0001$ ), secondary to a higher percentage of embryos with 8 blastomeres after ICSI (52.2% vs. 13.2%,  $p < 0.0001$ ). Conclusion: In PCO patients, IVM treatment after unsuccessful ICSI yields fewer oocytes and embryos but similar embryo quality per fertilised oocyte.

PCO patients who underwent IVM unsuccessfully can expect significantly improved embryo quality in subsequent conventional ICSI cycles, reflected by a higher embryo developmental rate. Whether this is a consequence of improved endocrine profiles after repeated IVM oocyte retrieval remains to be investigated.

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### **GDF-9 INDUCES CONNEXIN 43 AND ENZYMES OF CHOLESTEROL BIOSYNTHESIS IN HUMAN GRANULOSA CELLS**

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Bi-directional paracrine signaling between oocyte and surrounding granulosa cells is required for ovarian follicle growth. Genetic studies in mice demonstrated critical roles of GDF-9 expressed exclusively by oocytes. GDF-9 has been considered to act as a signal mediator to granulosa cells surrounding oocytes. However, the effect of GDF-9 on human granulosa cells is not fully understood. Therefore, we performed microarray analysis using a GDF-9-stimulated human granulosa cell line that we established from non-luteinized granulosa cells with oncogenic genes. Results of microarray showed that GDF-9 increased mRNA levels of connexin 43 and enzymes required for cholesterol biosynthesis, such as *hmgcr*, *hmgcs*, *mvk*, *fdft1*, *sqle* and *lss*. We confirmed these results by quantitative RT-PCR and/or western blotting. These results are consistent with previous studies indicating that GDF-9 promotes cholesterol biosynthesis in mouse cumulus cells, possibly as compensation for deficiency in cholesterol production of oocytes. Earlier studies have shown that greater than 85% of the metabolites present in follicle-enclosed mouse oocytes were originally transferred from granulosa cells via gap junctions. Connexin 43 has been reported to be primarily located where gap junction-like plaques form between human cumulus cells, and that high connexin 43 levels in granulosa cells are linked to a good prognosis in human oocytes. All things considered, our results suggest that GDF-9 might play significant roles in the regulation of cholesterol biosynthesis in human cumulus cells and metabolite transportation via gap junction to oocytes.

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### **THE ESSENTIAL ROLE OF THE PLACENTA AND FETAL MEMBRANES AT THE FETAL-MATERNAL INTERFACE FOR A SUCCESSFUL PREGNANCY AND INITIATION OF LABOUR**

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The fetal-maternal interface in early pregnancy is essential for the establishment of a pregnancy with a subsequently successful outcome, where the fetus undergoes its normal development and growth and parturition occurs at term. An inappropriately formed interface may result in a spectrum of problems from early pregnancy loss or miscarriage, pre-eclampsia, intra-uterine growth restriction, and incorrect timing of labour either preterm or post-dates. Nevertheless, despite much research effort, the interactions and control at this interface remain enigmatic. The regulatory elements involved are complex, including multiple systems including cell cycle and developmental control, immune and inflammatory function and endocrine regulation, interacting with environmental and genomic influences. Tissue samples can only be obtained at the time of delivery, by which time any problems at the fetal-maternal interface will likely be fully established and may have initiated a subsequent series of inappropriate responses that are then detected and investigated experimentally, masking the initial problems which may have occurred many weeks earlier in gestation. This is compounded by the lack of accurate predictors of problems arising during pregnancy which require large resources to sample prospectively and obtain adequate sample groups. This talk will examine some of the novel findings emerging from genome-wide and experimental studies at the laboratory-clinical interface, both recent and also re-examining and updating some earlier studies. It will focus upon cytotrophoblast control for correct placental and fetal membrane trophoblast development and establishment of the fetal-maternal interface. It will also discuss paracrine and endocrine responses and interactions, including novel oestrogen and progesterone receptors and signalling pathways. Finally, the talk will encompass the complexity and interplay between these multiple pathways, and emerging studies from other fields, to look at future opportunities to better understand and improve pregnancy outcomes.

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### **ABERRANT DNA METHYLATION IN UTERINE LEIOMYOMAS**

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The pathogenesis of uterine leiomyomas remains unclear. Women with African descent, high BMI, meat consumption, and early menarche have greater risk for uterine leiomyomas. These findings suggest that uterine leiomyomas develop not only by inherited genomic abnormalities but also by unfavorable environmental exposures that cause epigenetic abnormalities. DNA methylation is one of the most characterized epigenetic marks. Here, we describe the epigenetic features of uterine leiomyomas.

Informed consents were obtained from all patients and the study was approved by IRB of Yamaguchi University. To investigate the genome-wide DNA methylation profile of uterine leiomyomas, a paired sample of leiomyoma and normal myometrium was subjected to a microarray-based DNA methylation analysis with restriction tag-mediated amplification (D-REAM). In the leiomyoma, D-REAM identified an aberrant DNA methylation status for 781 genes; 463 hypomethylated and 318 hypermethylated genes. We examined the chromosomal distribution for the detected

hypomethylated or hypermethylated genes. Intriguingly, 45 genes out of 463 hypomethylated genes were preferentially ( $p < 0.000000384$ ) located on X chromosome. Methylation-sensitive quantitative real-time PCR revealed that 14 genes showed common hypomethylation profiles in 6 cases with uterine leiomyomas. We next studied whether aberrant DNA hypomethylation status on X chromosome is associated with an alteration of X chromosome inactivation. The mRNA expression levels of XIST, which is required for X chromosome inactivation, were not decreased in leiomyomas, but the methylation status of FMR1 and AR genes, which show the typical DNA methylation pattern in X chromosome inactivation, were hypomethylated in leiomyomas compared with corresponding myometrium.

In conclusion, epigenetic aberration of the genes broadly distributed on X chromosome is one of the characteristics of uterine leiomyomas. The better understanding of the involvement of epigenetic aberration in uterine leiomyomas may shed light on the research of pathogenesis and lead new strategies for prevention or prediction of prognosis of uterine leiomyomas.

## NOVEL FACTORS INVOLVED IN THE CROSS-TALK BETWEEN MOTHER AND FETUS DURING PREGNANCY: MULTINUCLEATION AND FUNCTIONS OF BOVINE TROPHOBLASTIC CELLS FOR IMPLANTATION.

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The bovine implantation process is a unique and comparative model of placentation in mammalian species because various steps of implantation occur simultaneously in one uterus. This study investigated two major questions; functional and morphological differentiation of trophoblast cells. How and when is binucleate cell (BNC) established? Is morphological differentiation engaged involved in these functions? However, one general theory is that as trophoblast BNC/giant cells may be raised with endoreduplication, endogenous retrovirus genes may be involved in fusion and multinucleation of trophoblast cells in ruminant placenta. Some endogenous retrovirus genes (ERVs); endogenous retrovirus envelope element-like transcript, bovine endogenous retrovirus K and endogenous jaagsiekte sheep retrovirus, have been reported to be expressed in trophoblastic cells. Establishment and differentiation of BNC and/or multinucleate cell may be a necessary process for regulating specific trophoblastic cells and placentomal functions in cows. However, although the process for establishing BNC and/or multinucleate cell has not yet been clarified, these genes seem to be involved in this event. In situ hybridization and immunohistochemical examination have shown that bovine BNC/multinucleate cells are first found in the adhesion area on the caruncle around day 20 of gestation. ERVs were expressed with trophoblast specific molecules like PL, PRPs, PAGs, in newly developed trophoblastic cell lines. Why does bovine placenta need an abundance of the PRL/GH family like PL, PRPs? PRP1 specifically binds type IV collagen around implantation and the cleaved PRP1 with either Cathepsin D or matrix metalloproteinases has a proliferation activity of the endothelial cells in vitro. The cleaved PRP1 may be shared in specific bovine placentomal angiogenesis. These findings imply that trophoblastic multinucleation is a key process in implantation and maintenance of placentomal functions during gestation in cows. The ERVs may play a vital role in multinucleation. These studies provide new insights for studying bovine implantation.

## TROPHOBLASTS DO NOT MIGRATE RETROGRADE TO FLOW IN LOW SHEAR STRESSES: CONSEQUENCES FOR SPIRAL ARTERY REMODELLING IN THE FIRST TRIMESTER OF HUMAN PREGNANCY

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Background: In early human pregnancy trophoblast migrate along uterine spiral arteries (SA) and remodel these vessels into wide bore conduits. Inadequacies in SA remodelling have been associated with pre-eclampsia and IUGR. Until 10-12 weeks of gestation trophoblast plug SA, preventing maternal blood flow into the intervillous space, resulting in slow, high resistance flow in these vessels. This work examined the consequences of these low shear stress conditions on trophoblast migration, adhesion molecule expression and attraction to chemotactic factors. Methods: SGHPL-4s were cultured alone or on HUVECs for 6-12hrs under 0.5-6dyne/cm<sup>2</sup> of shear stress using the BioFlux200 system, and imaged by time-lapse microscopy. Computer-based imaging algorithms were developed to quantify the speed and direction of migration. Adhesion molecule expression was determined by immunohistochemistry and confocal microscopy. Chemotaxis assays were run using parallel flow in BioFlux channels. Results: 1) SGHPL-4s cultured either alone or on HUVECs did not undergo directional migration in 0.5 and 2dyne/cm<sup>2</sup> cultures, however in 4 and 6dyne/cm<sup>2</sup> SGHPL-4 were stimulated to migrate with the direction of flow (n=4, p<0.001). 2) Low shear stresses did not affect the speed of SGHPL-4 migration, or the expression or distribution of the adhesion molecules E-selectin,  $\alpha_4$ ,  $\beta_1$  or  $\alpha_5\beta_3$  integrin. 3) SGHPL-4s cultured on HUVECs migrated into media containing IL-8, MCP-1 or RANTES (n=5, p<0.05). Conclusions: This work challenges the dogma that trophoblasts migrate down spiral arteries retrograde to flow by demonstrating that as shear stress increases trophoblasts are in fact stimulated to migrate in the same direction as blood flow. Thus, low shear stresses generated by trophoblast plugging of SA in the first trimester may favor SA remodeling by preventing the migration with flow seen at higher shear stresses, allowing trophoblast to migrate down the arteries in response to alternate stimuli such as uterine or endothelial cell derived chemotactic factors.

## THE ROLE OF ADRENOMEDULLIN IN TUBAL ECTOPIC PREGNANCY

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Adrenomedullin (ADM), a novel vasodilator, exerts diuretic, natriuretic, angiogenic, antioxidative, anti-apoptotic and anti-inflammatory effects in various body systems. It has important regulatory functions in the reproductive system. In the female reproductive tract, ADM was highly expressed in the human and rat oviducts. ADM has been shown to regulate the ciliary beat frequency (CBF) and contractility in the oviduct, affecting the transport of spermatozoa, ova and pre-implantation embryos. The oviduct is the most frequent site of ectopic pregnancy which is an important cause of pregnancy morbidity. In this study, we investigated the role of ADM in tubal ectopic pregnancy (tEP). Oviducts from women undergoing salpingectomy for tEP and those from women undergoing hysterectomy for benign diseases were collected. The latter oviducts served as controls after treatment for 16h with estradiol, progesterone and human chorionic gonadotrophin to simulate the early first trimester hormonal milieu of human pregnancy. ADM expression was studied at the peptide and mRNA levels by immunohistochemistry and RT-PCR respectively. After treatment of the oviducts with ADM and its receptor antagonists, CBF was measured using a photometric method and the contraction of the oviduct was measured using the organ-bath technique. The tEP oviducts expressed significantly less *Adm* mRNA than the control. The CBF was lower in tEP oviducts and in both groups the CBF increased with graded concentrations (1-100nM) of ADM and were specifically inhibited by a hADM<sub>22-52</sub> receptor antagonist. Treatment with ADM increased the basal tone and the frequency of contraction but decreased the amplitude in both the control and tEP groups. Contractility in both groups was not sensitive to the treatment with receptor antagonists. All the results indicate that in the oviduct, the low ADM expression, which may affect the embryo transport through ciliary beat and contraction, may be a causative factor in tubal ectopic pregnancy.

## TIME SPACE AND ENERGY IN THE MAMMALIAN OOCYTE

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Oocytes maturation is a remarkable transition that takes place in the final hours in the life of an oocyte. The timing of oocyte maturation is governed by a network of regulatory mechanisms that modulate activity of the cell cycle kinases. One of these mechanisms involves the anaphase promoting complex (APC) which targets key cell cycle proteins for destruction by the proteasome. The ability to control APC activity and its selection of substrates as meiosis progresses plays a major role in governing the timing of meiotic progression.

This APC-regulated entry into meiosis I is integrated with a dynamic reorganization of the oocyte cytoplasm that culminates in a highly asymmetric first meiotic division. The developing meiotic spindle orchestrates this reorganization then polarizes the cortex to ensure only a tightly defined region can participate in polar body formation. Cytoplasmic reorganization involves the trafficking of organelles to the spindle such that it becomes enveloped in a matrix of endoplasmic reticulum, mitochondria and secretory vesicles. A strict balance between activities of dynein and kinesin is responsible for the formation of this cytoplasmic super-structure that then migrates to the cortex in an actin-dependent process. Arrival at the cortex initiates a signalling pathway involving the small GTP-binding proteins, Ran, Rac and Cdc42 that results in actin polymerization and cortical polarization. This polarization serves to ensure the polar body is restricted in size and that cytoplasmic organelles are retained in the oocyte.

These processes that control the timing and spatial organization of oocyte maturation are fuelled by mitochondrial production of ATP. Mitochondrial localization and ATP levels show dynamic changes during oocyte maturation and inhibiting mitochondrial function causes rapid de depolymerization of the meiotic spindle and dysregulation of calcium signalling. Successful integration of the events that control timing, spatial organization and energy production is an essential requirement for the production of a fertile mature oocyte.

## GENETIC AND EPIGENETIC LANDSCAPE OF MOUSE PREIMPLANTATION DEVELOPMENT REVEALED BY LIVE-CELL IMAGING

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Mammalian fertilization and subsequent preimplantation development are irreversible processes of the dynamic conversion of two highly specialized gametes into totipotent zygotes, and of the rapid and frequent duplication of the cell with the specific cell cycle progression. They are achieved by the tightly coordinated regulation of a great variety of temporal and spatial changes in epigenetic and genetic (including chromosome structure level) events in nucleus. Here, we developed a live-cell imaging method specified for the preimplantation embryo. Importantly, by optimizing the devices and conditions for imaging, the procedure itself was not detrimental, 'minimum-damage' to full-term development, although it is a prolonged imaging process. Then, by using this technique, we assessed genetic and epigenetic status of 'ill-fated' embryos such as intracytoplasmic sperm injection (ICSI)-generated embryos and somatic nuclear transferred (SCNT) embryos.

First, we assessed DNA methylation status using EGFP-MBD-NLS probe that consists of methyl-CpG binding (MBD) domain and nuclear localization signal (NLS) of human methyl-CpG binding domain protein 1. We found that the centromeric/pericentromeric regions of the chromosomes were hypermethylated in SCNT embryos. Concentrations of the hypermethylated DNA remained throughout preimplantation development, indicating that this DNA from the somatic nucleus was not reprogrammed correctly in the cloned embryo.

Next, to observe the histone modifications in living cells, fluorescently labeled Fab fragment of specific monoclonal antibody against modified histone was injected into the embryo. We demonstrated that different acetylation levels of H3K27 between ICSI and SCNT embryo nuclei may at least partially account for their different development efficiencies.

Finally, we assessed chromosome integrity of cleavage state embryo. One major finding was that abnormal chromosome segregation (ACS) frequently occurred during mitotic divisions at early cleavage stage of both ICSI and SCNT embryos; and the incidence was significantly higher in SCNT embryos. Interestingly, when these embryos were transferred to recipient mother, no pups were obtained, suggesting that ACS might be a reason for the high incidence of gestational loss seen in ICSI and SCNT protocols.

## RNA POLYMERASE II CTD REGULATION IN MAMMALIAN OOCYTES AND EARLY EMBRYOS

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Embryonic genome activation results in replacement of both oocyte and embryo common transcripts with new transcripts that are necessary for further development. Nuclear translocation of RNA polymerase II (Pol II) and phosphorylation of its largest subunit at the C-terminal domain (CTD) are considered as major determinants of embryonic genome activation. The CTD of Pol II, composed of a highly conserved tandemly repeated heptapeptide motif (YSPTSPS), undergoes extensive phosphorylation and dephosphorylation during the transcription cycle. P-TEFb (CDK9/Cyclin T1), the Pol II CTD kinase, regulates transcription elongation by phosphorylation of serine 2 residues of the CTD. In this experiment, the expression and subcellular localization of Pol II and CDK9 were evaluated in mouse and porcine oocytes or preimplantation embryos. Also, the effects of CDK9 specific inhibitor, flavopiridol, on preimplantation development were examined. Both Pol II and CDK9 were detected in mouse and porcine oocyte and preimplantation embryos. CDK9 translocated into the pronuclei at late 1-cell stage and predominate into the nuclei at 2-cell or 4-cell stage. Treatment with flavopiridol resulted in mislocalization of CDK9 and phosphorylated Pol II as well as developmental arrest in 2-cell and 4-cell of mouse and porcine embryos, respectively. Also, flavopiridol-treated embryos showed obviously aberrant nuclear localization of phosphorylated forms of Pol II CTD. To directly measure of Pol II-dependent transcription in fertilized embryos in the presence of flavopiridol, nascent RNA chains were labeled in situ by incorporation of bromouridine 5'-triphosphate (BrUTP). When one-cell embryos were cultured in the presence of 100 mM flavopiridol, BrUTP labeling in treated embryos was dramatically decreased compared with that of untreated embryos. Our results indicate that CDK9 could play an important role in embryonic genome activation.

## EMBRYOS GENERATED FROM OOCYTE LACKING COMPLEX N- AND O- GLYCANS HAVE COMPROMISED IMPLANTATION AND DEVELOPMENT.

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Appropriate oocyte development is important for subsequent embryo development. Mice generating oocytes lacking complex N- and O-glycans undergo premature ovarian failure (POF) and produce small litters when mated at 6 weeks and are infertile 3 weeks later. In this study we investigated how oocyte development in this model of POF affects embryo development and implantation. Control (*C1galt1<sup>FF</sup>Mgat1<sup>FF</sup>*) and mutant (*C1galt1<sup>FF</sup>Mgat1<sup>FF</sup>:ZP3Cre*) females were mated with control males. Ovulation rate determined from flushed oviducts at 0.5 days post coitum (dpc). Zygotes were cultured for 4 days and development evaluated. Implantation sites, post-implantation embryo development and ovaries were analysed at 5.5, 6.5 and 9.5dpc. Despite mutants producing small litters when mated at 6 weeks, ovulation rate, fertilization and *in vitro* development to blastocyst stage did not differ to controls. These findings suggest that decreased fertility in mutants at 6 weeks results from abnormalities in implantation or post implantation embryo development. Mutants exhibited lower numbers of implantation sites at both 5.5 (1.25±0.5 vs 7.14±1.68; n=4 and 7 respectively) and 6.5dpc (3.2±2.17 vs 7.75±0.50; n=5 and 4 respectively). Moreover less decidia were also found in mutants at 9.5dpc (3.0±1.55 vs 7.6±1.14; n=6 and 5 respectively). Mutant ovaries also contained more corpora lutea (CL) than controls at all three time points (5.5dpc: 11.75±2.22 vs 8.29±0.95, P=0.005), however, most mutant CL were smaller and more varied in size than control CL. An increased number of CL in mutants with a normal ovulation rate indicates a defect in CL regression. Therefore, oocyte development when lacking N- and O-glycans affects implantation, postimplantation embryo development and CL regression. However further studies are required to elucidate if abnormal implantation and embryo development are due to defective signalling by the implanting embryo or if the uterine environment is not receptive due to an abnormal endocrine profile resulting from poor CL regulation.

## ATTACHMENT, BUT NOT TENSION OR POSITION, MEDIATED SATISFACTION OF THE SAC UNDERLIES THE SUSCEPTIBILITY OF MAMMALIAN OOCYTES TO CHROMOSOME SEGREGATION DEFECTS.

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Errors in segregating chromosomes during the first meiotic division of human eggs are very common, and following fertilization generate aneuploid embryos. In humans, aneuploidy is the leading cause of early embryo loss, and is thought to affect at least 30% of eggs. Here, using 4-D live cell imaging of mouse oocytes, we provide a reason for this susceptibility. The Spindle-Assembly Checkpoint (SAC), which is composed of members of the Mad and Bub family of proteins prevents aneuploidy in mitotic cells by inhibiting activity of the Anaphase-Promoting Complex/Cyclosome until all chromosomes are bi-orientated on a metaphase spindle. In oocytes we found that SAC satisfaction, assessed by the loss of Mad2 from kinetochores, was complete in oocytes 3-4h before anaphase, and was associated with the establishment of stable k-fibres and activation of the Anaphase-Promoting Complex/Cyclosome (APC/C). However, we found that the SAC did not monitor chromosome position or amphitelic kinetochore attachment. Therefore, following APC/C activation chromosome oscillatory movements on and off the spindle equator were common and could occur with duration of several hours. These non-aligned chromosomes became more Mad2 positive, but not enough to inhibit the APC, and they could persist past anaphase generating aneuploid embryos. Once the APC/C is activated the duration of prometaphase was then determined by CDK1 activity. Our data show that segregation errors arise as a result of what the SAC fails to monitor, rather than any primary defect in the SAC pathway. In conjunction with an age associated cohesin loss, this permissive SAC provides an attractive explanation for aneuploidy in female meiosis.

## CALCINEURIN AND NFAT ARE NEW MEMBERS OF THE GNRH TRANSCRIPTIONAL NETWORK

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Gonadotropin-releasing hormone (GnRH) signals through its receptor via coupling with Gq/11 proteins that leads to activation of several signaling pathways that culminate in regulated expression of at least 76 genes in gonadotropes. Four of these genes (*Cga*, *Lhb*, *Fshb*, and *Gnrhr*) are unique to gonadotropes and required for full functional activity. Four immediate early genes (*Egr1*, *Jun*, *Atf3*, and *Fos*) are direct transcriptional targets of GnRH signaling pathways. These immediate early genes encode DNA-binding proteins that render the four signature genes responsive to GnRH. Earlier work from our laboratory indicated that functional interactions between TCF7L2 and  $\beta$ -catenin mediated GnRH stimulated transcription of *Jun* and *Cga* but not that of *Egr1* or *Atf3*. Our present studies extend these findings by demonstrating that GnRH regulated expression of *Jun* also requires activation of JNK, calcineurin, and nuclear factor of activated T cells (NFAT). Since NFAT is a DNA-binding protein, we suggest that it acts in concert with JUN to confer GnRH responsiveness to *Cga* and other downstream targets of JUN including *Fshb* and *Gnrhr*. Together, these data suggest that calcineurin and NFAT are new members of the GnRH transcriptional network that regulates gonadotropin synthesis and secretion.

## FSH REGULATION OF LHCGR EXPRESSION IN GRANULOSA CELLS.

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Ovarian follicles are restrained at the preantral stage until they are stimulated by the pituitary hormone follicle stimulating hormone (FSH). In response to FSH, granulosa cells produce steroid hormones, protein hormones, and growth factors that regulate the hypothalamic/pituitary axis and promote oocyte maturation, development of follicles to a preovulatory phenotype, and uterine receptivity. FSH signals through its surface G protein-coupled receptor on granulosa cells to activate protein kinase A (PKA) that in turn is the key modulator of a number of downstream signaling pathways including the phosphatidylinositol-3 kinase (PI-3K) and extracellular signal-regulated/mitogen-activated kinases (ERK). The gene that encodes the receptor for LH/hCG (*Lhcgr*) is one of the most crucial FSH targets that characterizes the preovulatory phenotype. However, the cellular mechanism by which FSH induces expression of the *Lhcgr* gene in granulosa cells is incompletely understood. We utilized both mouse *Lhcgr*-luciferase reporter and real time quantitative PCR to evaluate *Lhcgr* gene expression in primary rat granulosa cells. Results show that *Lhcgr* gene expression is dependent on PKA. In addition, the PKA-dependent regulation of *Lhcgr* gene expression is mediated at least in part via  $\beta$ -catenin-dependent co-activation of the orphan nuclear receptor Nr5A1 (aka, steroidogenic factor 1 [SF1]) and Tcf711 (aka, T-cell factor). Moreover, one of the PKA targets responsible for *Lhcgr* gene expression appears to be  $\beta$ -catenin. Together these results indicate a novel pathway by which FSH via PKA signals to induce *Lhcgr* gene expression in granulosa cells. Supported by NIH RO1 HD062503 (MHD/JHN).

## ENVIRONMENTAL EXPOSURES AND REPRODUCTIVE DEVELOPMENT AND FUNCTION IN THE FEMALE

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It has become increasingly evident that in Environmental Chemicals (ECs), especially Endocrine-Disrupting Compounds (EDCs) contribute to reproductive dysfunction in wildlife, domestic animals and humans. ECs are associated with a range of conditions, ranging from intersex to gender-inappropriate behaviour, and in humans, falling sperm counts. The latter observations have contributed to an imbalance in research effort towards the male. However, it is evident that there may be problems with female fertility and reproductive health. In-utero exposures to ECs are associated, at a varying degree of data robustness, with reduced age at menarche or menopause, reproductive tract abnormalities, reduced fecundity and increased incidences of some cancers, eg breast cancer. There is also emerging evidence of epigenetic modifications following EC exposures. This presentation will review the current data for EC-exposure effects on female reproductive development and outline emerging data from collaborative projects using: (i) sheep pastured on fields fertilised with processed human sewage sludge, a highly relevant exposure for human health and (ii) mice exposed to environmentally-relevant doses of selected ECs, especially the phthalate DEHP. There are changes in fetal follicle and germ cell numbers, and ovarian proteome, following in-utero exposure to dietary intake of sewage sludge ECs. We have then studied whether the timing of exposure, before or after conception, or during specific, ovarian, developmental windows, affect the fetal ovary and brain (including KISS and GnRH systems). In addition, females exposed in-utero and studied in early adulthood show dramatic alterations in proportions of health and unhealthy follicles. Mice exposed in-utero to DEHP have significantly disturbed ovarian follicle numbers. The dramatic finding is that some of these effects persist in the F3 generation although only the pregnant F0 received direct dietary DEHP. These studies demonstrate that there is the potential for marked developmental disruption by EC exposures in the female and this area requires more research. Much of this work is supported by the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no 212885 and the Wellcome Trust grant number 080388.

## THE OVARY FROM BASIC SCIENCE TO CLINICAL TRANSLATION

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'Advances in our understanding of the developmental and cell biology of folliculogenesis are delineating the follicular physiome through which somatic cellular functions influence oocyte development and vice versa. It is becoming increasingly clear from pre-clinical studies and in-vitro modelling that oocyte 'quality'—in terms of maternal contribution to embryogenesis and early development—is programmed before ovulation and therefore potentially amenable to endocrine and paracrine manipulation. It is therefore surprising that so little attention has been paid to translating this basic science into clinical practice. This is all the more so given the many patient scenarios that are now potentially amenable to ART. Here the natural follicular lifespan is tracked to locate paracrine checkpoints tractable to endocrine manipulation that are likely to influence oocyte quality. Emphasis is placed on signalling by members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily of growth and differentiation factors, which contains the quintessential gonadal paracrine activin and inhibin. Armed with this knowledge, it is suggested that intelligent administration of FSH

and LH activity (either LH or hCG) can allow fine-tuning of ovarian stimulation on a patient-by-patient basis to improve individual responses and benefit treatment outcome.' Reproduced from Hillier 2009.

(1) Hillier SG. Paracrine support of ovarian stimulation. *Mol Hum Reprod.* 2009 Dec;15(12):843-50.

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## OVARIAN FOLLICLE METABOLISM AND CULTURE FOR FERTILITY PRESERVATION.

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The culture of follicles in vitro following cryopreservation of ovarian tissue is a desirable fertility preservation option for cancer patients. The growth of follicles in a 3-dimensional matrix shows promise in phenocopying in vivo folliculogenesis however oocytes generated from this system have a reduced capacity to complete nuclear maturation and be fertilized compared to oocytes matured in vivo. An increasing body of work points to the importance of metabolism and metabolic rate in determining oocyte quality and developmental competence and in particular the role of ATP. Lipids are a potent source of energy; however their utilisation by the follicle is poorly understood. Generation of ATP from lipids occurs in mitochondria via beta-oxidation and requires the metabolite carnitine. We have recently shown that beta-oxidation of lipids is essential for oocyte developmental competence during in vitro maturation of cumulus oocyte complexes. We have sought to understand how modulation of lipid metabolism during in vitro follicle culture and oocyte maturation impacts on follicle growth and oocyte developmental competence in a 3-dimensional follicle culture system. Inhibition of beta-oxidation in the presence of lipids significantly inhibits follicle growth in vitro with follicles not significantly altering in size over the culture period. Conversely, provision of lipid substrate during culture significantly increases follicle growth compared to the absence of lipids. Interestingly, modulation of beta-oxidation with the co-factor L-carnitine while significantly upregulating beta-oxidation, did not alter follicle survival, growth, antrum development or differentiation but did however, markedly improve oocyte quality with significantly more oocytes completing nuclear maturation, fertilization and development to blastocyst. This is the first identification that beta-oxidation is essential during follicle culture and that modulation by L-carnitine is beneficial. This indicates that key metabolic requirements are lacking in current follicle culture systems as L-carnitine supplementation increased lipid metabolism and improved oocyte developmental competence.

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## PARENTAL GENOTYPE AND RISK FOR PREECLAMPSIA

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Preeclampsia occurs in 7-8% of pregnancies and can be life threatening to the mother and/or her baby. Defects in early differentiation and invasion by placental trophoblasts predispose pregnancies to this complication. Furthermore, how the mother adapts to the dynamic state of pregnancy is critical to her risk for preeclampsia. This is a cardiovascular disease characterised by hypertension and proteinuria with systemic endothelial dysfunction and inflammation. Women afflicted by preeclampsia are more likely to suffer hypertension and stroke in later life suggesting common genetic and environmental contributing factors. Men who were born to a preeclamptic pregnancy are more likely to father a preeclamptic pregnancy and some men have fathered preeclampsia in more than one woman suggesting a paternal genetic contribution transmitted by the placenta. However, in first pregnancies clinicians have no idea which women are at risk of developing preeclampsia until symptoms arise. We have genotyped 100 single nucleotide polymorphisms (SNPs) in DNA extracted from blood samples collected from 3234 parent-infant trios from pregnancies recruited to the International SCOPE study in Adelaide Australia and Auckland New Zealand. In 2123 Caucasian pregnancies a number of SNPs in genes involved in placental invasion, the renin angiotensin system, the Insulin-like growth factor (IGF) axis and angiogenic gene family among others in the mother, father or baby are associated with risk for preeclampsia. Some of these associate with maternal blood pressure at 15 weeks gestation and with birth weight of the baby. Interactions between clinical and dietary risk factors such as maternal and paternal BMI and maternal fruit and vegetable consumption and socioeconomic status with SNPs were identified. An algorithm that predicts risk has been developed that if used as a screening tool in early pregnancy could change antenatal care such that women at high risk could be streamed into a dedicated high risk clinic.

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## OBTAIN COMPETENT OOCYTES FROM PRESERVED OVARIAN TISSUE

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Assuring an adequate fertility preservation for the female cancer patient implies expertise in a whole range of laboratory techniques. Before starting cancer therapy the possibilities are 1°) to obtain fertilisable MII oocytes, after using an adapted ovarian stimulation scheme and to vitrify the metaphase II oocytes; 2°) if no stimulation is feasible: to obtain immature germinal vesicle oocytes out of unstimulated small antral follicles to apply in vitro maturation (IVM). In case the patient has a partner, embryos could be cryopreserved. With the current vitrification protocols for MII oocytes or embryos, clinical pregnancy rates as high as 30 % per warmed gamete or embryo have been reported in donor cycles.

Additionally, a ovarian cortical tissue biopsy or an entire ovary should be laparoscopically retrieved for cryopreservation, as it contains thousands of primordial follicles.

A well documented procedure for cryopreserving thin ovarian cortex slices using a "slow freezing" protocol has been generally used in most centers. Today, there are the first pregnancies from vitrified ovarian tissue. Freezing of an entire ovary with its pedicle is another option, still in an experimental phase.

The culture of ovarian follicles and ovarian tissue needs to be developed within the scope of fertility preservation programmes. In recent years the mechanisms regulation the awakening of primordial follicles out of the resting pool have been partially unravelled. Recent work from Aaron Hsueh's laboratory has shown proof of principle data on synchronous activation of primordial follicles in mouse and human. Once a follicle has grown in vitro to the early secondary stage, it can be dissected out of the tissue and cultured as a single unit, by applying a variety of systems. A fully grown oocyte can be obtained after several weeks of culture. A crucial question is how to evaluate oocyte maturity within the in vitro cultured follicle and how to determine the right moment to reinitiate meiosis. Application of molecular biology and advances with biomaterials will help to establish robust culture systems for large mammal- and human oocytes.

## THE INTERFACE BETWEEN REPRODUCTIVE BIOLOGY AND REGENERATIVE MEDICINE

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The generation of human organs from pluripotent stem cells (PSCs) is one of the ultimate goals of regenerative medicine. The recent success in growing a rat pancreas from induced pluripotent stem (iPS) cells in the body of a host Pdx1(-/-) pancreatogenesis-disabled mouse (Kobayashi et al., 2010) has stimulated an increased expectation of the generation of functional human organs from PSCs using the inherent ontogenic mechanisms of animals. This breakthrough in xenogenic organ regeneration from PSCs also involved chimeric animal production, a technology that is now well-established in reproductive biology. Research into the xenotransplantation of animal organs, particularly those of the pig, for organ transplantation in humans has progressed remarkably in the last decade. Furthermore, with respect to the development of therapeutic methods and drugs for curing intractable diseases, disease model pigs are thought to have a higher potential than rodent models for extrapolation of research outcomes to humans. To overcome the various challenges in the development of therapeutic methodologies, it will be essential to create specifically designed and genetically modified pigs. This can be achieved by use of somatic cell cloning technology, a groundbreaking outcome of reproductive biology research. Thus, reproductive biology plays an indispensable role in the development of regenerative medicine therapies that are expected to bring about a revolution in human health. From our standpoint at the interface of reproductive biology and regenerative medicine, we have been tackling important issues that are directly related to human health. Here, I present an overview of our latest challenges aiming at making the medicine of the future a reality through translational research using as a platform pigs that have a number of similarities with humans.

## GLYCODELIN-C AND THE ROLE OF CUMULUS COMPLEX IN NATURAL SPERM QUALITY SELECTION

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To fertilize an oocyte, spermatozoa have to pass through the cumulus oophorus. During this passage, the spermatozoa encounter the cumulus cells and their extracellular matrix. Therefore, the cumulus oophorus is the ideal site for final tuning of sperm functions and/or selection of spermatozoa before fertilization. Consistently, spermatozoa that have passed through the cumulus oophorus swim faster, have higher percentages of spermatozoa with normal morphology, reacted acrosome and higher zona binding capacity when compared to those before cumulus penetration. These data suggest that the population of spermatozoa that can penetrate the cumulus oophorus has higher fertilizing capacity. While the changes in motility and acrosomal status after cumulus penetration can be partly explained by the actions of cumulus matrix components and progesterone, the action of glycodelin-C accounts for the change in the zona binding capacity of the penetrated spermatozoa, which can be abolished by pretreatment of cumulus oophorus with anti-glycodelin antibody.

Spermatozoa are exposed successively to glycodelin isoforms in the female reproductive tract. Glycodelin-S keeps the spermatozoa in an uncapacitated state before entering the uterus. Glycodelin-A primes spermatozoa for zona pellucida-induced acrosome reaction. Glycodelin-F prevents progesterone-induced acrosome reaction. Both glycodelin-F and -C are present in the cumulus oophorus. The cumulus cells use glycodelin-F in the follicular fluid as substrate for the production of glycodelin-C in the cumulus matrix. The cumulus cells also convert granulosa cell-derived  $\alpha 2$ -macroglobulin into glycodelin-interacting protein, which binds to glycodelin-C and hyaluronic acid and thus help to retain glycodelin-C in the cumulus matrix.

Glycodelin-C within the cumulus matrix displaces the glycodelin-F with zona-binding inhibitory activity, thereby restoring the zona binding capacity of spermatozoa passing through the cumulus mass. Glycodelin-C also promotes the zona binding capacity of the penetrated spermatozoa via increasing the zona pellucida glycoprotein binding sites.

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## OOCYTE SOMATIC CELL INTERACTIONS PROMOTING FOLLICLE ACTIVATION AND DEVELOPMENT

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Unavailable at time of print

## ABERRANT DNA METHYLATION OF IMPRINTED LOCI IN SPERM FROM OLIGOSPERMIC PATIENTS

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Recent studies indicate the possibility of excess imprinting disease occurrence in babies by assisted reproductive technologies (ART), but such a risk of ART treatment cannot be simply evaluated because the patients who receive ART may differ both demographically and genetically from the general population at reproductive age. In this study, we examined the DNA methylation status of seven imprinted genes by bisulphate-based methylation analyses in sperm DNA from male ART patients. We found 24 patients whose sperm had abnormal DNA methylation among the sperm samples of men from 97 infertile couples. Furthermore, only one sperm sample, from severe-oligospermic patient, with both paternal and maternal methylation errors of imprinted genes was successfully used to fertilize an ovum using ICSI and resulted in normal pregnancy. The newborn was normal and did not show any abnormality in the methylation patterns of all examined imprinted genes. The results showed that the abnormal methylation pattern seen in the father's sperm DNA was not inherited by the neonate in this one case and indicate that there are two possibilities explanations: one is that the fertilizing sperm prepared by ART was a rare sperm with a normal DNA methylation pattern and the other is that the imprint methylation abnormalities reverted to normal after fertilization.

## ENVIRONMENTAL MODIFIERS OF MALE REPRODUCTIVE FUNCTIONS

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Male reproductive organ development and functions are programmed during fetal life. Disruption of the programming at critical time results in birth defects, such as cryptorchidism, hypospadias, and micropenis, and origin of carcinoma in situ that later gives rise to testicular germ cell tumours. Sperm production capacity depends on the length of seminiferous tubules which is determined by the number of Sertoli cells. Since the Sertoli cells cease proliferation at puberty, adult sperm production capacity depends on fetal and childhood development of these cells. Genetic defects, such as certain nuclear receptor mutations, and environmental insults affecting hormonal regulation of sex differentiation (endocrine disruptors) can cause birth defects, testicular cancer and/or impaired spermatogenesis. Because these male reproductive problems appear to share causal factors, they are called together as testicular dysgenesis syndrome (TDS). Tobacco smoke contains chemicals that affect both adult and fetal testis harmfully, but the adverse effect appears only in sperm production capacity. Synthetic estrogen diethyl-stilbestrol (DES) has been linked to all features of TDS on the basis of follow-up studies of the double-blind randomized studies, in which DES was tested as a miscarriage preventing drug in the 1950s. These studies directed the interest to estrogens before it was realized that anti-androgenic compounds have a much higher potency to cause developmental damage in males. In experimental animals anti-androgens were shown to induce TDS. Whether human fetuses are affected by current environmental exposures to anti-androgens remains an open question, although the number of identified anti-androgenic compounds is increasing and these show dose-additive effects in mixture experiments. We have identified a positive association between cryptorchidism and exposure to polybrominated flame retardants and a combined exposure to chlorinated pesticides. However, it is apparent that we have not yet identified most of the compounds (out of tens of thousands) that contribute to adverse effects.

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## MOUSE GAMETIC DNA METHYLOMES SHOW THE ROLE OF INTRAGENIC DNA METHYLATION IN THE ESTABLISHMENT OF OOCYTE-SPECIFIC HERITABLE MARKS

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Genome-wide dynamic changes in DNA methylation—which are indispensable to germline and embryonic development—occur throughout gametogenesis in mammals. Here, we show the first extensive, high-resolution DNA methylome maps and transcriptome maps at single-base resolution of mouse germ cells. Our methylome profiles revealed sequence-specific and CpG-density-dependent methylation patterns of each cell, and *Dnmt3l*-deficient oocytes showed global hypomethylation with residual partial methylation in LINE and LTR retrotransposons at high CpG density. Furthermore, transcriptome analysis showed a strong positive correlation with methylation of the transcribed region in wild-type oocytes, however, the intragenic methylation was not observed with genome-wide hypomethylation in *Dnmt3l*-deficient oocytes. Together with the identification of a great number of *Dnmt3l*-dependent germline differentially methylated regions, these data suggest a potential rule of the *Dnmt3l*-mediated intragenic methylation for the establishment of genomic imprints and other oocyte-specific DNA methylation marks during germline epigenetic reprogramming.

## RNA BINDING MOTIF 5 DYSFUNCTION CAUSES MALE INFERTILITY IN MICE BY DISRUPTING SPERMATID MATURATION

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To comprehensively uncover novel male fertility regulators, we utilised an unbiased forward genetic screen, ENU mutagenesis. Using this approach, we have identified several novel infertile mouse lines including a male-specific infertile line that we designated “Joey”. The mutant *Joey* mice produced no sperm due to an arrest of male germ cells at the round spermatid stage. The mutation was identified in the RNA binding motif 5 (*Rbm5*) gene that resulted in an arginine to proline substitution within a highly conserved RNA recognition motif of the protein. The substitution of proline is likely to interfere with RNA binding and/or recognition. *Ex vivo* studies have suggested that RBM5 is a tumour suppressor, apoptosis modulator and RNA splicing regulator. To date, the role of *Rbm5* has never been linked to male fertility and the *Joey* line is the only mouse model of *Rbm5* dysfunction. Using our RBM5-specific antibody, we showed that RBM5 is expressed in pachytene spermatocytes and round spermatids. Based on the protein localisation, the proposed role of RBM5 in mRNA processing, the onset of the *Joey* phenotype, and the site of the identified mutation, we hypothesise that the *Rbm5* mutant allele results in a hypomorphic protein, and that RBM5 has an essential role in regulating male germ cell mRNA storage, transport and/or translational regulation of mRNAs that are critical for spermatid maturation. Further, we generated mice compound heterozygous of the *Joey Rbm5* mutation and *Rbm5* null alleles. We showed that the compound heterozygous males are infertile due to spermatid maturation arrest resembling the *Joey* mutant males. This result further confirmed the identification of the *Rbm5* mutation as a cause of infertility in the *Joey* mice and a crucial role of *Rbm5* in male fertility.

**CHROMATIN IN HUMAN SPERM, PACKAGING GENES FOR EMBRYO DEVELOPMENT****D. Carrell***Urology, Ob-Gyn, Physiology, University of Utah School of Medicine, Salt Lake City, UT, United States*

Unavailable at time of print

**STRUCTURAL AND FUNCTIONAL ANALYSES OF HUMAN NUCLEOSOMES CONTAINING TESTIS-SPECIFIC HISTONE VARIANTS****H. Kurumizaka***Graduate School of Advanced Science & Engineering, Waseda University, Shinjuku-ku, Tokyo, Japan*

In eukaryotes, genomic DNA is packaged into the nucleus as chromatin, and nucleosome is the fundamental repeating unit of chromatin. In nucleosome, two of each core histones, H2A, H2B, H3, and H4, form a histone octamer, and about 150 base-pair DNA is wrapped about 1.5-2 turns around the histone octamer. Histones H2A, H2B, and H3 have nonallelic isoforms called histone variants. Histone variants highly expressed in testis are also identified, and are suggested that they may play an important role in the chromatin reorganization required for meiosis and/or spermatogenesis. However, structures and functions of these testis-specific histone variants have not been extensively studied. To understand the structural basis of the functional chromatin, we bacterially expressed human histone variants, and reconstituted the human nucleosomes containing various histone variants including testis-specific variants. We then determined the crystal structures of the nucleosomes containing these histone variants. The structures and biochemical properties of these nucleosomes containing testis-specific histone variants will be presented, and their functions in chromatin reorganization during spermatogenesis will be discussed.

**NUCLEOSOME RETENTION DURING CHROMATIN PACKAGING IN HUMAN SPERMATOZOA.****K. McEwan<sup>1,4</sup>, A. T. Reid<sup>1,2,4</sup>, D. M. Campbell<sup>1,2</sup>, D. A. Jans<sup>2,3</sup>, S. D. Roman<sup>1,2</sup>**<sup>1</sup>*Biology, University of Newcastle, Callaghan, NSW, Australia*<sup>2</sup>*ARC Centre of Excellence in Biotechnology & Development, Australia*<sup>3</sup>*Biochemistry & Molecular Biology, Monash University, Clayton, VIC, Australia*<sup>4</sup>*These authors contributed equally, Australia*

In contrast to the histone packaging of somatic cells, spermatozoa are predominantly packaged by the protamine proteins. However, human spermatozoa retain ~15% histone packaging. Regions that are left nucleosome bound are either genes that are active shortly after fertilisation or genes that are transcribed late during spermatogenesis. DNA damage at histone bound loci would be of consequence to spermatogenesis and/or to a resulting embryo post-fertilisation. The transition from histone to protamine involves transition proteins and prior acetylation of histones.

Western blot analysis confirmed the presence of acetylated H3 and H4 in sperm. Interestingly, we identified the presence of these modified histones in isolated good quality sperm considered to have complete packaging. Using a combination of ChIP (chromatin immunoprecipitation) and tiling arrays we have identified regions bound by acetylated histone 3 (ac-H3). ChIP-PCR confirmed histone retention at several of these loci.

We found differential acetylation across the nucleosomes retained at a locus identified in exon 1 of the TNP-2 gene. Ac-H3 was limited to one nucleosome whereas ac-H4 was present in three retained nucleosomes. This supports the hypothesis that ac-H3 is retained after late transcription in spermatogenesis.

Using a modified form of ChIP known as carrier ChIP we examined histone retention in individual ejaculates. We demonstrate that humans consistently retain acetylated histones at the same loci. Ac-H4 retention was more widespread than ac-H3 retention. We propose a model whereby ac-H4 precedes the retention/repackaging decision. This indicates that histone acetylation is not sufficient to result in chromatin repackaging.

Furthermore, we have identified individuals who produce ejaculates that consistently differ from the common retention pattern and individuals who produce varying ejaculates. Thus ChIP-PCR may be a valuable tool in identifying apparently normal individuals with aberrant chromatin packaging that is of consequence to male fertility or developmental outcomes in the offspring.