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WCRB 2014

Plenary Lectures

SRF Plenary Lecture

S001

Polycystic ovary syndrome: follicles and fertility

Stephen Franks
Imperial College London, London, UK.

Polycystic ovary syndrome (PCOS) is the most common cause of anovulatory infertility but typically is also associated with insulin resistance, disordered energy balance and a predisposition to develop type 2 diabetes mellitus. Anovulation is characterised by arrest of antral follicles but there is compelling evidence for disordered regulation of the early stages of preantral follicle development in the ovaries of women with PCOS. Dysregulation of local growth factor function appears to play a significant part in abnormal preantral follicle development. Paradoxically, although PCOS causes ovulatory dysfunction, the fertility and fecundity of women with symptoms of PCOS in the general population is not compromised to the degree that might be expected. Family size is slightly smaller but women with PCOS have at least one child as often as those in the normal population cohort. The metabolic dysfunction associated with PCOS may have an evolutionary role in maintaining fertility in the population at times of food shortage (Corbett & Morin-Papunen 2013 *Mol Cell Endocrinol* 2013 **373** 29–50).

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KSAR Plenary Lecture

S008

Generation of transgenic pigs with modified immune systems

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Pigs are an excellent model for human diseases with their physiological similarities to human. In addition, they are assumed to be the most suitable organ donor to meet acute shortage of allogenic human organs. However, prior to their use in clinical studies, several genetic modifications are required to generate human diseases and to overcome immunological rejection of the xenograft, and specific breeding scheme of them is needed to avoid the transmission of zoonotic diseases. While the former can be addressed by introduction and/or knock-out (KO) of relevant genes by somatic cell nuclear transfer (SCNT), the later can be achieved by producing and breeding gnotobiotic pigs. Recently, we have been successful in generating and breeding the MHC class II homozygous pigs in a barrier-sustained, gnotobiotic miniature pig facility. Here, we describe a rapid single-step procedure to produce the specific gene KO pigs for xenotransplantation. We have been established a suitable transfection and selection procedures to produce efficiently KO somatic cells and utilized them as donor cells in producing KO pigs with SCNT technology. First, we produced CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) KO pigs by this rapid single-step ZFN procedure. Second, we generated the SCID pigs with KO of RAG2 gene. Interestingly, these KO pigs supported the formation of teratomas in the injection sites of human stem cells. Therefore, our gnotobiotic pig facility and transgenic pigs may be used as a valuable resource for xenotransplantation and human diseases.

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SRB Plenary Lecture

S009

Immune-mediated quality control of embryo implantation and reproductive investment

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Maternal and embryonic factors integrate to determine the success of embryo implantation and progression to pregnancy, with major impact on later placental and fetal development, and long-term consequences for offspring health. The female immune response is a major force in this peri-conception environment. In contrast to conventional wisdom, the immune response is not passive or suppressed in pregnancy. Instead, immune cells are centrally engaged with all steps of the reproductive process from conception to implantation and placental development. Through an active role in the decision pathway that permits pregnancy progression, the immune system acts in synergy with the reproductive system to ensure healthy reproduction. The biological benefit of a robust immune contribution is to discriminate between good and bad reproductive opportunities and execute an appropriate response at conception – to sustain and nurture an implanting embryo, or alternatively to actively suppress pregnancy. Emerging information is expanding our understanding of the underlying mechanisms – the immune system has an active function in sensing and evaluating parameters of male gamete quality and embryo viability, as well as markers associated with seminal fluid and the embryo that are relevant to embryo-maternal compatibility. This is mediated by immune contributions to pre-implantation embryo development, progesterone synthesis, and endometrial receptivity. Since the immune response is modulated by infectious, inflammatory, stress, nutritional and metabolic status, immune influence on progression or disruption of pregnancy can be disturbed by environmental stressors and resource availability. From an evolutionary perspective, such a 'quality control' function ensures appropriate investment of limited female resources and opportunities for reproduction. The challenge now is to define the critical cytokines and seminal fluid signals that control this nexus. Understanding immune decision-making at conception will offer new ways to promote fertility and will shed light on infertility disorders with an immune aetiology.

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SRD Plenary Lecture

S019

Kisspeptin-GPR54 signaling controlling reproductive functions in mammals

Hiroko Tsukamura
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Kisspeptin-GPR54 signaling plays a key role to govern reproductive function through regulating GnRH/gonadotropin release in mammals, including rodents, ruminants, reflex ovulators, and primates. Surge and pulse modes of GnRH/gonadotropin secretion are responsible for ovulation and gametogenesis/steroidogenesis, respectively. It has been suggested that kisspeptin neurons located in the anterior hypothalamus, such as the anteroventral periventricular nucleus (AVPV) and preoptic area (POA), are responsible for the estrogen-positive feedback action to induce GnRH/gonadotropin surge and then ovulation. On the other hand, kisspeptin neurons in the arcuate nucleus (ARC) are considered to be involved in regulation of GnRH/gonadotropin pulses and estrogen negative feedback. The present paper reviews the role of two populations of kisspeptin neurons with a focus on the epigenetic mechanism mediating the estrogen action on *Kiss1* gene expression in the brain to understand the mechanism underlying regulation of GnRH/gonadotropin release by kisspeptin. This work was supported in part by the Research Program on Innovative Technologies for Animal Breeding, Reproduction, and Vaccine Development.

DOI: 10.1530/repabs.1.S019

CSRB Plenary Lecture

S020

Generation of mammalian haploid embryonic stem cells

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Haploid cells are amenable for genetic analysis because they contain only one set of chromosomes. Haploid cells usually exist in simple organisms, such as the budding yeast and fern, leaving an intriguing question of whether haploid cell lines in be established in mammalian systems. Here, we report that the generation of mouse haploid embryonic stem cells (haESCs) via androgenetic blastocysts and monkey haESCs via parthenogenetic blastocysts.

Mouse and rogenetic blastocyst-derived haESCs, designated as AG-haESCs, partially maintain paternalimprints, express classical ESC pluripotencymarkers,

and contribute to various tissues, including the germline, upon injection into diploid blastocysts. Strikingly, live mice can be obtained upon injection of AG-haESCs into MII oocytes, and these mice bearhaESC-carried genetic traits and develop into fertile adults. Furthermore, gene targeting via homologousre combination is feasible in the AG-haESCs. These results demonstrate that AG-haESCs can be used as a genetically tractable fertilization agent for the production of live animals via injection into oocytes[1].

We then derive haESCs from parthenogenetic blastocysts of *Macaca fascicularis* monkeys. These cells, termed as PG-haESCs, are pluripotent and can differentiate to cells of three embryonic germ layers *in vitro* or *in vivo*. Interestingly, the haploidy of one monkey PG-haESC line (MPH1) is more stable compared with that of the other one (MPH2), as shown by the existence of haploid cells for more than 140 days without fluorescence-activated cell sorting (FACS) enrichment of haploid cells.

Importantly, transgenic monkey PG-haESC lines can be generated by lentivirus- and piggyBac transposon-mediatedgene transfer. Moreover, genetic screening is feasible in monkey PG-haESCs. Our results demonstrate that PGhaESCscan be generated from monkeys, providing an ideal tool for genetic analyses in primates[2].

References

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SSR Plenary Lecture

S030

Animal models, reproduction and ovarian cancer

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The research in my laboratory is focused on defining the essential factors that regulate the hypothalamic–pituitary–gonadal axis in women and men. To investigate this axis, we have been using multiple *in vitro* and *in vivo* strategies. In particular, we have created over 60 knockout mouse models, have begun to study over a dozen conditional knockout mouse lines, and have produced several important recombinant growth factors and small molecules. In the process, we have uncovered interesting genes and small RNAs in germ cells and somatic cells of the ovary and testis as well as key proteins in the pituitary, oviduct, and uterus that have important implications for fertility and reproductive cancers. Our approaches have lead us down exciting paths and in fascinating directions, allowing us to identify candidate targets for infertility, contraception, and cancer treatments. A major translational goal is to create therapeutics for clinical use.

Studies in the Matzuk Laboratory have been supported by the National Institutes of Health, the Ovarian Cancer Research Fund, and the Marsha Rivkin Center for Ovarian Cancer Research.

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Symposia

Epigenetic reprogramming in reproduction

S002

High-resolution DNA methylome analysis of mouse germ cells

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Dynamic epigenetic reprogramming occurs during mammalian germ cell development, whereas the targets of this process including DNA methylation/demethylation remain poorly understood. Here, we examined genome-wide methylation profiles in developing and fully developed germ cells of mice using whole-genome bisulfite sequencing (WGBS). We scaled down the construction and analysis to nanogram quantities of DNA by generating a new WGBS library, termed the post-bisulfite adapter tagging (PBAT) method. Thus, we could provide complete maps of cytosine methylation in mature sperm cells, fully-grown oocytes, and developing male/female primordial germ cells (PGCs). Our methylome study demonstrated genome-wide DNA demethylation, with erasure of genomic imprinting and X-inactivation during gonadal sex determination, and gender-specific differences in genome-wide and gene-specific DNA methylation levels in developing germ cells. Some of these global/local changes in DNA methylation during germ cell progression were consistent with previous studies. However, our complete DNA methylome maps revealed important and novel details of DNA methylation/demethylation processes. Some of the new findings from this study include the following: i) DNA methylomes exhibited sex- and chromosome-specific differences in genome-wide CpG methylation during early to late PGC development; ii) LINE and LTR retrotransposons were resistant to DNA demethylation during PGC migration; iii) non-CpG methylation occurred in male gonocytes during mitotic arrest and fully-grown oocytes; iv) identification of over a thousand germline differentially methylated CpG islands; and v) strong gene-body methylation in fully-grown oocytes. Our present data establish the basis for future studies on the role of epigenetic modifications in germline development and other biological processes.

DOI: 10.1530/repabs.1.S002

S003

The origin and fate of epimutations in offspring produced by assisted reproductive technologies

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Epimutations are heritable defects in epigenetic programming that do not involve changes in the underlying DNA sequence and may or may not impact gene expression. Epimutations can occur naturally, but are more likely to be induced by environmental factors that disrupt the normal epigenome. Previous studies have shown that the use of assisted reproductive technologies (ART) can induce epimutations in the offspring produced. We chronicled the occurrence of epimtuations in mice produced by natural reproduction, intracytoplasmic sperm injection (ICSI) or somatic cell nuclear transfer (SCNT) by analyzing allele-specific DNA methylation and expression at three imprinted genes, *H19*, *Snrpn* and *Peg3*, in somatic cells from juvenile and adult mice generated by each method. No epimutations were detected in naturally conceived mice, but epimutations were detected in ~50% of ICSI mice and in ~40% of SCNT mice. We allowed the ICSI mice carrying epimutations to reproduce naturally and found no epimutations in the offspring. We then examined germ cells from the ICSI mice and found that the epimutations present in the somatic cells had been corrected by germline-specific epigenetic reprogramming. These are therefore examples of environmentally-induced epimutations that are not transgenerational. Potential differences in these and other environmentally-induced epimutations that do show transgenerational transmission will be discussed. Key differences between transgenerational and non-transgenerational epimutations may include – i) the developmental window during which, or ii) the sequence context in the region of the genome in which the epimutations are induced. Thus, epimutations induced during the period of the 'epigenetic ground state' unique to fetal germ cells, and/or those induced in particularly CpG-poor regions of the genome appear to have a higher likelihood of manifesting as transgenerational than those induced at other developmental windows or in other regions of the genome.

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S004

Effects of maternal obesity and diabetes on DNA methylation in germ cells and offspring

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Introduction
Obesity and diabetes have adverse effects on germ cell quality, embryo development, fertility, and the health of offspring. However, the underlying mechanisms responsible for the negative effects of obesity and diabetes are little known.
Materials and methods
A high-fat-diet (HFD)-induced maternal obese mouse model, streptozotocin (STZ)-induced and nonobese diabetic (NOD) maternal diabetic mouse models, and HFD combined with STZ-induced paternal prediabetic mouse model were employed. We investigated the DNA methylation status of imprinted genes and/or metabolism-related genes in germ cells and offspring by using combined bisulfite restriction analysis and bisulfite sequencing, as well as MeDIP-Seq analysis.
Results and discussion
DNA methylation of imprinted genes in oocytes was not altered in either obese dams or their offspring; however, DNA methylation of metabolism-related genes was changed not only in oocytes of obese mice but also in oocytes and liver of their offspring. The methylation pattern of maternall imprinted gene *Peg3* differential methylation regions (DMR) was affected in a time-dependent manner, and evident demethylation was observed on day 35 after STZ injection. In NOD mice, the methylation pattern of *Peg3* was similar to that of STZ-induced mice. Embryo development was adversely affected by maternal diabetes; however, no evident imprinting abnormality was observed in oocytes from female offspring derived from a diabetic mother. Offspring of prediabetic fathers exhibited altered gene expression patterns in the pancreatic islets, with down-regulation of several genes involved in glucose metabolism and insulin signaling pathways. Paternal prediabetes altered overall methylome patterns in sperm, with a large portion of differentially methylated genes overlapping with that of pancreatic islets in offspring. Our data may contribute to the understanding of adverse effects of obesity and diabetes on reproduction and health of the offspring.

DOI: 10.1530/repabs.1.S004

Testis biology and spermatogenesis

S005

The contributions of importin proteins to spermatogenesis: roles in signalling, gene transcription and stress responses

Yoichi Miyamoto[1], Penny Whiley[1], Julia Young[1], Arash Arjomand[1], Yoshihiro Yoneda[2], David A Jans[1] & Kate L Loveland[1]
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Importin protein family members are best known for serving critical roles in mediating regulated nucleocytoplasmic transport, and many are essential to developmental events including gametogenesis. We proposed that coordinated expression of an importin and a cell-specific transcription factor would drive a developmental switch by enabling transcription and other nuclear factors to enter the nucleus appropriately. Analyses of importin alpha (IMPα) and beta (IMPβ) mRNAs and proteins revealed their synthesis and subcellular localization are tightly regulated throughout spermatogenesis. Although normally cytoplasmic, certain IMPαs are nuclear-localized in meiotic and haploid male germ cells. IMPα-specific binding partners differ between these germ cells; intriguingly, many are not nuclear proteins, and several are essential for male fertility. IMP proteins localize to distinct regions in mature sperm, highlighting their potential to serve as adaptors for protein trafficking to sites other than the nucleus. Our HeLa cell work showed that IMPα proteins become nuclear-localised sequestered following transient exposure to cellular stressors and that importins can function in the nucleus to control gene transcription. This led to identification of *STK35* as a unique transcriptional target for nuclear-localized IMPα proteins that is also highly expressed in testes, and *Stk35* transcripts are relatively high in germ cell subtypes that contain nuclear-localized IMPα proteins. In addition, our data implicate importins in germ cell stress responses. Interrogation of our unique mouse models with higher and lower IMPα4 levels in spermatids has provided *in vivo* evidence that nuclear IMPα proteins affect cell fate in response to oxidative stress. Thus interrogation of IMPα functions during spermatogenesis showed that importins function at several levels to modify cellular fate. Our growing understanding is that this can occur through regulating transcription factor access to the nucleus, by acting within the nucleus to directly influence

transcription, and through modulating subcellular localization of proteins required for fertility.

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S006

What have we learned about the testis from cell ablation studies

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Cell ablation is a powerful technique for examining the role of a particular cell type in organ development and function. The utility of the technique is dependent upon the ablation being specific to one cell type and for those of us interested in the testis we have been fortunate that methods have existed for some time to ablate the germ cells and the Leydig cells. Recently we have also developed a transgenic mouse model which expresses the diphtheria toxin receptor (DTR) specifically on the Sertoli cells allowing controlled ablation of the Sertoli cells through injection of diphtheria toxin (DTX). This presentation will concentrate largely on the consequences for testis development and function of either Leydig cell or Sertoli cell ablation. In the rat a single injection of ethane dimethane sulphonate (EDS) leads to complete Leydig cell loss within 24 h. These initial studies led to the important observation that Leydig cell stem cells exist in the adult testis which will regenerate the Leydig cell population after ablation. The EDS model remains an important tool in the study of Leydig cell differentiation. Treatment with EDS has also been particularly useful for identification of Leydig cell transcripts/proteins and we recently carried out a microarray analysis using EDS which has identified a group of Leydig cell specific transcripts in the testis. Studies using the new model of Sertoli cell ablation have shown that these cells are essential for maintenance of the peritubular myoid cells in the neonate and for development of the adult population of Leydig cells. In the adult animal Sertoli cell ablation also leads to a 75% loss of Leydig cell number emphasising the continuing role of the Sertoli cell in maintaining/orchestrating adult testis function.

DOI: 10.1530/repabs.1.S006

S007

The role of autophagy in spermatogenesis

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Acrosome is a specialized organelle that covers the anterior part of the sperm nucleus and plays an essential role in the process of fertilization. The molecular mechanism underlying the biogenesis of this lysosome-related organelle (LRO) is still largely unknown. Here we show that germ cell specific Atg7 knockout mice were infertile due to defect in acrosome biogenesis and displayed a phenotype of human globozoospermia, and this reproductive defect was successfully rescued by intracytoplasmic sperm injections. Furthermore, we found that the depeletion of Atg7 in germ cells did not affect the early stages development of germ cells until round spermatids, but at later stages of spermatogenesis, the proacrosomal vesicles failed to fuse into a single acrosomal vesicle at Golgi-phase and finally resulted in irregular or nearly round-headed spermatozoa. We revealed that autophagic flux was disrupted in Atg7 depleted germ cells, and finally led to the failure of LC3 conjugation to Golgi apparatus-derived vesicles. In addition, we found that Atg7 partially regulates another globozoospermia-related protein PDZ- and coiled-coil motif-containing protein (GOPC), during acrosome biogenesis. Finally, we demonstrated that the injection of either autophagy or lysosome inhibitors into testis resulted in a similar phenotype to germ cell specific Atg7 knockout mice. Altogether, our results uncover a new role for Atg7 in the biogenesis of acrosome, and we provide evidences to support acrosome's autolysosome origination hypothesis.

DOI: 10.1530/repabs.1.S007

Stem cells and development

S010

Oxygen environment and the regulation of human embryonic stem cells

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Human embryonic stem cells (hESCs) derived from the inner cell mass of the blastocyst, propagate by self-renewal and can give rise to all cells of the body. Thus, they are a useful model to investigate a range of degenerative disorders such as type 1 insulin-dependent diabetes, multiple sclerosis and Parkinson's disease. However, hESCs are notoriously difficult to maintain in culture as colonies have a propensity to spontaneously differentiate making it difficult to uniformly direct all cells down a specific lineage pathway. Thus, to expedite the use of these cells in regenerative medicine, fundamental research is required to understand the mechanisms that regulate hESC maintenance. *In vivo*, the blastocyst is thought to reside in a low oxygen atmosphere and thus, the routine use of atmospheric oxygen to culture hESCs may be detrimental to pluripotency. Research in my lab has found that reducing the oxygen tension to a more physiological level supports the maintenance of a highly proliferative, pluripotent population of cells; a response mediated by a family of hypoxia inducible factors. Moreover, highly pluripotent hESCs cultured at a low oxygen tension display an altered metabolism compared to early differentiating cells, or those maintained under atmospheric oxygen; a reduction in expression of pluripotency markers is associated with a more oxidative metabolism. Our data suggest that a low environmental oxygen tension is an important regulator of energy metabolism and intrinsic to hESC self-renewal.

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S011

Pluripotency of embryo-derived stem cells in domestic animals – a pig view

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The establishment of ES cells in domestic animals will help us to understand embryonic biology and improve the studies of regenerative medicine using stem cells. Among these domestic animal, pig can be particularly useful to study human diseases, because of similarities in size, immunology and physiology to human. In domestic animals including pig, however, authentic ES cells could not be derived from pre-implantation embryos. Although several attempts to establish ES cells have been tried from porcine pre-implantation embryos of the various stages (E6-E10), it could not derive the ES cells with characteristics shown in mES cells. Most of ungulate ES cell's papers, including porcine ES cells, have shown similar characteristics to hES cells with monolayer colony morphologies, FGF culture condition and Activin/Nodal signaling pathways. Recently, we addressed the comparative study using ES-like cell lines derived from porcine embryos of various origins. Our results showed that stem cell lines derived from porcine pre-implantation embryos of various origins have a flattened morphology and FGF and Nodal/Activin signaling and belong to the category of EpiSC-like ES cells rather than authentic ES cells. It should be considered that defining the optimum stage of embryos for stem cell derivation is an important key to obtain stable ES cell lines with identifying optimum pluripotent markers and the key signaling pathways that regulate the pluripotency of ES cells in domestic animals. This work was supported by the BioGreen21 Program (PJ0094932013), RDA, Republic of Korea.

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S012

Stem cells in the endometrium: clues to health and disease

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The human endometrium undergoes >400 cycles of growth, differentiation and shedding during a woman's reproductive years. Endometrial regeneration is likely mediated by stem/progenitor cells. Emerging evidence suggests that disorders associated with abnormal endometrial proliferation (endometriosis, endometrial cancer) may involve endometrial stem/progenitor cells. In human endometrium, colony forming units (CFU), side population (SP) cells and tissue reconstituting cells have been identified. Individual CFUs undergo self-renewal

and have high proliferative capacity. Epithelial CFUs differentiate into gland-like structures and stromal CFUs have a mesenchymal stem cell (MSC) phenotype and differentiate into mesodermal lineages, typical of MSC. This suggests that human endometrium contains small populations of epithelial progenitors and MSC. Gene expression profiling of purified endometrial epithelial cells from pre- and post-menopausal women has identified a candidate marker for endometrial epithelial progenitor cells. Endometrial MSC (eMSC) can be prospectively isolated from tissue in the CD140bCD146 fraction or using a novel single marker, W5C5. These markers show that the epithelial progenitors are found in the endometrial basalis while eMSC are located perivascularly in both functionalis and basalis. Using these markers we show that epithelial progenitors and eMSC are shed in menstrual blood, and in women with endometriosis, endometrial W5C5 MSC are preferentially shed into the pelvic cavity during menstruation, where they may initiate endometriosis lesions. We are examining the utility of autologous eMSC as a cell-based therapy for pelvic organ prolapse, an intractable disorder resulting from herniation of the pelvic organs into the vagina due to childbirth injury. In a nude rat model of fascial repair, we have shown that polyamide mesh seeded with eMSC promoted early neovascularisation, an M2 macrophage wound healing response, deposition of non-scarring collagen and improved biomechanical properties of the mesh/tissue complex compared with mesh alone. These studies demonstrate that human endometrium provides an alternate source of MSC for cell-based therapies.

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Ovary and folliculogenesis

S013

Ovarian androgen: poison or panacea?

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The 'old' science base generally casts androgens as 'bad' for folliculogenesis: agents of follicular atresia and directly involved in reproductive pathologies such as PCOS. A more recent literature has mapped cellular sites of androgen formation and action within the ovarian follicle, pinpointing theca as the source and granulosa its target. Molecular studies have located the androgen receptor (AR) to granulosa cells, functionally coupled to FSH-regulated (cAMP-mediated) gene expression. Experiments show an initial capacity of androgen to enhance FSH action, which is lost or becomes suppressive as follicles mature. Granuolsa cell AR levels decline in the preovulatory follicle, possibly as a protective device against over-sensitisation to FSH. This may be a mechanism for supporting follicular dominance and determining which follicles ovulate. AR knockout studies in mice confirm that androgens regulate follicle progression from pre-antral into the antral stage and benefit oogenesis. Thus androgens are neither good nor bad for folliculogenesis: simply essential. Successful clinical ovarian stimulation regimes take account of this reality.

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S014

Cumulus cells are essential mediators of LH induced ovulation stimuli from granulosa cells to the oocyte

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The LH surge induces ovulation and events associated with this process: oocyte meiotic maturation, cumulus cell–oocyte complex (COC) expansion, and release of mature (MII arrested) oocytes within the expanded cumulus cell complex matrix. Oocytes and cumulus cells express few if any LH receptors whereas mural granulosa cells exhibit high levels of this gonadotropin receptor. Therefore, in response to the LH surge granulosa cells are presumed to secrete growth factors that mediate the LH receptor activation to the cumulus cells. We determined that not only the EGF receptor (EGFR, ErbB1) but also ErbB2 and ErbB3 were activated in cumulus cells following the LH surge. A possible role for EGFR ligands, amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC) in the ovulation process was strengthened by recent observations (Park *et al.* 2004, Shimada *et al.* 2006). We also identified neuregulin 1 (NRG1) that selectively activates ErbB3 and ErbB2 in mouse periovulatory follicles (Noma *et al.* 2011). To determine the role of NRG1 in oocyte maturation, we generated a granulosa cell specific *Nrg1* knockout mouse (*Nrg1*$^{flox/flox}$*; Cyp19a1Cre* mice; gc*Nrg1*KO, Kawashima *et al.* 2014). In these mutant mice, the number of oocytes that were ovulated was normal. However, the progression of meiosis in these ovulated gc*Nrg1*KO oocytes was abnormal and was related to the inappropriate activation of PKC and phosphorylation of connexin-43 in cumulus cells of these mutant mice. The abnormal meiosis progression (failure to arrest at MII) decreases successful fertilization and fewer pups were born per litter in gc*Nrg1*KO mice. We conclude that NRG1 is induced by LH in mural granulosa cells and exerts an important regulatory role on oocyte meiotic maturation and competence by the modification of the EGF lignad-EGFR signaling in cumulus cells and preventing premature progression to the MII stage that leads to abnormal fertilization and fertility.

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S015

Regulation and dysregulation of primordial follicle formation and activation

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There are several key stages in the development of a reproductively competent ovary. These include: i) successful migration of the primordial germ cells and associated precursor somatic cells to the genital ridge, ii) the entry and maintenance of the developing germ cells in meiotic arrest, iii) the accumulation of pre-granulosa cells by oocytes to form primordial follicles, and iv) the maintenance of the primordial follicles in a quiescent state. This population of inactive primordial follicles will then represent the female mammal's, or woman's, ovarian reserve and will be a key component of her lifetime reproductive potential.

This presentation will outline some of the key stages and controlling mechanisms in the formation of the primordial follicle. Some of the differences and similarities between key models, rodents, sheep and human fetus will be addressed. In doing this considerable focus will be placed on the human, partially informed by non-human primate studies, because of the emergence of a number of new studies over recent years. Primordial follicle formation and activation occur against a background of complex intra- and extra-ovarian signalling, including steroid hormones, gonadotrophins and local inhibin/activin signalling.

In addition, there is concern over the potential adverse effects of *in-utero* exposures to toxicants such as environmental chemicals, endocrine disrupting compounds and maternal cigarette use. The nature of adverse effects on the development of the ovary will be discussed and will include examples from electively terminated fetuses exposed to cigarette smoke during pregnancy. Animal models of chemical and cigarette exposure will also be outlined.

Finally, aspects of normal follicle activation will be put into perspective against the evidence for activation and progression of some primordial follicles even before birth. Transcription repressors such as PTEN and local AMH signalling have a key role to play in the regulation of primordial follicle activation.

DOI: 10.1530/repabs.1.S015

Embryo

S016

Characterization of gene expression in 1-cell stage embryos

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Before fertilization, oocyte genome is not transcribed and this transcriptionally silent state continues after fertilization. It is known that in mice, the first gene expression in the life occurs at mid-1-cell stage and the expression pattern dramatically alters during development following the program of gene

expression. However, the profile of transcribed genes and the mechanism regulating their expression at the 1-cell stage has not been clarified, since the amount of zygotic transcripts are too small compared to the maternal mRNA which has been accumulated during oocyte growth. Here, we show the unique characteristics of gene expression in 1-cell stage embryos. RNA sequence analysis revealed that the transcripts were deficiently spliced. Based on the expression of introns, we identified 4575 genes transcribed at the 1-cell stage and found that their expression pattern was unique. We also found that intergenic regions are pervasively expressed. Analysis for the regulation of the expression of those genes and reporter gene assay revealed that precocious gene expression, i.e. core promoter elements-independent, cryptically initiated and terminated transcription, occurs in 1-cell stage embryos. The first gene expression during life, which is the initiation of transcriptional cascades, is thus loosely regulated. The significance of this unique gene expression will be discussed.

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S017

Effect of transcriptional regulation on early embryo development

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A large number of embryos are produced both in human and domestic animals using assisted reproductive technologies (ART) including *in vitro* fertilization, but the majority of embryos fail to develop to the blastocyst stage and/or implant during early embryo development. However, the causes of the developmental failures and early miscarriage are not well defined in human and other mammals. Blastocyst stage is critical to the manipulation and/or selection of embryos for embryo transfer (ET) to produce offspring. Studies in animal models, particularly mice, revealed that members of several gene families such as tight junction proteins, claudins, occludins, aquaporins and Na/K ATPases are responsible for the fblastocoel, however the underlying transcriptional mechanisms of these genes are largely unknown. Moreover, the majority of existing research has focused on lineage specific transcript factors (TFs) such as Oct4, Nanog, Sox2, and Cdx2 since establishment of embryonic stem cells. In this context, there is only a limited amount of research in regulation of TFs responsible for genes required for the blastocyst formation including tight junction (TJ) biogenesis and cavity formation. A recent study reported a novel and essential role for transcription activating protein 2 c (Tcfap2c/AP-2γ) in blastocyst formation in a mouse model. The AP-2γ has been shown to regulate a diverse group of gene families critical for TJ biogenesis, ion gradient, water channel and cell proliferation. However, the spatial and temporal expressions of the AP-2γ are not consistent with those in other species, suggesting that a wider and more comparative studies of biological roles and mechanisms of AP-2γ in early embryo development is need.

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S018

Epigenetic reprogramming to form the pluripotent inner cell mass

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Reprogramming of DNA methylation patterns in the early embryo is required for normal development. A longstanding paradigm of global loss of 5′-methylcytosine (5meC) over the first few cell-cycles of early development has recently been challenged (Li & O'Neill 2012, Li & O'Neill 2013)[1,2]. Here we show that immuno-detectable levels of 5meC and 5′-hydroxymethylcytosine (5hmC) persist throughout zygotic maturation and the first cell-cycles (to the eight-cell stage). The inner cells of the morula showed a marked reduction in global levels of both 5meC and 5hmC and further loss occurred in the pluripotent inner cell mass (ICM) of the blastocyst stage embryo. High levels of both modifications persisted in the outer cells of the morula and the resulting trophectoderm of the blastocyst. This lineage-dependent differential pattern of cytosine modifications was dependent upon the activity of DNA methyltransferases. The low level of both cytosine modifications persist as cells of the ICM differentiate into the epiblast and hypoblast lineages after implantation, while the high levels of 5meC and 5hmC in the trophectoderm persisted as it differentiated into trophoblast and then into trophoblast giant cells. Our results redefines the dynamics of programming covalent modifications to cytosine in early development and shows that the global changes in the pattern of nuclear 5meC and 5hmC are early markers of commitment of cells to the pluripotent lineage in the early embryo.

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Uterine and placental function

S021

Immunotrophic promotion of pregnancy and of healthy pregnancy outcomes

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Introduction

Several subsets of uterine natural killer lymphocytes (uNK cells) home to early decidua basalis in rodents and humans. UNK cells peak at midgestion when uNK cells synthesizing the angiokines placenta growth factor (PGF), vascular endothelial growth factor (VEGF) and Delta-like ligand 1 (DLL1) are dominant. UNK cell-driven angiogenesis regulates the timing of both uterine lumen closure and early, post-implantation conceptus development. UNK cells contribute to orthogonal branching and pruning of nascent decidual vessels and initiate spiral arterial remodeling. Physiological impact of the absences of uNK cells or of PGF were addressed and localized to maternal and fetal/offspring cardiovascular systems.

Materials and methods

Pregnancies were compared between mice without lymphocytes, with partial or full lymphocyte sufficiency or with genetic deletion of *Pgf*. All studies were conducted under approved animal utilisation protocols and in compliance with Canadian guidelines. Techniques used included histology, ultrasound or chronic continuous radiotelemetry recordings over pregnancy, analyses of cerebral and renal circulations and cognitive behavioural testing.

Results and discussion

The normal pattern of gestational blood pressure regulation (stable to gestation day (gd)5, decline to nadir at gd9, rebound towards baseline at gd10 and stable to term) was present in alymphoid mice lacking spiral arterial modification. In $Pgf^{-/-}$ mice, blood pressure decline was twice as large and nadir was 5 days, implicating PGF in normal gestational blood pressure stabilization. Ultrasonographic cardiac parameters in pregnant alymphoid or $Pgf^{-/-}$ mice differed from controls by gd14 and suggested left ventricular damage. In alymphoid but not $Pgf^{-/-}$ pregnancies, fetal cardiac function was additionally altered. $Pgf^{-/-}$ fetuses and offspring had poorly interconnected cerebral vessels that impacted on stroke susceptibility and cognitive functions. These studies highlight the importance of very early uNK cell functions and of PGF in normal *postpartum* health.

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S022

Maternal–conceptus interactions for the establishment of pregnancy in pigs

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The implantation process requires well-coordinated interactions between the maternal uterus and the developing embryo. In pigs, embryo implantation begins around day (D) 12 of pregnancy. During this period, the conceptus undergoes a dramatic morphological change and the elongated filamentous conceptus secretes various biological products such as estrogens and cytokines, interleukin-1β (IL1B), interferon-γ (IFNG), and IFN-δ (IFND). In response to these conceptus signals as well as the ovarian hormone, progesterone, the uterine endometrium becomes receptive to the conceptus. Estrogen acts as the signal for maternal recognition of pregnancy, and increases expression of many uterine endometrial genes, including *AKR1B1*, *FGF7*, *IL1RAP*, *LPAR3*, *SPP1*, *STAT1*, and *TRPV6*. IL1B of conceptus origin also affects endometrial functions. Especially, IL1B affects synthesis and transport of endometrial prostaglandins (PGs) by increasing endometrial expression of PG-synthetic enzymes, *PTGS1*, *PTGS2*, and *AKR1B1*, and PG transporters, *ABCC4* and *SLCO2A1*. IL1B induces endometrial expression of IL1B receptors. IL1B in concert with estrogen also increases endometrial production of salivary lipocalin 1, a lipid-binding protein, during early pregnancy. It is known that IFNG and IFND, which are produced by the conceptus with the highest levels on D14 – D16 of pregnancy, do not have an anti-luteolytic effect as IFN-τ in ruminants does, but the detailed function of IFNG and IFND in the uterus is not well understood in pigs. Recent data from ours and others indicate that IFNG induces endometrial expression of the IFN signaling molecules, *STAT1*, *STAT2*, *IRF1*, and *IRF2*, and *CIITA*, *SLA-DQA*, and *SLA-DQB*. Further analysis of the molecules derived from the conceptus and the endometrium will provide insights into the cellular and molecular basis of maternal–conceptus interactions during early pregnancy in the pig.

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S023

Modulation of progesterone action in the endometrium

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Endometrial function is orchestrated by endogenous ovarian steroid hormones, oestradiol (E) and progesterone (P). P plays a crucial role in the functional modification of the endometrium in preparation for pregnancy (differentiation of stromal fibroblasts, maturation of spiral arterioles and influx of immune cells). In the peri-menstrual phase cyclical tissue 'injury and repair' in the local endometrial environment is a consequence of P-withdrawal which initiates a cascade of changes including focal hypoxia, vessel breakdown and tissue shedding (menstruation). Initiation of menses may be reversed with P add-back up to 36 h following its withdrawal but beyond 36 h menstruation is inevitable. A spectrum of synthetic therapeutics exist that are capable of activating (agonists), or blocking (antagonists) the action of P. Persistent exogenous local delivery of progestogen (agonist) to the endometrial cavity (levonorgestrel-releasing intrauterine system; LNG-IUS) has dramatic impacts on endometrial function and menstrual bleeding. Selective progesterone receptor modulators (SPRMs) for example, Asoprisnil and Ulipristal acetate (UPA) are compounds with mixed agonist/antagonist properties. Administration of ligands (LNG; Asoprisnil; UPA) for the progesterone receptor (PR) also has a dramatic impact on the function and architecture of the endometrium (a complex multicellular tissue). The SPRMs induce unique PRM-associated endometrial changes (PAEC), the full significance of which is yet to be determined. Each of these PR ligands results in a profound suppression of uterine bleeding. The mechanisms that underpin these impressive clinical impacts remain a crucially important area for research especially if such compounds are to achieve their full clinical utility.

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Preserving fertility

S024

Dissecting gene regulation network in human early embryos at single-cell and single-base resolution

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Measuring gene expression in individual cells is crucial for understanding the gene regulatory network controlling human embryonic development. Here we apply single-cell RNA sequencing (RNA-Seq) analysis to 124 individual cells from human preimplantation embryos and human embryonic stem cells (hESCs) at different passages. The number of maternally expressed genes detected in our data set is 22 687, including 8701 long noncoding RNAs (lncRNAs), which represents a significant increase from 9735 maternal genes detected previously by cDNA microarray. We discovered 2733 novel lncRNAs, many of which are expressed in specific developmental stages. To address the long-standing question whether gene expression signatures of human epiblast (EPI) and *in vitro* hESCs are the same, we found that EPI cells and primary hESC outgrowth have dramatically different transcriptomes, with 1498 genes showing differential expression between them. We also profiled DNA methylome dynamics during preimplantation development of human embryos. Our work provides insights of critical features of the transcriptome and DNA methylome landscapes of human early embryos, as well as the functional significance of DNA methylome to regulation of gene expression and repression of transposable elements.

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S025

Testing male fertility

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The diagnosis of male fertility is of importance in humans and animals. Therefore several laboratory techniques have been developed for the evaluation of male fertility. Initial evaluation of male fertility generally depends upon semen analyses. While these tests provide valuable quantitative data, they yield no information concerning the functional competence of the spermatozoa.

To cope with the weak points of conventional semen analyses, several tests relating sperm function have been issued. Although these tests may provide preliminary information on male fertility, very few individual tests showed significant correlations with the successful pregnancy. None of tests have reached their clinical validity. Therefore, more accurate testing tools for male fertility are a well-timed request.

As indicated by several researchers, the establishment of new diagnosis tools requires the development of genomic and proteomic studies in a clinical setting. Recently comparative studies between fertile and subfertile/infertile spermatozoa have been initiated to investigate male fertility at the gene and protein levels.

To identify the true biomarkers for the diagnosis of male fertility, we suggest three strategies in a proteomic approach. First, a comprehensive proteomic study will be necessary to identify the differential quantities of proteins present in spermatozoa at different maturation stages, fertility statuses, and species levels for using these tools. Second, the validity of these protein markers will have to be confirmed through rigorous clinical tests by using an abundant number of samples. Finally, the functions of these markers will be investigated to understand the mechanisms by which proteins are associated with male fertility.

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S026

Protecting female fertility during anti-cancer treatment

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A common side-effect of standard cytotoxic cancer therapy is infertility and premature menopause resulting from collateral damage sustained by the ovaries during treatment. The survival rates for many cancers has improved greatly

during recent years and consequently there has been considerable interest in the development of new and innovative strategies to protect the fertility and health of women post-cancer treatment. Towards this end, we are investigating the cellular mechanisms that regulate oocyte apoptosis and primordial follicle loss during cancer treatment. We have identified the pro-apoptotic BH3-only proteins, PUMA and NOXA, as essential regulators of oocyte death and primordial follicle loss following exposure to γ-irradiation. Furthermore, our studies in mice demonstrate that elimination of PUMA, either alone or together with NOXA, protects a cohort of primordial follicles from γ-irradiation induced loss and preserves fertility without compromising the health of offspring. We are now extending our findings to determine if PUMA and NOXA are also responsible for the loss of primordial follicles that occurs during exposure to commonly used chemotherapy agents, such as cyclophosphamide, cisplatin and doxorubicin. Our work provides support for the concept that preventing oocyte death, by blocking key members of the apoptotic pathway, such as PUMA and NOXA, may represent a viable new stategy for protecting future fertility and ovarian function in girls and women being treated for cancer.

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Control of meiosis

S027

Environmental effects on gametogenesis: it all depends on sex

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Gametogenesis is a complex process with striking sexual dimorphism. Although oogenesis and spermatogenesis share several global features (e.g., the production of haploid cells through meiotic divisions and extended periods of gamete maturation), the timing of events, their order and the propensity for errors during the process differ markedly. Our interest is in the factors – both endogenous (e.g., age) and endogenous (e.g., environmental exposures) – that influence the genetic quality of the resultant gamete. Increasing evidence suggests that both male and female reproduction are susceptible to the effects of endocrine disrupting chemicals (EDCs), and our focus has been understanding potential sex-specific effects of these chemicals on gametogenesis. Data from our studies in mouse, monkey and sheep suggest that exposure to BPA in the female and to either BPA or other exogenous estrogens in the male can induce meiotic effects. Temporal differences in the timing of events, however, result in different developmental windows of vulnerability in the two sexes. Further, our findings suggest that EDCs induce meiotic effects by altering the germline epigenome and, importantly, demonstrate strikingly different consequences in males and females. These findings underscore the necessity of considering sex-specific differences in developmental timing when devising accurate methods of measuring the impact of exposures and evaluating the reproductive risk posed by chemicals with endocrine disrupting potential.

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S028

Making it through meiosis: APC/C FZR1 has an essential role in meiotic prophase I in germ cell development

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Fizzy-related 1 (FZR1) is one of two known activators of the anaphase promoting complex (APC) and a well-established important regulator of the mitotic cell cycle. In a germ-cell-specific (DDX4-cre) conditional knockout model we examined the role of FZR1 in germ cell development with particular emphasis on the entry into meiosis and early meiotic events. Loss of APC FZR1 activity in the male germline led to both a mitotic and a meiotic cell defect resulting in sterility through the complete absence of mature spermatozoa. Spermatogonia in the prepubertal testes displayed abnormal proliferation and delayed entry into meiosis. Although early meiotic recombination events were initiated, male germ cells failed to progress beyond zygotene stage of meiosis and underwent apoptosis. APC FZR1 loss was also associated with elevated cyclin B1 levels in spermatogonia and spermatocytes, indicating that CDK1 may trigger apoptosis. In contrast, the FZR1 null female mice were subfertile, with premature ovarian failure by 20 weeks of age. Initially a large loss of oocytes occurred embryonically, around the time of the zygotene-pachytene transition, similar to that observed in males. In addition, in post natal folliculogenesis the transition of primordial follicles into the growing follicle pool was abnormal and resulted in premature depletion of the primordial follicle pool. We have established that APC FZR1 is an essential regulator of spermatogonial proliferation and in early meiotic prophase I in both the male and female germline, and in post natal follicular development, and conclude that FZR1 is important in establishing and maintaining the reproductive health of adult male and female mammals.

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S029

Major causes of age-related chromosome segregation errors at meiosis I in oocytes

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Chromosomes must be properly segregated during meiosis to transmit the correct set of the parental genome into gametes. Incorrect chromosome segregation produces aneuploid gametes, fertilization of which results in pregnancy loss and congenital diseases such as Down syndrome. However, it is known that the frequency incorrect chromosome segregation is extremely high at meiosis I in oocytes (20–40% in humans), compared to other cell divisions. Moreover, the frequency of the errors increases with maternal age. Why chromosome segregation is so error-prone and age-related in oocytes is not fully understood. In this study, we established a high-throughput and high-resolution imaging of chromosome dynamics in live oocytes from naturally aged mice. Our 4D recording with automated microscopy detected nearly 100% of kinetochores and chromosomes at every timepoint from germinal vesicle breakdown to anaphase of meiosis I, at a high spatiotemporal resolution sufficient to robustly track all kinetochores and chromosomes. Our high-throughput approach yielded the datasets of >200 oocytes from aged mice, including >10 oocytes that underwent chromosome segregation errors at meiosis I. Thus, these datasets provide the first quantitative analysis of 'at-risk' single chromosome dynamics and a comprehensive resource to identify the major causes of age-related chromosome segregation errors at meiosis I in oocytes.

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Oral Presentations

Embryo

P001

Restoring mitochondria in oocytes of obese mice normalizes embryo development

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Obesity in females causes altered fetal growth during pregnancy and permanently 'programs' the metabolism of offspring; however the origin of these changes and whether they are reversible is not known. We now show that in obese female mice the oocytes exhibit endoplasmic reticulum (ER) stress, high levels of intracellular lipid, reduced Ptx3 matrix production and fail to ovulate. The oocytes from obese mice contain normal levels of mtDNA but have reduced mitochondrial membrane potential and a high degree of autophagy compared to oocytes from lean mice. After IVF, the oocytes of obese female mice demonstrate their reduced developmental potential and they form blastocysts with reduced mtDNA. Analysis at e14.5 showed that obese oocytes gave rise to fetuses that were heavier than controls and had reduced liver and kidney mtDNA per cell indicating that maternal obesity altered the mitochondrial set-point of offspring. Treatment of the obese females with ER stress inhibitors for just 4 days prior to conception completely restored oocyte, embryo and fetal physiology to normal. These results show that obesity prior to conception imparts a legacy of mitochondrial loss in offspring due to ER stress that is reversible in the pre-conception period.

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P002

CHD1 is the zygotic determinant of *Oct4* and *Cdx2* expression via the *Hmgpi* pathway during preimplantation development

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Chromatin remodeling factor chromodomain helicase DNA binding protein 1 (CHD1) is a protein belonging to the family of ATPase dependent chromatin remodeling factors and recognizes tri-methylated lysine 4 of histone H3 (H3K4me3) in chromatin. Histone methylation is one of the epigenetic modifications involved in gene regulation and H3K4me3 is associated with a transcriptionally permissive state of chromatin. It has been shown that CHD1 facilitates pre-mRNA splicing, and is required for the maintenance of pluripotency in mouse ES cell via *Oct4* activation. However, the role(s) of CHD1 in mouse preimplantation embryos has not yet been addressed (s1). In the present study, we investigated the expression pattern of *Chd1* in mouse preimplantation embryos and the effects of RNA interference (RNAi)-mediated *Chd1* repression on the development of mouse embryos. Here, we showed that *Chd1* was zygotically expressed at the late two-cell stage and *Chd1*-knockdown at the two-cell stage led to early embryonic lethality due to the loss of ICM pluripotency. In *Chd1*-knockdown embryos, the levels of mRNAs and proteins of both *Oct4* and *Cdx2* were dramatically decreased. Furthermore, the mRNA and protein of *Hmgpi* which regulates the zygotic expression of Oct4 and Cdx2 were also significantly decreased. Based on the results, when HMGPI was rescued by microinjection of *Hmgpi* mRNA in *Chd1*-knockdown embryos, the levels of mRNAs and proteins of both *Oct4* and *Cdx2* and pluripotency of ICM cells were recovered. These results suggest that CHD1 has important roles in mouse embryonic development via the *Hmgpi* pathway.

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P003

Genome-wide DNA methylation analysis of single and pooled bovine blastocysts

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Introduction
Genome-wide analysis of DNA methylation in mammalian preimplantation embryos, particularly at the single embryo level, will be of particular use for assessing the epigenetic status and health of *in vitro*-derived embryos, including humans. To date, single embryo DNA methylation analysis has been limited to a small number of genes (<5). In this study we used an adaptation of reduced representation bisulfite sequencing (RRBS) to i) perform genome-wide analysis of the DNA methylome of the bovine blastocyst using pooled blastocysts and ii) to assess whether the same aim could be achieved in single blastocysts.
Materials and methods
Embryonic genomic DNA was isolated from bovine *in vitro*-derived blastocysts. Two pooled blastocyst samples were generated (pool 1, $n=17$ blastocysts; pool 2, $n=5$ blastocysts). In addition, DNA from three single bovine blastocysts was isolated. RRBS reads were mapped using Bismark and the Bioconductor package BiSeq for methylation calling, clustering, and analysis.
Results and discussion
Assessment of RRBS read clusters revealed that greater than 34 000 CpG clusters were commonly methylated between the two pooled blastocyst samples and >13 000 CpG clusters were common between the three single blastocyst samples (clusters contain >20 CpGs). Genome-wide plots for methylated CpG clusters and methylated CpG islands will be presented. Using DAVID analysis, the highly methylated loci include members of the MAPK signalling pathway, members of the pathways in cancer and several miRNAs as examples. This report is the first genome-wide analysis of DNA methylation in the bovine blastocyst and the first to show that comprehensive genome-wide methylation analysis in single mammalian blastocysts is possible.

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P004

Regulation of nuclear size in mammalian embryos

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Introduction
The mechanisms that dictate nucleus size are largely mysterious. Experiments in fission yeast suggest that nuclear size is dictated by the size of the cell, whereas studies in *Xenopus* indicate that nuclear import rates control nuclear volume. Here we set out to exploit the reductive divisions of early development present to what regulates nucleus size in mammalian embryos.
Materials and methods
We generated three-dimensional confocal images of preimplantation mouse embryos to determine nuclear/cytoplasmic volume ratio. Fluorescent recovery after photobleaching (FRAP) of GFP-tagged nuclear localization signal (GFP-NLS) was used to establish nuclear-import rates. Micromanipulation was used to probe the influence of cell volume upon nuclear size.
Results and discussion
Nuclear size decreases with successive cleavage divisions from one-cell stage to blastocyst, and nuclear/cytoplasmic ratio was relatively constant at any given developmental stage, suggesting a direct relationship between nuclear and cell size. Experimental cytoplasmic removal reduced significantly nuclear size ($P<0.01$), further suggesting that cell size directly influences nuclear volume. However, intriguingly, the nuclear/cytoplasmic ratio set-point changes progressively through development (from 0.057 in two-cells to 0.141 in morulae), revealing that factors other than cell volume influence nuclear size. Nuclei in experimentally-generated embryos with 'double-sized' blastomeres were of normal size, also suggesting that nuclear size is controlled by a developmental programme. The rate of nuclear import was identical in two-, four-, and eight-cell stage embryos ($P>0.1$), revealing that in contrast to other systems, nuclear import does not explain the developmental programme. This nuclear volume in mammalian embryos is determined cooperatively by cell size and developmentally-regulated cytoplasmic factors.

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P005

Generation of diploid cloned embryos from porcine-induced pluripotent stem cells synchronized to metaphase

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Pigs provide outstanding models of human genetic diseases due to the striking similarities to human anatomy, physiology and genetics. Pig induced pluripotent

stem cells (piPSCs) have been generated for several years but the cloning efficiency using unsynchronized piPSCs was extremely low. Here, we reported a method to produce diploid cloned embryos from piPSCs which were synchronized to metaphase. The piPSCs cell line was established using a drug-inducible system and exhibit similar morphology to mouse embryonic stem cells with normal karyotype. After synchronized by a two-step block method with aphidicolinand nocodazole, 77.6% of the cells were arrested at G_2/M phase. Round cells ranged from 17–19 μm were selected and fused with enucleated MII oocytes. After activation, 81.3% of reconstructed embryos extrudedone pseudo-second polar bodies (p2PB). The immunofluorescent results confirmed that half chromatids were extruded with the p2PB. However, 2 mM 6DMAP treatment post activation blocked the p2PB extrusion. Moreover, immediately activation method yielded significantly more blastocysts than delayed activation (31.3 vs 16.0%, based on fused embryos). 6DMAP treatment post p2PB extrusion also didn't improve the blastocyst formation rate. Karyotyping of the blastocysts indicated that 61.1% blastocysts were diploid. This study demonstrated a new efficient way to produce cloned embryos from piPSCs which synchronized to mitotic metaphase. Further studies will be focused on the pluripotent gene expression of these embryos and the feasibility of using these embryos to produce cloned pigs.

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Uterus

P006

Estrogen-dependent modulation of neuroinflammation in endometriosis

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Introduction

Endometriosis is an estrogen-dependent inflammatory disorder, associated with debilitating pelvic pain. We believe the pain is due to neuroinflammation: growth of sensory neurons and their subsequent hypersensitisation by macrophage-derived cytokines within the lesion. We explored the regulation of neuroinflammation by estrogen receptor (ER) modulators.

Materials and methods

Peritoneum and lesions were collected from women with and without endometriosis and from a novel *MacGreen* (transgenic Cfs1r-EGFP) mouse model of endometriosis. Immunofluorescence was used to locate macrophages and nerves, and QPCR to measure macrophage-derived cytokines (IL6, IL1β, and TNFα). Human peripheral blood monocyte derived macrophages and mice with endometriosis were exposed to ER agonists.

Results and discussion

IL6 and *IL1β* mRNAs were elevated in peritoneum and lesions of women with pain or endometriosis ($P<0.05$) compared to women without disease. Levels were reduced ($P<0.05$) in women taking oral contraceptives/GnRH analogues. IL6 and TNFα correlated with pain scores in women with pain but no endometriosis ($P<0.05$ and $P<0.01$), highlighting the importance of cytokines in pelvic pain, and a possible role for estrogenic manipulation. In mice with endometriosis, macrophage chemotactic ligands were elevated ($P<0.001$) in lesions compared to peritoneum. Using *MacGreen* mice we identified macrophages originating from peritoneum and shed endometrium. IL1β and TNFα were elevated ($P<0.001$) in mouse lesions and exposure of mice to DPN (ERβ agonist) abrogated this effect. In isolated macrophages IL1β and IL6 were estrogen-regulated. Macrophages and nerve fibres were closely associated in mouse lesions. Modulation of ERs may reduce neuroinflammation in lesions and offer a new opportunity to manage endometriosis-associated pain.

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P007

Oestrogen-driven changes in the bovine uterus are associated with activation of the unfolded protein response

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The endometrium plays a central role in fertility and pregnancy. It undergoes changes in function and histological morphology over the oestrus cycle, driven by dynamic changes in progesterone and oestrogen. To understand the changes resulting from elevated oestrogen levels around oestrus, we profiled the transcriptome of the bovine uterus. Endometrial tissue was collected from synchronized beef heifers in six cohorts: i) 12 h after controlled intravaginal progesterone-releasing device (CIDR) removal; ii) 24 h after CIDR removal, iii) oestrus onset, iv) 12 h after oestrus onset, v) 48 h after oestrus onset, and vii) 7 days after oestrus onset. Gene expression levels were measured using RNA-Seq. Corresponding blood samples were submitted for hormonal analysis. The 200 genes most highly correlated with oestradiol were analysed using the Ingenuity Pathway Analysis (IPA) tool. IPA identified XBP1 (the key transcriptional regulator of the unfolded protein response (UPR)) as the top ranked upstream regulator. The UPR is usually activated in cells in response to endoplasmic reticulum stress, however it is also active in the biogenesis of the secretory machinery in exocrine cells. XBP1 exists as two isoforms XBP1(u) and XBP1(s). XBP1(s) is a potent activator of the UPR. RT-PCR indicated that XBP1(s) decreased 57% from 12 h after CIDR removal until 12 h after oestrus, then rose again in the luteal phase, indicating that XBP1(s) was induced during the progesterone-dominated phase of the cycle. Our results show that the UPR/XBP1 pathway is under hormonal control in the bovine endometrium and influences the secretory activity of the endometrium during the peri-oestrus period.

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P008

Conditional deletion of progesterone receptor membrane component 1 (*Pgrmc1*) and *Pgrmc2* results in subfertility and aberrant endometrial epithelial cell proliferation

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Introduction

Since progesterone receptor membrane component 1 (PGRMC1) and PGRMC2 are highly expressed in the uterus, this study was designed to evaluate the impact of *Pgrmc2* and *Pgrmc1/2* deficiency on female fertility and uterine epithelial cell proliferation.

Materials and methods

Uterine PGRMC2 and Ki67 expression were assessed by immunohistochemistry. Mutagenesis studies were completed in which *Pgr-cre* was used to conditionally ablate *Pgrmc* genes.

Results and discussion

PGRMC2 was predominantly expressed in the cytoplasm of uterine epithelial and stromal cells with limited change in expression in response to steroid hormones. Breeding trials showed that *Pgrmc2* conditional knockout (cKO) and *Pgrmc1/2* double cKO (dcKO) female mice experienced a significant reduction in the number of pups/litter (control 7.7 ± 0.80 vs *Pgrmc2*, cKO 4.1 ± 0.43, $P=0.0001$; control 8.4 ± 0.28 vs *Pgrmc1/2*, cKO 5.2 ± 0.67, $P=0.0006$). The reduction in pup numbers, which was due to post-implantation embryonic loss, was not the result of luteal insufficiency, since serum P4 levels were higher in cKO mice ($P=0.007$). Reductions ($P<0.05$) in both the number of litters over the 6-month breeding trials and number of pups surviving to weaning were also observed. Uteri obtained from cKO and dcKO females showed development of cystic hyperplasia starting around 4 months of age. Compared to controls, this phenotype also showed an increase in mitotically-active uterine epithelial cells, as judged by Ki67 immunostaining. These findings demonstrate that members of the PGRMC family are required for normal pregnancy and that their deletion leads to uterine hyperplasia. This work is supported in part by NIH RR030264.

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P009

Label retaining stromal cells are involve in uterine remodeling after parturition

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Introduction

Mouse endometrium undergoes extensive postpartum remodeling. To accomplish the extensive cellular turnover processes, we hypothesize that the endometrial cells with quiescent properties (stem/progenitors) are involved in the regeneration of the tissue after parturition.

Methods
Prepubertal C57BL/6J female mice were labeled with BrdU, followed by a chase period of up to 11 weeks. Mice in the remodeling group were mated and sacrificed at gestational day (GD) 7, 14 and *postpartum* day (PPD) 1, 2, 7, 14, and 21. Age-matched virgin mice were used as control.
Results and discussion
Endometrial label retaining stromal cells (LRSC) were identified after 7-week chased as there was similar percentage of BrdU+ stromal cells between virgin ($1.2 \pm 0.6\%$) and pregnant (GD7: $1.4 \pm 0.4\%$, $P = 0.76$) mice. During gestation, quiescent LRSC are localized to the inter-implantation loci. Immediately after parturition BrdU-labeled epithelial cells were detected in the luminal epithelium. At PPD1, $43.9 \pm 5.4\%$ of the LRSC proliferated (BrdU+Ki-67+) and by PPD3 declined to $18.2 \pm 0.8\%$ ($P > 0.05$). Total and active β-catenin were expressed in 34.5 ± 13.2 and $8.0 \pm 4.6\%$ of the proliferating LRSC at PPD1 respectively. After 11-week chased, only $0.41 \pm 0.14\%$ of LRSCs remained in the remodeling mice compared to $1.0 \pm 0.4\%$ in virgin mice ($P > 0.05$). LRSC were primarily observed beneath the luminal epithelium, adjacent to blood vessels and near endometrial–myometrial junction from mice which have undergone pregnancy. Our findings suggest that LRSC participate in the remodeling of the mouse endometrium and possibly involve the activation of the wnt/β-catenin pathway.

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P010

Vital uNK growth factors are reduced in endometrial tissue from women with endometriosis resulting in an accumulation of uNK progenitor cells

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Introduction
Uterine natural killer (uNK) cells have a largely recognised role in normal uterine function, particular successful pregnancy. We have previously found that infertile women have more uNK progenitor cells than fertile women. We aim to study the differentiation of uNK cells in women with endometriosis.
Material and methods
Numbers and phenotype of uNKs (CD45+, CD56+, CD3+, and CD16−) were defined using flow cytometry: CD10, CD34, CD94, CD117, CD161, integrinβ7, and NKp46. Levels of interleukin 15 (IL15), stem cell factor (SCF), and IGF1 were analysed using qRT-PCR and western blot. CD56+CD34+ cells were detected using double immunohistochemistry. CD34+ (progenitor marker) cells were isolated from endometrial biopsies and cultured with IL15, SCF, and IGF1.
Results and discussion
CD56+ cell numbers were higher in women with endometriosis when compared with unaffected women. This appears to be because UNK progenitors (CD56+CD34+) at all phases of development were also higher in women with endometriosis. Additionally, NK growth factors (GFs) IL15, SCF, and IGF1 are reduced in women with endometriosis. Culturing CD34+ endometrial cells from women with and without endometriosis with these GFs resulted in higher numbers of mature NK cells from women with endometriosis.
This study shows for the first time that altered uNK maturation, as evidenced by accumulated progenitor cells and abnormal levels of significant GFs, occurs in endometriosis. Replacement of these GFs in cell culture restores NK maturation. Stunted development of a cell vital for fertility, uNK cells, provides an explanation for endometriosis-related infertility.

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Testis

P011

Delayed puberty onset and reduced sperm quality in male rats prenatally exposed to betamethasone

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Introduction
Betamethasone is a drug of choice for antenatal treatment. The aim of the present work was to evaluate the age of puberty onset and sperm quality in male rats whose mothers received betamethasone during gestation.
Material and methods
Pregnant Wistar rats were allocated into control group and treated with 0.1 mg/kg betamethasone on gestational days 12, 13, 18, and 19. Maternal weight gain and the following parameters of male offspring ($n = 10$/group, two males/litter) were evaluated: body weight and anogenital distance on postnatal day (PND) 1, body weight on PND21 (weaning) and age of preputial separation (evaluated from PND30 onwards). One male from each litter was killed on PDN45 and reproductive organ weights and testosterone levels were obtained. The other male (PDN90) was used to perform fertility tests. Sperm retrieved from the epididymal cauda was also utilized to evaluate sperm motility and morphology. Student's *t*-test and Mann–Whitney *U* test were used to statistically compare the results.
Results and discussion
The following results, observed in the treated group, significantly differ from the control group ($P < 0.05$): maternal weight gain and the weight of male litters on PND1 were reduced; preputial separation was delayed; on PDN45 testosterone levels were decreased; testis weight increased and seminal gland weight decreased. At adulthood these alterations remained and additionally the ventral prostate weight decreased in the treated group; the incidence of abnormal sperm increased; sperm motility and fertility potential were decreased. Thus, our findings show that intrauterine betamethasone exposure delayed puberty installation and reduced sperm quality in male rats.
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P012

Mitochondrial activity and reactive oxygen species production define distinct subpopulations of human sperm with different functional properties

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Human ejaculates are heterogeneous, and can be comprised of different subpopulations. We have shown that mitochondrial activity varied within ejaculates, and that sperm with high mitochondrial activity were more functionally relevant. Mitochondria are also known to produce reactive oxygen species (ROS), which have been implicated both in sperm function, and in causing gamete dysfunction, depending on amounts and timing of production. We have applied several fluorescent probes in order to quantify ROS production in human sperm samples using flow cytometry. We show that human ejaculates are heterogeneous in terms of ROS production, with several subpopulations clearly detectable, comprised of sperm that produce increasing amounts of ROS (ROS−, ROS+, ROS++, etc.). In the case of the probe MitoSOX™ Red (MitoSOX), the sperm subpopulation producing the lowest amount of ROS represented the most functional subset of male gametes within the ejaculate, as it was correlated with the highest amount of live and non-apoptotic sperm, and increased both in samples with better semen parameters, and in samples processed by both density gradient centrifugation and swim-up, both known to select for higher quality sperm. Importantly the MitoSOX ROS− subpopulation was clearly more prevalent in samples that gave rise to pregnancies following Assisted Reproduction, regardless of other characteristics. Currently we are characterizing human sperm subpopulations with distinct mitochondrial properties at the molecular level. Our work therefore suggests that mitochondrial function may represent a strategy to both evaluate sperm samples, and isolate the most functional gametes for Assisted Reproduction.

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P013

Exogenous androgens reduce the expression of INSL3, a hormone involved in normal testicular descent, in fetal Leydig cells

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Introduction
Hypothalamo–pituitary–testicular feedback is intact in the ovine fetus. Exogenous testosterone suppresses fetal LH and testicular steroidogenesis to

maintain normal circulating testosterone concentrations. INSL3 is a non-steroidal hormone secreted by the fetal testis whose roles include facilitating testicular descent. We hypothesised that the homeostatic response to increased androgens would have a detrimental effect on fetal *INSL3* expression.
Materials and methods
Pregnant Scottish Greyface sheep were treated twice weekly with testosterone propionate (TP: 100 mg) or vehicle control from day (d) 62 gestation and male fetuses collected at d70 (TP = 5, C = 8) and d90 (TP = 14, C = 12). In some, testis explants (d90) were cultured for 1hr then LH or control was added for 1hr. Other fetuses (d90) had direct injection of TP (20 mg) or vehicle at d62 and d82 (TP = 6, C = 11). *INSL3* expression was analysed by quantitative RT-PCR and localised by immunohistochemistry.
Results and discussion
INSL3 was localised to Leydig cells in the fetal testis. Maternal TP administration reduced the expression of *INSL3* in the fetal testis at d70 ($P < 0.05$) and d90 gestation ($P < 0.05$). In addition, direct injection of TP into the fetus also reduced testicular *INSL3* expression ($P < 0.05$). *In vitro* expression of *INSL3* in cultured explants was much lower after maternal TP exposure ($P < 0.005$) and there was a non-significant increase in explant *INSL3* expression after exposure to LH ($P = 0.06$). *INSL3* expression in Leydig cells in the ovine fetus is altered by testosterone, probably though LH regulation. This is further evidence that INSL3 is a marker of endocrine disruption in the developing fetus.

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P014

Suppressive effects of ovarian steroid hormones on the expression of synaptonemal complex protein 1 in organ-cultured testis of neonatal mice

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Introduction
Onset timing of meiosis is different between the two sexes in mammals. In the mouse, meiosis in the ovary initiates during late stages of the second trimester, whereas germ cells in the testis at the same stage enter mitotic arrest and do not initiate meiosis until *postpartum*. In the present study, I have studied effects of ovarian steroids on the formation of synaptonemal complex in the neonatal mouse testis.
Materials and methods
Testis was collected aseptically from neonatal C57BL/6N mice, de-capsulated, placed atop an agarose block placed in wells of multi-well plates, and then cultured with control medium containing fetal bovine serum, transferrin and insulin, and either with or without steroid hormones. After culture for a few weeks, testis was recovered and assayed for SYCP1 and its transcripts, by immunofluorescence (IF) and qRT-PCR respectively.
Results and discussion
SYCP1-specific IF signals were detected in the testis from days 6–8 *postpartum* that was cultured for 1 week or longer with no addition of steroids, whereas specific signals with reduced intensities were detected in the testis that was cultured with progesterone. Estradiol β added alone showed no or little effects but an additive effect with progesterone in suppression of SYCP1. QRT-PCR confirmed that progesterone reduced the amount of *Sycp1*-specific transcripts, and that estradiol β further reduced when added with progesterone. Removal of these steroids from culture medium induced the resumption of SYCP1 expression. These results indicate that ovarian steroid hormones are likely to be candidates for physiological suppressors of meiosis in the testis.

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P015

Identification of a novel, ancient family of germ cell enriched proteins

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Introduction
For two decades, an antibody recognizing germ cell nuclear antigen (GCNA) has been widely used to identify mouse germ cells. The antigen, highly germ cell specific and expressed coincident with a key developmental transition as migrating germ cells enter the gonad, has heretofore not been identified. Here, we reveal GCNA as the founding member of a novel, ancient family of germ cell enriched proteins found across eukaryotes, including all major animal classes, plants, and fungi.
Materials and methods
Immunoprecipitation, mass spectrometry, and computational analysis.
Results and discussion
We have determined that GCNA is the product of an X-linked gene encoding an acidic and highly repetitive protein. Largely due to the unique amino acid composition and rapid evolution of proteins in this family, orthologs in most species, including mouse, have been overlooked due to poor annotation. All GCNA orthologs contain a 300–800 amino acids domain that is likely intrinsically disordered. Furthermore, GCNA orthologs outside of the rodent lineage share a well-conserved uncharacterized domain. In species for which expression data is available, gene products of GCNA orthologs are enriched in reproductive organs or specifically in germ cells, and in some species, they are required for fertility. GCNA-related genes are more ancient than the classical germ cell genes *Dazl/Boule* and *Vasa*, which are confined to metazoa. The tremendous evolutionary depth of this family suggests its involvement in a fundamental aspect of germ cell biology.

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Ovary

P016

Rho-GTPase effector ROCK regulates mouse oocyte maturation and embryo development

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Introduction
ROCK (Rho kinase) is a Rho-GTPase effector that is involved in various cellular functions, such as stress fiber formation, cell migration, tumor cell invasion, and cell motility. In this study, we investigated possible roles for ROCK in mouse oocyte meiosis and embryo development.
Materials and methods
Time lapse microscopy, immunofluorescence staining, western blotting, RNAi and inhibitor treatment were adopted to analyze the roles of ROCK in mouse oocytes and embryos.
Results and discussion
ROCK was localized around spindles after germinal vesicle breakdown (GVBD) and was co-localized with cytoplasmic actin and mitochondria. Disrupting ROCK activity by RNAi or an inhibitor resulted in oocyte polar body extrusion and embryo development failure. Time lapse microscopy showed that this may have been due to spindle migration and cytokinesis defects, as chromosomes segregated, but failed to extrude a polar body, and then re-aligned. Actin expression at oocyte and embryo was significantly decreased after these treatments. Actin caps of oocytes were also disrupted, which was confirmed by a failure to form cortical granule-free domains (CGFDs). In addition, the phosphorylation levels of LIMK1/2 and Cofilin, two downstream molecules of ROCK, decreased after disrupting ROCK activity in both oocytes and embryos. Thus, our results indicated that a ROCK-LIMK1/2-Cofilin-actin pathway regulated cytokinesis during mouse oocyte maturation and embryo development.

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P017

Phosphatidyl-inositol-3-kinase/AKT regulation is involved in oocyte developmental competence

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Introduction
We recently demonstrated that somatic follicular cells finely regulate the translation of stored mRNAs during oocyte maturation. Amphiregulin and other

EGF-like peptides expressed by follicular cells trigger the translation of specific transcripts in the oocyte, seemingly by indirect activation of the Phosphatidyl-inositol-3-kinase PI3K/AKT/mTOR cascade. The present study aims to better characterize the role of PI3K/AKT during oocyte maturation by using a genetic model where (phosphatase and tensin homologue) Pten, which negatively regulates PI3K, is specifically ablated in oocytes.
Materials and methods
Cumulus–oocyte complexes were collected from 21-day-old $Pten^{fl/fl}$:ZP3-CRE mice after PMSG-priming. AKT phosphorylation was assessed by western-blot. Translational activity was measured by luciferase assay after intraoocyte injection of dual luciferase translation reporters. Developmental competence was assessed by *in vitro* maturation and IVF (IVM–IVF). Oocyte diameter and the kinetics of meiotic resumption were also analyzed.
Results
In $Pten^{fl/fl}$:ZP3-CRE oocytes AKT was phosphorylated to the same extent in GV stage oocytes and after 2.5 h culture either with or without amphiregulin, while AKT in WT oocytes was only transiently phosphorylated after 2.5 h culture with amphiregulin. Similarly, the specific translation of the luciferase reporters was no longer dependent on amphiregulin in $Pten^{fl/fl}$:ZP3-CRE oocytes. Interestingly, Pten conditional knock-out oocytes showed a higher developmental competence after IVM–IVF. Oocyte diameter and the re-entry into cell-cycle were not affected.
Discussion
Our results demonstrate that PI3K/AKT activation is a key factor for oocyte developmental competence and confirm its involvement in downstream signaling regulating the execution of the oocyte translational program during maturation.
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P018

Intrinsic oxidative stress increases meiotic chromosome misalignment and aneuploidy in *in vitro* matured oocytes from SOD1-deficient mice

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Introduction
Aneuploidies in mammalian embryos usually originate from oocyte chromosome segregation errors in meiosis I and are a major genetic cause of pregnancy loss. But the mechanism remains poorly understood. We have previously shown total two-cell arrest of IVF embryos derived from superoxide dismutase 1 (SOD1)-deficient mouse oocytes under atmospheric oxygen conditions. This study was conducted to investigate effects of intrinsic oxidative stress by a SOD1 deficiency during *in vitro* maturation (IVM).
Materials and methods
Immature cumulus–oocyte complexes (COCs) were retrieved from SOD1-deficient and WT ICR mice after administration of equine chorionic gonadotropin (eCG), while IVM COCs were recovered after administration of eCG and human CG. Immature COCs underwent IVM for 18 h under 20 or 5% O_2 culture conditions. The resulted IVM and IVM oocytes were subjected to examination on spindle formation, aneuploidy and expression of BubR1 that is one of the spindle assembly checkpoint (SAC) proteins.
Results and discussion
SOD1-deficient oocytes under 20% O_2 IVM substantially increased chromosome misalignment (45.8%) and a withering spindle assembly compared with WT oocytes or *in vivo* oocytes (3.3–10%). Under 20% O_2 IVM, SOD1-deficient oocytes accelerated the timing of germinal vesicle break down and progression of anaphase I compared with WT oocytes. The percentage of aneuploidy was two times higher in SOD1-deficient oocytes than in WT oocytes under 20% O_2 IVM while *in vivo* oocytes showed similar percentage regardless of genotype. BubR1 signals on kinetocore were apparently weaken in SOD1-deficient oocytes compared with WT oocytes. Our results suggest that intrinsic oxidative stress during oocytes meiotic maturation impairs spindle assembly, regular timing of meiosis progression and localization of BubR1, which would consequently lead to aneuploidy.

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P022

Wnt5a is a crucial regulator of ovarian follicle development and female fertility in mice

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Introduction
The WNT signaling pathway plays important roles in sex determination and embryonic development. Previous results indicated that WNT4 is required for normal ovarian follicle development and fertility and that WNT4 and WNT5a play redundant roles in regulating oocyte entry into meiosis during embryogenesis, suggesting potential redundant roles of WNT4 and WNT5a in the ovary. The objective of this study was to determine the effect of granulosa cell-conditional knock-out of *Wnt5a* on follicle development.
Methods
Fertility was determined in 6-month mating trials with *Wnt5a*(flox/−); Amhr2(cre/+) and control mice. Ovaries were obtained at six and 32 weeks of age, and every fifth section used to count the number of follicles. TUNEL was used to assess apoptosis in adult ovaries. Microarray and RT-PCR were used to determine the effect of WNT5a on granulosa cell function.
Results
Wnt5a(flox/−);Amhr2(cre/+) mice had reduced fertility ($P<0.05$), reduced numbers of healthy follicles ($P<0.05$) and increased apoptosis in adult ovaries. *Wnt5a*(flox/−);Amhr2(cre/+) mice also had a decreased ovulation rate ($P<0.05$). To determine the mechanism of action of WNT5a, primary granulosa cells were treated *in vitro* with WNT5a, which decreased the expression of genes associated with granulosa cell differentiation (*Cyp19a1*, *Lhcgr*, *Prlr*, and *Fshr*). Consistent with this, the expression of these same genes in granulosa cells was higher in *Wnt5a*(flox/−);Amhr2(cre/+) mice compared to controls *in vivo*. Together, these results indicate that *Wnt5a* is required for normal female fertility and may act by regulating genes critical for granulosa cell differentiation and follicle development.

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P023

Functional evaluation of miRNAs during bovine ovarian follicular/luteal development

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Little is known about the involvement of miRNAs during terminal follicle differentiation in the monovular ovary. This study aimed to characterise miRNAs involved in the follicle-luteal transition in bovine. Microarray analyses were performed on RNA from ovulatory-size follicles ($n=6$) and early corpora lutea ($n=6$) obtained at an abattoir. Exiqon's miRCURY LNA microRNA Array, sixth generation was used and results were validated by qPCR. A total of ten and 24 miRNAs were upregulated and downregulated (greater than twofold; Benjamini and Hochberg adjustment, $P<0.05$), respectively, in luteal relative to follicular tissues. Among top upregulated miRNAs in the CL (greater than fivefold) were the cluster miR-182-96-183 and miR-132. To investigate the functional involvement of these miRNAs we used an *in vitro* model of forskolin-induced bovine granulosa cell luteinisation. Levels of miR-132 and miR-96 increased more than twofold within the first 4 days of luteinisation. Transfecting these cells with specific LNA inhibitors or mimics miR-132 and miR-96 led, respectively, to abolished expression and a significant increase in the levels of these miRNAs ($P<0.001$) within 2 days. However, the induced changes in miRNA levels during luteinisation did not have any significant effect on granulosa cell growth curves or on transcript levels of predicted mRNA targets including FOXO1, ADCY6, and CDKN1A. In summary, we have identified the miR-182-96-183 cluster and miR-132 as candidate miRNAs for an involvement in the follicular–luteal transition in the monovular bovine ovary. We are currently testing further the effects of these miRNAs during luteinisation *in vitro*, and we are investigating the actual distribution of these miRNAs within luteal tissues.

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Stem cells and reprogramming

P019

Diabetes alters the epigenetic landscape of the oocyte and early embryo

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Early embryo development is an exquisitely sensitive period of the developmental continuum, profoundly influencing neonatal and adult health. Maternal and paternal hyperglycaemia is associated with long-term health complications including increased risk of miscarriage, altered birth weight, and increased incidence of diabetes and metabolic syndrome in adult life. The mechanisms underlying this intergenerational transmission of disease remain unclear. New evidence has implicated the ten-eleven-translocation (TET) family of chromatin-modifying enzymes in *O*-linked glycosyltransferase (OGT)-dependent histone glycosylation. This led us to assess these systems in the mouse oocyte and early embryo. We demonstrated co-precipitation of TET3 (the key TET family member in the oocyte and early embryo) and OGT. In oocytes matured under hyperglycaemic conditions, we identified significant alterations to histone glycosylation (H3/H4). Given *O*-linked glycosylation competes for residues also targeted by phosphorylation, we examined the abundance of known phospho-modifications on both H3 and histone variant H3.3. Hyperglycaemia during IVM dramatically decreased the abundance of phosphorylation at H3S10P and H3S28P. Interestingly, hyper-phosphorylation at H3.3S31, a serine only present in the H3.3 variant, occurred following oocyte maturation under diabetic conditions. We also assessed alterations in histone landscape in the early embryo (pronuclear and two-cell) and identified changes in a number of key histone modifications, as well as the localisation of a number of glycosylated proteins in the nucleus. This data represents the first evidence that maternal hyperglycaemia alters the histone landscape of the early embryo and identifies a novel, direct mechanism between maternal hyperglycaemia and intergenerational transmission of disease.

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P020

Involvement of GLP in the development of mouse pre-implantation embryos *in vitro*

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Introduction
G9A-like protein (GLP) plays an important role in early mouse embryonic development as GLP-deficient embryos display severe growth retardation and defects, leading to lethality around embryonic day 9.5. In this study, we investigated the early role of *GLP* in controlling cell fate decisions by employing siRNA, which depleted both maternal and embryonic *GLP* mRNA during the mouse pre-implantation embryo. Our study demonstrates that *GLP* deficiency in the early mouse embryo led to long-term adverse effects on embryonic growth and development, including increased cell apoptosis. Furthermore, immuno-fluorescence staining revealed alterations in epigenetic modification due to *GLP* deficiency.
Materials and methods
IVF, mouse embryos, *GLP* siRNA, microinjection, immuofluoresence staining, qRT-PCR, western blotting, and TUNEL assay.
Results
In this study, we investigate the effect of microinjecting *GLP*-specific siRNA into mouse zygotes on *in vitro* embryonic development. Our results showed that *GLP* knockdown induced abnormal embryonic development and reduced blastocyst formation. Expression of the pluripotent markers *Oct4, Sox2,* and *Nanog* were also decreased significantly in *GLP*-deficient embryos. However, the apoptotic index and the expression of two pro-apoptotic genes, namely *Caspase 3* and *Caspase 9*, were increased in *GLP*-deficient embryos. Moreover, the methylation levels of H3K27me2 and H3K9me2 were decreased in *GLP* knockdown embryos. In conclusion, these results suggest that *GLP* deficiency leads to abnormal blastocyst development and apoptosis due to changes in histone modification that may affect genes expression.

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P021

Epigenetic programming in the male germline: novel mechanisms for paternal inheritance?

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Disruptions during epigenetic reprogramming in developing germ cells may result in the introduction of germline epimutations. These epimutations may cause aberrant gene expression in the developing germline and in the parent's offspring. This project investigates the impacts of functionally depleting the essential epigenetic modifier polycomb repressive complex 2 (PRC2) on germline formation and development in the next generation. To achieve this we are examining a mouse model with a point mutation in the *Eed* gene, an indispensible component of PRC2. Preliminary experiments have revealed stochastic variation in somatic and germ cell phenotypes. Fertility tests show that some surviving homozygous mutant males are subfertile and exhibit germ cell loss in the adult testis, which may be due to compromised testis and/or germ cell development. Fetal germ cells display variagation of transgene silencing, indicating that transcriptional control in *Eed* mutant germ cells is compromised. Moreover, we observed paternal affects on transcriptional control in offspring from *Eed* mutant fathers. Using whole genome transcriptional analyses we identified over 2000 genes that are differentially expressed in E8.5 day embryos fathered by *Eed* homozygous males compared to heterozygous males. These expression differences are likely to be due to aberrant epigenetic patterning in the sperm of the father. These data provide the first evidence that PRC2 regulates transmission of epigenetic effects from the father to his offspring. Greater understanding of epigenetic mechanisms in the developing germ cells is critical for determining how epigenetic defects in the germline influence the inheritance of complex diseases in a parent's children.

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P024

Identification of the gene for germline sex determination in medaka

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Introduction
Sex determination is an essential process for the production of dimorphic gametes, sperms and eggs. The sex determination genes in gonadal somatic cells have been well studied, but nothing is known about the mechanism of germline sex determination in vertebrates. We have tackled a totally unaddressed issue using medaka.
Materials and methods
Using transgenic medaka in which germ cells were labeled with EGFP, three different types of germ cells, stem-type, cystic, and meiotic germ cells, were isolated by flowcytometry, followed by RNA-Seq analysis. The expression of candidate genes was screened by *in situ* hybridization, and the functional analysis was performed.
Results and discussion
We identified an oocyte–sperm switch gene, which was initially expressed in germ cells of indifferent gonad in both XX and XY embryos but totally disappeared during male development. The loss-of-function of homozygous XX mutants produced sperms instead of oocytes during the sex differentiation. These results suggest that germline sex determination occurs by repressing the spermatogenesis. This is the first report of the mechanism of germline sex determination in vertebrates and provides an important insight into the sex determination processes between germ cells and somatic cells.

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P025

The role of maternal low protein diet on neural stem cells and neurogenesis in the offspring brain?

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Globally, malnutrition is the single greatest threat to public health. Maternal malnutrition during pregnancy is detrimental to foetal development and increases the risk of many chronic diseases in later life. Neurological consequences include increased risk of schizophrenia and abnormal anxiety-related behaviour. Previous studies have shown that maternal protein restriction has a negative effect on foetal brain development *in vivo* and we previously showed an effect on neural stems cells (NSCs) *in vivo*. With this in mind we investigated if a maternal low protein diet affects NSC number and proliferation in the foetal brain and if any impairment to neurogenesis will persist in the adult brain. Female mice were fed different diets from conception: normal protein diet (NPD), low protein diet (LPD), or embryonic LPD (Emb-LPD: LPD for 3.5 days, NPD thereafter). Offspring were maintained on standard chow after weaning at 3 weeks. Immunostaining of E14.5 brain sections showed a decrease in NSCs (Sox2 positive) in both LPD and Emb-LPD compared with NPD. Furthermore, decreased cellular proliferation (Ki67-positive cells) was demonstrated in both LPD and Emb-LPD, compared with NPD. Adult brains (6 months) were analysed by western-blot. Trends for a decrease in β3-tubulin (early neuronal marker) and an increase in NeuN (mature neuronal marker) were revealed in the cortex of LPD, compared with NPD. Taken together our data suggests that neurogenesis is profoundly altered in the embryonic brain following maternal protein restriction and this may result in permanent neuronal architectural changes in the adult brain.

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Pregnancy

P026

Kisspeptin signalling is required to maintain progesterone levels during mouse pregnancy

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Kisspeptin neuropeptides stimulate release of GnRH to maintain mammalian fertility. Kisspeptins are encoded by the *Kiss1* gene and directly stimulate GnRH neurons via the G-protein coupled receptor54 (GPR54). Transgenic mice with inactivating mutations of *Kiss1* or *Gpr54* are sterile and have underdeveloped gonads (hypogonadism) and low GnRH levels caused by a failure to secrete GnRH. The aim of this study was to determine whether the sterility of mutant female mice was solely caused by absence of central kisspeptin signalling or whether there are additional defects in the ovaries. To study this, we used *Kiss1* mutant female mice and applied a hormone replacement regime to mature the reproductive axis and induce ovulation. These mutant mice were bred with fertile males and evaluated for maintenance of pregnancy. Although, the mutant mice could ovulate and fertilized eggs develop into blastocysts and implant into the uterus, none of the mutant female mice maintained pregnancy past E6.5. It was found that the mutant mice had significantly lower levels of progesterone at E10.5 compared to WT pregnant mice. Ovary transplantation studies showed that mutant ovaries implanted into WT mice could support pregnancy to term indicating that there was no intrinsic defect in the mutant ovaries. Progesterone replacement allowed pregnancy to proceed normally up to E10.5. We hypothesise that the failure of the mutant mice to maintain progesterone levels during pregnancy is caused by inadequate luteotrophic stimulation of the corpus luteum due to central kisspeptin signalling defects at the level of the GnRH neuron. This work was funded by a BBSRC Case Award (BB/FO1936X/1).

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P272

The role of placental growth factor in regulating fetal brain vascular development

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Introduction
Maternal plasma in many pre-eclamptic (PE) pregnancies is low in the placenta product "Placental growth factor" (PGF). Offspring of PE compared to uncomplicated pregnancies show higher blood pressure, cognitive impairment and stroke; mechanisms explaining these differences are undefined. In $Pgf^{-/-}$ mice, decidual vessels have limited branching and connectivity. We asked if PGF deficiency diminishes brain vascular development, impairs cognition and elevates stroke risk.
Materials and Methods
$Pgf^{-/-}$ and $Pgf+^{/}+$ brain vasculatures were compared by resin casts (adults) or whole-mount immunohistochemistry (gestation day (GD) 10.5 fetal hindbrains). In adults, cognitive functions were compared by the tail-suspension test (TST) and Y-maze alternation test (YMAT) and 30 min left common carotid artery occlusion was used to compare stroke susceptibility. Brains were recovered, stained with 2,3,5-triphenyltetrazolium chloride (TTC) to identify ischemic regions or processed for RNA collection and qRT-PCR analyses.
Results and Discussion
Adult $Pgf^{-/-}$ brain arteries were less connected than in controls often showing an incomplete Circle of Willis. Fetal $Pgf^{-/-}$ hindbrain vessels were less mature and more disorganized than in controls. $Pgf^{-/-}$ were twice as depressed (TST) and had 50% less spatial learning ability (YMAT) than $Pgf+^{/}+$. Large brain infarcts were detected in all $Pgf^{-/-}$ mice after occlusion while controls lacked infarcts. Thus, PGF is essential for normal fetal cerebral vasculogenesis and normal adult brain perfusion and cognitive functioning. In PE, PGF may be deficient in the fetal as well as placental compartment leading to disturbed brain vascular development that elevates stroke risk and impairs cognitive functioning.
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P028

Targeting placental trophoblast leukemia inhibitory factor with a unique inhibitor: a novel treatment strategy for ectopic pregnancy

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Introduction
Ectopic pregnancy is unique to humans and a leading cause of maternal morbidity/mortality however the etiology remains unknown. Leukemia inhibitory factor (LIF) has roles in extravillous-trophoblast adhesion/invasion and is expressed in ectopic pregnancy. We hypothesised that LIF facilitates blastocyst adhesion/invasion in the fallopian tube (FT), contributing to ectopic pregnancy. LIF blockade could serve as a treatment strategy.
Methods
We used an oviduct epithelial cell line-OE-E6/E7 and HTR-8/SVneo cell-line (trophoblast-derived) spheroid co-culture to model blastocyst attachment to the FT. LIF signaling was determined by western blot. The effect of LIF/LIF inhibition (using a unique PEGylated-LIF-antagonist (PEGLA)) on first-trimester placental outgrowth was determined. To demonstrate the effect of LIF blockade to reverse trophoblast invasion *in vivo*, pregnant mice were administered with PEGLA (500 μg×2/day PEGLA/PEG) on gestation days (D)8–10 or 10–13. Implantation sites at D10/13 were stained with cytokeratin (trophoblast), isolectin-B4 and α-SMA (vascular).
Results and discussion
LIF receptor (R) was immunolocalised to villous and extravillous trophoblast and FT epithelium in ectopic pregnancy. LIF activated STAT3 but not ERK in OE-E6/E7 and stimulated HTR-8/SVneo-spheroid adhesion to OE-E6/E7 which was blocked by PEGLA. LIF promoted placental-explant outgrowth, which was blocked by PEGLA. In mice, PEGLA blocked LIF action, reduced decidua (D10), and reduced placental spongiotrophoblast/labyrinth and vascular cells (D13). Our data suggests LIF facilitates the development of ectopic pregnancy by stimulating blastocyst adhesion and trophoblast outgrowth in the FT.

Conclusion
Ectopic pregnancy is usually diagnosed after six weeks gestation; pharmacologically targeting LIF-mediated trophoblast-outgrowth may be useful as a novel treatment for ectopic pregnancy.

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P029

IGF1 increases blastocyst attachment to endometrial epithelial cells *in vitro* and regulates fibronectin expression

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Introduction
Implantation is a highly coordinated event in which the receptive endometrium is primed to receive adhesion-competent blastocysts. Implantation relies on the bridging of integrins on the endometrium and the blastocyst by extracellular matrix proteins, such as fibronectin. Factors produced in the female reproductive tract are thought to support development of the blastocyst to an adhesion competent state and to ensure coordination of blastocyst implantation ability with uterine receptivity.
Materials and methods
Murine blastocysts were cultured on Ishikawa cells, a human endometrial cell line, in the presence or absence of IGF1 and assessed for attachment 48 h later. Western-blotting, immunofluorescence, and flow cytometry were used to determine levels of expression of fibronectin, integrins, and focal adhesion kinase (FAK) in Ishikawa cells and blastocysts.
Results and discussion
Attachment of blastocysts to Ishikawa cells *in vitro* was increased in the presence of IGF1. Treatment of blastocysts with IGF1 increased apical expression of fibronectin. Apical expression of integrin $\alpha v\beta 3$, $\beta 3$, and $\beta 1$ in Ishikawa cells was unaltered by IGF1 treatment. However, IGF1 treatment increased phosphorylation of FAK and total FAK expression in Ishikawa cells. FAK signalling is known to be linked to integrin activation and this may affect the integrins' ability to bind and recognise ECM proteins such as fibronectin. This study shows the importance of IGF1 in implantation. Failure of the embryo to implant successfully is the major cause of IVF failure. Understanding how the embryo implants successfully and how this is influenced by growth factors is essential to improve IVF treatment.

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P030

Maternal metabolic syndrome, induced by increased fructose consumption, is associated with subfertility and impaired fetal growth in mice

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Introduction
The CDC reports that roughly one-third of USA adults suffer from metabolic syndrome. Amongst other health complications, metabolic syndrome is associated with subfertility and complications later in pregnancy. Successful implantation and pregnancy require the priming of both embryo and uterus during a short window of time called the 'window of implantation'. At this time, hormones stimulate uterine stromal fibroblasts to differentiate into specialized secreting cells in a process termed endometrial decidualization.
Materials and methods
Here, we hypothesized that metabolic syndrome, induced by 6 weeks of high fructose (HFrD) feeding in mice, would impair endometrial decidualization leading to subfertility and altered fetal growth. To study endometrial decidualization, induced deciduomas were used. Additionally, embryos were cultured in 4 mM D-fructose or L-glucose (two-cell to blastocyst) and transferred into normoglycemic dams (CON) to examine possible effects of HFrD on embryonic competence.
Results and discussion
HFrD feeding induced metabolic syndrome, characterized by decreased glucose tolerance, hepatic steatosis, and elevated circulating lipids. Litter size was decreased by 37% and birth weights were significantly reduced in HFrD mice compared with CON. Induced deciduoma weights were 50% smaller in HFrD mice. Conversely, implantation rates were similar between D-fructose (36%) and L-glucose exposed embryos (30%), suggesting that reduced litter size in HFrD mice may be a consequence of impaired uterine receptivity as opposed to altered embryonic competence. However, at E14.5 crown-rump lengths of D-fructose exposed embryos were significantly shorter and their placentas were smaller compared to L-glucose embryos, suggesting that embryonic exposure to HFrD may alter fetal–placental development.

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Poster Presentations

P031

Mitochondrial dynamics controlled by mitofusins direct organelle positioning and movement during meiotic division

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Introduction
Mitochondria are abundant in fully-grown mammalian oocytes with a unique spherical morphology, but the manner in which the behavior of mitochondria is controlled is not well understood. We explored the spatiotemporal control of mitochondrial morphology by mitochondrial fusion and fission mechanisms and the functional impact of that control on meiotic division, using mouse oocytes.
Materials and methods
Immature oocytes were collected from the ovaries of CD1 female mice. Oocytes were microinjected with mRNAs encoding mitochondrial fusion and fission proteins, i.e. Mfn1, Mfn2, Opa1, and Drp1. Oocytes were matured *in vitro* for 16 h. Mitochondria were labeled with green fluorescence protein and the subcellular distribution of mitochondria during meiotic division was examined using confocal microscopy. To investigate the colocalization of mitochondria with endoplasmic reticulum (ER) or chromosomes, mCherry-tagged ER and histone H2B were expressed in oocytes respectively.
Results and discussion
Overexpression of Mfn1 or Mfn2 causes marked mitochondrial aggregation, particularly in the perinuclear region during meiotic progression. This aggregation occurs because of the contact between mitochondrial outer membranes, but not of the interconnected tubules. Tracking of mitochondria with chromosomes or ER throughout the first meiotic division demonstrates that Mfn-promoted mitochondrial aggregation disturbs the spatiotemporal dynamic of the ER and chromosomes. Our findings suggest that organelle dynamics are coordinately controlled during meiotic division, and an imbalance of mitochondrial fusion/fission leads to disorganization of the organelle compartments.

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P032

The detailed localization of meiotic cohesin subunits, RAD21L and REC8, in mouse spermatocytes

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Introduction
In meiosis, homologous chromosomes pair, synapse, and recombine with their partners in parallel with the formation of the synaptonemal complex, a tripartite structure with two axial elements connected by transverse filaments. It has been demonstrated that two meiosis-specific cohesin subunits, RAD21L and REC8 are essential for the formation of the axial elements and homologous chromosome recombination. However, it is unknown how they are involved in synapsis and crossover recombination between homologous chromosomes. To get a clue about individual functions of different types of mieotic cohesins, in the present study, we tried to determine the detailed localization of RAD21L and REC8 on the synaptonemal complex.
Materials and methods
Mouse spermatocytes were fixed and immunofluorescently labeled with the antibodies against cohesin subunits (RAD21L and REC8), the synaptonemal complex proteins (SYCP3 and SYCP1), and a component of recombination intermediates (MSH4). The signals were observed by three-dimensional structured illumination microscopy (3D-SIM).
Results and discussion
At pachytene stage, two well-separated axial elements labeled with anti-SYCP3 antibody were observed by 3D-SIM. Numerous dot-like signals of RAD21L and REC8 were detected inside the synaptic axial elements rather than right along the axial elements. Some sinals of RAD21L, but not REC8, were seen as if they formed a bridge between axial elements. Furthermore, the ratio of overlapping areas between RAD21L and MSH4 signals was greater than the one between REC8 and MSH4. From these results, we propose a hypothesis that RAD21L might coordinate synapsis and recombination between homologous chromosomes by connecting non-sister chromatids from homologous chromosomes.

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P033

Atrazine exposure of adult mice affect spermatogenesis

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Declining human fertility has become a serious public health problem in many countries around the world. There is a rapidly growing number of evidence that human reproductive health is negatively affected by various environmental factors including life style and exposure to chemical compounds such as pesticides, phthalates, and bisphenol A (BPA). The aim of our study is to understand how commonly used pesticides can affect meiosis, the central event of mammalian gametogenesis. We hypothesize that pesticides change DNA histone modifications and gene expression that finally lead to a decrease of fertility. To test this idea we are using the mouse as a model organism. We treated 4–5 weeks old adult males with the different doses of atrazine during different time intervals. Morphology analysis of the treated mouse showed a reduction of the spermatozoa number in epididymis compared to the control. In some severe cases we found abnormal seminiferous tubules upon atrazine treatment. By using Affymetrix microarray approach we found around 50 genes are differentially expressed including some histone modifying enzymes, transcription factors and regulatory proteins. Immunostaining of tubule sections and chromosomal spreads revealed increased histone gamma H2AX staining which may reflect DNA damage sites or double stranded breaks persistence. Our preliminary data showed that atrazine affect meiosis and have a possible impact on spermatogenesis.

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P034

C-type natriuretic peptide stimulates resumption of meiosis via a cGMP-dependant mechanism in porcine oocytes

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Research conducted in recent years has led to great advances in our understanding of the participation of cGMP in meiosis. It is clear that increased intra-oocyte concentrations of cGMP inhibit meiosis in mouse models. Like cAMP, cGMP may also have a meiotic stimulatory function, possibly via cGMP/PKG. Abattoir derived gilt porcine ovaries were collected, antral follicles aspirated and oocytes collected and cultured in TCM-199 + 3 mg/ml BSA. Natriuretic peptide receptor 2 (*NPR2)* mRNA expression was two-fold higher in cumulus cells compared to granulosa cells. After 24 h of culture CNP increased the proportion of oocytes that resumed meiosis compared to control (main effect). Both 100 nM CNP and 1 μM 8pCPTcGMP (cGMP analogue) significantly reduced oocyte–cumulus gap junction communication (GJC), as measured by dye transfer, from 12 h by 1.7- and 1.9-fold respectively, compared to control ($P < 0.05$). CNP or 8pCPTcGMP significantly increased ERK1/2 phosphorylation (western blot), 2.6- and 3.1-fold respectively, above control ($P < 0.05$). This increase was eliminated by 10 μM AG1478 a selective EGF receptor inhibitor. Neither CNP nor 8pCPTcGMP had any effect on phosphorylation of cAMP-response binding protein (CREB) nor did they effect mRNA expression of EGF-like peptides at 2 h culture, compared to control. These results demonstrate that CNP stimulates the phosphorylation of ERK1/2, possibly via a cGMP-dependant mechanism requiring EGFR signalling in porcine COCs. This subsequently leads to a decrease in oocyte–cumulus GJC and the resumption of meiosis.

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P035

Zinc regulate meiotic resumption and metaphase arrest in porcine oocyte

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Introduction
Zinc is an extremely important trace element that play important roles in several biological processes. In this study, we investigated the role of zinc during meiotic resumption and metaphase arrest in *in vitro*-matured porcine oocytes.

Materials and methods
Oocytes which arrest at GV or MII stage were treated with TPEN, a Zn^{2+} chelator, respectively. Meiotic resumption and activation were assayed. Effect of PMA, a PKC activator, on GVBD blocking and oocytes activation results from TPEN treatment were checked.
Results and discussion
Depletion of zinc with TPEN blocked meiotic resumption and results in failure of metaphase II arrest. The $p34^{cdc2}$ activity in both MII oocytes and GVBD oocytes which treated with TPEN was decreased. Phosphorylated MAPK also be decreased in GVBD stage after TPEN treatment which might be explained by the low expressions of *C-mos*, *Cyclin B1*, and *Cdc2* in GVBD stage. But treated the oocytes with PKC agonist PMA rescued the meiotic resumption and increased MAPK and increases $p34^{cdc2}$ activity. Treatment oocytes with PMA in GV stage also increased the zinc content in cytoplasm, showed that zinc regulate meiotic resumption is a PKC-dependent event. However, although TPEN treatment reduced phosphorylation of PKC substrates in both meiotic resumption and MII stage, rescue the PKC substrates phosphorylation with PMA didn't prevent the activation of oocytes caused by zinc depletion. These data demonstrate zinc regulate meiotic resumption via a PKC dependent pathway, but independent of that in maintain metaphase arrest in porcine oocytes.

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P036

Morphological markers to select populations of oocytes with different cultural needs for dedicated pre-maturation systems

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Introduction
Several studies support the notion that *in vitro* pre-maturation treatments aimed to improve the developmental capability of immature oocytes have a different effect depending on the oocyte metabolic status at the time of its removal from the follicle. We demonstrated that changes in large-scale chromatin configuration within the germinal vesicle (GV) of fully-grown bovine oocytes are related to the acquisition of developmental competence. In particular, only a limited percentage of GV1 oocytes reached the blastocyst stage, while GV2 and GV3 oocytes showed a higher embryonic developmental potential. The present study aimed at identifying possible correlations between cumulus–oocyte complex (COC) morphology and chromatin configurations.
Methods
COCs were collected from 2 to 6 mm follicles, selected according to commonly accepted criteria for *in vitro* embryo production and those with evident signs of atresia were discarded. Selected COCs were further divided into three groups based on the texture of the ooplasm (homogenous or granulated) and on the morphology of the cumulus oophorus (layers number, degree of expansion of outer layer). After cumulus cell removal, chromatin configuration was assessed by DAPI staining.
Results and discussion
Our study indicate that GV1 is present only in group 1 (homogenous ooplasm and absence of outer layer expansion), representing one third of group 1 total COCs, while groups 2 and 3 are similarly enriched of GV2 and GV3. This can providea non-invasive approach to select populations of oocytes with different cultural needs to be subjected to dedicated *in vitro* development protocols. This hypothesis is currently under investigation.

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P037

Abstract withdrawn.

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P038

The gametic synapse; transferring RNA to the oocyte

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Introduction
Oocytes must effectively grow, accumulate reserves, and mature prior to fertilization. Errors in any of these processes affecting nuclear, cytoplasmic, or molecular maturation of the oocyte will result in poor embryonic development.
Materials and methods
Immunofluorescence, direct fluorescent staining, and autoradiography were combined with confocal, epifluorescent, and electron microscopy to study the transzonal projections (TZPs) between the cumulus cells and the oocyte. Total RNA and *de novo* synthesized transcripts were detected in the TZPs by selective staining. Long transcripts present in the TZPs were identified by RNA-Seq. Functional analyses of potential RNA transfer from the cumulus cells to the gamete was done by RT-qPCR, polyribosomal extraction and exposure to diverse inhibitors.
Results
We have demonstrated that long poly(A) bearing RNA molecules are shuttled to the oocyte by the cumulus cells down their TZPs during *in vitro* maturation. Vesicles existing between the TZP and the oolemma present a means of large molecule transfer capable of carrying RNA to the oocyte. Inhibition of vesicle function and RNA synthesis negatively impacted oocyte maturation. We also observed a time dependant increase in TZPs RNA content post-slaughter suggesting induction from a change in follicular status. Maturing oocytes collected pre-induction experienced significantly diminished maturation rates.
Discussion
We present a new perspective of how the cumulus cells influence the acquisition of the oocyte's maturation potential by delivering exogenous RNA. Hormonal control of the follicle including the responsive cumulus cells will relay transcriptional support and directly influence the quality of the oocyte.

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P039

Dissecting the meiotic gene network in female embryonic germ cells

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Context
In mammalian female germ cells, meiotic entry occurs during fetal life. The current model places retinoic acid (RA) as a key signal inducing meiotic initiation by activating the expression of stimulated by retinoic acid 8 (*Stra8*), a necessary factor for pre-meiotic chromosomal replication. However, the requirement of RA for meiotic initiation has been recently questioned in the female germ cells.
Material and method
To clarify whether RA receptors regulate *Stra8* expression in murine fetal ovaries, we performed treatments with RAR-antagonists in organotypic cultures and analyzed the expression of RA target genes and meiotic genes. We performed a fine study of meiotic genes expression around the time of meiosis initiation in fetal gonads. Meiosis inducer candidate genes were studied using siRNA and plasmids in a cell line.
Results and discussion
Treatments with RAR-antagonists evidenced that *Stra8* expression may not be directly driven by endogenous RA in the fetal ovary. The precise study of meiotic gene expression revealed that meiotic genes are expressed in three distinct waves and that some are expressed prior to *Stra8*. Interestingly, we observed that other genes previously identified in transcriptome analyses as specific of female germ cells were expressed before *Stra8*. These may be considered as new meiosis inducing factors and their role was investigated using F9 cells that express many meiotic genes. Altogether our work challenges the place of *Stra8* as the only gatekeeper of the induction of the meiotic program. In conclusion, the gene network regulating female meiotic entry appears more complex than expected.

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P040

The oocyte influences cumulus expansion and glucose metabolism during IVM in cattle

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Oocyte–cumulus communication is essential for COC metabolism and oocyte developmental competence. Although the oocyte appears not to be absolutely required for cumulus expansion in cattle, it is not clear whether it influences this process. We tested the effects of oocytectomy on expansion and glucose metabolism of bovine COCs submitted to *in vitro* maturation (IVM). Follicles 3–8 mm were aspirated from abattoir ovaries and grades 1 and 2 COCs were selected. Intact COCs, oocytectomized COCs (OOXs), and OOXs added to denuded oocytes (OOXs + DOs; 1 DO/μl) were matured in groups of 20 (four replicates). IVM was performed at 38.5 °C; 5.5% CO_2 for 22 h in 96-well plates with 100 μl of TCM-199 supplemented with piruvate (22 μg/ml), amicacin (75 μg/ml), FSH (1 μg/ml), and BSA (4 mg/ml). After IVM, expansion degree was visually assessed (grades 1–3) and lactate and glucose concentrations were measured in the medium by dry chemistry. Oocytectomy did not prevent expansion but led to a lower percentage of structures with maximum expansion (grade 3; OOX: 66.25% vs COC: 81.6%). The addition of DOs stimulated expansion in OOXs, leading to values equivalent to those observed in intact COCs (83.75% fully expanded). Oocytectomy decreased glucose uptake (909.1, 669.2, and 732.3 pmol/COC/H for COCs, OOxs, and OOXs + DOS respectively) and lactate production (2210.2, 1409.1, and 1619.3 pmol/COC/H for COCs, OOXs, and OOXs + DOS respectively). Addition of DOs did not alter glucose uptake and lactate production of OOXs. These data suggest that the oocyte regulates expansion and glucose metabolism in bovine cumulus cells submitted to IVM. While the effects on expansion appear to be mediated by oocyte secreted factors, the regulation of cumulus metabolism appears to require direct oocyte–cumulus contact.

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P041

Plk1 plays a role in cortical actin polymerisation during meiosis I

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Female meiosis involves a highly asymmetrical division to form a large secondary oocyte and a small polar body. Polo-like kinase I (Plk1) is a serine/threonine kinase which is highly conserved from yeast to human and is a potent regulator of mitosis including cytokinesis. Plk1 is known to regulate myosin via the activation of RhoA which leads to the contraction of the cleavage furrow in mitotic cells. Indirect evidence has also shown that Plk1 may regulate Cdc42 activity through CLIP-170 phosphorylation.

To investigate the role of Plk1 during the first meiotic division oocytes were microinjected with three probes to visualise DNA, microtubules, and actin. Oocytes were then treated with 5 μM BI2536 at 9.5 h post release and imaged throughout meiosis I using 4D confocal microscopy or fixed and stained at different time points after treatment.

Live cell imaging revealed that oocytes were arrested at telophase I with no polar body extrusion. We also observed that prior to anaphase onset cortical actin over the region of the meiotic spindle was significantly reduced compared to control. However, there was no significant difference in the distance between chromatin and the cortex. It was also observed that N-WASP was unable to localise properly on the cortex when Plk1 was inhibited. As N-WASP has been found to be involved with actin cap formation through its interaction with Cdc42, PLK1 may have a role in Cdc42-mediated cortical actin polymerisation that complements its role in myosin activation and thereby provides a mechanism for co-ordinating events of cytokinesis.

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P042

Cyclin A2 is essential for the chromosome segregation during the meiosis I of the mouse oocyte maturation

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Cyclin A, the first cyclin ever cloned, is thought to be an essential component of the cell-cycle engine. Mammalian cells encode two A-type cyclins, a testis-specific cyclin A1 and a ubiquitously expressed cyclin A2. Cyclin A2 is an essential mitotic CDK regulatory partner and is attributed with a wide range of effects early in the G2–M transition. Although its role in mitosis has been extensively investigated, research into the role of cyclin A2 in meiosis is lacking. In order to investigate the functions of cyclin A2 in meiosis we have developed two models: the cyclin A2 overexpression model with GFP-tagged cyclin A2 protein microinjection and conditional knockout model with created *cyclin A2$^{f/f}$; Zp3 Cre* mouse. With the GFP-tagged cyclin A2 protein microinjection in the GV stage mouse oocytes, and together with live cell imaging indicate the subcellular localization of cyclin A2. The conditional knockout mice were created by crossing cyclin A2 floxed mice with the Zp3 Cre mice. With the microinjection of the cyclin A2–GFP protein, we found that the overexpression of cyclin A2 could delay the MI–AI stage transition. The fertility experiment and superovulation experiment with the *cyclin A2$^{f/f}$; Zp3 Cre* mice indicated that the depletion of cyclin A2 could cause the aneuploidy during the first polar body extrusion and finally reduce the infertility of the mice. In conclusion, we have found that cyclin A2 is necessary to create a cellular environment that ensures normal oocyte growth and the faithful segregation of the chromosomes.

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P043

Effect of cAMP regulators on bovine cumulus–oocyte communication and embryo development *in vitro*

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Introduction

Preventing spontaneous maturation *in vitro* by adding cAMP regulators is thought to maintain the communication between oocyte and *cumulus* cells via gap junction communication (GJC), hence promoting developmental competence. We aimed to assess the integrity of gap junction maintenance in cumulus oocyte complexes (COCs) after pre-maturation with IBMX and Forskolin (FSK) and the influence on bovine embryo development.

Materials and methods

Immature COCs were cultured in VitroMat (IVF Vet Solutions) + 4 mg/ml FAF–BSA and divided into the following treatments groups: i) IBMX + FSK: pre-treatment with IBMX (500 μM; Sigma–Aldrich) and FSK(100 μM; Sigma–Aldrich) for 2 h (pre-IVM) following 22 h maturation with rhFSH (0.1 IU/ml) and ii) spontaneous IVM: rhFSH (0.1 IU/ml) for 24 h. Oocytes were inseminated and zygotes were cultured for 5 days in VitroCleave (IVF Vet Solutions) and transferred into VitroBlast (IVF Vet Solutions), untilblastocystassessment (days 7and 8). Lucifer yellow (3% in 5 mM lithium chloride) dye was microinjected into the ooplasm to assess GJC.COC GJC was scored as open (+2), partially open (+1), and closed (0) communication.

Results and Discussion

Embryo development did not differ between groups probably due to the short period of maturation after pre-IVM (22 h). Functional GJC were 1.6-fold increased in IBMX + FSK group. In summary, pre-treatment with IBMX and FSK improved communication between oocyte and cumulus, but 22 h of maturation seems insufficient to improve embryo development.

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P044

Insight into progesterone receptor membrane component 1 action during bovine oocyte meiosis by means of siRNA-mediated gene silencing

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Introduction
Previous studies suggest that progesterone receptor membrane component 1 (PGRMC1) plays an essential role during bovine oocyte meiosis, since it i) localizes to the centromeres at metaphases I and II and ii) concentrates between the separating chromosomes at ana/telophase I. Moreover, injection of an antibody to PGRMC1 significantly impairs completion of meiosis. The aim of the present study is to expand these findings by using siRNA (RNAi)-mediated gene silencing.
Methods
Cumulus–oocytes complexs were microinjected to deliver PGRMC1 or CTRL-RNAi into the oocytes cytoplasm, kept in meiotic arrest for 18 h with 10 μM cilostamide and then *in vitro*-matured (IVM) for 24 h. After IVM, efficacy in depleting PGRMC1 expression was assessed by quantitative RT-PCR and western blotting. Finally, the oocyte capability to extrude the first polar body (PBI) and the morphology of the MII plates were assessed.
Results and discussion
PGRMC1 expression following PGRMC1-RNAi treatment was significantly reduced by 30%. This was accompanied by a 22% reduction of the oocytes that extruded the PBI ($P<0.05$). Surprisingly, PGRMC1-RNAi treatment did not affect MII plate formation or morphology. However, a significantly higher proportion of PGRMC1-RNAi injected oocytes possessed clumps of DNA scattered throughout the ooplasm in addition to the MII plate ($P<0.05$). This is consistent with PGRMC1 localization at the midbody and with a putative role in cytokinesis. We hypothesize that lower PGRMC1 expression impairs the process of PBI formation. As a consequence, DNA that should be extruded with the PBI is retained in the cytoplasm and degraded.
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P045

Maturation conditions do not affect *Myst1*, *Hat1*, and *Sirt1* mRNA abundance in horse oocytes

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Introduction
We recently demonstrated that *in vitro* maturation (IVM) is associated with defects in histone H4 lysine 16 (H4K16) acetylation in horse oocytes, together with a higher incidence of spindle anomalies and aneuploidy. In the present study we investigated whether maturation conditions can alter the abundance of transcripts involved in histone acetylation and deacetylation. The specific H4K16 acetyl-transferase *Myst1*, the general acetyl-transferase *Hat1* and the specific H4K16 deacetylase *Sirt1* were examined in GV-stage oocytes and after IVM.
Methods
Follicle growth was followed by daily ultrasound scanning in adult mares. When a follicle >33 mm emerged, hCG was injected. After 35 h the IVM oocyte was collected by ultrasound-guided transvaginal aspiration. For IVM, oocytes were collected from follicles 5 to 25 mm and cultured for 28 h with EGF and serum. GV-stage oocytes and follicular cell samples were also collected from follicles 5 to 25 mm. Samples were retro-transcribed using random hexamers and analyzed by real-time Q-PCR. *Gapdh* and *Renilla* Luciferase served as internal and external housekeeping respectively.
Results and discussion
Our study reports for the first time the expression of *Hat1*, *Myst1*, and *Sirt1* in horse oocytes and follicular cells. *Hat1*, *Myst1*, and *Sirt1* were expressed at the same extent in GV-stage oocytes and after maturation, independently from the maturation conditions. Our findings show that the transcript abundance of *Hat1*, *Myst1*, and *Sirt1* does not decrease during horse oocyte maturation, strongly suggesting that IVM does not promote/accelerate the degradation of transcripts, but rather affects H4K16 acetylation machinery at translational or post-translational level (L'Oreal 2012).
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P046

Inhibition of mTOR signaling induces cumulus expansion and stimulates meiotic maturation of oocytes in mice

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The mammalian target of rapamycin (mTOR) signaling pathway functions as a central regulator of cell growth, proliferation, and survival. We previously reported that during meiotic maturation, the expression levels of mTOR in oocytes remain similar from the germinal vesicle (GV) stage to metaphase II (MII). To investigate the role played by mTOR during meiotic resumption, we cultured murine cumulus – oocyte complexes (COCs) in the presence of mTOR inhibitors. mTOR expression was detected in the cumulus cells. The COCs were cultured for 18 h in a medium containing dbcAMP with or without the mTOR inhibitor PI-103 or rapamycin. We observed cumulus expansion but the oocytes were arrested at the GV stage. These oocytes were then transferred to fresh maturation medium containing FSH with or without an mTOR inhibitor before culturing for 8 more hours. We found premature development of the first polar body in oocytes treated with the mTOR inhibitor. This result suggests that mTOR inhibition induces early progression of oocytes. Further, when GV-stage oocytes were cultured for 18 h in maturation medium lacking FSH but containing the mTOR inhibitor PI-103 or rapamycin, the cumulus cells expanded and the first polar body successfully developed. In addition, we found that the mRNA expression of hyaluronan synthase (HAS) in the cumulus cells increased after treatment with the mTOR inhibitor. In conclusion, our data suggest a role for mTOR signaling during cumulus expansion and meiotic maturation in mice. In the presence of an mTOR inhibitor, cumulus expansion occurred and meiotic maturation progressed without gonadotropin stimulation.
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P047

Inhibition of DMRTA2 impairs human female germline development in xeno-grafted ovaries

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Context
DMRTA2 belongs to a family of genes coding for proteins containing a DM-domain that are conserved among vertebrates and widely involved in gonadal differentiation. We recently identified *Dmrta2* gene expression through transcriptome analysis performed in murine female embryonic germ cells and retrieved its expression in human fetal ovaries. The role of *DMRTA2* is poorly documented and we thus conducted this study to clarify its implication in human fetal ovaries.
Material and methods
Human fetal ovaries (8 – 11 weeks post fertilization) were harvested from material available following legally induced abortions and were grafted in two immunodeficient NMRI:Nu/Nu mice. Recipient mice were treated with siRNA targeting specifically the sequence of human *DMRTA2*.
Results and discussion
The xenograft model did recapitulate all the stages of female germ cell development with no overt change when compared to ungrafted ovaries. After 10 days of treatment with siRNA, we observed of robust inhibition of *DMRTA2*. This inhibition did not alter germ cell density or apoptosis. RT-qPCR and immunostaining analyses indicated that the expression of markers of undifferentiated germ cells was unchanged while the expression of markers of differentiating, pre-meiotic and meiotic germ cells were systematically decreased. This study reveals for the first time the requirement of *DMRTA2* for the normal development of human female embryonic germ cells. *DMRTA2* appears required to allow the differentiation of oogonia shortly prior their entry into meiosis. Additionally, we set up an original model of xenograft that should prove useful for future investigations of the human germline development.
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P048

Abnormal behavior of lysine acetylation during one-cell stage mouse cloned embryos

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It has been proven that treatment of cloned embryos with a histone deacetylase inhibitor (HDACi) such as trichostatin A (TSA) following somatic-cell nuclear transfer (SCNT), significantly improves subsequent development to a blastocyst as well as full-term development (Kishigami *et al. BBRC* 2006). Recently, we have shown that lysine acetylation levels in both the nucleus and cytoplasm are significantly increased after oocyte activation (Matsubara *et al. BBRC* 2013) or with oocyte aging (Lee *et al. JRD* 2013), which are more enhanced by TSA treatment. To get insight into the mechanism underlying HDACi requirement for SCNT embryos, we looked into the dynamics of lysine acetylation during one-cell stage mouse cloned embryos. First, we observed that high acetylation levels in somatic cells disappeared within 3 h after SCNT without oocyte activation, suggesting high HDAC activities in oocytes erasing the lysine acetylation derived from somatic cells. Next we found lower accumulation of acetylation in SCNT nuclei than control embryos (parthenogenetically-activated oocytes), which is consistent with previous reports showing lower acetylation of histones in SCNT embryos. Further, to directly compare the acetylation levels of nuclei within the same oocyte cytoplasm, we injected somatic cells into oocytes without enucleating, revealing that somatic-cell derived nuclei showed significantly lower acetylation level than the female pronuclei. Thus, lysine-acetylation in SCNT embryos is dynamically changed as in fertilized embryos but distinct at the acetylation level, suggesting an intrinsic property of SCNT nuclei which require HDACi for further development.

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P049

Nucleoli are formed in developing mouse embryos without nucleolus precursor bodies

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Introduction
The large, compact oocyte nucleoli, sometimes referred to as nucleolus precursor bodies (NPBs), are essential for embryonic development in mammals. It has been convincingly documented that zygotes inherit the oocyte nucleolar material and form NPBs again in pronuclei. During early embryonic development, the compact zygote NPBs gradually transform into reticulated nucleoli of somatic cells type. Here we show that zygote NPBs are not required for embryonic and full-term development in the mouse.
Materials and methods
We microsurgically aspirated NPBs from both female and male pronuclei (enucleolation) at 10 h after ICSI. Zygotes from which a small amount of nucleoplasm was aspirated and removed were served as controls (sham-operated). After enucleolation, cleavage, and morulae/blastocysts formation rates were recorded at 24 h and 3.5 days after ICSI respectively. Moreover, after transfer of two-cell stage embryos, we examined full-term development of embryos originating from enucleolated zygotes. To examine the nucleolus formation in developing embryos originating from enucleolated zygotes, live-cell imaging, and immunostaining were performed.
Results and discussion
When NPBs were removed from late-stage zygotes by micromanipulation, the enucleolated zygotes developed to the blastocyst stage, and after transfer to recipients, live pups were obtained. We also presented *de novo* formation of nucleoli in developing embryos. After removal of NPBs from zygotes, they formed new nucleoli at after several divisions. These results indicate that zygote NPBs are not essential for embryonic development and not required for the formation of nucleoli in developing embryos.

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P050

Fingerprints on oocyte and embryo lipid profiles caused by *in vitro* embryo production system and fatty acid diet supplementation in bovine

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Introduction
Lipid content of oocytes and early embryos is critical for embryonic and fetal development. Indeed, the success of assisted reproductive techniques (ARTs) such as cryopreservation and *in vitro* embryo production is heavily affected by the lipid content of preimplantation embryos.
Materials and methods
Lipid profiling of single *in vitro* (IVTB) and *in vivo* (IVVB) produced bovine embryos was performed by desorption electrospray ionization mass spectrometry (DESI – MS). The relative abundance of transcripts related to oocyte development capacity (IGF1R and GJA1) and lipid metabolism (FASN, SCAP, SRBP1, and CPT1b) was assessed by RT-qPCR. Similar analyses were performed from oocytes collected from Holstein – Friesian Heifers after long-term supplementation with rumen-protected stearic conjugated linoleic (CLA) and stearic (SA) acid of the daily diet. Follicular fluid and blood samples were collected from supplemented animals for monitoring lipid profiles by gas chromatography (GC).
Results and discussion
Results showed effects of *in vitro* culture conditions on the lipid profile and gene expression of embryos when comparing to their *in vivo* counterpart. The rumen-protected fatty acid supplementation experiments showed modification of the lipid profile of oocytes collected from supplemented donors. Oocytes collected from CLA supplemented Heifers showed significant accumulation of triacylglycerols of unsaturated fatty while oocytes from the SA supplemented group accumulated higher amounts of palmitic acid and plasmalogen species. These results pave the way for improving embryo culture conditions and for identifying fertility impairments associated to the female nutrition. Results support the role of the bovine experimental model for improving ARTs technologies in humans and other mammals.

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P051

Degradation of estrogen receptor α in activated blastocysts is associated with implantation in the mouse

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Introduction
Implantation of a blastocyst into a receptive uterus involves a series of highly coordinated cellular and molecular events directed by ovarian estrogen and progesterone. In particular, estrogen is essential for on-time uterine receptivity and blastocyst activation in mice. Although estrogen receptor α (ERα) is expressed in blastocysts, its targeted disruption leaves embryonic development and implantation unaffected. Therefore, the role of ERα in implanting blastocysts remains unclear.
Materials and methods
Delayed implantation was induced and maintained on daily injections of P_4. Implantation-induced (activated) blastocysts were collected 23 h after the E_2 injections. Immunohistochemical analysis was performed to examine the protein expressions. Blastocysts were transferred into recipient mice on the morning of Day 4 of pseudopregnancy (Day 1 = vaginal plug).
Results and discussion
Expression of ERα was increased in activated blastocysts; however, this ERα expression in activated blastocysts decreased within 6-h culture. In contrast, breast cancer 1 (Brca1) was maintained in the blastocysts during the culture. The treatment of activated blastocysts with the proteasome inhibitor MG132 demonstrated that proteolysis is associated with down-regulation of ERα expression in activated blastocysts. Embryo transfer of MG132-treated activated blastocysts showed a decreased implantation rate, whereas combined treatment with MG132 and the ER antagonist, ICI 182 780, resulted in recovery of the rate of implantation. This study has revealed that down-regulation of ERα in activated

blastocyst is associated with completion of blastocyst implantation. Our results also suggest that selective protein turnover, such as that of ERα, occurs in activated blastocysts, while expression of other proteins, including Brca1, is maintained at the same stage.

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P052

Production of prion gene knockout cow to prevent spontaneous bovine spongiform encephalopathy

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Introduction
In 1986, typical borine spongiform encephalopathy (BSE) was found in UK. Cattle were orally given abnormal prion protein in the brain of sheep with Scrapie. Orally administered abnormal prion protein causes variant type Creutzfeldt-Jakob disease infection in human. In 2003, spontaneous BSE was found in many countries. Spontaneous BSE is transmissible from cattle to monkey. In 2012, United States Department of Agriculture reported that spontaneous BSE cow was found in California. To make prion gene homo knockout (KO) cattle is only solution against BSE problem.
Materials and methods
We made 11 prion gene homo KO Japanese Black cow and revealed her characteristics as follows: firstly, prion gene was knockouted in fibroblast cells prepared from embryo. The somatic cell nucleus of prion gene hetero KO fibroblast was transferred into oocyte with removal of nucleus (somatic cell nuclear cloning: SCNC). After activation and *in vitro* culture, the blastocysts were transplanted into the uterus. Secondary, prion gene was knockouted in the embryo fibroblast cells, and then the 2nd SCNC was performed. Prion gene homo KO calves were born.
Results and discussion
No obvious difference was observed between KO cows and controls (SCNC cows) at the 0, 3, 6, 12 or 18 months of age. In the brain tissue of KO cows, anti-oxidative function was decreased at 24 months of age. To produce the prion gene homo KO cattle is useful and the only way to ensure the safety of bovine derived products.

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P053

The effect of lysophosphatidic acid during *in vitro* maturation of bovine cumulus–oocyte complexes: cumulus expansion, glucose uptake and expression of expansion-related genes

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The role of cumulus cells during *in vitro* maturation is essential for proper oocyte maturation and acquisition of its developmental competence. During development of ovarian follicle, cumulus cells undergo expansion, leading to extracellular matrix production (mainly hyaluronic acid), regulated by various intracellular signaling cascades. Moreover, cumulus cells metabolize glucose to pyruvate or lactate, substrates used by oocyte for its growth and maturation. We demonstrated before that lysophosphatidic acid (LPA) improved cumulus–oocyte complexes (COCs) quality via the influence on oocyte quality markers expression. Thus, the objective of the present study was to examine the effect of LPA on cumulus expansion, glucose uptake and expression of genes involved in cumulus expansion and glucose metabolism (AREG, EREG, BTC, ADAM, EGFR, TNFAIP6, PTGS2, PTX3, HAS2, GFPT, PFK, and LDH).
COCs were obtained by aspiration from subordinate ovarian follicles and matured *in vitro* in presence or absence of LPA (10^{-5} M) for 24 h. Following maturation, cumulus expansion was visually assessed, cumulus cells were separated from oocytes and used for gene expression analysis (real-time PCR). Glucose, lactate and lactate dehydrogenase (LDH) levels were determined in the maturation medium.
Although, cumulus expansion was similar in the control and the LPA-treated COCs, LPA simulated mRNA expression of genes critical for cumulus expansion from the epidermal growth factor (EGF)-like family: amphiregulin (AREG) and epiregulin (EREG). Moreover, LPA stimulated glucose uptake, lactate production and LDH concentration in COCs. These results suggest that LPA stimulates cumulus expansion by enhancement of glucose metabolism and controlling the expansion-related genes: AREG and EREG in cumulus cells.

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P054

Essential roles of condensins in chromosome organization during mouse early embryogenesis

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Introduction
Multi-subunit protein complexes, called condensin I and condensin II, play a pivotal role in construction and segregation of mitotic chromosomes in many organisms. In mammals, however, previous studies examining condensins' function are limited to siRNA-mediated knockdown experiments using some established cell lines. Hence the roles of both condensins in mammalian development have remained to be solved. In the present study, we have investigated defective phenotypes of knockout (KO) of *Smc2,* one of the core subunits for both condensins I and II, during mouse early embryogenesis.
Materials and methods
Heterozygous *Smc2* KO mice were mated, and fertilized eggs were collected from the oviducts and cultured *in vitro* for 4 days. The embryos were fixed, immunofluorescently labeled with various antibodies, and observed with a confocal microscope.
Results and discussion
We found that SMC2 is essential for mammalian embryogenesis since no homozygous *Smc2* KO mice were born. SMC2-deficient embryos showed a slight developmental delay after 4-day culture. In the embryos, the mitotic index was significantly higher than that in control embryos. Furthermore, all mitotic cells in SMC2-deficient embryos exhibited a variety of chromosomal defects: chromosome individualization, sister chromatid resolution, centromere organization and chromosome segregation were impaired. These results strongly suggest that condensins are essential for proper formation and segregation of chromosomes in mouse early embryos. Notably, we also noticed that heterochromatin organization in interphase nuclei was disordered in the SMC2-deficient embryos, implying that condensins might regulate chromatin architecture not only in M phase but also in interphase.

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P055

Effect of downregulating CDX2 transcript by RNA interference on early development of bovine embryos

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In Cdx2-deficient mouse embryos, Oct-4 and Nanog expression are not restricted to the inner cell mass (ICM), and trophectoderm (TE) development is impaired. These results suggest that Cdx2 regulates Oct-4 and Nanog expression in mouse embryos, and plays a key role in TE development. The objective of this study was to investigate the role of CDX2 in the early development of bovine embryos. We attempted CDX2 downregulation in bovine embryos by short interfering RNA (siRNA), and evaluated the effects of CDX2 suppression on developmental competencies and expression of the genes involved in the segregation and function of ICM or TE tissues. Bovine IVF embryos injected with or without siRNA were cultured for 8 days. Gene expressions were evaluated at the morula stage. CDX2 siRNA injected embryos were able to develop to the morula stage. However, the blastocyst developmental rate of CDX2 siRNA-injected embryos (13.1%) was lower ($P<0.05$) than that of siRNA uninjected or control siRNA-injected embryos (36.6 and 34.4%, respectively) on day 6. On day 8, the embryos downregulated CDX2 were able to form blastocoel. However, the expanded blastocyst formation rate (20.0%) of embryos injected with CDX2 siRNA was lower ($P<0.05$) than that of control siRNA-injected embryos (33.9%). The CDX2 downregulation resulted in a distinct decrease in *GATA3*. In contrast, the *NANOG* expression level in the CDX2 siRNA-injected embryos was higher ($P<0.05$) than that in control siRNA-injected or siRNA uninjected embryos. Our results suggest that CDX2 is essential for proliferation and functionalization of TE in bovine embryos.

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P056

Fine cryopreservation method of porcine blastocysts produced by *in vitro* fertilization

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Introduction
Cryopreservation has been applied successfully in many mammalian species. Nevertheless, pig embryos have shown a reduced ability to be a lower developmental competence, because of their greater susceptibility to cryoinjuries. The aim of this study was to evaluate the survival status of vitrified–warmed porcine blastocysts.
Materials and methods
Forced collapse blastocoel (FBC) and non-FBC blastocysts are vitrified and concomitantly cultured in culture media which were supplemented with/without fetal bovine serum (FBS). Porcine vitrified–warmed embryos were examined in four different methods: group A, non-FBC and non-FBS; group B, non-FBC and FBS; group C, FBC and non-FBS; group D, FBC and FBS.
Results and discussion
After culture, differences in survival rates of blastocysts derived from vitrified–warmed porcine embryos were found in group A–D (39.7, 56.7, 56.5 and 70.9%, respectively, $P<0.05$). Reactive oxygen species (ROS) level of survived blastocysts was lower in group D than that of another groups ($P<0.05$). Moreover, total cell number of survived blastocysts was higher in group D than that of another groups ($P<0.05$). Otherwise, group D showed significantly lower number of apoptotic cells than other groups ($P<0.05$). Taken together, these results showed that FBC and FBS treatment improves the developmental competence of vitrified porcine embryos by modulating intracellular levels of ROS and the apoptotic index during the vitrification/warming procedure. Therefore, we suggest that FBC and FBS is an effective treatment technique during the vitrification/warming procedures of porcine blastocysts.

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P057

***In vivo* embryo production in queens treated with deslorelin acetate (Suprelorin)**

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Introduction
Reversibility after use of deslorelin acetate as a contraceptive for queens has been described. However no data concerning the ability of *in vitro* embryo production after treatement was published.
Objective
This study aimed to evaluate the *in vitro* embryo production in queens treated with deslorelin acetate (Suprelorin).
Methodology
Twenty queens and one tomcat were used. Ten queens were treated with deslorelin 4.7 mg/animal, the other females were not treated. After 6 months of treatment all queens were spayed, the ovaries were kept during 2 h maximum at 4 °C immersed in PBS. The ovaries were placed in petri dishes and sliced for cumulus oocyte complex (COCs) releasing. Grade I COCs were submitted to *in vitro* maturation, fertilization and the possible zygotes were maturated for 6 days, according to protocol described by previously (Rascado, 2009). The number of morulae and blastocysts produced were counted on day 3 and 8, respectively. No formal analysis was done.
Results
A total of 87 I COC, 50 (57.47%) morula and 18 (36%) blastocysts (four queens did not produced any blastocyst) were obtained from treated group. From control group we obtained 184 I COC, 103 (55.87%) morula and 23 (22.33%) blastocysts. Clearly the number of grade I COC recovered from treated queens was lesser than the control group, however the percentage of morulae obtained was very similar in both groups and the percentage of blastocysts was higher in the treated group. We concluded that ovaries from queens under contraceptive treatment with deslorelin may use for *in vitro* embryo production with rates similar to non-treated queens, although individual variations should be expected.
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P058

***O*-GlcNAcylation in pig embryos during preimplantation development**

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Protein modification with *O*-linked β-N-acetylglucosamine (*O*-GlcNAcylation) is essential for eukaryotic cells. There are many reports concerned with *O*-GlcNAcylateion, but little is known about it in preimplantation development. The objective of this study was to examine the presence of *O*-GlcNAcylation and the role of *O*-GlcNAc cycling in pig preimplantation development using parthenogenetic diploids. *In-vitro* matured oocytes were subjected to an electro-stimulation and cytochalacin B treatment to obtain parthenogenetic diploids. Diploids were cultured to 144 h after electro-stimulation. At first, mRNA expression of glutamine-fructose-6-phosphate transaminase (GFPT) that is the rate limiting enzyme for production of UDP-GlcNAc (substrate for *O*-GlcNAcylation), *O*-GlcNAc transferase (OGT) that catalyzes *O*-GlcNAcylation of proteins, and *O*-GlcNAcase (OGA) that removes *O*-GlcNAc from *O*-GlcNAcylated proteins was examined in diploids at various stages using RT-PCR. Secondly, *O*-GlcNAcylated proteins were detected by immunostaining using anti-*O*-GlcNAc antibodies (CTD110.6 and RL2). Finally, effect of inhibition of *O*-GlcNAc cycling on the preimplantation development was examined. Diploids were cultured for 144 h in PZM3 containing an OGA inhibitor, PUGNAc (0-300 μM) and observed every 24 h. GFPT and OGT mRNA were detected in diploids from 2-cell to expanded blastocyst stage. OGA mRNA was also observed in diploids at all preimplantation stages, but not 4-cell. *O*-GlcNAcylated proteins were detected throughout preimplantation development. Furthermore, almost all diploids cultured with 300 μM PUGNAc did not develop beyond the 4-cell stage. These results suggest that *O*-GlcNAcylation and *O*-GlcNAc cycling are present in preimplantation development and inhibition of OGA results in developmental arrest of embryos at the 4-cell stage in pigs.

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P059

Importance of amino acids in the development of preimplantation mouse embryo

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The composition of the external environment can have a significant impact on intracellular signaling pathways during early embryonic development. Components of the culture media, especially amino acids, are known to improve embryo development. We have shown that L-proline and L-glutamine improve development to the blastocyst stage by acting in a growth factor-like way. The aim of this study is to investigate the effect of amino acids on signaling pathways in the embryo.
Zygotes were cultured in the presence or absence of either all 20 amino acids, or L-pro alone, for between 15 min and 48 h and collected for western blotting and immunofluorescence. Rapamycin (10 nM) was used to investigate the role of the mTOR pathway in the developmental effects of amino acids.
Culture of embryos in the presence of rapamycin prevented the L-pro-induced improvement in development, suggesting mTORC1 activity is up-regulated by L-pro. However, western blotting did not show any change in phosphorylation of 4E-BP1 or rpS6, which are downstream of mTORC1, following treatment with L-pro for 48 h. Similarly, L-pro had no effect on activity of ERK1/2 or AKT pathways. However, the presence of all 20 amino acids for 6 h increased the phosphorylation of AKT and ERK1/2 in a cell cycle dependent way. These results indicate that L-pro-mediated developmental improvement in cultured embryos occurs via signaling pathways, including activity that may be cell cycle dependent and independent.

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P060

Thermoprotective action of IGF1 in embryos hamster 4-cells subjected to heat stress *in vitro*: effect of the redistribution of E-cadherin

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Introduction
IGF1 has been used as a thermoprotective during *in vitro* culture, however, its function is dependent on the cleavage embryos. Furthermore, the localization of E-cadherin dependent cleavage embryos. It is unknown whether there is a relationship between the thermoprotective action of the IGF1 and localization of E-cadherin.
Materials and methods
Ten female hamster young were used. Hamster embryo 4-cells were obtained. Groups of embryos 4-cells were incubated in the presence or absence of 6- DAMP (compacted and uncompacted). Both groups of embryos were incubated for 24 h under conditions of heat stress. The medium was supplemented with IGF1, the end of the culture period the development stage and the rate of apoptosis was determined.
Results and discussion
The percentage of embryos that reached the 6-cell stage embryos was significantly higher than 4-cells compacted or non-compacted (63.57 vs 38.81 respectively). The intensity of Annexin-V (apoptosis) embryos was significantly higher than non-compacted compacted (33 vs 25 respectively). The anti-apoptotic activity of the IGF1 is via the PI3K/Akt pathway, evidence of a relationship between the activation of this pathway and the formation of cell junctions dependent E-cadherin. In conclusion, the heat protection of the IGF1 activity is favored by a change in the localization of E-cadherin in embryos of four cells when subjected to heat stress *in vitro*.

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P061

Effect of blastocyst artificial collapse prior to vitrification on pluripotency-specific genes expression in mouse embryos

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Introduction
Cryopreservation is possible for all stages of pre-implantation embryos. It has been reported that survival rate of blastocyst is comparably lower than other stages. There is a high volume of fluid in blastocoel cavity that can be a good ground for ice crystals formation, resulting in damage to the cell structure. In this study, the effects of artificial collapseand reduction of the fluid volume in blastocyst cavity before vitrification process on the survival rate and quality of blastocysts were assessed by estimating the expression levels of *Oct4*, *Nanog*, *Sox2*, and *Klf4*.
Materials & Methods
Mouse blastocysts divided into 5 groups including: A) Vitrified- thawed blastocysts, B) Vitrified- thawed blastocysts after artificial collapse, C) Collapsed blastocysts, D) Immersed blastocysts in vitrification/warming solutions and E) Fresh blastocysts as control group. The survival and hatching rate of embryos were evaluated and the expression of pluripotency-specific genes was assessed by Real Time PCR technique in comparison to control group.
Results and Discussion
Although there was a significant reduction in hatching rate of groups A and B, the survival rate in group B increased significantly compared with other groups ($P < 0.05$). In addition, there were no significant changes in expression of genes of all groups ($P > 0.05$). It can be concluded that artificial collapse of blastocyst could be a simple and effective way to contribute to the successful blastocyst vitrification.

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P062

The role of histone H3 variants in the alteration of chromatin structure after fertilization

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After fertilization, differentiated oocytes become totipotent embryos. In this transition, a drastic change in chromatin structure is known to occur, although the mechanism underlying this change remains to be elucidated. Recent studies have revealed that one of the major factors that contribute to the change in the chromatin structure is the exchange of histones with their variants. In mammals, there are three main non-centromeric histone H3 variants: H3.1, H3.2, and H3.3. Using transgenic mice and the microinjection method, we have previously shown that H3.3 is incorporated before and after fertilization. In contrast, there was limited incorporation of H3.1. However, the nuclear localization of H3.2 is not clarified. Here, using transgenic mice and microinjection method, we aim to investigate the role of H3 variants in embryonic development.
To examine the nuclear localization of H3.2, we generated transgenic mice that ubiquitously express Flag-tagged H3.2. Furthermore, to determine the biological significance of the composition of H3 variants at the 1-cell stage, we performed an overexpression analysis of H3.1, H3.2, and H3.3 to examine their effect in development.
We have found that H3.2 was not detected as well as H3.1 in 1-cell embryos, suggesting that H3.3 is the major histone variant incorporated at this stage. In addition, the embryos overexpressing H3.1 and H3.2 showed developmental delay and did not reach the blastocyst stage. These results suggest that limited incorporation of H3.1 and H3.2 into chromatin is essential for the change in chromatin structure at the 1-cell stage.

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P063

Roles of the untranslated regions in the translational regulation of cyclin A2 in murine oocytes and embryos

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After the resumption of meiosis and fertilization, differentiated oocytes dramatically change their nature to generate totipotent zygotes. However, since transcription does not occur in oocytes and early 1-cell stage embryos, gene expression is controlled only by post-transcriptional regulation. There are huge mRNAs stored in full-grown oocytes (FGO). A part of them is not translated in FGO and MII stage oocytes, and become translated after the resumption of meiosis and fertilization, respectively. Generally, translation of mRNA is regulated by its untranslated regions (UTRs). In the present study, we examined that the role of UTRs in the alteration of cyclin A2 (CcnA2) translation after the resumption of meiosis and fertilization.
FGO and MII stage oocytes were microinjected with cRNA encoding luciferase with 5'UTR and/or 3'UTR of CcnA2. They were allowed to resume meiosis and fertilized *in vitro* and examined for luciferase activity.
Both of 5'UTR and 3'UTR decreased the translation of Luc cRNA in FGO. However, when meiosis was resumed, 3'UTR increased the translation, while 5'UTR decreased it. The Luc cRNA with both of two UTRs increased the translation. After fertilization, the up-regulation by 3'UTR was also observed, whereas 5'UTR showed no significant effect. These results suggest that UTRs of CcnA2 bear time-specific translational regulation, and that this regulation is largely dependent on 3'UTR.

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P064

The effects of PHE mixture, theophylline, and sperm concentrations on fertilization and development of bovine oocytes in vitro

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Introduction
We aimed to establish an efficient *in vitro* fertilization (IVF) system without optimization of sperm concentration of individual bulls, which can obtain stable fertilization and development. We examined the effects of PHE mixture (20 μM D-penicillamine, 10 μM hypotaurine, and 1 μM epinephrine), theophylline (2.5 mM), and sperm concentrations (1, 2, or 5×10^6 spermatozoa/ml) on fertilization and development of bovine oocytes in vitro.
Materials and Methods
Firstly, matured cumulus-oocyte complexes (COCs) were co-incubated with 1 or 2×10^6 spermatozoa/ml with or without PHE mixture and/or theophylline for 12 or 18 h, and fertilization was examined. Secondly, matured COCs were co-incubated with different sperm concentrations (1, 2, or 5×10^6 spermatozoa/ml) from 3 bulls with PHE mixture and/or theophylline for 18 h, and fertilization and development were examined. Finally, matured COCs were co-incubated with 2×10^6 spermatozoa/ml from 8 bulls with PHE mixture and theophylline for 18 h, and development was examined.
Results and Discussion
When PHE mixture and theophylline were added to IVF medium, normal fertilization (two pronuclei) for 12 h at 1×10^6 spermatozoa/ml became higher, and we obtained stable normal fertilization (70–80%) from 3 bulls, cleavage (80–90%), and blastocyst rates (30–50%) from 8 bulls at 2×10^6 spermatozoa/ml. However, 5×10^6 spermatozoa/ml was necessary when only theophylline was added to fertilization media to obtain high fertilization. In conclusion, fertilization was synergistically enhanced by PHE mixture and theophylline, and the blastocyst development became stable even if 2×10^6 spermatozoa/ml from any bulls used for IVF.

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P065

The effects of mesenchymal stem cell- conditioned medium on the fertilization rate, embryo development and OCT-4 gene expression of 8-cell embryo in NMRI mice

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Normal growth of oocyte, embryo and also successful Implantation depend on environmental factor such as the secretion of cumulus cells and composition of follicular and tubular fluids.
Therefore, addition of serum, conditioned medium (CM) to the culture medium and also co-culture with somatic cells improve mammalian embryo development. So, in the respect to the secretion of different growth factors and cytokines by mesenchymal stem cells (MSc), the present study shows the effects of MSc-derived CM on fertilization rate, early embryo development and OCT-4 expression.
Materials & Methods
60 female and 10 male NMRI mice were used to obtain the MII oocytes and capacitated sperm for IVF in the following groups:
The basic fertilization medium (BFM) in control group contained T6 + BSA 15 mg/ml, for treated groups 1–3 was added respectively 25, 50 and 75% of MSc-derived CM to BFM.
The fertilized oocytes transfered to embryo developmental medium (T6 + BSA 4 mg/ml) for 72 h, and then 8-cells embryo were obtained to extract total RNA and assay OCT-4 gene expression.
Results and Discussion
The study showed statically a difference on fertilization rate in the treated group 3 in comparison with other ones.
The results didn't show any difference on embryo developmental rate; Howevere, there was a higher expression of Oct-4 in the treated groups particularly in the presence of 50% MSc-derived CM, in comarision of control group. It concluded that adding MSc-derived CM (50%v/v) may improve OCT-4 gene expression and subsequently early embryo cell division.

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P066

The regulatory roles of let-7 in embryo dormancy in mice

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Introduction
MicroRNAs interact with multiple mRNAs resulting in their degration and/or translational repression. Embryo diapause is a widespread phenomenon in which temporarily arrest occurred in embryo development. Our previous data showed that the levels of let-7 are relatively high in diapause embryos compared to reactivated embryos by E_2 in mice. However, it is still not clear whether or not let-7 is involved in embryo diapause.
Materials and methods
Firstly, blastocysts electroporated with pre-let7a were cultured for 3-9 days *in vitro*, and then checked metabolic parameters (such as glucose, lactate et al) and shape of embryos. Secondly, we transferred the embryos cultured *in vitro* into the uteri of 2-Fl-E_2 induced pseudopregnant mice to confirm whether these embryos are dormant or not. Finally, we applied microarray methods to dectect which genes are mediated the roles of let-7 in embryo dormancy.
Results and discussion
The embryos electroporated with let-7 can still be alive and induce the formation of implantation sites even after the culture for up to Day 13. Further studies showed that the activated embryos changed into diapause state if these embryos were electroporated with let-7. Interestingly, 12.5% of diapause embryos induced by let-7 *in vitro* developed to term after culture for 4 days (ie Day 8). Survival mechanisms of diapause embryos induced by let-7 are involved in decreasing the apoptosis and cell cycle of embryos. In conclusion, the levels of let-7 are positive correlated with the diapause state of embryos. This study will provide information on understanding difference in embryo dormancy between animals.

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P067

Plasma growth hormone decline during early mammalian development is the result of expanding blood volume

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Growth hormone (GH) is necessary to grow to normal adult size. While GH plasma concentrations are highest during early development, there is no evidence that GH influences somatic growth until after birth when GH-receptors are upregulated in peripheral tissues. The gradual decline in plasma GH during fetal life in a range of mammals suggests that negative feedback to the developing pituitary occurs progressively to decrease pituitary GH output. We have tested this hypothesis using a marsupial model in which we can rapidly accelerate growth of the young by fostering day 60 post-partum tammar wallabies to females at day 120 of the lactation cycle, thereby significantly increasing growth rate relative to controls. Foster and control young were then killed at 120 days of chronological age (180 days of the lactation cycle in the recipient female) and plasma GH concentrations and pituitary mRNA expression compared with control young. Foster young had significantly lower plasma GH concentrations, indicative of temporally advanced growth axis maturation, but these differences were absent when calculated relative to body weight. There were no differences in pituitary *GH* expression or hepatic *GH-binding protein (GHBP)* expression between groups. Young of small mothers, which are smaller at the same chronological age, had higher plasma GH concentrations but no difference relative to body weight and no difference in pituitary *GH* expression or hepatic *GHBP* expression relative to controls and fosters. This data suggests that GH decline during early life is an effect of dilution by a greater blood volume as the animal grows.

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P068

Single-step generation of rabbits carrying a targeted allele using CRISPR/Cas9

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Introduction
The type II bacterial clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein (Cas) have been proven to be an effective gene targeting system. Genome editing of non-rodent mammalian species is a promising strategy for generation of animal models for human diseases. Mashiko et al. reported a high efficacy of direct injection of a plasmid DNA, encoding humanized Cas9 and sgRNA (single-guide RNA) in mice. Here we show successful gene targeting in rabbit via the same strategy.
Materials and methods
To establish a CRISPR/Cas9 system in rabbit, we selected the *TYROSINASE* gene as a target using Dutch-belted rabbits. Plasmid pX330 was inserted with a candidate gRNA for the evaluation of endonuclease activity by single strand annealing (SSA) assay. Circular plasmid containing a selected gRNA was microinjected into the pronucleus of rabbit fertilized embryos. The embryos were cultured for 24 h and then transferred into the oviducts of pseudo-pregnant Japanese White rabbits.
Result and discussion
Four candidate gRNAs were evaluated by SSA assay and then circular prasmid pX330 containing *TYROSINASE* CR2 as a gRNA sequence was microinjected into 77 embryos. Of them, 67 (87%) were cleaved and transferred into recipients. Among 9 (13%) pups obtained at term, 2 (3%) had targeted alleles. One heterologous and one homologous mutant pups were obtained, although the latter was still born. This study demonstrated that the CRISPR/Cas9 system by direct injection of plasmid DNA into zygotes can also be potentially applicable to rabbits.

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P069

Supplementation of 6-diazo-5-oxo-l-norleucine improves *in vitro* maturation and *in vitro* developmental competence in pigs

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Activators of protein kinase A (PKA) signaling pathway have been frequently used to transiently stall meiosis at early phase of *in vitro* maturation (IVM) for balancing between cytoplasmic and nuclear maturation of mammalian embryos. Despite of the tight association of 6-diazo-5-oxo-l-norleucine (DON), an inhibitor of hyaluronan synthesis, with PKA cascade, few evidences has been provided concerning the role of DON as an IVM supplement with meiosis-stalling activity during IVM of porcine oocytes. Thus, the current study was conducted to examine the effect of DON on nuclear/cytoplasmic maturation of porcine oocytes and subsequent early embryonic development. From the observation of nucleus patterns, we found that treatment of DON successfully retarded meisis progression. Unlike the results from the treatment of 5 and 10 mM DON, IVM rate of porcine oocytes was increased only in 1 mM DON treatment group compared to control (81.9 ± 0.1 vs 71.5 ± 1.3, $P < 0.05$). Consistent with this, blastocyst formation rate (44.2 ± 1.9 vs 30.5 ± 7.4, $P < 0.05$) and TE proportion (34.5 ± 3.8 vs 23.8 ± 2.0, $P < 0.05$) were greatly increased in 1 mM DON treatment group compared to control. Consistent with this, pathenogentic activation of MII oocytes matured under 1 mM DON greatly increased blastocyst formation rate (54.4 ± 5.2 vs 31.5 ± 6.1, $P < 0.05$). From the results, we concluded that 1 mM DON can be efficiently used to massively yield MII oocytes with high nuclear/cytoplasmic maturation and developmental competence.

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P070

Treatment of fetal bovine serum improves early development of porcine embryos by alleviating oxidative stress

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Despite of the application of numerous supplements to improve *in vitro* culture (IVC) of mammalian cells, few studies have been conducted concerning the effect of fetal bovine serum (FBS) on early development of mammalian embryos. Thus, the current study was carried out to determine the effect of FBS on the developmental competence of porcine embryos and to establish the optimal treatment conditions. Unlike the treatment during full-term (1–6 days) or early phase (1–3 days) of IVC, the beneficial effect of FBS supplementation was only found in late phase (4–6 days) of IVC. The rates of blastocyst formation and hatching were significantly increased by addition of FBS during the late phase of IVC compared to control, which was further evidenced by the improvement of cellular survival and total cell number in blastocysts. Moremore, reactive oxygen species (ROS) levels were markedly reduced in the late-phase FBS treatment group compared to control. Interestingly, supplementation of hydrogen peroxide aggravated the early development of porcine embryos, which was greatly ameliorated by treatment with FBS during the late phase of IVC. Taken together, these results suggest that FBS can be efficiently used as a useful supplement for massive production of porcine embryos with high developmental competence.

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P071

Hollow fiber vitrification of *in vitro* produced bovine embryos at early developmental stages

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The hollow fiber vitrificaiton (HFV) method, we developed (Matsunari et al., JRD 58, 2012) has been shown to be very effective in the cryopreservation of highly cryosensitive embryos, such as *in vitro* matured-fertilized pig morulae. The objective of this study was to verify the effectiveness of the HFV method to the *in vitro* matured-fertilized bovine embryos in early developmental stage. Crossbred (Holstein×Japanese Black) *in vitro* matured-fertilized bovine embryos were cultured in IVD101 medium (IFP, Japan) for 8 days after insemination (day-0). Vitrification was performed on the embryos at the 2-4 cell (day-1), 8-16 cell (day-3), and morula (day-5) stages, respectively. Survival of the vitrified embryos were evaluated by *in vitro* development to blastocysts. Vitrificaiton of the embryos was performed according to our previous report. Seven to 19 embryos were loaded in cellulose triacetate hollow fibers (internal/external diameter 185/200 μm, length 25 mm), and vitrified with 15% DMSO, 15% ethylene glycol, and 0.5M trehalose in liquid nitrogen. After thawing at 38.5 °C in 1M sucrose solution, the cryoprotectants were removed by a stepwise manner. The blastocyst formation rates of the vitrified embryos at each developmental stage were similar to those of the control embryos (2-4 cell: 71.4% (10/14) vs 85.7% (12/14), 8-16 cell: 70.0% (7/10) vs 77.8% (7/9), morula: 89.5% (17/19) vs 84.2% (16/19)). These data show that the HFV method is effective for cryopreserving early stage *in vitro* matured-fertilized bovine embryos.
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P072

Treatment of histone deacetylase inhibitor increases *in vitro* developmental competence of bovine cloned embryos through suppression of endoplasmic reticulum stress

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Although the beneficial roles of HDAC inhibitors (HDACis) in somatic cell nuclear transfer (SCNT) were known, few studies have conducted concerning the role of valproic acid (VPA) as an efficient HDACi for SCNT and its associated developmental event(s). Thus, the present study was carried out to determine the effect of VPA on developmental competence of bovine SCNT embryos and the underlying mechanism(s). We showed that the VPA treatment restored the histone acetylation level of SCNT embryos similar to IVF embryos, with optimal results obtained by treatment with 3 mM VPA for 24 h. Importantly, although blastocyst formation rate and ICM and TE cell numbers were not different between the VPA and trichostatin A (TSA) treatment groups, cell survival was significantly improved by VPA, indicating the improvement of developmental competence of SCNT embryos by VPA. Interestingly, VPA markedly reduced the transcript levels of endoplasmic reticulum (ER) stress markers, including *sXBP-1* and *CHOP*. In contrast, the levels of *GRP78/BiP*, an ER-stress-alleviating transcript, were significantly increased by VPA. Furthermore, VPA greatly reduces apoptotic cells in SCNT blastocysts, which was further evidenced by the increased levels of anti-apoptotic transcript *Bcl-xL* and decreased level of the pro-apoptotic transcript *Bax*. Collectively, these results suggest that VPA enhances the developmental competence of bovine SCNT embryos by alleviating ER stress and its associated developmental damage.

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P073

Early development of bovine embryos depends on the cooperative action between oxidative and endoplasmic reticulum stresses

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The coupling of reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress has been explored using a variety of biological systems, but little is known regarding their role in the early development of mammalian embryos. Here, we demonstrated that early embryonic development of *in vitro*-produced (IVP) bovine embryos was governed by the cooperative action between ROS and ER stress. Compared to 5% O_2 tension, 20% O_2 significantly decreased the blastocyst formation rate and cell survival, accompanied with increases in ROS and *sXBP-1* transcript levels, which is an ER stress indicator. In a 20% O_2 environment, treatment with glutathione (GSH), a ROS scavenger, decreased ROS levels, which resulted in increased blastocyst formation and cell survival rates. Importantly, the levels of *sXBP-1* and ER stress-associated transcripts were reduced by GSH treatment during *in vitro* culture (IVC) of bovine embryos. Consistent with the effects of GSH supplementation, tauroursodeoxycholate (TUDCA), an ER stress inhibitor, improved blastocyst development rates, trophectoderm proportions, and cell survival. Moreover, ROS and *sXBP-1* transcript levels were markedly decreased by supplementation of TUDCA into IVC medium (similar to the GSH treatment group), suggestive of the possible mechanism governing the mutual regulation between ROS and ER stress. Based on these results, developmental competence of IVP bovine embryos was highly dependent on the coupled response between oxidative and ER stress. These results increase our understanding of the mechanism(s) governing early embryonic development and may improve strategies for the generation of IVP embryos with high developmental competence.

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P074

Novel porcine OCT4 variants identification in blastocyst and discriminative expression analysis in adult tissues

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Introduction
OCT4 has been known to master gene for maintaining pluripotency in mouse inner cell mass and embryonic stem cell. However, the variant of OCT4 was false-positively detected in somatic cells and this confuses the result in stem cell and embryo development research. So in this study, we aimed to identifying novel OCT4 variants in pig and examined their expression in various adult tissues.
Material and method
By comparing the sequence between human OCT4 variants and pig genome, the candidate OCT4 variant coding region was searched and the region was confirmed by RT-PCR in pooled *in vitro* fertilized blastocyst ($n=10$). The amplified porcine OCT4 variant region was sequenced. After identifying full sequence of porcine OCT4 variants, their expression was examined in following somatic tissues, liver, lung, kidney, spleen, muscle, heart, brain, testis, and ovary to confirm the possible relation between novel variant transcripts and pluripotency.
Result and discussion
Porcine OCT4 variant was expressed in blastocyst and their gne structures were similar to human OCT4 variant. Porcine OCT4B transcript contains differential N-terminal coding region, about 400bp upstream region of the second OCT4 exon, and OCT4B1 span cryptic exon which is normally the second intron of porcine OCT4A and OCT4B transcript. Porcine OCT4B transcript was expressed in somatic tissues like spleen and heart as well as testis and B1 form was only detected in testis. This result showed that the porcine OCT4 should be analyzed discriminatively considering the variant and OCT4B1 could be related to the pluripotency in pig.This research was supported by the Next BioGreen 21 program (PJ009493), Rural Development Administration, Republic of Korea.

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P075

Chromatin remodelling gene *Cecr2* in murine gametogenesis, fertilization, and early embryonic development

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Mammalian reproduction is dependent on a myriad of genes, all of which must be carefully regulated spatially and temporally to ensure successful fertilization and embryonic development. Chromatin remodellers are able to affect nuclear processes such as gene transcription, DNA replication, recombination and repair through modulation of chromatin structure. Mice with hypomorphic mutations in the chromatin remodelling gene *Cecr2* have normal reproductive histology and their gametes have no obvious abnormalities, yet both sexes produce significantly decreased litter sizes (by ~50%) when mated to normal mice. When combined with a more deleterious *Cecr2* mutation, the subfertility phenotype increases in severity. The defect in mutant males occurs around the time of fertilization: after *in vivo* matings, fewer oocytes/zygotes show visible pronuclei or progress to blastocyst in culture. To address whether fertilization itself is impaired or early post-fertilization remodelling events are affected, zygotes are being examined within hours of mating using phalloidin, tubulin and DAPI staining to visualize the fertilization cap and polar body formation as well as early chromatin remodelling events. In contrast, oocytes from mutant females do not show reduced pronuclei formation, indicating that the female subfertility is not due to a defect at the time of fertilization but rather to embryonic loss during development. However, an increase in embryonic resorptions is not observed, suggesting that embryos are being lost before or during implantation. Investigating the cause of subfertility in both sexes will advance our understanding of the various roles that chromatin remodelling plays in gametogenesis, fertilization, and early development.

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P076

Are cumulus cells additional players in calcium signalling during cattle oocyte fertilisation?

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Upon sperm entry within the oocyte, pulsatile calcium release occurs, facilitating activation of the oocyte. While calcium signalling has been characterised in mouse oocytes, patterns within the whole cumulus oocyte complex (COC) and in larger mammals such as cow, are yet to be determined. The aim of this study was to investigate calcium changes in media, cumulus and oocytes during *in vitro* fertilisation in cattle oocytes.

Cattle COCs were inseminated *in vitro* following IVM. Approximately 3 h post-insemination (negative control = no sperm), COCs were cultured for 30 min in 5 uM Fluo-4AM (calcium indicator), washed and transferred into glass-bottomed confocal dishes maintained at 38 °C. Fluorescence intensity was captured using confocal microscope every 2 min, between 200–510 min post-sperm addition (8.5 h). Fluorescence intensity was determined within regions of the oocyte, cumulus vestment and surrounding medium. Successful fertilisation was determined by DAPI fluorescence and visualisation of female/male pronuclei.

A peak and then decrease in Fluo-4AM intensity occurred within the oocyte and then cumulus vestment, corresponding with previously reported timing of sperm entry in cattle (399–405 min), followed by 2–4 fold increase 8–16 min later in intensity of probe in the media of fertilised COCs. In contrast, oocyte, cumulus and media fluorescence remained relatively constant in the no sperm group. These results suggest the intensity and pattern of calcium release from the oocyte, cumulus and into the media indicates successful fertilization, and demonstrates that calcium signalling at fertilization extends beyond the oocyte.

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P077

Pregnancy of bovine somatic cell nuclear transfer embryos reconstructed by using donor cells: iPS cell vs AID transfected cell*

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SCNT technology provides potential applications for biomedical and agriculture. However, the efficiency of SCNT is still low. Incomplete epigenetic reprogramming of transferred somatic cell is believed to be one of main causes of developmental problems. Hanwoo is considered as only bovine species with mainly reddish color but quite a few of mini-, white-colored, black-colored, striped cows. The objective of this study was to produce cloned offspring from endangered mini-Hanwoo SCNT embryos reconstructed by using either iPS or AID gene transfected cells. These reprogrammed cells may enhance development of SCNT embryos. biPS cells were prepared from transfection with six reprogramming factors. After the transduction iPS cells were placed in DMEM, LIF and bFGF. pAcGFP1-C1 vectors included AID genes were transfected. *Oct4* and *Nanog* were expressed after transfection of AID gene. The pregnancy of SCNT embryos produced by two cell lines was diagnosed by a ultrasound scanning. Mini-Hanwoo SCNT embryos (blastocysts: $n = 20$) reconstructed by using either iPS or transfected AID cells were transferred into uterus of each synchronized recipients ($n = 10$). One recipient in each group was diagnosed as pregnancy (10 vs 10%). However, control SCNT embryos did not show the pregnancy (blastocysts: $n = 13$; recipients: $n = 10$). This result indicates that bovine SCNT embryos reconstructed by using the reprogrammed cells as iPS and transfected AID cells are able to induce the successful pregnancy.

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P078

The effect of cRNA concentration of artificial nuclease microinjected cytoplasmically to pronuclear porcine embryos on survival and development *in vitro*

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Although GH receptor (GHR) gene deletion causes a hereditary dwarfism and also shows a longer lifespan, the fact was shown only in mice. Because it was difficult to generate knockout (KO) animals other than mice. Recently, the generation of KO animals became possible without difficulty due to advances in genome editing technologies such as transcription activator-like effector nuclease (TALEN). In the present study, we examined the effect of cRNA concentration microinjected cytoplasmically to pronuclear porcine embryos on survival and development of embryos *in vitro*. Porcine follicular oocytes were matured and then fertilized (IVF) *in vitro*. At 10 h after IVF, oocytes were centrifuged to visualize the pronuclei. Embryos with two or three pronuclei were injected cytoplasmically with 2 or 100 μg/ml of GHR TALEN cRNA. TALEN cDNAs for GHR were transcribed *in vitro* using mMESSAGE mMACHINE T7 Ultra kit and the products were diluted with RNase free water. After microinjection, embryos were cultured and observed survival at 15 min after microinjection and development at 48 and 134 h after culture. There were no significant differences in survival rates between 2 and 100 μg/ml groups (80.3 and 87.3%) ($P > 0.05$). The rates of cleavage and blastocyst formation in 2 μg/ml group (50.9 and 35.1%) were significantly higher ($P < 0.05$) than those in 100 μg/ml group (25.8 and 16.1%). The results suggested that 100 μg/ml of GHR TALEN cRNA may negatively affect development of porcine embryos. On the basis of the results, further study is required to generate GHR gene KO pigs.

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P079

Abstract withdrawn.

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P080

Isolation and characterization of porcine mesenchymal stem cell as a donor for efficient nuclear transfer

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Introduction

Nuclear transfer (NT) has used for generating cloned animals or genetically modified animals. However, the efficiency has remained low, because of epigenetic errors that occur during donor cell reprogramming. Mesenchymal stem cells (MSCs) were known as undifferentiated state cell compared to somatic cells. Thus MSCs can reduce the chance of error that can be occurred during reprogramming process and can be easily isolated from adult, while embryonic stem cells are not. For these reasons, undifferentiated stem cells may be more suitable as donor cells for NT than fully differentiated somatic cells. To increase the rates for cloned animal production, MSCs will be a good donor cell source for successive NT.

Materials and methods

In this study, we isolated MSCs from porcine fetal femur and explants on the plate directly. Putative femur-derived MSCs were first observed after 5–6 days of culture. Cells at passage 3–5 were characterized by RT-PCR for marker gene expression and differentiation capacity of osteogenesis and adipogenesis and then those cells were used as a donor cells.

Results and discussion
Femur-derived MSCs showed fibroblast-like morphology. Positive markers for MSC (CD29, CD44, CD90, CD105, CD144) was highly expressed in this cell, whereas negative markers (CD14, CD34, CD45) were not detectable. Especially, expression of major MSC surface molecules (CD90, CD105) were observed by FACS. According to the osteogenesis and adipogenesis from MSCs, differentiation potential was verified. These results suggest that MSCs from porcine fetal femur were successfully isolated, and might be a good donor cells for producing porcine NT embryo.

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P081

Establishment of efficient system in the DNA microinjection into porcine *in vitro* embryos

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To investigate the efficient laboratory techniques for the injection of DNA, pEGFP-N1 commercial plasmid were microinjected into porcine parthenogenetic and IVF embryos to explore the injection time, volume and concentration, for the efficient blastocyst production. In experiment 1, to investigate injection time, compared four different time durations (2, 4, 6 and 8 h) after post activation and 6 h of co-incubation with sperms. There were no significant difference ($P<0.05$) between four groups regarding the percentage of cleavages. However there were significant difference ($P<0.05$) in development (4.4, 8.9, 3.9, 0.6%), GFP expression (1.3, 5.7, 2.3, 0.0%) which injected after post activation of 4 h compared with another three groups. IVF embryos after 2 and 4 h were expressed GFP significantly higher than rest of two groups. In experiment 2, two concentrations of 20 and 50 ng/μl were injected to the embryos and observed blastocysts after 7 days of incubation. There were significant difference ($P<0.05$) between two treatments which has higher cleavage (58.8 vs 41.9%), blastocysts rate (13.0 vs 11.1%) and GFP expression (5.7 vs 0.0%) in 20 ng/μl. In IVF embryos, only 20 ng/μl injected embryos were expressed GFP (4.2%) after 7 days of incubation and 77.3 vs 64.7% of cleavage, 26.4 vs 23.5% development. In experiment 3, three different volumes (5, 10 and 20 pl) were microinjected into porcine embryos. Out of three groups, significantly higher development rates of cleavage (68.3, 58.0, 29.3%), blastocysts (11.7, 12.7, 0.5%) and GFP expressed blastocysts (2.9, 7.8, 0.0%) were shown in the 10 pl group ($P<0.05$).

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P082

The effect of development *in vitro*

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Although mammalian embryo culture is important for research in early development as well as reproduction engineering, rat embryo culture is still infant other than that of mice. The aim of the present study was to improve rat embryo culture *in vitro*, we compared modified rat-one cell embryo cuture medium (R1ECM) with potassium simplex optimized medium with amino acids (KSOMaa) and that effect of addition of EDTA (0.013 mM) and glutamine (1 mM) to mR1ECM on develop to 2 cell stage embryos and blastocysts. Pronuclear embryos were collected at 29 h after hCG injection from oviducts of superovulated females (4–5 weeks old of Wistar) that naturally mated with males (12–24 weeks old of Wistar). The embryos were cultured at 37.5 °C for 120 h. When cultured with mR1ECM or KSOMaa, 2-cell block was observed in embryos cultured in KSOMaa and that no development to blastocysts.

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P083

Comparison of survivin gene expression between porcine SCNT and iSCNT-derived normal and arrested embryos

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Introduction
Interspecies somatic cell nuclear transfer (iSCNT) is an useful tool to produce cloned embryos using livestock oocytes instead of the animal with peculiar reproductive system to produce endangered animal and rescue incurable disease. There are many researches about iSCNT such as porcine-bovine, mouse-porcine, mouse-bovine and so on, in contrast, canine-porcine SCNT especially have been almost no reports.
Materials and methods
Porcine fetal fibroblast (pFFs) and canine ear skin fibroblast (CES) were injected to enucleated porcine oocytes to understand why iSCNT embryos were not developed to the blastocyst stage. The development rates of embryos were investigated during culture for 7 days in porcine embryo culture medium. Gene expression levels of survivin were analyzed by quantitative real-time PCR. TUNEL analysis was conducted for arrested embryos.
Results and discussions
This study showed that iSCNT development rates and cell numbers were lower than those of SCNT and the rate of apoptotic-positive cells were significantly increased in iSCNT embryos ($P< 0.05$). When anti-apoptotic gene expression, survivin levels compared with SCNT and iSCNT arrested embryos were lower than normal embryos from both SCNT and iSCNT. Therefore, these findings explained the underlined mechanism why developmental arrests of SCNT and iSCNT embryos were caused at early stages.
Keywords
SCNT, interspecies, survivin, apoptosis, embryonic arrest.

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P084

Involvement of poly(ADP-ribosyl)ation and autophagy in porcine pre-implantation development

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Introduction
The poly(ADP-ribosyl)ation (PARylation) has involvement with pro-survival autophagy. However, there has been no report about PARylation and autophagy during pre-implantation development. Here, we investigated the mechanism between PARylation and pro-survival autophagy in pre-implantation development.
Materials and methods
IVF embryos were cultured in the medium containing 3-aminobenzamide (3ABA), parp inhibitor. Autophagy and PARylation were evaluated by immunocytochemistry and quantitative real time-PCR in pig embryos.
Results and discussions
The expression level of Parp-1 mRNA was increased in 6 hours of post-insemination (hpi) and 12 hpi embryos and decreased from 20 hpi to blastocyst stage. The distribution of PAR was diffused in cytoplasm at metaphase II oocytes. After fertilization, PAR localized in nucleus was transiently induced at 6 and 12 hpi, however, the PAR was restored in cytoplasm again from 20 hpi to blastocyst. To investigate the effect of PARP on embryo development, the treatment of 3ABA from one cell stage to blastocyst. There was no significant difference of development to the morula stage (20.77 ± 4.19 vs $24.55\pm5.19\%$), whereas the blastocyst development was significantly reduced (3.63 ± 2.65 vs $12.26\pm3.37\%$). The blastocyst formation was significantly reduced by inhibition of PARP in morula stage (19.64 ± 4.61 vs $41.39\pm5.26\%$), especially expanded blastocysts (4.69 ± 2.99 vs $28.1\pm6.06\%$). The expression level of autophagy related genes (Atg5, Beclin1 and Lc3) and LC3 protein synthesis were reduced in 3ABA treated blastocysts. In addition, total cell number of 3ABA treated blastocysts was significantly lower than control group and apoptosis was increased. Collectively, these data suggest that PARylation may be involved in pro-survival autophagy and embryo quality in pig IVF embryos.
Keywords
Autophagy; Poly(ADP-ribosyl)ation; pig embryos; blastocyst.

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P085

Transporters for L-proline in the pre-implantation mouse embryo

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The inclusion of amino acids in pre-implantation embryo culture media is known to improve the rate of development and embryo viability. Our studies have shown that embryos cultured in L-proline from the zygote to the blastocyst stage develop better than embryos cultured in the absence of amino acids. This study aimed to identify the pre-implantation stage(s) at which L-proline transport is required for development to be improved and then to characterise the amino acid transporter(s) responsible for L-proline uptake into the embryo.
Mouse embryos were cultured in medium containing 400 mM L-proline and scored for development. Competitive substrates, immunostaining and embryos from SLC6A19 null mice were used to identify L-proline transporters in early embryos.
Embryos cultured with L-proline from the 2-cell stage developed better than embryos cultured with L-proline in earlier stages, suggesting that the later stages are crucial for L-proline's transport and subsequent effect. The presence of excess L-leucine or glycine in the medium prevented the improvement in development induced by L-proline, implicating the transporters SLC6A19 and SNAT2 since they transport L-proline, L-leucine and glycine. Immunostaining showed that SLC6A19 was present in the pre-implantation embryo and that it appeared to be expressed in the membrane from the 8-cell stage onwards. SLC6A19 null mice showed poor development to the BL stage, which was not improved by addition of L-proline to the medium. These results suggest that SLC6A19 may be responsible for L-proline uptake in the pre-implantation embryo and for the subsequent improvement in embryo development.

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P086

Treatment of antioxidant (acteoside) in fetal fibroblats improves the efficiency of canine cell cloning

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This study was designed to investigate whether acteoside (antioxidant) improves canine SCNT efficiency. Cell cycle of canine fetal fibroblasts was synchronized by culturing to contact inhibition, serum starvation and treating with acteoside. Cell cycle synchronization, apoptosis and reactive oxygen species (ROS) detection were analyzed using flow cytometry. The fibroblasts, prepared by confluent-cell culture or treating with 30 μM acteoside for 48 h, were reconstructed in enucleated oocytes. Embryos using acteoside-treated cell were surgically transferred into oviducts of estrus cycle synchronized recipient dogs. Based on flow cytometry, there was no significant difference between serum starvation (88.2%), contact inhibition (84.6%) and most acteoside groups (82.43–84.48%, $P > 0.05$). The rate of ROS and apoptosis in acteoside group (42.75 and 4.17%) showed significantly decrease between contact inhibition (54.33 and 10.63%) and serum starvation (99.5 and 62.37%). After SCNT, fusion rate of acteoside group was 66.7%, which was higher than those of contact inhibition (52.5%). Developmental rate to the 8 cell and 10 cell stages was higher in acteoside-treated groups (14.8 and 4%) than those of contact inhibition (5 and 0%). Total 38 SCNT embryos using acteoside-treated fibroblasts were transferred into three recipient dogs and one recipient finally delivered one puppy. In conclusion, this study demonstrated that canine fibroblasts could be arrested at the G0/G1 stage with reduced ROS and apoptosis after acteoside treatment. And reconstructed canine embryos with acteoside-treated cells forwarded subsequent embryo development. These results may contribute to improve the efficiency of canine SCNT. This research was supported by 'Cooperative Research Program for Agriculture Science & Technology Development (No. PJ009333)', Rural Development Administration, Republic of Korea.

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P087

Semi-quantification and elucidation of bovine embryo biomarkers by mass spectrometry imaging

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In the field of 'single cell analysis', many classical strategies like immunofluorescence and electron microscopy are the fundamental choices. However, these methodologies are time-consuming and do not permit direct identification of specific molecular classes such as lipids. A novel mass spectrometry-based analytical approach has been applied to bovine oocytes and embryo. This new metabolomics-based application uses mass spectrometry imaging (MSI), efficient data processing and multivariate data analysis. Metabolic fingerprinting (MF) was applied for the analysis 2-, 4- and 8-cell embryos. Additionally, a semi-quantitative strategy for sphingomyelin [SM (16:0) Na]+ (m/z 725) and phosphatidylcholine [PC (32:0) Na]+ (m/z 756) was developed, showing that lipid concentration was important in order to elect the best biomarkers. This report demonstrates that the combination between MF strategy along with MSI feature and chemometric analysis can be applied in discriminating embryo stages, characterizing specific biomarkers and relating them to pathways that allow the understanding pre-implantation events during bovine embryo development.

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P088

Artificial activation enhances pre-implantation embryo development after intracytoplasmic injected with evaporative-dried sperm

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Evaporative-drying is one alternative to cryopreservation. It has the advantages that during sample preparation it does not require liquid nitrogen and a freeze-dryer machine; however, the results have not been as successful as freeze-drying. The objectives of this study were to determine whether artificial activation (electrical and chemical activation) could improve the development of porcine embryos *in vitro* after intracytoplasmic injected with evaporative-dried sperm, to optimize the activation protocol for porcine ICSI. The results show that, cleavage and blastocyst rate were significantly improved by oocytes activated with ionomycin for 5 min followed by treatment with 6-DMAP for 4 h (81.0 and 17.4%) or with an output voltage of 1.1 KV/cm for 30 μs (85.5 and 24.0%) after ICSI. When the oocytes activated with combination of chemical and electrical activation significantly increased activation, pronuclear formation, and cleavage, blastocyst formation rates. The diploidy of blastocysts was no significant difference in ICSI groups. Finally, we found that total apoptosis, fragmentation and TUNEL index of blastocysts were significantly decreased, the total cell number was significantly increased and ROS level did not increased when the oocytes underwent artificial activation stimuli. These results collectively indicate that artificial activation after ICSI is effective in elevating the embryos development, and improves the quality of blastocysts, while ensuring normal chromosome composition. Our findings suggest that artificial activation might contribute to the optimization of techniques for *in vitro* production of porcine embryos. This study was supported by a grant from the Next-Generation BioGreen 21 program (No.PJ009624) funded by Rural Development Administration, Republic of Korea.

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P089

Construction of porcine FoxN1 knockout system by RNA-guided endonucleases

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Introduction
Forkhead box protein N1 (FoxN1) regulates development, differentiation, and function of thymus epithelial cells (TECs), both in the prenatal and postnatal thymus. In mice, knockout of the FoxN1 results in two well-known defects as hairlessness and athymia. However, knockout of FoxN1 in other species has not been discovered yet. RNA-guided endonucleases (RGENs), derived from the prokaryotic type II CRISPR-Cas system, enable targeted specific genes. We successfully targeted FoxN1 in pig by using CRISPR-Cas system.
Materials and methods
RGEN design: We designed specific single-guide RNA (sgRNAs) which targets exon 2 of porcine FoxN1, and constructed Cas9 vector system.
Transfection and Microinjection: Cas9-FoxN1 vectors were transfected intoporcine fibroblastusing lipofectamin 2000 reagent according to manufacturer's protocol. Also to induce parthenogenesis, Cas9-FoxN1 vectors (4, 8, and 16 ng/μl) were microinjected into the porcineoocytes and these were activated by BTX.
T7 endonuclease I assay: The region of DNA containing the target site was amplified by PCR using the specific primer set. The PCR products were hybridized to form heteroduplex DNA, and digested by T7 endonuclease I for 1 h at 37 °C. The DNA was analyzed by gel electrophoresis using 2% agarose gel.
Results and discussion
To confirm the knockout of FoxN1, we analyzed the DNA cleavage activities by T7 endonuclease I assay both fibroblasts and oocytes. As a result, there was no significantly difference in knockout efficiency among injected DNA concentrations, 4 ng (20.7%), 8 ng (15.4%) and 16 ng (20%), respectively. However, we checked that Cas9-FoxN1 could successfully knockout the porcine FoxN1. This study suggests that knockout pig is also producible using Cas9-system by DNA microinjection into zygotes, directly.

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P090

Expression pattern and function of claudins during tight junction maturation in the mouse pre-implantation embryo

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Introduction
The pre-implantation embryo has two distinct cell lineages at the blastocyst stage. An outer epithelial monolayer called trophectoderm and the non-polar inner cell mass. Tight junctions (TJ) form continuous intercellular contacts between neighbouring trophectoderm cells and are crucial in establishing a blastocoele cavity. TJs are composed of several transmembrane proteins including Claudins, a large family of proteins that play a central role in the formation of the epithelial barrier. Claudins (Cl) show distinct distribution patterns among epithelial tissues and several isotypes can exist in the same TJ.
Methods
mRNA extracted from different stages of mouse embryos was reverse-transcribed before amplification of cDNA for particular Claudin isotypes. Embryos at 2-cell, 8-cell, morula and blastocyst stage were fixed for immunofluorescence and confocal microscopy. Zygotes were microinjected with siRNA and development to blastocyst observed.
Results
RT-PCR showed that Cl-4 and 7 were transcribed from compaction to blastocyst stage, while mRNA for Cl-3 and 6 were present from late morula stage. mRNA for Cl-12 was only present in blastocysts and mRNA for Cl-2 and 15 was absent throughout. Immunofluorescence showed Cl-4 and 7 proteins assembling at cell–cell contacts after compaction in a dis-continuous pattern. By late blastocyst stage, Cl-3, 4, and 7 all displayed continuous staining at trophectoderm TJs, whilst Cl-12 and 15 were absent at TJs. siRNA inhibition of Cl-4 and 6 reduced percentage of blastocysts formed, whilst cavitation was not affected by Cl-7 siRNA. Our study shows that five different claudins are expressed in the early embryo and two play a crucial role in blastocyst formation. Funding by BBSRC to TPF.

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P091

Human granulocyte–colony stimulating factor enhances viability of porcine embryos in defined oocyte maturation medium

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Granulocyte colony-stimulating factor (G-CSF) enhances the proliferation, differentiation and survival of cells. In addition, G-CSF is a non-invasive biomarker of human oocyte developmental competence for embryo implantation. In human follicle G-CSF concentration increased during the ovulatory. At present experiment, the cumulus oocyte complexes (COCs) were aspirated from superficial follicles (1–3 and 3–6 mm). COCs from small and medium follicle were matured in protein-free maturation medium supplemented with concentrations of hG-CSF (0, 10 and 100 ng/ml). After 44 h of IVM, the ratios of nuclear maturation have no difference. The intracellular ROS levels of oocytes from both follicle groups matured with 10 ng/ml were significantly ($P<0.05$) decreased. However, all groups showed no significantly difference in GSH levels. After PA, the cleavage and blastocyst stage was significantly ($P<0.05$) high in 100 ng/ml with small and 10 ng/ml with medium follicle. Cell numbers of blastocyst from both follicle groups were significantly high in 10 ng/ml. After IVF, the blastocyst stage was significantly ($P<0.05$) increased in 10 ng/ml with medium follicle. Monospermy and fertilization efficiency were significantly high in the 100 ng/ml of small and 10 ng/ml of medium follicle. We examined the *Has2* and *Bax* transcript levels were significantly decreased in cumulus cells from small follicle treatment and the *Bcl2* and *ERK2* were significantly increased from medium follicle treatment. In conclusion, these results indicate that hG-CSF improve the viability of porcine embryos. Research supported, in part, by a grant from the National Research Foundation of Korea Grant Government (NRF-2012R1A1A4A01004885, NRF-2013R1A2A2A04008751), Republic of Korea.

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P092

Chromatin remodelling and histones mRNA accumulation in bovine germinal vesicle oocyte

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In several mammalian species, a major remodelling of the germinal vesicle chromatin is known to occur towards the end of the oocyte growth. Various chromatin configurations have been identified. However, the mechanisms involved in this remodelling process are yet not completely understood. In the bovine species, four distinct and progressive states of chromatin compaction have been characterized and are linked to a gradual acquisition of the developmental potential. Germinal vesicle oocytes were collected and separated in four groups according to their degree of chromatin condensation, ranging from a diffused state to a fully compacted configuration. To better understand the molecular changes undergoing in the oocyte during that critical period, transcriptomic analysis was performed with the EmbryoGENE microarray platform (custom Agilent 44K) in order to identify mRNA modulations occurring during the remodelling process. An important proportion of genes showed a reduced mRNA level as the chromatin becomes more compacted, which strongly correlates with the decreased transcriptional activity at the end of oocyte growth. However, among the transcripts presenting an increased mRNA level, many were associated with the histone genes. Depending on the specific histone (H2A, H2B, H3, H4 or linker H1), an important mRNA accumulation occurs in the oocyte before ovulation. This dataset then offers a unique opportunity to picture the stock of accumulated histone mRNAs either to complete the build-up of a compacted chromatin, but also to ensure the protamine-histone replacement following fertilization and the completion of the first three cell cycles.

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P093

Effect of ganglioside GT1b treatment during porcine *in vitro* maturation on embryonic development and mRNA expression pattern in cumulus cell

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Ganglioside is an acidic glycosphingolipid with sialic acid residues. The b-series ganglioside GT1b was reported that suppressing damage of mtDNA by reactive oxygen species (ROS) in mouse brain. The purpose of this study is to investigate the effect of exogenous addition of GT1b on *in vitro* maturation (IVM) of porcine oocytes and to confirm the related bradykinin 2 receptors (B2R). GT1b were treated on IVM that concentration was 0 (control), 5, 10 and 20 nM. After IVM, we evaluated intracellular ROS levels in matured oocytes. The 10 and 20 nM groups showed a significant ($P<0.05$) decrease in intracellular ROS levels compared with control group. To analyze the mechanism of GT1b effect on IVM, we identified the existence of B2R known to increase calcium concentration stimulated by GT1b. As a result, we identified the expression of B2R in cumulus cell but not in oocyte. To examine the expression of apoptosis-associated genes (Bax, BCl2, Caspase-3), B2R and CaMK2G gene in matured cumulus cells. The treatment of 20 nM GT1b significantly ($P<0.05$) decreased the expression of PCNA and CaMK2G, and BCl2, an anti-apoptotic gene, was increased significantly ($P<0.06$). GT1b significantly ($P<0.05$) decreased expression of B2R. In conclusion, these results indicated that GT1b may play an important role in decreasing the intracellular ROS levels during IVM. Further studies are needed to show the mRNA expression of apoptosis-associated genes and CaMK2G in matured oocytes. This work was supported, in part, by a grant from the National Research Foundation of Korea Grant funded by the Korean Government (NRF-2012R1A1A4A01004885, NRF-2013R1A2A2A04008751), Republic of Korea.

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P094

The antioxidative effect of carboxyethylgermaniumsesquioxide (Ge-132) on *in vitro* maturation of porcine oocytes and subsequent embryonic development after *in vitro* fertilization and parthenogenetic activation

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The carboxyethylgermaniumsesquioxide (Ge-132) is an organogermanium compound known to have anti-oxidative effect. In this study, we examined the effect of Ge-132 on *in vitro* maturation (IVM) of porcine oocytes analyzing intracellular glutathione (GSH) and reactive oxygen species (ROS) levels, mRNA expression levels and subsequent embryonic development after *in vitro* fertilization (IVF) and parthenogenetic activation (PA). After 40 h of IVM, intracellular GSH levels in oocytes treated with 200 μg/ml Ge-132 increased significantly ($P<0.05$) and the 200- and 400 μg/ml Ge-132 treated groups showed a significant ($P<0.05$) decrease in intracellular ROS levels compared with the control. Oocytes matured with 200 μg/ml Ge-132 during IVM had higher total cell numbers after IVF than the control group. Furthermore, oocytes matured with 200- and 400 μg/ml Ge-132 during IVM had significantly higher cleavage rates and the 200 μg/ml Ge-132 treated group had higher blastocyst formation rates and total cell numbers after PA than the control group. We evaluated mRNA expression levels of *PCNA* and *Nrf-2* in the cumulus cells of each group. The 100- and 400 μg/ml Ge-132 group showed significantly higher mRNA expression levels of *PCNA* ($P<0.05$) and *Nrf-2* ($P=0.062$) respectively compared to those of the control group. Our results suggested that Ge-132 treatment during IVM improved the developmental potential of PA and IVF porcine embryos by decreasing the intracellular GSH level, there by increasing the intracellular ROS level and regulating gene expression of cumulus cells during oocyte maturation. This work was supported, in part, by a grant from the National Research Foundation of Korea Grant Government (NRF-2012R1A1A4A01004885, NRF-2013R1A2A2A04008751), Republic of Korea.

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P095

The effect of vitrification on subsequent mouse embryo development and postnatal health

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Short-term effects of cryopreservation on embryo development, survival and after embryo transfer (ET), resulting pregnancy rate have been documented. However, long-term effects on offspring remain relatively unexplored.

Materials and methods

Using a mouse model, we investigated the effect of vitrification on blastocyst trophectoderm (TE) and inner cell mass (ICM) cell numbers by differential staining and postnatal growth and cardiovascular and metabolic health. Embryos were vitrified with equilibration (8 min) and vitrification media (1 min) followed by vitrification with liquid nitrogen. Embryos were warmed subsequently with thawing solutions (5 min) and cultured until blastocyst before ET. Offspring were weighed weekly, monitored for systolic blood pressure (SBP; weeks 9, 15, 21) and culled for organ allometry and serum collection (week 27).

Results and discussion

There is no significant differences ($P<0.05$) on survival rate between control ($n=50$) or vitrified ($n=70$) embryos. Vitrification reduced TE and ICM cells compared to controls but not significantly. Currently, offspring body weight between naturally-mated (controls; $n=81$), non-vitrified ET (IVC-ET; $n=37$) and vitrified ET (IVFr-ET; $n=25$) showed significant differences from week 3–8 to week 3–5 ($P<0.05$); respectively. Mean SBP showed no significant differences ($P>0.05$) in male and female offspring between control, IVC-ET and IVFr-ET treatments in week 9. We conclude that embryo vitrification may affect long-term growth of offspring into adulthood. Funding EU FP7 EpiHealth and MARA, Malaysia.

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P096

Insulin and branched-chain amino acid depletion during mouse *in vitro* preimplantation development alters postnatal growth and cardiovascular physiology

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Introduction

Maternal depletion of insulin in serum and branched-chain amino acids (BCAA) in uterine luminal fluid occurs in response to dietary protein undernutrition during the periconceptional period which leads to adverse offspring growth and cardiovascular phenotype (e.g. high blood pressure). This study aimed to determine whether insulin and/or BCAA depletion might be inductive *in vivo* factors for postnatal phenotype using an *in vitro* embryo culture model.

Materials and methods

Two-cell embryos were cultured to the blastocyst stage in KSOM medium supplemented with insulin and BCAA at normal (N, 100%) or low (L, 50%) concentrations: N-Insulin + N-bcaa, L-Insulin + N-bcaa, N-Insulin + L-bcaa, and L-Insulin + L-bcaa. Control medium (N-Insulin + N-bcaa) was supplemented with serum insulin (1 ng/ml) and uterine luminal fluid concentrations of 19 amino acids, including BCAA (valine (0.46 mM), isoleucine (0.21 mM), leucine (0.32 mM)), found in well-fed mice. Blastocysts were transferred to pseudopregnant recipients.

Results and discussion

Males and females from the L-Insulin + L-bcaa group showed an increased birth weight and higher body weight at weeks 5–7 and 4–6 in males and females respectively compared to the control group. Mean blood pressure (taken at weeks 9, 15 and 21) was not affected in females. However, males from the L-Insulin + L-bcaa group showed a higher mean blood pressure than controls at week 9. Our data show that the combined depletion of insulin and BCAA during early embryo development can induce increased growth and high blood pressure in offspring during early postnatal development, independent of litter size, mimicking in broad terms effects caused by maternal dietary protein restriction. Funded by BBSRC BB/1001840/1.

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P097

Supplementation with sunflower seed increases conception rates in recipient beef heifers

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In a previous study (Peres *et al.*, 2008 *Acta Scientiae Veterinariae* **36** 639), sunflower supplementation during 22 days improved conception rates by 20.4% in Timed AI programs in beef cows. We aimed to evaluate the effects of sunflower seed supplementation on circulating cholesterol (CHOL) and progesterone (P_4) concentrations, and the conception rate of recipient beef heifers after timed embryo transfer (TET). Heifers were synchronized using a P_4/estradiol-based TET protocol. After device removal (D−2; D0= 2 days after P_4 device removal), heifers receive 1.7 kg/animal per day of 40% soybean meal 44% crude protein (CP) and 60% sunflower seed (Sunflower Group; $n=106$) or 53% of soybean meal with 44% CP and 47% corn (Control Group; $n=111$) for 22 days (from D−2 to D19), both balanced with 72% TDN and 24% CP. Heifers received an *in vitro*-produced embryo on D7. Conception rate on D30 was greater ($P=0.01$) in the Sunflower Group (55.66%) than in the Control Group (36.94%). Greater ($P<0.04$) CHOL concentration was observed in the Sunflower Group than in the Control Group on D7 (306.0±11.6 vs 277.1±11.9 mg/dl, respectively) and on D19 (260.5±8.0 vs 232.0±8.0 mg/dl, respectively). Plasma P_4 concentrations were not different between groups on D7, but were greater ($P<0.0001$) in the Sunflower Group (5.75±0.39) than in the Control Group (3.48±0.38 ng/ml) on D19. These results indicated that sunflower seed supplementation also increases conception rate in recipient beef heifers after TET.

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P098

The role of vitamin C in *in vitro* early embryonic development: verification using aldehyde reductase gene knockout mice

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Introduction
Supplementation of appropriate amount of vitamin C (VC) improves oocyte fertilization and subsequent embryo development while high concentrations of VC rather reduce the developmental potency. To clarify the role of VC, oocytes were collected from aldehyde reductase (AKR1A) knockout mice that are unable to synthesize VC and subjected to analyses from view point of developmental potency and gene expression involved in VC metabolism.
Materials and methods
Three to 4 -week-old WT and AKR1A knockout (AKR1A-KO) female ICR mice were injected i.p. with each adequate hormones, and then super-ovulated oocytes and immature oocytes were collected. Oocytes at the MII stage were obtained after *in vivo* maturation or *in vitro* maturation (IVM). Expressions of mRNA for genes involved in VC biosynthesis and VC transport were quantified. VC contents of the oocytes were determined.
Results and discussion
IVM oocytes showed lower VC contents than *in vivo*-matured oocytes (0.05 pmol/oocyte) regardless of their genotypes. Concerning the *in vivo*-matured oocytes, VC contents in the AKR1AKO oocytes were significantly lower compared with those in the WT oocytes (0.1 and 1.3 pmol/oocyte, respectively). However after IVF, no difference was observed in the developmental potency in *in vivo*-matured oocytes. IVM oocytes from AKR1A-KO mice developed with significantly higher rate than those from WT mice. Expression levels of VC biosynthesis-related genes were not different between *in vivo*-matured and IVM oocytes. mRNA of VC transporter SVCT1 was expressed significantly higher in the IVM WT oocytes than other groups. These results suggest that VC within the oocytes may not directly support the early embryonic development, but instead VC plays beneficial roles in the culture medium most likely by its antioxidative function.

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P099

Selection of reference genes in mouse preimplantation embryos of different ploidies at various developmental stages

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Real-time RT quantitative PCR (qPCR) has become the most frequently used system for studies of gene expression. Many studies have provided reliable evidences that the transcription levels of reference genes are not constant at different developmental stages and in different experimental conditions. However, suitable reference genes which are stably expressed in polyploid preimplantation embryos of different developmental stages have not yet been identified. In the present study, we examined the expression levels of 12 candidate reference genes in preimplantation embryos of four different ploidies at six developmental stages. Stability analysis of the reference genes was performed by four independent software programs, namely, geNorm, NormFinder, the comparative ΔC_t method, and RefFinder. The stability of the selected three reference genes was evaluated by comparison with the *Oct4* expression level during preimplantation development in diploid embryos. The expression levels of most genes in the polyploid embryos were higher than that in the diploid embryos, but increasing degree were disproportionate with the ploidies. There were no significant differences in reference genes expression among embryos of different ploidies when they reached the morula stage, and the expression level remained flat until the blastocyst stage. We conclude that *Ubc*, *Ppia*, and *Pgk1* are the most stable reference genes for gene expression analysis of mouse diploid and polyploid preimplantation stage embryos.

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P100

Competence of porcine first polar body for normal development

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To determine whether chromosomes in porcine first polar body (PB1) can complete the second meiotic division and participate in subsequent normal embryo development, we examined developed competence of chromosome in PB1 that had been injected into enucleated MII stage oocytes by nuclear transfer method (chromosomes replacement group, CR group). After parthenogenetic activation (PA) and *in vitro* fertilization (IVF), the cleavage rate of reconstructed oocytes in IVF group (CR-IVF group, 34.2±3.5%) was significantly lower than in PA group (CR-PA group, 54.7±8.2%). The blastocyst formation rate was 7.0% in CR-PA group, but there was no blastocyst formation in the CR-IVF group. For producing tetraploid parthenogenetic embryos, intact MII stage oocytes injected with chromosomes in PB1 were electrically stimulated, treated with 7.5 μg/ml cytochalasin B for 3 h (MII oocyte+PB1+CB group), and then cultured without cytochalasin B. The average cleavage rate of reconstructed oocytes was 72.5% (48 of 66), and blastocyst formation rate was 18.7% (nine of 48). Chromosome analysis was shown that the similar proportion of chromosome status (haploid and diploid) were found between control (normal MII oocyte) and CR groups after PA. Overall 23.6% of blastocysts were tetraploid in MII oocyte+PB1+CB group. Our results demonstrated that chromosomes in porcine first polar body can participate in normal embryo development when injected into enucleated MII stage oocyte; Tetraploid PA blastocyst were produced when PB1 chromosomes were injected into intact MII stage oocyte, although the proportion of tetraploid was low. This result indicates that chromosomes within the first polar bodies can participate in embryonic development in intact MII stage oocyte.

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P101

Association between birth weight and age at menarche: a meta-analysis

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Low weight at birth may affect the onset of sexual maturation. Early puberty timing in those being small for gestational age might be explained by fetal

reprogramming that helps to adapt for the poor-resource postnatal life and be ready earlier for reproduction. Biological plausibility for this association prompted for many epidemiological studies aimed to test a relationship between low birth weight and age at menarche (AAM) as a reliable indicator of puberty timing.
Materials and methods
Following the MOOSE guidelines for meta-analysis of observational studies, we found 27 relevant studies in the MEDLINE database. Among them, we identified seven studies with the data of good quality where birth weight was assigned as continuous variable and AAM was treated as categorical variable. The total sample included 1232 subjects with early and 2214 with late AAM. R statistical software was used for analysis. Due to the high heterogeneity of the data, the random-effects model was used.
Results and discussion
The meta-analysis under the random-effects model showed no significant association between birth weight and AAM (std diff $= -0.503$, $P = 0.409$). Although this meta-analysis did not confirm the association between birth weight and AAM, but it helped to clarify problems with data and the design of studies that had been analysed. It seems that the real effect of low birth weight was underestimated due to misclassification of early and late AAM. We found that the data gave conflicting results due to the inappropriate study design, insufficient adjustments and lack of biological plausibility to establish causality.

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P102

Effect of downregulating GLIS1 transcript on early development and gene expression of bovine embryos

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GLIS1 markedly enhances the generation of iPS cells from both mouse and human fibroblasts. In mouse, *GLIS1* is enriched in unfertilized oocytes and 1-cell stage embryos. Therefore, it is possible that GLIS1 plays a critical role in early development through epigenetic regulation. We investigated the role of GLIS1 during the early development of bovine embryos using siRNAs targeted for GLIS1. *GLIS1* transcript levels in unfertilized oocytes and early embryos at 1-cell to blastocyst stage were evaluated. GLIS1 siRNA (B-GLIS1-1 or B-GLIS1-2) or nonsilencing (control) siRNA were injected at the 1-cell stage. Some embryos were not injected with siRNA (Uninjected). *PGK1*, *XIST*, *PDHA1* and *HSPA8* levels at 8- to 16-cell stage were evaluated. Higher levels of *GLIS1* were detected in unfertilized oocytes, 1-cell and 2-cell stage embryos. *GLIS1* transcripts were decreased at 4- to 8-cell stages. No difference in developmental rate for the 2-cell to 16-cell stage was observed between GLIS1 siRNA injected and control embryos (Uninjected and control siRNA injected). However, B-GLIS1-1 injected embryos showed developmental arrest at 16-cell stage. The blastocyst rate of B-GLIS1-1 injected embryos (6.5%) was significantly ($P < 0.05$) lower than those of the uninjected (43.9%), B-GLIS1-2 (37.2%) and control siRNA (34.5%) injected embryos. *PGK1* and *PDHA1* levels in B-GLIS1-1 injected embryos were lower ($P < 0.05$) than uninjected embryos. Similarly, *HSPA8* transcript was decreased in B-GLIS1-1 injected embryos. Our results indicate the critical importance of GLIS1 for early development of bovine embryos, and raise the possibility that GLIS1 is an important factor for epigenetic regulation of bovine embryos.

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P103

Genome-wide reprogramming by DNA demethylation during mouse oocyte growth and early development

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Introduction
Genome-wide demethylation reprograms the genome after fertilization and re-establishes totipotency: 5-methylcytosine (5 mC) in the paternal pronucleus is rapidly converted to 5-hydroxymethylcytosine (5 hmC) by the dioxygenase 10–11 translocation (TET) 3, while 5 mC in the maternal pronucleus is protected by the binding of developmental pluripotency-associated (DPPA) 3 to histone H3 dimethyl Lys9 (H3K9me2). This process is essential for normal development, because deletion of TET3 or DPPA3 in the maternal germline alters 5 mC and 5 hmC levels in parental pronuclei, resulting in lethality. This study examined the developmental significance of demethylation events by evaluating 5 hmC and H3K9me2 levels in oocytes and preimplantation embryos.
Materials and methods
Oocytes and embryos were fixed, pretreated with 2N HCl, and labeled with primary antibodies against 5 mC, 5 hmC, and H3K9me2, and then washed and treated with fluorophore-conjugated secondary antibodies and visualized by confocal microscopy.
Results and discussion
The signal of 5 hmC was detected at heterochromatin regions from growing stage oocytes, and the signal was strengthened up to fully grown oocytes, spreading into whole chromatin. Interestingly, 5 hmC was mainly localized to retrotransposon regions such as IAP and MLV during oogenesis, but was restricted to the male pronucleus in zygotes, and was not detected after the 4-cell stage. In contrast, the H3K9me2 signal was constant during early development, and was associated with a copy number increase via retrotransposon reactivation. These results suggest that demethylation process of female genome begin from oocyte growth stage, and the conversion of 5 mC to 5 hmC contributes to retrotransposon activation after fertilization.

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P104

Expression profile of imprinted genes and DNA methyl transferases in uniparental sheep placenta

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Epigenetic programming has a crucial role in mammalian development. It is well described that disarrangement of epigenetic programming is associated with impaired embryonic development, compromised pregnancy (ie. abortion or preeclampsia) and several imprinting disorders (i.e Prader Willi and Beckwith-Wiedemann syndromes). A useful tool to better understand the etiology of compromised pregnancy is represented by uniparental embryos, characterized by only maternal (parthenogenotes – PAR) or only paternal (androgenotes – AND) genome, not compatible with full-term development. To this aim, we *in vitro* produced uniparental (AND, PAR) and biparental (*in vitro* fertilized Control – CTR) sheep embryos, transferred blastocyst stage embryos in recipient female and subsequently collected early placenta tissues at 20 day of pregnancy. Subsequently, we evaluated by qPCR 1) the expression of a selected panel of maternally (H19, IGF2R) and paternally (IGF2, MEST, DLK1) expressed imprinted genes and 2) the expression of the main enzymes involved in DNA methylation maintenance (DNMT1) and *de novo* methylation (DNMT3A, DNMT3B). Our data revealed that imprinted genes were expressed according to uniparental genomic composition (only maternal – PAR, only paternal AND). Moreover we observed that DNMTs mRNA levels were mainly upregulated in PAR placenta ($P < 0.05$) compared to AND and CTR ones. Taken together, these results revealed that the parental specific expression of the analyzed imprinted genes is maintained in sheep placenta and suggested a possible rescue mechanism to correct improper epigenetic programming in PAR tissues but not in AND one, probably because at day 20 of pregnancy they are severely compromised and failed further development.

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P105

Genome editing technology using ZFN and TALEN is effective on cultured porcine ST cells knockout pig

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In recent years, advances in molecular biology and reproductive engineering make generation of knockout (KO) animals possible. KO animals are useful and powerful tools for agriculture as well as biomedical research. Zinc finger nuclease

(ZFN) and transcription activator-like effector nuclease (TALEN) genome editing technology enables site directed engineering of the genome. The aim of the present study was to ZFN and TALEN system could work on editing genome of cultured porcine Sertoli (ST) cells. DNAs of the ZFN or the TALEN were designed for introducing mutations to the genomes of GH receptor (GHR) and myostatin (MSTN). The ST cells were cultured *in vitro* for 24 h at 38.5 °C, 5% CO_2 and saturated humidity. The transfections of DNAs of the ZFN or the TALEN into ST cells were performed by using Lipofectamine™ LTX. We examined whether the transfections were successful or not by immunostaining with anti-flag ABM2, and that gene mutations were identified by using detection kit (Cel I assay). The results of immunostaining regardless of the target in both ZFN and TALEN, showed introduction into about 10% of the ST cells was observed, and that we confirmed the mutation by using Cel I assay. Genome editing technologies using the ZFN and TALEN are effective on cultured porcine ST cells.

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P106

Genome-wide DNA methylation analysis of bovine clones derived from the same donor cells

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Introduction
What is nature versus nurture? To what extent do genetic inheritance and non-genetic factors contribute to one's character? This is one of the oldest issues in psychology. To answer this historic question, cloned animals are good models because they have identical genetic information to the donors. Previous studies indicated that cloned animals showed different coat color patterns, noseprints and characters compared to the donors. However, no molecular work or genome-wide analysis has been done using genetically identical clones. Here we analyzed DNA methylation patterns of cloned cows derived from the same donor cells and compared with those of non-clones, to describe epigenetic differences in the same genetic background (clones) or in a different genetic background (non-clones).
Materials and Methods
Five cloned cows (Japanese Black, age: 68 to 82 months) and six non-cloned cows (Japanese Black, age: 52 to 129 months) were used for the study. Genomic DNA was extracted from various tissues and DNA methylation analysis was carried out by two methods. Imprinted genes H19, PEG3 and XIST were analyzed by bisulfite sequencing method. Genome-wide DNA methylation analysis (in liver tissues only) was carried out by MeDIP-chip (Methylated DNA Immunoprecipitation microarray) method.
Results and Discussion
By bisulfite sequencing experiments, the variation in DNA methylation levels is not different between clones and non-clones in most of all tissues and genes analyzed. However, MeDIP-chip analysis indicated more variability in non-clones, suggesting that genetic variation influences epigenetic differences. The identification and validation of differentially methylated regions are currently under way.

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P107

DNA methylation analysis in sperm from boars exhibiting normal and altered spermograms

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In the last decade, more and more studies have reported aberrant pattern of methylation in sperm DNA of patients with an altered spermogram. It is also admitted that epigenetic reprogramming during germ cell development is a key mechanism for the production of functional gametes and the proper development of the embryo. As poor semen quality is also a key issue for farm animals' productivity, we studied the methylation profile of pig sperm DNA of fertile and poor-quality sperm boars. We first described the methylome of control sperm cells using MeDIP-Seq. We then focused on the methylation level of imprinted genes in poor-quality semen by MeDIP-qPCR analysis, and then perform single base methylation analysis by bisulfite conversion and pyrosequencing in regions of interest. Methylome analysis and comparison with mouse and human data revealed the high conservation of the methylation pattern of sperm cells between mammals: sperm DNA is highly methylated with the exception of CpG islands and promoters. We nevertheless observed some discrepancies between species, like in the promoter of the developmental gene POU5F1 which was highly methylated in human sperm only, suggesting a different dynamics of activation of this gene following fertilization. Global methylation level, assayed by LUMA, did not vary between control and poor-quality sperm DNA but local analysis revealed an increased methylation level in the promoter of NESP55 and GNASXL transcripts of the GNAS locus in some boars presenting an altered spermogram, indicating a possible new role for this locus in the development of efficient gametes.

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P108

Effect of the way in which ewes were handled during mid-gestation on testicular and ovarian development in their lambs

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Introduction
This study determined whether the way in which pregnant ewes were handled affected gonad development in their offspring.
Methods
Twin-bearing Scottish Blackface × Border Leicester ewes were either handled minimally (control, C, $n=20$), positively (predictable timings, soft vocal tones etc. P, $n=20$) or negatively (random timings, shouting and whistling etc. N, $n=20$) between day 65 and 100 of pregnancy. Following euthanasia of a sub-set of lambs, gonads were collected from one lamb on the day of birth and from its twin at 7 months of age for histological analyses. Oestrous cycles of 7 month old female lambs were synchronised and ovaries collected on Day 8 ± 1 of the following cycle. Cell proliferation was assessed by Ki-67 immunohistochemistry. Data were analysed using ANOVA.
Results and Discussion
There was no effect of prenatal treatment on ovarian or testicular morphology at birth. At 7 months, ovaries from all female P lambs contained antral follicles, whereas no antral follicles were observed in ovaries from C or N lambs. Seminiferous tubular area was greater in testes from 7 month old N ram lambs compared to P or C lambs (80146, 45561, 46409 μm^2, for N, C and P, respectively s.e.d. $=10974$; $P=0.045$). N lambs had a higher percentage of proliferating testis cells compared to P lambs (4.5 ± 0.8 vs 1.8 ± 0.9% respectively; $P=0.049$). In summary, prenatal handling had no effect on gonad morphology at birth, but gonad development in post-pubertal animals was altered, with gender differences in response.
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P109

Postovulatory ageing influences methylation marks and development in bovine oocytes and early embryos

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Introduction
Oocytes that are not fertilized in a specific time-frame after ovulation undergo a time-dependent process of degradation and loss of developmental potential that

can be referred to as postovulatory ageing. This process occurs *in vivo* and under specific *in vitro* settings. Here, we studied epigenetic changes related to postovulatory ageing.
Materials and methods
Bovine oocytes were collected from slaughterhouse ovaries and matured *in vitro* either for 24 h (standard protocol) or 48 h (postovulatory ageing) in TCM199 medium supplemented with BSA and Suigonan®. Maturation, fertilization, cleavage and blastocyst rates were determined. Correct spindle formation was determined by immunhistochemistry. Matured oocytes and 4-8 cell stage embryos were used for gene specific methylation analysis using the limiting dilution assay and direct sequencing. RT-qPCR was performed with developmentally important genes in single oocytes and early embryos.
Results and discussion
Maturation rates did not differ between the two groups; spindle formation was not impaired, but aged oocytes showed distinct detachment of cumulus cells. Fertilization rates did not differ significantly between the two groups; presumptive zygotes derived from aged oocytes showed significantly reduced cleavage rates and very low development to the blastocyst stage (1.3%). Analysis of relative transcript abundance of a panel of genes did not reveal significant differences. Gene specific methylation analysis of DNMT3Lo revealed a significant increase of aberrantly methylated alleles in postovulatory aged oocytes ($P=0.03$), other imprinted and non-imprinted genes were not affected. Results indicate that postovulatory ageing severely influences development and may affect offspring due to aberrant epigenetic marks.

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P110

Epigenetic regulation of Gata-6 transcription factor expression in mouse primitive endoderm extra-embryonic lineage induced by maternal protein restricted diet

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Introduction
Maternal low protein diet exclusively during preimplantation mouse development (Emb-LPD) induces compensatory changes in extra-embryonic lineages including primitive endoderm (PE) to enhance nutrient delivery during later gestation, for example, stimulation of endocytosis. Whilst such responses protect fetal growth, adult offspring exhibit increased cardiometabolic disease risk. Here, we investigate epigenetic mechanisms underlying maternal dietary programming, using embryonic stem (ES) cell lines derived from Emb-LPD and control blastocysts to assess expression and histone epigenetic regulation of Gata-6 and Gata-4, lineage-specific transcription factors for PE differentiation.
Materials and methods
Mouse ES cell lines were derived from blastocysts from each diet treatment and used for embryoid body (EB) formation. qPCR, immunoblotting, fast chromatin immunoprecipitation (ChIP) and siRNA protocols were undertaken with appropriate normalisation and technical controls.
Results and discussion
Emb-LPD EBs with outer PE proliferate to larger diameter than control EBs. Gata-6 down-regulation is known to stimulate proliferation and reduce differentiation in cancer and cardiomyocyte models. Gata-6 mRNA and protein expression are down-regulated in Emb-LPD EBs and conceptus yolk sac with similar trend for Gata-4. ChIP analysis revealed Gata-6 promoter in Emb-LPD EBs was hypoacetylated for histone H3 and H4, reduced in H3K4Me3 and associated RNA polymerase II versus control EBs, all recognised mechanisms to suppress gene expression. Histone deacetylase (HDAC-1) expression was elevated in Emb-LPD EBs which may contribute to Gata-6 hypoacetylation and reduced expression, currently investigated by HDAC-1 siRNA manipulation. Our study indicates extra-embryonic lineage responses to maternal diet are mediated through epigenetic control of key transcription factors.
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P111

Lactation litter size differentially affects satiety hormone concentrations and gut adaptations in Wistar rat dams

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Introduction
Peptide-YY (PYY) is a satiety hormone secreted by the colon. We have previously shown that PYY significantly increases in early lactation in rats, despite hyperphagia. Increased PYY may be associated with significantly increased gut growth by late lactation in dams with average-sized litters. Our aim was to determine whether feeding different litter sizes influences postpartum changes occurring during the maternal adaptation to lactation.
Materials and methods
Wistar rats had litter sizes adjusted to 4, 8 (standard) or 12 pups by 1 day postpartum and were sacrificed at day 25 of lactation. Colonic PYY was quantified using radioimmunoassay. Gut tissue measurements were standardised by free-floating gut for length measurements and using nicardipine for circumference measurements.
Results and discussion
Dams feeding 4 pups had significantly shorter and lighter small and large intestines and lighter caecums compared with dams feeding 8 pups and we hypothesise that this is due to reduced suckling demand. Significantly increased descending colon PYY in dams with 8 pups was associated with increased gut size in these dams. Despite no increase in gut length or wet weight, dams feeding 12 pups had significantly increased gut circumferences, which may represent an adaptation to lactation that requires less energy. Energy insufficiency for structural modifications may be why dams feeding 12 pups showed different adaptations to dams feeding 8 pups. This study is the first time such differential adaptations have been shown in dams due to litter size; further work is being carried out to establish if pups from different sized litters have altered endocrine profiles or gut structure that influence feeding.

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P112

TAF4b promotes oocyte survival and proper ovarian chromatin state in the mouse

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Introduction
The condition of primary ovarian insufficiency (POI) affects 1% of women worldwide under the age of 40, and is associated with premature ovarian follicle depletion. TAF4b is a gonadal-enriched TFIID subunit that promotes healthy ovarian aging and fertility. Female TAF4b-deficient mice experience POI hallmarks including infertility, poor oocyte quality, and dramatic gene expression changes. Understanding TAF4b's role in promoting healthy ovarian aging is critical for future treatments of POI.
Methods
Immunofluorescence – Mouse ovarian sections were fixed with 4% formaldehyde and stained with antibodies against Tra98 and DDX4. They were then incubated with fluorescently-conjugated secondary antibodies for visualization. Neonatal Ovary Culture – Wild-type and TAF4b-deficient mouse ovaries were harvested from embryonic-day 18.5 embryos for organ culture until postnatal-day 2. One ovary per mouse was cultured in 'control media', while the other was cultured in media treated containing ZVAD-fmk or β-estradiol, prior to sectioning and staining.
Results and Discussion
Here we present that TAF4b-deficient ovaries suffer accelerated primordial follicle depletion immediately after birth. This depletion is due to caspase-dependent apoptosis and coincides with delayed cyst nest breakdown. Additionally, germ cell loss in these ovaries can be reduced by culturing embryonic ovaries in the presence of 50 uM pan-caspase inhibitor ZVAD–FMK or 10 nm β-estradiol, further implicating apoptosis as a primary mechanism for germ cell loss. Furthermore, TAF4b-deficient ovaries experience dramatic ovarian epigenetic deregulation as well as gene expression alterations in meiosis and kinetochore assembly genes, all of which may underlie the observed meiotic defects. This research will help refine our understanding of TAF4b's role in normal ovarian aging.

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P113

Combinations of 17β-estradiol and androgen support bovine oocyte growth *in vitro* by maintaining physical connections with granulosa cells

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Introduction
Recent studies have revealed the contribution of steroid hormones to *in vitro* growth of oocytes in different species. However, exactly how these steroid hormones support oocyte growth is not well understood. This study was conducted to examine the effect of various steroid hormones on *in vitro* growth of bovine oocytes and to determine the contribution of steroid hormones to oocyte growth by assessing the connections between oocytes and granulosa cells, transzonal projections (TZPs).
Materials and methods
Oocyte-granulosa cell complexes (OGCs, oocyte: 94.3 ± 0.1 μm) were collected from bovine early antral follicles (0.4–0.7 mm in diameter) and cultured for 14 days with 17β-estradiol (E_2), androstenedione (A_4), testosterone (T) and dihydrotestosterone (DHT) either alone or in combination. After 14 days of culture, oocytes were subjected to *in vitro* maturation culture or fluorescence staining of TZPs.
Results and discussion
During growth culture, OGCs cultured with E_2 showed high survivability. Meanwhile, oocytes grown with androgens matured to metaphase II at high rates in maturation culture. When OGCs cultured with combinations of E_2 and androgens (A_4 or DHT), OGC survivability and maturation rate were increased compared to that cultured with one steroid hormone, and these oocytes grew to the similar size (120.0 ± 0.6 or 118.8 ± 0.7 μm) as *in vivo* grown oocytes. Moreover, the number of TZPs was maintained in these oocytes after growth, while oocytes grown without steroid hormones had few TZPs. The results suggest that steroid hormone support oocyte growth by maintaining oocyte-granulosa cell connections during growth culture. The results also indicate the different roles of steroid hormones in oocyte growth.

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P114

Anti-Mullerian Hormone (AMH) regulates the growth and maturation of primate antral follicles during the menstrual cycle

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Introduction
Studies in primates indicated that AMH promotes preantral follicle growth, but inhibits antral follicle estradiol (E2) production in vitro. Thus, experiments were designed to investigate the role of AMH on primate follicular development in vivo during the spontaneous menstrual cycle.
Materials and methods
Hemi-ovariectomized, adult rhesus macaques ($n = 5$) first received vehicle (control) treatment by intraovarian infusion from cycle day 1–4 to the midcycle E2 peak (follicular phase) via a catheter connected to an osmotic pump placed subcutaneously in the abdomen. After a recuperation cycle, the same ovary was infused with anti-human AMH antibody (AMH-Ab; 800 ng/h). Daily blood samples were collected for E2 and progesterone (P4) assays. Doppler 3D/4D ultrasonography was performed at the midcycle E2 peak to assess antral follicles.
Results and discussion
E2 peak levels tended to be higher during the AMH-Ab treatment compared with controls (251 ± 68 vs 166 ± 18 pg/ml). P4 levels were 2-3-fold higher ($P < 0.05$) at the mid-to-late luteal phase following the AMH-Ab treatment compared with controls. Three of the five AMH-Ab-treated ovaries displayed multiple ($n = 2$–9) medium-to-large (up to 8 mm) antral follicles at the midcycle E2 peak, whereas only one large (4-7 mm) antral follicle was observed during all control cycles. Thus, blocking endogenous AMH actions may promote macaque antral follicle growth during the follicular phase. Corpora lutea may develop from the multiple mature antral follicles and increase circulating P4 levels during the luteal phase. AMH may contribute to selection of the dominant follicle during the menstrual cycle by inhibiting antral follicle growth and maturation.
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P115

Critical role of decoy receptor 3 in regulation of granulosa cell apoptosis in pig ovarian follicle

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Introduction
During follicular development in mammalian ovaries, the majority of follicles undergo atresia. One of the characteristics of this process is apoptotic cell death in granulosa cells. Death ligands and receptors, including Fas ligand (FasL) and Fas, have been detected in follicles and also demonstrated to be capable of inducing apoptosis in follicular cells. Decoy receptor 3 (DcR3) competes with Fas to bind FasL but lacks intracellular death domains, thus inhibiting the induction of apoptosis by FasL.
Materials and methods
Total RNA and proteins were extracted from granulosa cells of porcine tertiary follicles at healthy, early atretic and progressed atretic stages. The localization and expression of pDcR3 mRNA was demonstrated by *in situ* hybridization and real time RT-PCR, respectively. The localization and expression of pDcR3 protein was demonstrated by immunohistochemistry and western blot respectively.
Results and discussion
In situ hybridization showed that pDcR3 mRNA expression was confirmed in granulosa cells of healthy. Quantitative RT-PCR showed that the expression of pDcR3 mRNA was extremely weaker in granulosa cells of early atretic follicles than those of healthy follicles. No pDcR3 mRNA was detected in granullosa cells of progressed atretic follicle. Protein expression profiles were similar to the profiles of mRNA. These findings suggest that DcR3 plays a significant role in the regulation of apoptosis in granulosa cells during atresia.

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P116

Expression and function of lysophosphatidic acid in theca cells of the bovine ovarian follicle

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In cow, lysophosphatidic acid (LPA) stimulated synthesis and secretion of luteotropic factors in ovary, which affected growth and development of ovarian follicles. Theca cells, among other components of ovarian follicle, play an essential role in ovulation, which depends on intrafollicular prostaglandin synthesis. In bovine reproductive tract, modulation of prostaglandin synthesis occurs under influence of LPA. Thus, the aim of this study was to determine possible correlation between LPA signaling and expression of genes involved in preovulatory follicular development and ovulation in theca cells in different types of ovarian follicles. Theca cells were separated from healthy, transitional and atretic ovarian follicles, based on intrafollicular estradiol:progesterone ratio. mRNA expression for enzymes responsible for LPA synthesis (ATX, PLA2), receptors for LPA (LPAR1–4) and factors involved in ovulation and preovulatory follicle development (PGES, DBI, BTC, TFG, RABGAP-1 and CD-36) were determined. Obtained results confirm the presence of LPAR1-4, PLA2 and ATX mRNA in theca cells in all examined types of follicles. We also detected follicle type-dependent mRNA expression of all examined factors involved in preovulatory follicular development and ovulation. Moreover, we found that LPAR2-4 and PLA2 mRNA expression was positively correlated with mRNA expression of PGES, DBI, BTC, TFG, RABGAP-1 and CD-36 in theca cells gained from healthy follicles. These results suggest that theca cells are the place of LPA synthesis and target of its action in all examined types of follicles. Correlations between mRNA expression for LPARs, PLA2 and factors participating in follicle development and ovulation indicate potential role of LPA in those processes in cow.

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P117

Expression and function of lysophosphatidic acid in granulosa cells of the bovine ovarian follicle

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Mural granulosa cells participate in development and differentiation of ovarian follicle. In bovine reproductive system, LPA is involved in autocrine and paracrine signaling, through LPA receptors (LPAR)s. We demonstrated before that LPA modulated prostaglandin (PG) synthesis in uterus and ovary and stimulated estradiol (E_2) synthesis in granulosa cells in bovine follicle. As the ovarian follicle development and differentiation leading to oocyte ovulation depend on PGs, in the present study we examined possible link between LPA signaling and expression of genes involved in ovulation in mural granulosa cells in three different types of ovarian follicles. Granulosa cells were recovered from healthy, transitional, and atretic follicles, based on intrafollicular E_2: progesterone ratio. Transcription profiles of enzymes responsible for LPA synthesis (PLA2, ATX), four LPARs and factors involved in ovulation and preovulatory follicle development (PTGS2, PGES, DBI, BTC, TFG, RABGAP-1 and CD-36) were determined in the cells. We found mRNA expression for PLA2, ATX and LPARs in granulosa cells. We found dynamic profile of mRNA abundance for factors involved in ovulation and preovulatory follicle development in granulosa cells depending on the type of follicle. Positive correlations between mRNA expression for PLA2 and RABGAP-1, PLA2 and TFG as well as LPAR2 and BTC were detected in granulosa cells in healthy follicles. Summarising, granulosa cells are the place of LPA synthesis and action in the bovine ovarian follicle. Positive correlations between mRNA expression for LPA synthesizing enzymes, LPARs and factors participating in ovulation and preovulatory follicle development suggest LPA involvement in those processes in cow.

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P118

Evaluation of oocyte quality and ovarian function in aged female cystine-glutamate transporter gene-deficient mice

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Introduction
Cystine-glutamate transporter (xCT) regulates cysteine levels by limiting uptake of cystine and consequently determines glutathione (GSH) contents in the cells. This study was performed to clarify effects of xCT deficiency (KO) on oocyte quality and ovarian function during maternal aging.
Materials and methods
Ovaries and plasma were collected from young (2 months) and aged (12 months) C57BL/6 xCT-KO and WT mice at 44 h after PMSG treatments. Follicle numbers at each classification stage were counted, and the estradiol levels were quantified by ELISA. The ovulated oocytes from each group were fertilized and cultured under 2% O_2/5% CO_2/93% N_2. Contents of ATP and GSH in the MII oocytes were measured. Also mRNA expressions of genes related to GSH synthesis, antioxidation, and aging were evaluated by real-time RT-PCR.
Results and discussion
The number of primordial follicles from young xCT-KO was significantly higher than that from WT (WT: 378.9±26.0, xCT-KO: 471.7±45.7). The number of total follicles from xCT-KO also tended to be higher than that from WT. The estradiol levels were not significantly different among the experimental groups. The fertilization rate of aged xCT-KO oocytes was lower than that of WT oocytes. However developmental potency of the fertilized oocytes to blastocysts was the same between the aged xCT-KO oocytes and the young WT oocytes. Both ATP and GSH contents were higher in the aged xCT-KO oocytes than the aged WT oocytes. Expression of several antioxidant-related genes was enhanced by aging. These results suggest that long-term deficiency of xCT does not affect oocyte quality from aged mice but unexpectedly raises potential to maintain the primordial follicle number.

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P119

Ovarian and hormonal responses of female goats to feed restriction and intermittent administration of neurokinin B analog

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The aim of this study was to determine the effect of feed restriction on follicular dynamics and hormone secretion patterns in goats, and to examine the effect of neurokinin B analog (senktide) under this experimental setting. Goats were administered 2 mg of dinoprost during the luteal phase, and CIDR was inserted for 10 days. Feed was provided at the level of 50% (feed-restricted; $n=9$) or 100% maintenance (control; $n=4$) for 15 days, beginning on the day of CIDR insertion. At 12 h after CIDR withdrawal, feed-restricted goats were administered 200 nmol senktide ($n=4$) or saline ($n=5$) at 4 h intervals for 24 h. Feed restriction caused 13.9±5.1% body weight loss, accompanied by lowered glucose and increased non-esterified fatty acid levels in plasma. On the day of CIDR withdrawal, feed-restricted goats had fewer follicles (2.6±0.9 vs 3.8±1.9 follicles), with smaller diameter (3.5±0.3 vs 3.9±0.5 mm) than control goats. However, occurrence of estrus, ovulation rate, and related hormone profiles in the feed-restricted goats were similar to those in the control goats. In senktide-treated goats, there was a rapid increase in LH after each injection, followed by an increase in plasma estradiol. Three of four goats treated with senktide did not show estrus. The interval from the beginning of treatment to ovulation tended to be shorter in senktide-treated goats (60.0±24.0 h) than in saline-treated goats (86.4±13.1 h). Maximal diameters of ovulatory follicles in senktide-treated goats were smaller than those in the control group (4.4±0.8 vs 5.1±0.6 mm). These results showed that 50% feed restriction negatively affected follicular development and that senktide could enhance LH secretion and accelerate the ovulatory process.

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P120

Differential changes in LH secretion after administration of investigational metastin/kisspeptin analog TAK-683 to goats

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The aim of the present study was to evaluate ovarian and hormonal responses to the administration of metastin/kisspeptin analog, TAK-683, under the endocrine environments of luteal and follicular phases in goats. At 7–10 days after the confirmation of ovulation by ultrasonography, six goats received a prostaglandin $F_2\alpha$ ($PGF_2\alpha$) injection (Day 0) followed by 10 days of CIDR-G® treatment. $PGF_2\alpha$ was injected again at CIDR removal. Fifty μg of TAK-683 was intravenously administered (hour 0) on Day 3 (luteal phase condition: LC) and at 12 h after CIDR removal on Day 10 (follicular phase condition: FC). Blood samples were collected daily or every other day from Day −1 to Day 10, and then collected at 10-min (hours −2–6), 2 h (hours 6–24), or 6 h (hours 24–48) intervals for analysis of endocrine profiles in both trials. Ovarian ultrasonographic images were assessed daily or every other day from Day −1 until the second ovulation in three of six goats. In the LC trial, small amplitude increases in the basal level of LH were observed after TAK-683 administration during hours 0–6 associated with a rise in estradiol concentration, followed by a surge-like release of LH with the peak time at 12.4±0.9 h ($n=5$) after TAK-683 administration. In the FC trial, in contrast, a surge-like release of LH occurred immediately after TAK-683 administration with the peak time at 5.0±1.1 h ($n=5$) which was significantly faster than that in the LC trial ($P<0.05$). Ovulation was detected within 3 days after TAK-683 administration in both trials. These results suggest that TAK-683 treatment induces a surge-like release of LH under the conditions of luteal and follicular phases with different peak times.

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P121

Endocrine responses and ovarian dynamics in goats treated with low dose of investigational metastin/kisspeptin analog TAK-683 in follicular phase

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The aim of the present study was to evaluate ovarian and hormonal responses to follicular phase administration of a metastin/kisspeptin analog, TAK-683, with a low dose, determined as the minimally effective dose for LH stimulation in ovariectomized goats. At 7–10 days after ovulation, goats received a prostaglandin $F_2\alpha$ ($PGF_2\alpha$) injection (day 0) followed by 10 days of CIDR-G® treatment. A $PGF_2\alpha$ was injected again at CIDR removal. Then, they received 5 μg of TAK-683 (0 h) intravenously ($n=5$, I.V.) or subcutaneously ($n=3$, S.C.) at 12 h after CIDR removal. Vehicle was given intravenously in the same manner ($n=4$, Cont). Blood samples were collected at 10-min (2–6 h), 2-h (6–24 h) or 6-h (24–48 h) I.V. for analysis of endocrine profiles. Ovarian ultrasonographic images were assessed daily to confirm ovulation after the treatment. A surge-like release of LH was immediately observed after the injection in all animals in I.V. (peak time: hours 4.2 ± 0.6, peak concentration: 73.3 ± 27.5 ng/ml) and S.C. (peak time: hours 4.6 ± 0.4, peak concentration: 62.6 ± 23.2 ng/ml) groups. Estradiol concentrations at hours 24 and 30 in I.V. and S.C. groups were significantly lower than those in the Cont group. Ovulation was detected within 3 days after TAK-683 injection in all animals in I.V. and S.C. groups. There was no significant difference in the changes in the luteal diameter and blood progesterone levels after ovulation between I.V. and S.C. groups. These results show that follicular phase administration of the minimally effective dose of TAK-683 to ovariectomized goats induces a surge-like release of LH followed by ovulation in ovary-intact goats.

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P122

TLR4-mediated signaling pathway is modulated by melatonin through MyD88-dependent pathway in ovarian carcinoma of ethanol-consuming rats

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Introduction
Toll-like receptors (TLRs) are active molecules expressed on the surface of ovarian cancer (OC) cells, but the consequences of TLR2/TLR4 signaling pathways in these cells remain unclear. Because melatonin (mel) act as an immunomodulatory hormone and has been reported to modulate TLRs in some aggressive tumor cells, we investigated the effective role of long-term mel therapy on TLR2- and TLR4-mediated molecule myeloid differentiation factor 88 (MyD88)-dependent signaling pathway in OC of ethanol-preferring rats.
Materials and methods
After developing OC, half of the animals received i.p. injections of mel (200 μg/100 g BW per day) for 60 days. Four experimental groups were established: Group C, rats bearing OC; Group EtOH + C, rats voluntarily consuming 10% (v/v) EtOH and bearing OC; Group C + M, rats bearing OC and receiving mel; and Group EtOH + C + M, rats with OC consuming EtOH and receiving mel. The target proteins/factors were investigated through immunohistochemistry and western blot analysis.
Results and discussion
While mel therapy was unable to reduce TLR2 levels, it was able to suppress TLR4, MyD88, inhibitor of nuclear factor kappa beta alpha (IKKα), nuclear factor kappa B (NFkB p65), and inhibitor of NFkB (IkBα) in OC. In addition, mel significantly attenuated the elevation of IkBα and NFkB p65 during ethanol intake, which were involved in TLR4-mediated signaling in OC. Together, our results suggest that mel modulates TLR4-induced MyD88-dependent signaling pathway in OC of ethanol-preferring rats.

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P123

Relative transcript abundance in porcine cumulus cells collected from different size follicles

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Introduction
Bi-directional communication between the oocyte and cumulus cells (CCs) is essential for the production of competent oocytes. Previous studies analyzed the relative transcript abundance in oocytes from small follicles (SF) and medium follicles (MF) to determinate the likely use of oocytes from SF in artificial reproductive techniques. The aim of this study was to examine the relative transcript abundance of CCs from cumulus–oocyte complexes (COCs) from SF and MF.
Materials and methods
CCs were obtained from COCs derived from SF (<3 mm) and MF (3–6 mm). We studied genes involved in oocyte developmental competence: hormonal receptors (GHR, BMPR2, LHR, and FSHR), genes related with oocyte maturation (INHBA and FST), genes related with CCs expansion and oocyte meiotic resumption (AREG and CD44), one gene related with gap-junction communication (ARID1B) and another related with hormonal response (NR2F6). RNA was extracted from CCs (30 COCs) and cDNA was synthesized and used in quantitative PCR.
Results and discussion
Significant effect of follicular size was detected in INHBA and GHR transcripts ($P<0.05$). INHBA was more expressed in CCs from MF whereas GHR was more abundant in the CCs from SF. Rest of the genes didn't show significant differences. The higher expression of INHBA might reflect the oocyte's degree of maturation since COCs from MF are closer to resumption meiosis and complete maturation than SF-derived COCs. The abundance of GHR transcripts in CCs from SF follicles might be related to the preparation of these follicles for a better response of the somatotropic axis.

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P124

Real-time observation of hormone-secretion using CFP, YFP-fused gonadotropin expression vector

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Introduction
FSH and LH are glycoprotein hormones and are synthesized in the anterior lobe of the pituitary gland and plays critical roles for maintenance of vital functions. FSH and LH are secreted to pituitary capillary vessel and exert their effects on reproductive organs of both sexes to stimulate steroidogenesis and gametogenesis. Secretion of FSH and LH is controlled mainly by GnRH released from hypothalamus. Many investigations on secretion of FSH and LH were performed by measurement of the hormone level in blood or in cultured medium of the pituitary primary cells or cell lines requiring much time. Herein, we developed a monitoring system by using the expression of fluorescence protein-fused FSH and LH.
Materials and methods
Expression vectors were constructed by fusing rat cDNA of FSHβ- and LHβ-subunit to the 5′ of CFP or YFP of the vector and transfected into gonadotrope-lineage cell line, LβT2, with FuGENE6. After time-laps observation of fluorescence microscopy, immunocytochemistry was performed with antibodies against α-, FSHβ-, and LHβ-subunit. Secretion was achieved with forskolin or phorbol ester followed by determination of the molecular sizes.
Results and discussion
By transfection of expression vectors, fluorescent signals were observed in the cytoplasmic granules and the presence of hormones were verified by immunocytochemistry. Addition of western blotting analysis of transfected cell lysates using anti-GFP antibody showed success of the synthesis of fluorescence-fused hormones. Thus, the expression vectors for CFP, YFP-fused FSH and LH are useful tool for real-time observation of the secretion of hormone.

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P125

BMP15 plus GDF9 and cAMP modulator promote EGF receptor signaling to increase oocyte developmental competence in porcine cumulus–oocyte complexes from small follicles

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Introduction
Oocytes acquire developmental competence with progressive folliculogenesis. Cumulus–oocyte complexes (COCs) from small antral follicles are commonly used in IVM but have inherent low competence and are poorly responsive to amphiregulin (AREG) which normally mediates oocyte maturation. Here we examined effects of bone morphogenetic protein 15 (BMP15), growth differentiation factor 9 (GDF9), and dbcAMP on the maturation and subsequent developmental competence of oocytes derived from small follicles in AREG-induced IVM.
Materials and methods
Gilt COCs were aspirated from 2 to 4 mm follicles and cultured in porcine oocyte medium supplemented with 100 ng/ml AREG, ±1 mmol/l dbcAMP, ± 100 ng/ml pro-mature human GDF9, ±100 ng/ml pro-mature human BMP15 for 22 h and then in additive-free medium for an additional 22 h. Following IVM, chromosome configurations, developmental competence and mRNA of matrix related genes were determined. Phosphorylation of ERK1/2 (pERK1/2) in COCs was assessed 1 h following AREG-stimulation.
Results and discussion
In AREG-induced IVM, the addition of dbcAMP + BMP15 + GDF9 increased the rate of nuclear maturation and blastocyst formation compared to BMP15 + GDF9 alone. Furthermore, the combination of BMP15 + GDF9 and dbcAMP enabled AREG-stimulated cumulus expansion because of increased matrix related genes such as *HAS2*, *TNFIPA6* and *PTGS2*. Additionally, the combination enhanced pERK1/2. An EGF receptor phosphorylation inhibitor decreased nuclear maturation and blastocyst formation rates in the IVM combinational treatment. These results indicate that BMP15 + GDF9 and dbcAMP may synergistically promote EGF receptor signaling stimulated by AREG in small follicle derived COCs, resulting in enhanced developmental competence of oocytes that are otherwise developmentally compromised.

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P126

TGFβ regulation of the human fetal ovarian matrix

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Introduction
Human germ cells develop in synchronised nests in the fetal ovary, interspersed with somatic cells prior to reorganisation to form primordial follicles. We have isolated several somatic cell lines from disaggregated human fetal ovaries as a model for understanding their contribution to the germ cell niche. Fetal dysregulation of TGFβ signalling has been linked with fibrosis in polycystic ovary syndrome (PCOS): effects of TGFβ on extracellular matrix (ECM) components in these somatic cells have therefore been investigated.
Materials and methods
Expression of key somatic cell markers was assessed by qRT-PCR across a number of passages. Changes in the expression of ECM and epithelial to mesenchymal transition (EMT) associated genes in response to 5 ng/ml TGFβ1 or its inhibitor SB431542 (10 μM) for 24 h were also determined.
Results and discussion
Expression of markers associated with pre-granulosa cells (e.g. FOXL2, ID2, and AR) was retained for at least eight passages. TGFβ-signalling pathway associated genes showed up-regulated expression in these cells and many were reciprocally sensitive to exogenous TGFβ1 (*TGFB1, LTBP1, LTBP2, TGFBI, INHBA* up-regulated) and SB431542 (aforementioned plus *LTBP4, FBN1* down-regulated). Several other ECM and EMT-associated genes (*CTGF, THBS1, FN1, ITGA5, ITGB1, SNAI1, CDH2, and ACTA2*) were also down-regulated by SB431542 while *SNAI2* was up-regulated. *CTGF, THBS1*, and *SNAI1* were up-regulated by TGFβ1. CDH2, ITGA5, and ITGB1 are associated with cell adhesion while increased CTGF, and TGFB1 can lead to tissue fibrosis. This suggests a key role for the TGFβ pathway in normal ovary development.

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P127

Abstract withdrawn.

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P128

Effects of the mycotoxin, deoxynivalenol, on bovine granulosa cells

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Introduction
Deoxynivalenol (DON) is a major mycotoxin found in animal feed, one of its major actions is to activate the ribotoxic stress response (RSR) in different cells types. DON has been shown to inhibit progesterone secretion from porcine granulosa cells, but its effect in the cow is unknown. The objective of this study was to determine the effects of DON on bovine granulosa cells *in vitro*.
Materials and methods
Bovine granulosa cells from follicles 3–5 mm diameter were placed in serum-free culture. Dose- and time-course studies with DON were performed with a maximum dose of 100 ng/ml. Oestradiol and progesterone secretion was measured by RIA, apoptosis was measured by Annexin Flow Cytometry, activation of two major RSR pathways (ERK1/2 and p38) was assessed by western blot, and target gene mRNA levels were measured by real-time PCR.
Results
Treatment with DON resulted in a significant inhibition of oestradiol ($P<0.05$) and progesterone ($P<0.05$) secretion, and an increase in the proportion of apoptotic cells ($P<0.05$) after 4 days of treatment. Western blot demonstrated significant up-regulation of ERK1/2 ($P<0.05$) and p38 ($P<0.005$) phosphorylation within 15–30 min of adding DON. We then determined the effect of DON on ERK1/2 target genes; EGR1 and FOS mRNA levels were transiently stimulated with maximum levels at 1 h of adding DON, whereas COX2 and GADD45B mRNA levels were upregulated but not until 24 h after DON treatment ($P<0.05$). Taken together these results suggest that DON activates the RSR in bovine granulosa cells and negatively impacts cell health.

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P129

Transcriptome in small antral follicles of monkeys on a western-style diet with/without testosterone

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Introduction
Recent studies indicate that dietary habits and hyperandrogenemia influence ovarian function in women. However, data are limited regarding the effects of western-style diet (WSD) and androgen (e.g. T) on follicle development, especially during the early follicular stage, in primates. The current study investigated the impact of WSD alone and with chronic T exposure on gene expression in small antral follicles (SAFs) of macaques.

Materials and methods
Macaques ($n=3$/group) received s.c. implants without or with T (to increase serum levels three to four fold; $P<0.05$) beginning at 1 year of age, followed by a WSD (high fat/fructose) at 5.5 years. A control group ($n=3$) without T treatment was fed a regular diet. Ovaries were collected at 7 years. SAFs ($\sim$1 mm in diameter) were isolated for total RNA extraction. RNA samples were amplified, labeled, and hybridized to a Rhesus GeneChip for microarray. Data were analyzed using GeneSifter and one-way ANOVA.
Results and discussion
The mRNA levels for 1334 genes changed >1.5-fold ($P<0.05$) among the three groups. The top z-scores of biological processes were 4.80 (cellular component organization or biogenesis), 3.71 (cellular processes), and 3.58 (metabolic processes). Compared with controls, AR, ERα, and FSHR levels were significantly lower in WSD and WSDT groups, while ERβ, PR, and StAR levels did not change. Gene expression levels of LHCGR were significantly lower in WSD group than those of control and WSDT groups. The results indicate that WSD with/without T affects SAFs biogenic and metabolic processes. Changes in hormone receptors in SAFs may affect follicular development and dominant follicle selection.

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P130

The effects of estradiol and ECG combination on ZP_3 gene in mouse oocyte

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Introduction
The ovarian stimulators have a crucial role in steriodogenesis, follicular development, oocyte maturation and embryo maintenance by activating some genes in ovarian follicles. As, one of the most important cellular and molecular mechanisms involved in successful fertilization is the expression of sperm receptor binding protein or ZP_3 in zona Pellucida, therefore our attempts have been made to delineate the effects of combined Estradiol and eCG on ZP_3 gen expression here in.
Materials and methods
Sixty female NMRI mice in the three groups intraperitoneally received normal saline, eCG (10IU) and also eCG(10IU)+Estradiol (E2) (1 μg) in same injection volumes respectively.
After 48-h, they received hCG (10IU) due to ovulation induction. The half of yielded oocytes designed for achieving IVF and another one for measuring ZP_3 gene expression by RT-PCR.
Results and discussion
There was significant difference in ZP_3 gene expression of the group which were only received eCG in comparison with others. It concluded that ZP_3 gene expression may be responsive to eCG alone rather than eCG+E2.

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P131

Inhibin α induced porcine granulosa cells apoptosis through mitochondrial apoptotic pathway

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Inhibin is a heterodimeric gonadal glycoprotein hormone belonging to the TGFβ superfamily that named for its ability to suppress FSH synthesis and secretion from anterior pituitary via a negative feedback. It can regulate proliferation, differentiation, and steroidogenesis of granulosa cells (GCs) through paracrine and autocrine manners. The objective of this study was to investigate the interaction between inhibin α subunit (INHA) and apoptosis of porcine ovarian GCs by exploring i) the effect of INHA on mitochondrial membrane potential (MMP) of GCs and ii) the effect of INHA on Caspase-3 and Bax mRAN expression in GCs. In experiment 1, GCs were divided into two groups and cultured in serum-free medium for 12 h with 0 and 50 ng/ml INHA. Then, MMP were detected under fluorescence microscopy using mitochondrial membrane potential assay kit with JC-1. Treatment with INHA induced a disappearance of red fluorescence along with a diffusion of green fluorescence into the cytoplasm of GCs. This shift of JC-1 fluorescence from red to green indicated a collapse of MMP. In experiment 2, GCs were treated with different concentration of INHA (0, 10, 20, 50, and 100 ng/ml) for 6 and 18 h. Then, total RNA was extracted, Caspase-3 and Bax mRNA were detected by qRT-RCR. The expression levels of Bax and caspase-3 were significantly increased ($P<0.05$) after INHA treated for 6 h. For 18 h, only treatment with 50 ng/ml INHA significantly ($P<0.05$) increased Caspase-3 expression. In conclusion, INHA may have a role in regulating the apoptosis of GCs through mitochondrial apoptotic pathway. This research was supported by the State Key Development Program of Basic Researchof China (No. 2011CB944203).

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P132

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P133

The effect of bisphenol A on Notch signaling pathway in the follicular development of neonatal rat ovary

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Introduction
Neonatal exposure to bisphenol A (BPA) can impair reproductive physiology, but the specific mechanisms by which this occurs remain largely unknown. We aimed to study whether mRNA levels of Notch receptor and ligands are altered following BPA treatment during postnatal days.
Materials and methods
Neonatal rats were divided into two main groups as groups I and II according to treatment time intervals consisting of postnatal day (P)0–P3 and P4–P7. Each group was divided into further three subgroups as vehicle group, 50 μg/kg per day BPA group and 50 mg/kg per day BPA group. Group I received daily treatment from P0 to P3, whereas group II was given daily from P4 to P7. The animals were sacrified on P7 in all groups.
Results and Discussion
When compared with vehicle rats in group I, 50 mg/kg per day BPA treatment was found to suppress *Notch-1, 2, and 3* and *Jagged-2* gene expressions but enhanced *Notch-4* and *Jagged-1* gene expressions. Furthermore, 50 μg/kg per day BPA treatment increased the gene expression levels of all Notch receptors and their ligands. Compared to the vehicle rats in group II, although gene levels of Notch-1, 2 and 3, and Jagged-2 were elevated. *Notch-4* and *Jagged-1* gene expressions were decreased by 50 mg/kg per day BPA treatments. 50 μg/kg per day BPA treatment to neonatal pups at PND 4–7 was shown to reduce *Notch-4*, *Jagged-1*, and *Jagged-2* gene expressions but increased Notch 1, 2, and 3 gene expression levels. It was concluded that neonatal exposure to BPA can affect the early ovary development by disrupting Notch signalling pathway. This research was supported by Tubitak (Tubitak 1110751).

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P134

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P135

Interaction between galectin-1 and glycoconjugates is involved in hCG-mediated gene expression in cultured human luteinized granulosa cells

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Introduction
The corpus luteum (CL) is a temporal endocrine organ secreting large amount of progesterone essential for the establishment and maintenance of pregnancy. The CL undergoes luteolysis in a non-fertile cycle, while the CL is rescued from luteolysis by human chorionic gonadotrophin (hCG) secreted from the conceptus during pregnancy. We recently revealed that a β-galactoside-binding lectin, galectin-1, plays important roles in the regulation of luteal rescue in the human CL. To explore the function and the ligand glycoconjugates for galectin-1, we examined the effect of i) lactose, which is a competing saccharide for the binding of galectins to glycoconjugates and ii) hCG without *N*-glycans, on the hCG-mediated gene expression in cultured human luteinized granulosa cells (LGCs).
Materials and methods
LGCs were isolated from the follicular fluids obtained from the patients undergoing IVF treatment with informed consent. Cultured LGCs were treated by i) 100 ng/ml hCG with or without 30 mM lactose and ii) 100 ng/ml hCG pre-treated with PNGase F to remove *N*-glycans. LGCs were collected 24 h after the treatments and changes in the gene expression were analyzed by quantitative PCR.
Results and discussion
Both treatments diminished hCG-induced cAMP/PKA-mediated steroidogenic acute regulatory protein and prostaglandin (PG) E synthase expression, while enhancing the expression of PI3 kinase-mediated AKR1C1 and AKR1C2 which are involved in progesterone degradation and the conversion from luteotrophic PGE to luteolytic PGF. These results suggest that an interaction between galectin-1 to glycoconjugates on luteal cells or hCG is required for luteotrophic function of hCG through the cAMP/PKA pathway.

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P136

Expression of steriod hormone receptor in the ovary and oviduct during the canine estrus cycle

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Introduction
Accurate measurement of ovulation timing is difficult in the dog. In this study, we investigate the expression of steroid hormone receptors in the ovary and oviduct before and after ovulation in the dog.
Material and methods
Estrus and ovulation period were measured by serum concentrations of progesterone hormone and LH. LH surge was defined as day 0. The ovary and oviduct were isolated at the day before LH surge (day-1), 2 day after ovulation (day 1) and 5 days after ovulation (day 3) respectively. Real-time RT-PCR was conduct to measure mRNA expression of progesterone receptor (PR), androgen receptor (AR), estrogen receptor alpha (ERα), beta (ERβ), FSH receptor (FSHR), and LH receptor (LHR) mRNA.
Result and discussion
The ERβ is faster than ERα on day 1 was confirmed to be a very highly expressed. Expression of FSHR was very low in the day-1 and day 3 was higher. This is just before the LH surge is extremely lowered the expression of FSHR was found to be higher after ovulation. LHR expression was highly expressed at day-1, days 1 and 3 through and the lower. Most of the results are in oviduct was similar to the results of the ovary, except that there was a little difference in the date only. However, AR and PR showed a very different pattern. Therefore, the steroid hormone receptor mRNA expression was confirmed to be dynamically changing before and after ovulation.

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P137

Effect of progesterone levels and parity on the maturity of *in vivo* canine oocytes

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Somatic cell nuclear transfer (SCNT) in dogs has been used *in vivo*-matured oocytes due to low efficiency of *in vitro* maturation system. Actually, more information is needed to acquire good quality *in vivo* oocytes for the dog research. The objective of present study was to evaluate whether the progesterone (P4) on estimated ovulation time and the parity of donor bitches have any effect on the maturity of oocytes. *In vivo* oocytes were retrieved from 289 mixed breed bitchs by laparotomy. Each individual fallopian tube was flushed by Hepes-buffered TCM199 medium containing 10% FBS. Maturity of collected oocytes were categorized as immature, mature, and aged. Our results found a correlation between P4 levels on estimated ovulation time and the parity of donor bitch in the oocyte maturity. In nulliparous bitches, the percentage of mature oocyte was not different among the various P4 levels. However, the percentage of aged oocytes was significantly ($P<0.05$) increased in 8.01–10.0 and >10.01 ng/ml (22.3 and 18.2%) than in 4.01–6.0 ng/ml (9.6%). In multiparous bitches, the percentage of mature oocyte was significantly ($P<0.05$) decreased in 6.01–8.0, 8.01–10.0, and >10.01 ng/ml (48.8, 60.7, and 27.1%) than in 4.01–6.0 ng/ml (71.7%). On the contrary, aged oocytes was significantly ($P<0.05$) increased in 6.01–8.0, 8.01–10.0, and >10.01 ng/ml (31.1, 30.6, and 55.4%) than in 4.01–6.0 ng/ml (18.0%). This study suggests that a correlation between P4 levels on estimated ovulation time and the parity of donor bitch does exist in the maturity of canine oocytes collected by flushing fallopian tube.

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P138

The development of a novel mouse embryonic ovary culture

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Introduction
In vitro cultures are a widely used tool to study ovary development and assess reproductive toxicology of chemicals. However, establishing a culture system whereby mouse ovaries can be cultured from a pre-meiotic stage to a mature oocyte has proved challenging. We have developed a novel culture system that spans meiotic entry to meiotic arrest, germ cell nest break-down, follicle formation, and initiation of follicle growth.
Methods
E13.5 mouse ovaries were cultured for 12 days on an agar block. Follicle health and numbers was analysed histologically, and oocyte meiotic chromosome spreads were analysed to determine meiotic progression of cultured oocytes.
Results
Cultured ovaries contained follicles at stages in comparable ratios to those in P4 *in vivo* ovaries (77.1% primordial, 17.0% transitional, and 5.6% primary in cultured ovaries vs 88, 7.8, and 2.8% respectively, *in vivo*). Ovaries and follicles appeared morphologically normal and healthy; with only a slight, non-significant increase in unhealthy follicles in the cultured ovaries compared with P4 ovaries (3.6% increase, $P=0.38$). Furthermore, 23% of cultured oocytes had reached pachytene after 6 days in culture, which, compared with the 50% pachytene nuclei in an *in vivo* E18 ovary, demonstrates that the culture system supports development of oocytes pachytene, albeit with a slight delay.
Conclusion
Our results demonstrate that our novel culture system supports growth of pre-meiotic mouse germ cells through prophase I of meiosis to meiotic arrest, the formation of primordial follicles and initiation of follicle growth.

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P139

Regulatory role of resistin on ovarian function

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Introduction
We recently reported expression of resistin in porcine ovarian follicles and stimulatory on basal why inhibitory on gonadotropin-stimulated ovarian steroidogenesis by direct influence on 3β-HSD and 17β-HSD expression. The question is whether resistin, beyond the control of steroidogenesis, regulates apoptosis in ovarian follicles. The present *in vitro* study was designed to asses effect of resistin on basal and FSH or IGF1 stimulated caspases activity and protein expression.
Materials and methods
Medium size follicles were collected from ovaries of estrus cycling crossbred gilts. Coculture of granulosa and theca interna cells in ratio 4:1 were incubated with resistin (1 and 10 ng/ml), FSH (100 ng/ml), or IGF1 (30 ng/ml) in M199/10% FBS at 37 °C for 24 h. The activities of caspase-8, -9, and -3 were measured using fluorescent substrates (Ac-IETD-AMC, Ac-LEHD-AFC, and Ac-DEVD-AMC respectively). Western blot analysis was used to measurement protein expression.
Results and discussion
Caspase-3, an executor caspase can be activated by either caspase-8 (the death receptor pathway) or caspase-9 (the mitochondrial pathway). Resistin at both doses was without effect on basal caspase-8 why significantly decreased caspase-3 and -9 activity and protein expression, suggesting involvement of mitochondrial pathway in antiapoptotic action of resistin in ovary. No effect on FSH and IGF1 stimulated caspases activity and protein expression was noted. The presence of resistin in the porcine ovary and its direct stimulatory effects on steroidogenesis and inhibitory on apoptosis suggests that resistin is a new regulator of ovary function.
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P140

The effects of bisphenol A in *in vitro* neonatal rat ovary

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Introduction
Neonatal bisphenol A (BPA) exposures affect the developing reproductive tract of females, several distinct stages of oogenesis in the developing ovary. Notch signaling also plays an important role during oogenesis. We aimed to explain whether mRNA levels of Notch receptor and ligands are altered following BPA treatment during neonatal days *in vitro*.
Materials and methods
Wistar rats were sacrificed and ovaries were isolated on postnatal days 0 and 4. Whole ovaries were cultured on floating filters in ovary organ culture medium in a four-well culture plate. Ovaries were treated with %1 DMSO as vehicle, 1 μM BPA as low dose and 10 μM BPA as high dose at P0–P3 (group I) and P4–P7 (group II) time intervals. Following *in vitro* treatments, mRNA levels of Notch receptors and ligands were determined with qPCR.
Results and discussion
When compared with vehicle treatment in group I, 1 μM BPA treatment was found to enhanced Notch and their ligands gene expressions. In addition 10 μM BPA treatment increased the gene expression levels of Notch receptors and their ligands except for Notch-3. When compared with vehicle treatment in group 2, 1 μM BPA treatment was found to increased Notch-1, Notch-2, Notch-3, Jagged-1, and Jagged-2 but decreased Notch-4. 10 μM BPA treatment also suppressed Jagged-1 and Notch-4 mRNA levels. It was conclueded that neonatal exposure to BPA can impair the early ovary development by disrupting Notch signaling pathway This research was supported by Tübitak (Tovag 1110751).

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P141

Acquisition of meiotic competence in porcine secondary follicles following long-term culture

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The development of culture methods to produce oocytes from immature ovarian follicles *in vitro* would greatly contribute to livestock production and assisted human reproduction. However, successful *in vitro* culture to produce fully mature oocytes is very difficult in the case of large domestic animals. In the present study, we investigated the growth properties of *in vitro*-cultured porcine oocytes derived from early developmental stage follicles. Oocyte granulosa cell complexes (OGCs) were isolated from secondary follicles (250–300 μm in diameter) and individually cultured for 28 days. We examined antrum formation, the oocyte diameter, the chromatin configurations, the follicle cell numbers, and meiotic competence of the *in vitro*-cultured oocytes. During *in vitro* culture, the rate of antrum formation was the highest on day 14, and gradually decreased afterwards. The diameters of the oocytes increased during the culture period (from 75.5 to 112.7 μm) and was significantly and positively correlated with the number of follicle cells derived the OGCs ($P < 0.01$), independent of the days in culture. As the culture progressed, the oocytes categorized as germinal vesicle (GV)1 increased in number, with a decrease in GV0 oocytes. At the end of the culture, oocytes derived from secondary follicles acquired meiotic competence. The cultured porcine oocytes, and may serve as a model for oocyte development for the propagation of large domestic animals.

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P142

Isolation of primordial and primary follicles from porcine ovarian medulla tissue

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Introduction
The ovarian follicle has a three-dimensional structure in which the oocyte is surrounded by tightly connected follicle cells that mediate the action of external signals and nourish the gamete during its maturation. *In vitro* follicle techniques provide a tool to model follicle development in order to investigate basic biology and are further being developed as a technique to preserve human fertility or to product transgenic animals for cell therapies. Main objective of this work was to establish an effective method for retrieving viable, preantral pig follicles from ovarian medulla.
Material and methods
Medulla was collected from 30, 4–5 months old prepubertal gilts. Primordial and primary follicles were isolated from ovarian medulla using different types of collagenase (types I, II, and IV) and Liberase (DH, TM, and TH). Different treatment protocols have been also used. Follicles quality was assessed by evaluating their morphology and viability after H&E and fluorescent staining.
Results and discussion
The number of fully isolated follicles recovered from medullas treated by Liberase TH was always higher than from collagenase group (independently of an enzyme type). Very high proportion of follicles were viable after Liberase TH isolation, and most of follicles were of good morphology with a complete granulosa cell layer. TUNEL method indicated that Liberase-isolated follicles showed signs of atresia only occasionally and that the oolemma-follicular cell interface was well preserved. It seems, Liberase TH preparation is promising alternative allowing use of isolating primordial and primary pig follicles for future reproductive applications.
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P143

Analysis of programmed cell death of porcine granulosa cells treated with vinclozolin

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Introduction
Androgens: testosterone, dihydrotestosterone (DHT), and a fungicide with antiandrogenic activity – vinclozolin (Vnz) served to study agonism and antagonism of the androgen receptor.
Material and methods
Granulosa cells (GC) isolated from pig follicles were cultured for 48 h with testosterone (10^{-7} M), DHT (10^{-7} M), and Vnz (1.4×10^{-5} M) separately or in combinations. Cells were then assessed for viability, cytotoxicity, and caspase activation events with ApoTox-Glo Triplex assay. Additionally, to determine the mechanism of GC cell death induced by Vnz, Apoptosis Antibody Array Kit has been used.
Results and discussion
Supplementation with Vnz resulted in a dramatical reduction in the viability of GC but suprisingly without an increase in cytotoxicity. This might result from the ability of Vnz to alter normal cell division (cell-cycle arrest) without producing membrane integrity changes. Moreover, caspase-3 activity was suppressed in cultures treated with a combination of Vnz and testosterone. Such an antiapoptotic effect of Vnz applied together with high concentration of androgens might be explained by the suppression of two intra-cellular proapoptotic proteins bad and bax. It seems that selective destruction of porcine follicles is a serious consequence of their exposure to Vnz leading to premature ovarian failure in the affected organisms.
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P144

Etoposide has a detrimental impact on mouse ovarian development when exposure occurs during early meiotic prophase

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Introduction
The use of the chemotherapeutic agent etoposide in pregnancy is considered to be relatively safe during the second and third trimesters. However, the drug does have detrimental effects on oocytes undergoing meiosis II. Similar effects on oocytes in meiosis I may have a clinical impact on the fertility of women exposed to the drug *in utero*, during critical stages of ovarian development. This study aims to examine the effects of etoposide exposure during early meiosis using a mouse embryonic whole ovary culture system.
Methods
Day 13.5 embryonic mouse ovaries were collected, with oocytes then initiating meiosis. Ovaries were cultured on agar blocks for a total of 12 days, and exposed to etoposide (50–150 ng/ml) for the first 6 days of culture; covering early prophase I. Newborn mouse ovaries, in which oocytes are already in meiotic arrest, were also exposed to etoposide (50–150 ng/ml) in a 6 day culture system. Immunofluorescent staining for γ-H2AX was carried out to localise double strand DNA breaks induced by etoposide exposure.
Results and discussion
Histological analysis revealed a detrimental effect of etoposide exposure on follicle numbers (150 ng/ml; $P<0.001$) in ovaries exposed during early prophase I, but not in those exposed after meiotic arrest. Double strand breaks were also visible in many cells, in ovaries exposed during early prophase I. Together, this indicates the possibility of effects on future reproductive potential of embryos exposed during early second trimester, but further work is required to determine the extent of damage which would occur *in vivo*.
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P145

The age-associated deterioration in the follicular fluid induces a decline in bovine oocyte quality

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Introduction
In general, the maternal age affects the quality of the oocytes as well as that of embryos. This study aims to examine whether changes in the follicular fluid (FF) can affect oocyte quality in aged cows.
Materials and methods
Oocytes were collected from ovaries of young (<120 months) or aged cows (≥120 months), and oocytes from animals in these two age groups were cultured in a maturation medium that contained 10% FF derived from young or aged cows (young-FF and aged-FF). In experiment 1, we compared progression of nuclear maturation, the fertilization outcome, and the developmental competence between the two groups. In experiment 2, we measured the levels of estradiol, progesterone, and advanced glycation endproducts (AGEs), and the antioxidant capacity of young-FF or aged-FF.
Results and discussion
Experiment 1: In oocytes from both the younger or older animals, nuclear maturation progressed faster when culture was carried out in the medium containing aged-FF compared to that in the medium containing young-FF. Moreover, for both groups, compared to the young-FF-cultured oocytes, the aged-FF-cultured oocytes showed a lower rate of normal fertilization and development to the blastocyst stage.
Experiment 2: The estradiol and progesterone levels were significantly lower while the AGE level was significantly higher in the aged-FF. In addition, antioxidant capacity in aged-FF was lower than that in young-FF, which resulted in higher ROS levels in these oocytes cultured with aged-FF.
These results suggest that age-associated deterioration in the FF induces a decline in oocyte quality.
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P146

Resveratrol enhances the *in vitro* development of oocytes derived from early antral follicles

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Introduction
The present study aims to investigate the effect of resveratrol supplementation on the *in vitro* development of porcine oocytes derived from early antral follicles.
Materials and methods
Oocyte-granulosa cell complexes (OGCs) were collected from early antral follicles that were 0.5–0.7 mm in diameter and cultured in the absence or present of resveratrol (2 μM) for 14 days. The effect of resveratrol on SIRT1 expression was determined by immunostaining, furthermore the *in vitro* development of the OGCs was examined by assessing the antrum formation rate, the nuclear maturation following *in vitro* maturation, and the developmental ratio to the blastocyst stage after activation. In addition, ATP levels and the mitochondrial DNA copy number in oocytes grown *in vitro* were examined.
Results and discussion
Supplementation of culture medium with resveratrol increased SIRT1 expression in oocytes, while neither the antrum formation rate nor the viability of surrounding granulosa cells was affected by resveratrol. The rate of nuclear maturation was not affected by resveratrol. The developmental competence of oocytes grown *in vitro* was however improved, with the blastocyst rate being higher than that for oocytes cultured in the absence of resveratrol (1.6 vs 7.4%). Furthermore, resveratrol supplementation significantly increased the ATP levels in the *in vitro*-cultured oocytes (3.0 vs 3.5 pM), but did not affect the mitochondrial DNA copy number. In conclusion, our findings indicate that resveratrol improves the developmental potential of oocytes derived from early antrum follicles by improving mitochondrial function.
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P147

Expression of pentraxin 3 transcript and protein in porcine preovulatory follicles: comparison of *in vivo* and *in vitro* conditions

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It has been previously shown that multimeric pentraxin 3 (PTX3) is a key component of the cumulus oophorus extracellular matrix. Noteworthy, cumuli from PTX3$^{-/-}$ mice were defective in matrix organization and fertility was impaired. PTX3 binds to both tumor necrosis factor alpha-induced protein 6 (TNFAIP6) and inter-alpha-trypsin inhibitor thereby likely serving as a cross linker for multiple hyaluronan molecules, stabilizing in this way the cumulus oophorus extracellular matrix. We investigated whether PTX3 is also expressed in porcine preovulatory follicle. Oocyte-cumulus complexes (OCC) and granulosa cells (GC) from gilts were either stimulated *in vivo* with PMSG and hCG (4, 8, 16, 24, and 32 h) or cultured *in vitro* (4, 24, and 44 h) with FSH/LH. Methods performed were real-time RT-PCR, western blot analysis and immunostaining. Expression of PTX3 transcripts was significantly increased 24 h after either *in vivo* hCG stimulation or *in vitro* FSH/LH treatment in both OCC and GC. Western blot analysis with PTX3 antibody revealed that not only matrix extracts from naturally cycling gilts contain high levels of PTX3 protein (~49 kDa) but also matrix extracts of FSH/LH-stimulated OCC cultured in medium supplemented with follicular fluid or porcine serum. Localization of PTX3 in the extracellular matrix was confirmed by immunohistochemistry. This research was supported as follows: 'collaboration via a fellowship (EN) under the OECD Co-Operative Research Programme: Biological Resource Management for Sustainable Agricultural Systems' and by Grant P502/11/0593 from GA of the Czech Republic.

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P148

Cell viability in ovarian follicles exposed to cisplatin and doxorubicin

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Introduction
This work adapts a novel ovary culture technique, devised by De Felici's group, for assessing the ovotoxicity of two chemotherapeutic drugs, cisplatin and doxorubicin. Previous work from our laboratory used cultures of intact neonatal ovaries to determine the effects of the two drugs, showing that both cisplatin and doxorubicin are moderately ovotoxic.
Methods
Here, ovaries collected from newborn WT mice were cultured as by De Felici. Briefly, ovaries were fragmented and seeded into the wells of a culture plate with serum containing medium. These were left for 3 days to allow the fragments to attach, with cisplatin and doxorubicin added into the culture on day 7 only, at patient serum concentrations of 0.5 and 1 μg/ml, 0.1 and 0.2 μg/ml respectively. Immediately before and after drug administration, tissue fragments were stained with trypan blue to assess cell viability and follicle health.
Results and discussion
From the total of 152 fragments analysed, the percentage of the growing follicles regarded as morphologically unhealthy by trypan blue staining, was significantly lower in the controls (1.98%) as compared to 22.1% ($P<0.05$) and 25.2% ($P<0.01$) seen in those treated with 0.5 and 1 μg/l cisplatin respectively, and 41.0% ($P<0.0001$) and 44.2% ($P<0.0001$) seen in those treated with 0.1 and 0.2 μg/ml doxorubicin respectively. The culture method used here offers a quick and easy way to evaluate the effects of toxic drugs on ovarian tissue without the need for either *in vivo* experimentation or extensive histological analysis.

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P149

Ghrelin, like adipokines produced by adipose tissue, acts on accelerate puberty, while in contrast to adipokines, during sexual maturity prevents from excessive secretion of steroids by ovarian follicles

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Introduction
In our previously published study we focused on actions of ghrelin on ovarian steroidogenesis in prepubertal period and indicated that as adipokines produced by adipose tissue (such as leptin or resistin), ghrelin acts on accelerate puberty. This data had been performing to determine whether also in cycling animals ghrelin acts on ovarian steroidogenesis similarly to leptin and resistin.
Material and methods
Small (SFs), medium (MFs), and large (LFs) ovarian follicles were collected on days 4–6, 10–12, and 16–18 of the estrous cycle from cycling pigs and exposed to 20, 100, and 500 pg/ml ghrelin for 24 h. In additional experiments, MFs were exposed to ghrelin plus 100 ng/ml FSH or LH. Levels of progesterone (P4), testosterone, and E_2 in culture medium were determined by ELISA, and the expression of the steroid pathway enzymes 3β-HSD, 17β-HSD, and CYP19 was evaluated by western blotting.
Results
Ghrelin at dose of 20 pg/ml had no effect on steroid secretion, whereas in dose of 100 pg/ml and 500 pg/ml ghrelin significantly decreased P4, testosterone, and E_2 secretion. Moreover, all concentrations of ghrelin decreased steroid secretion in FSH- and LH-stimulated follicles. Western blot analysis showed that ghrelin inhibited expression of 3β-HSD, 17β-HSD, and CYP19 proteins.
Conclusion
The presented study indicate very clearly that, as adipokines produced by adipose tissue, ghrelin acts on accelerate puberty, while in contrast to the adipokines, during sexual maturity prevents from excessive secretion of steroids by ovarian follicles.
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P150

Abstract withdrawn.

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P151

Detection of ZP-autoantibody in the sera from infertile women using a human multiepitope peptide as antigen

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Background
The role of autoantibodies against zona pellucida (ZP) in infertility and success of IVF is debatable. In the present study, a novel recombinant human zona protein is used to address this issue.

Materials and methods
Fourteen serum samples positive for porcine ZP, six from infertile patients, and 20 from healthy women were analysed. Sera were tested for their reactivity against a recombinant multi-epitope peptide (named ZPCP7) composed of ten predictable antigenic peptides from human ZP proteins by ELISA. In addition, seven identified fine epitope peptides (fused with a GST188 carrier protein) of human ZP3 and ZP4 were used as antigens for western blotting.
Results and discussion
Eight of 14 porcine ZP antibody positive sera and one out of six testing infertile patients showed positive reaction in ELISA when all sera were tested at 1:2560 dilution. Further, out of nine serum samples seven also showed reactivity to three to five of epitope peptides fused with truncated GST188 carrier protein in western blot respectively. Thus, seven sera samples were finally determined as ZP antibody positive in our two steps method of analysis. Also, the positive serum sample (no. S3) was able to recognize native human ZP in indirect immunofluorescence experiment. These results provide evidence that ZP antibodies are associated with infertility. The use of recombinant multi-epitope peptide based on human zona proteins may also overcome the problem of false positivity observed using porcine ZP as an antigen.

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P152

Occurrence of rare three types of chromosome configurations in a Murrah buffalo (*Bubalus Bubalis*)

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Introduction
Chromosomal abnormalities are deviations in normal genetic architecture and lead to disorders in bearer individuals. These can be both in number and structure of autosomes or sex chromosomes, usually inflict adverse effects on reproductive performance in domestic animals. Present report is on a female Murrah buffalo (*Bubalus bubalis*), which was among animals with inefficient reproductive performance and cytogenetic screening programme.
Material and method
The animal was a 9-year-old female buffalo with a long history of different types of reproductive problems including abortion, stillbirth, temporary anoestrus, irregular heat cycle, repeat breeding and even calving in early age of three parities. Metaphase chromosome preparations were obtained with whole blood cell cultures standard method. Slides were stained with Giemsa, treated for R-banding (RB-FPG technique) and C-banding. Karyotypes were constructed and abnormal chromosome was identified.
Result and discussion
Reproduction history covered a period from 2007 to 2013, which included three calving. Succeeding to second calving, buffalo conceived after six unsuccessful AIs from different bulls, inter-calving period between second and third calving was $\sim$ 3.5 years. Cytogenetic evaluation of 407 metaphase spreads revealed three types of chromosome configurations, viz. 49, 50, and 51 all with XX, overall frequency was 16.7, 76.7, and 6.6%, respectively. The R- and C- bandings confirmed autosome 11 both in monosomy ($2n=49$) and trisomy ($2n=51$) respectively. This unusual chromosomal constitution might have arisen due to non-disjunction during early stage of zygotic development of the buffalo. Nevertheless in young stage ovulations occur in such cases, subsequently follicular atresia becomes fast resulting in anoestrous condition as in this buffalo.

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P153

Effect of different diet fat sources on *in vitro* maturation of Anglo-Nubian goats oocytes

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There is a lack of information about effects of the diet fat source on goat reproduction. The aim of the present study was to evaluate the fat source effect on *in vitro* maturation (IVM) of oocytes. Eighteen goats were divided into three groups, on diets (40% concentrate/60% corn silage) with 4% (dry matter) of ether extract from different fat sources [soy oil (SG) and linseed (LG), Megalac® (MG)]. The does passed thru an adaptation period (15 days) and, then, an experimental period of 42 days. The laparoscopic ovum pick-up was performed on day 42 of the trial, after 36 h of an ovarian superstimulation protocol [FSH (80 mg) + eCG (300 IU) after 36 h of the last shot of GnRH from the Ovsynch protocol]. The recovered oocytes were classified as viable by morphological appearance. IVM and analysis were performed for each individual. The oocytes were incubated in maturation media (TCM 199 supplemented with 10% inactivated heat goat serum) at 38.5°C and 5% CO2, 27 h. Oocytes were subsequently fixed and stained on Hoechst 33342 and analyzed using a fluorescent microscope. Data were analyzed by ANOVA. The number of viable oocytes (means $\pm$ S.E.M) were significantly higher in SG (10.0 ± 2.03) than the others groups (LG: 5.17 ± 1.47 and MG: 5.83 ± 0.48). The nuclear maturation had no difference between groups (SG: 89.0 ± 7.96; LG: 79.3 ± 7.91; and MG: 92.5 ± 4.08). In conclusion, the fat source affect the number of goat viable oocytes, nonetheless, it does not affect the nuclear maturation rate. Financial support: FAPESP.

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P154

Chronic sympathetic stress during gestation delays puberty and follicular development of female offspring

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It is widely accepted in the literature that the exposure to stress during early stages of development could have permanent and severe effect in the developing organism. We studied the effect of maternal sympathetic stress to pregnant rats, and studied the permanent effects in follicular development of the ovary of the progeny.
Methods
Pregnant Sprague – Dawley rats were cold stressed (4°C 3 h/day) during all pregnancy. Control group was maintained at room temperature. The female offspring was randomly distributed in the prepuberal (30 days old) and adult's rat groups. Ovaries were used to morphometric analysis, real-time PCR, incubation assay, noradrenaline concentration, and EIA for determining plasma levels of ovarian steroids.
Results
Prenatal stress decreased the number of primordial, primary, and secondary follicles at neonatal stage (4 days old) and affected the early follicular development. These early changes translated into a decreased prepubertal antral follicular development and delayed puberty. The fact that ovarian NE was decreased could mean that the increased maternal NE plasma levels during gestation might induce a compensatory response in the progeny by decreasing the development of sympathetic nerves of the ovary. After an erratic beginning in estrous cycle activity, the rats were regulated but increased atretic follicles and decreased the number of corpus luteum most probably related by an early decrease in follicular population and hence an early reproductive senescence.
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P155

Proteins in porcine follicular fluid as potential biomarkers for fertility

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Introduction
Following IVF, blastocysts produced from oocytes recovered from gilts fed a high fibre diet for the preceding 19 days contained more cells than blastocysts from oocytes of control-fed gilts. Oocytes were collected on day 19 of the gilts' third oestrous cycle and matured in 10% of their own follicular fluid (FF), suggesting that FF may confer the reproductive benefits. The current study compared the protein composition of pooled FF from six high fibre-fed pigs whose oocytes produced blastocysts and 11 control-fed pigs whose oocytes did not produce blastocysts, in search of biomarkers for fertility or nutritional status.
Materials and methods
Abundant proteins including albumin and transferrin were depleted from FF samples. Remaining proteins were labelled with iTRAQ reagents and detected by liquid chromatography tandem mass spectrometry. Lactate dehydrogenase (LDH) activity and oestradiol concentrations were determined in FF of individual animals ($n=40$) by enzyme activity assays and RIA respectively. Statistical tests carried out included two-way ANOVA and correlation analyses.
Results and discussion
Out of 352 proteins detected, 21 were differentially expressed between the FF pools, including LDH, glutathione *S*-transferase and plasma kallikrein. LDH activity did not significantly differ between feeding groups or blastocyst yield but was negatively correlated with follicle size, FF volume and oestradiol concentration (all $P<0.05$).
Conclusion
This study has shown that the protein composition of FF could be a marker for prior nutrition and/or later fertility. A more comprehensive proteomic study is required to distinguish the effect of diet and later fertility.
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P156

Glucocorticoid metabolism in bovine cumulus–oocyte complex during *in vitro* maturation

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Introduction
Tone of glucocorticoid action in target organs is regulated by relative activity of 11βHSD type1 (11HSD1), a bidirectional oxidoreductase that mainly converts cortisone to active cortisol, and type 2 (11HSD2), an oxidase that inactivates cortisol to cortisone. We have demonstrated that bovine cumulus–oocyte complex (COC) expresses 11HSDs and both reductive and oxidative activities are present in COC undergoing IVM. In the present study we investigated relative role of these 11HSDs and their localization in the bovine COC.
Materials and methods
Bovine COCs were matured in M199. Activities of 11HSDs were determined by measuring conversion of ^{3}H-labelled cortisol/cortisone/dexamethasone to respective metabolites in intact COC/denuded oocyte (DO)/dispersed cumulus cell (DCC). To discriminate oxidative activities of 11HSD1 and 2, COCs were treated with 11-keto-progesterone (11KP), a selective inhibitor of 11HSD2. The presence of 11HSDs in the oocyte was examined using immunohistochemistry.
Results and discussion
The reductive activity led by 11HSD1 increased as IVM progressed while the oxidative activity was unchanged. 11KP equally suppressed oxidation of cortisol and dexamethasone, a selective substrate for 11HSD2. DO showed oxidative activity comparable to that of intact COC but not reductive activity. DCC did not show either activity. A strong signal of 11HSD2 was observed in oocyte. These results indicate that the bovine COC possesses both activating and inactivating abilities led by 11HSD1 and 11HSD2. Oocyte is the main site of oxidation where 11HSD2 is expressed while CC appears to be the site of 11HSD1 led reductive activity although integration of COC is necessary for 11HSD1 action.

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P157

A high concentration of polyvinylpyrrolidone in culture medium affects the number of tranzonal projections in bovine oocyte–granulosa cell complexes in long-term culture

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Introduction
A high concentration of polyvinylpyrrolidone (PVP, average molecular weight 360 000) supplementation in culture medium exerts a positive influence on the growth and morphological organization of bovine oocyte–cumulus/granulosa cell complexes in a long-term culture. The objective of the present study was to determine the effect of PVP supplementation on the number of transzonal projections (TZPs) traversing the zona pellucida from cumulus cells to the oocyte.
Materials and methods
Bovine oocyte–granulosa cell complexes with a mean oocyte diameter of ~100 μm were cultured on membrane inserts for 14 days, with culture medium that was supplemented with or without 4% PVP (w/v). After the 14-day culture period, the oocytes enclosed with cumulus cells were collected and fixed for immunofluorescence microscopy. F-actin of these cells was labelled with Alexa Fluor 488 phalloidin and visualized using a confocal laser-scanning microscope.
Results and discussion
Although numerous TZPs were observed irrespective of the presence or absence of PVP, there were significantly more TZPs after growth in medium with PVP than in control medium, as indicated by image analysis using ImageJ. This observation suggests that promoting effects of PVP supplementation on oocyte growth may involve modulation of connectivity between oocytes and their companion granulosa cells.

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P158

Mouse CD11c+ dendritic cells are crucial in folliculogenesis, luteinization, and luteal maintenance

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Introduction
The ovary shows cyclical events, such as folliculogenesis, ovulation, and luteinization. During these events, leukocytes are known to infiltrate in the ovary. However, the role of each type of leukocytes in the ovary is not fully determined. In order to determine the roles of dendritic cells (DC) in the ovary, we conducted following experiments.
Materials and methods
i) To analyze DC population, immature mice were injected with PMSG followed 48 h later by hCG to make ovarian cycle. Ovaries were taken before PMSG, and at 0, 12, and 24 h post hCG for flow cytometory. ii) To evaluate DC function, CD11c-DTR transgenic mice which enables depletion of CD11c+ DCs were used, and compared with control mice. For study of folliculogenesis, sera and ovaries were collected 48 h post PMSG to evaluate estradiol level and follicle count. For study of luteinization, these were collected at 24 h post hCG to evaluate progesterone level and corpus luteum count. iii) To observe recovery ovarian function after DC depletion, DC depleted mice were left for 2 or 6 weeks to create DC recovered, and ovaries and sera were collected.
Results and discussion
i) The number of DCs is increased in folliculogenesis and luteinization. ii) In DC depleted mice, the folliculogenesis and luteinization were impaired. iii) DC recovery did not restore ovarian function. These facts together with our findings suggest that dysfunction of DC may cause ovarian dysfunction such as luteal deficiency and premature ovarian insufficiency.

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P159

Investigating whether Kiss1 KO mice can be used as a model for PCOS and age-onset diabetes

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Polycystic ovary syndrome (PCOS) is generally thought to be a genetic disease, which affects 5–10% of women of reproductive age (approximately 11–47 years old). Typically, PCOS is characterized by the formation of cysts on the ovaries and ovulatory failure. Characteristically PCOS results in high levels of androgens such as testosterone and insulin resistance within the blood. The ovaries of *Kiss1* mutant mice have been shown to frequently form multiple cysts (Lapatto *et al.,* 2007, Endocrinology, 148:4927-4936) leading us to hypothesise that these mice might provide a model for PCOS. Mutant *Kiss1* mice have hypogonadotrophic hypogonadism and fail to ovulate, which may contribute to cyst formation. To test this hypothesis, we compared androgen levels in12 month old *Kiss1* mutant mice compared to age matched wild-type mice. Furthermore, the ovaries were collected for histological analysis to investigate the presence of cysts and immunohistochemical detection of proteins involved in the metabolism of androgens and estrogens. We also investigated glucose responses in these aged mice as it has recently been reported that kisspeptin production by the liver can impair insulin secretion (Song *et al.,* 2014, Cell Metabolism 19:667-681). Serial tail bleeds after an injection of glucose were taken from wild type ($n=14$) and mutant mice ($n=12$) and assayed for glucose clearance and insulin levels. *Kiss1* KO mice showed reduced clearance of glucose from the blood stream. We are currently investigating whether this results from impaired insulin release or insulin resistance.

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P160

Androgen action within the preantral follicle; a role for the epidermal growth factor receptor?

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Introduction
Polycystic ovary syndrome (PCOS) is the most common cause of anovulatory infertility affecting over 5% of the female population. PCOS is characterized by aberrant early follicle development in which hyperandrogenism is thought to play a key role. However, the molecular mechanisms of androgen action within the ovary remain largely unknown. Recent evidence suggests androgens may be acting, in part, through modulation of growth factor signalling. We have recently shown that members of the epidermal growth factor (EGF) family play an important role in promoting preantral follicle development in the mouse. The aim of this study was to investigate androgen–EGF interaction within preantral follicles.
Materials and methods
Preantral follicles obtained from PND 15 mouse ovaries (C57BL/6) were isolated and cultured in the presence of EGF (10 ng/ml), dihydrotestosterone (DHT) (10 nM) with or without the EGF receptor (EGFR) inhibitor AG1478 (10 μM). Follicle growth was monitored over 72 h, with samples processed for qPCR and immunohistochemistry at 24 and 72 h.
Results and discussion
EGF treatment significantly increased follicle growth from 24 h onwards ($P<0.001$). DHT increased preantral follicle growth from 48 h ($P<0.0001$), fitting with the PCOS phenotype. Combined incubation with EGF and DHT resulted in elevated growth above that of individual treatments ($P<0.05$), indicating an additive effect. The addition of AG1478, a specific EGFR tyrosine kinase inhibitor, not only reversed EGF regulated follicle growth but also attenuated the effect of DHT on growth at 48 h ($P<0.001$), suggesting that the effects of DHT on follicle growth are mediated, at least in part, through the EGFR.

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P161

Differential role of TGFβ2 during follicle activation and preantral follicle growth

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Introduction
Female mammals are born with a finite reserve of primordial follicles which dictates the reproductive lifespan. Despite this the factors responsible for primordial follicle activation remain largely unknown. The transforming growth factor beta (TGFβ) superfamily has been implicated in early follicle development however little attention has been given to the effects of TGFβ2, expression of which has been demonstrated in the ovary. This study aimed to examine the role of TGFβ2 on follicle activation and preantral follicle growth.
Materials and methods
Neonatal (PND4) mouse ovaries (C57BL/6) were treated with TGFβ2 (1, 10, and 100 ng/ml) for 6 days in a whole-ovary culture system. Cultured ovaries were fixed for immunohistochemical localization of vasa and laminin and image analysis was performed to classify and quantify follicle proportions. In addition, preantral follicles (two or more layers of granulosa cells) were mechanically isolated from PND15 mouse ovaries. Single follicles were cultured in 96-well plates with TGFβ2 (0.01, 0.1, and 1 ng/ml). Follicle area was measured at 0, 24, 48, and 72 h (ImageJ).
Results and discussion
TGFβ2 induced a dose-dependent suppression of follicle activation in whole ovary as demonstrated by reduced depletion of primordial follicles and fewer growing follicles. No observational differences in caspase-3 staining were demonstrated between treatment groups eliminating TGFβ2 toxicity. By contrast, TGFβ2 promoted a significant increase in growth of isolated preantral follicles at all concentrations after 48 h (two-way, ANOVA $P<0.0001$). These results highlight differential effects of TGFβ2 during primordial follicle activation and preantral follicle growth.

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P162

Mycotoxin-contaminated diet affects mouse oocyte quality

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Introduction
Mycotoxins, such as deoxynivalenol (DON), zearalenone (ZEN), and aflatoxin (AF), are commonly found in many food commodities and may impair the growth and reproductive efficiency of animals and humans. We investigated the effects of a mycotoxin-contaminated diet on mouse oocyte quality.
Materials and methods
Maize contaminated with DON (3.875 mg/kg), ZEN (1,897 μg/kg), and AF (806 μg/kg) was incorporated into a mouse diet at three different levels (0, 15, and 30%, w/w).
Results and discussion
After 4 weeks, oocytes from the mycotoxin-fed mice exhibited low developmental competence with reduced germinal vesicle breakdown and polar body extrusion rates, and the majority of embryos could not develop to the morula stage. Moreover, a large percentage of oocytes derived from mice that were fed a mycotoxin-contaminated diet exhibited aberrant spindle morphology, lower actin expression, a loss of the cortical granule-free domain, and abnormal mitochondrial distributions, which further supported the decreased oocyte quality. The fluorescence intensity analysis showed that the general DNA methylation levels increased in oocytes from high dose mycotoxin-fed mice. Mouse oocyte histone methylation was also changed, showing with altered H3K9, H4K20, H3K27, and H4K20 level. Thus, our results indicatethat naturally occurring mycotoxins have toxic effects on mouse oocyte quality.

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P163

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P164

Obesity depresses Toll-like receptor superfamily members in the ovary

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Introduction
Obesity and its associated metabolic disorders lead to ovarian failure and infertility. Nonetheless, the hierarchy of pathways leading to ovarian dysfunction is unknown. Presently, we studied the impact of obesity on ovarian innate immune response and steroidogenesis throughout the time.
Materials and methods
An *in vivo* study was conducted on C57Bl/6J mice ($n=8$/group) fed a chow-diet versus high-fat diet during 4- (C-4w; HFD-4w) or 16 weeks (C-16w; HFD-16w). Further on, whole ovaries were used for quantification of: i) Toll-like receptors (TLR)1, TLR2, TLR4, and TLR6 and downstream pathway proteins (MyD88, TRIF, Nfkβ2, and RELA); ii) tumor necrosis factor alpha (TNFα) and interleukin (IL)1β; and iii) steroidogenesis marker Star-protein mRNA transcription by Real-Time PCR.
Results and discussion
Consistently, both 4w and 16w HFD groups showed lower TLR1, TLR4, and TLR6 mRNA level ($P<0.05$). Regarding TLR2, mRNA was decreased only in HFD-4w, comparing with C-4w ($P<0.01$). Looking at TLR adaptors, MyD88 mRNA was decreased in HFD-4w and HFD-16w ($P<0.05$), while TRIF was diminished exclusively in HFD-16w ($P<0.01$). No changes were seen in Nfkβ2, whilst RELA presented lower mRNA in both HFD groups ($P<0.05$). Inflammatory markers IL1β and TNF were decreased in HFD-4w ($P<0.05$), but increased in HFD-16w ($P<0.05$), regarding respective controls. Star mRNA transcription was reduced in both HFD groups ($P<0.01$). Generally, the startling decrease in TLR and inflammatory markers mRNA after 4w may suggest HFD depresses local innate immunity, with a direct down-regulation of steroidogenesis. Further studies are being pursued to clarify TLRs role on ovarian failure due to obesity.

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P165

Space pup project: sperm preservation in space station

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Sustaining life beyond earth either on space stations or other planets will require a clear understanding of how the environment in space affects key phases of mammalian reproduction. However, because of the difficulty of conducting such experiments in live animals, most studies on reproduction in space have thus far been carried out in species such as fish or amphibians. Mammalian embryos can only be cultured for a few days and could pose technical challenges for the astronauts. For this reason experiments on mammalian reproduction in gamete cells was considered unfeasible using present-day technology. In a previous study, we found that freeze-dried spermatozoa can be preserved at room temperature for a few months without losing their fertilization capacity. This is advantageous for space experiments because the samples have lower mass and do not require a freezer for storage during launch or landing. Here, we propose to examine the effects of cosmic radiation on spermatozoa using freeze-dried samples. These freeze-dried spermatozoa collected from four different mouse strains were lunched at August 4th, 2013 and kept on board the ISS in the Japanese Experiment Module 'Kibo', then exposed to cosmic radiation during 2–3 years. We lunched three boxes of sample for space and first box will be return in April 2014. After came back to earth, we will try to make offspring from them by ICSI and examine the effects of cosmic radiation on sperm DNA. This will be the first step for studying mammalian reproduction in space.

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P166

Amino acid and glucose profiles of bovine oviduct epithelial cell secretions in response to 17β-oestradiol and progesterone

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Oviduct luminal fluid supports early embryo development. The composition this fluid has been debated despite studies using post mortem samples and direct sampling under anesthesia. The routine availability of such data would benefit *in vitro* embryo culture in addition to our understanding of the influences of maternal physiology on *in vivo* oviduct fluid composition.
We have devised an *in vitro* model for the determination of bovine oviduct epithelial cell (BOEC) fluid composition.
BOECs were isolated from reproductive tracts and seeded to the apical fascia of Transwell porous supports at 10^6 cells/ml. Epithelial disposition (>99%) was confirmed by FACS against CK18. At confluence – confirmed by TEER $>700\ \Omega\times cm^2$ – BOECs were maintained in an air–liquid interface for 24 h. The amino acid and glucose in content in the fluid accumulated on the apical fascia, termed *in vitro* Derived Oviduct Fluid (*iv*DOF), was analysed. To assess hormonal impact, basal medium was supplemented with 29.4 μM 17β-oestradiol and 6.4 nM progesterone.
17β-oestradiol and progesterone were antagonists for glutamine, phenylalanine, leucine, and lysine secretion ($n=6$). The concentration of glucose in ivDOF dropped from 2.5 to 1.2 mM following 17β-oestradiol exposure ($n=4$). Significance was tested by one-way ANOVA coupled with the Holm–Sidak method for overall significance ($P<0.05$).
A functional model of the bovine oviduct has been developed to study the composition of the luminal environment created *in vitro*. The results show 17β-oestradiol promoting the establishment of a comparatively hypoglycemic ivDOF microenvironment. This could relate to the subfertile physiology of females with metabolic disorders such as PCOS who ordinarily demonstrate hyperglycemia.

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P167

Generation of rats from vitrified oocytes with surrounding cumulus cells via IVF with cryopreserved sperm

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The aim of the present study was to evaluate fertility of vitrified rat matured-oocytes with surrounding cumulus cells after IVF with cryopreserved sperm. Oocytes with surrounding cumulus cells and cumulus cells denuded oocytes were vitrified with 30% (v/v) ethylene glycol + 0.5 M sucrose + 20% (v/v) FCS in PBS (PB1) by using Cryotop. After warming, oocytes were co-cultured with fresh or cryopreserved epididymal sperm for 10 h. Although the fertilization (two pronuclei formation: 2PN formation) of vitrified denuded oocytes were not observed after IVF with fresh sperm, some vitrified oocytes with surrounding cumulus cells ($32.7\pm4.3\%$) were fertilized. Fresh and vitrified oocytes with surrounding cumulus cells (62.9 ± 5.1 and $41.3\pm5.0\%$ respectively) were also fertilized after IVF with cryopreserved sperm. In addition, the 113 of vitrified oocytes after IVF with cryopreserved sperm were transferred into the oviducts of recipients. As the results, seven live pups derived from vitrified oocytes and cryopreserved sperm were produced. In conclusion, to our knowledge the present study demonstrates for the first time that vitrified rat oocytes can be fertilized *in vitro* with cryopreserved spermatozoa and that 2PN embryos obtained from cryopreserved gametes via IVF can develop to term after embryo transfer.

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P168

Comparative effects of administration of kisspeptin-10 and GnRH on LH secretion in buffalo cows

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Introduction
Previous studies have proven the efficiency of GnRH for stimulating both the pulse and amplitude of LH releasing in buffaloes, but not study of kisspeptin efficiency.
Materials and methods
There were three treatments: i) kisspeptin-10 (K-10; a single i.v. of 1000 pmol/kg b.w. dosage), ii) GnRH (a single i.m. injection of 10 μg/cow dosage), and iii) distilled water (DW; a 2 ml single i.v. injection). All six animals were treated and the early luteal phase (day 10 after onset of estrus) was induced by prostaglandin F2α. Blood samples were collected every 15 min between −2 and 3 h and every 30 min after that for the next 3 h for plasma LH analysis.
Results and discussion
The frequency of the LH pulse after treatment with K-10 (mean ± S.E.M.) trended greater than after GnRH and DW (2.2 ± 0.4, 1.3 ± 0.3, and 1.7 ± 0.3 peak/6 h respectively). The peak of LH pulsatility occurred after GnRH injection was highest (GnRH 28.2 ± 2.8, K-10 2.1 ± 0.7, and DW 1.3 ± 0.3 ng/ml) and buffalo injected with GnRH also had the the largest area under LH response curves 6 h after treatment (GnRH 2602.7 ± 398.2, K-10 360.1 ± 79.0, and DW 416.6 ± 112.6 min × ng/ml) ($P < 0.05$). In the luteal phase, GnRH treatment stimulated the ovulatory LH surge level but K-10 did not. This could indicate that the negative feedback control in the luteal phase may inhibit kisspeptin induced GnRH releasing in the hypothalamus which might not be enough to activate an LH surge.
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P169

Ovulation induction in prepubertal Sahiwal × Friesian cross bred heifers by the use of clomiphene citrate and hCG

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Introduction
Clomiphene citrate belongs to group of drugs known as selective estrogen receptor modulators. In this study, an attempt was made to induce ovulation in crossbred prepubertal heifers by the use of clomiphene citrate and hCG. It was hypothesized that clomiphene citrate, being antiestrogen, would remove negative feedback effect of estrogen on the release of pituitary gonadotropins. Elevated gonadotropins would support follicular growth to a point where hCG would induce ovulation.
Materials and methods
Six prepubertal Sahiwal × Friesian heifers were orally given clomiphene citrate at 300 mg/heifer for 9 days. Another six heifers served as control. On the 10th day, hCG was given to all heifers at 2500 IU. Blood was collected on alternate days up to 14 days after hCG injection, starting from 1 day before start of treatments and plasma was assayed for progesterone and estradiol concentrations.
Results and discussion
Mean estradiol level prior to start of treatment was below 3 pg/ml in heifers of the two groups. This pattern persisted for the first 6 days of treatment, during last 3 days of treatment estradiol level rose, reaching peak of 16.20 ± 8.74 pg/ml on day of hCG injection in four heifers of treatment group. After hCG injection, mean estradiol level of treated group gradually dropped and progesterone level rose. In control heifers, mean estradiol concentration remained >4 pg/ml throughout the experiment. Progesterone assay showed that four heifers (67%) in treated group ovulated (P4 >1 ng/ml plasma) compared to one heifer (16%) in control group. In conclusion, clomiphene citrate along with hCG can be used to induce ovulation in prepubertal heifers.

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P171

Evaluation of the cyclophosphamide gonadotoxicity and the protective effect of GnRH analogues in mice model

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Introduction
While gonadotoxicity of cyclophosphamide (Cy) has been well demonstrated in mice model, the kinetic and effect at different follicular stages are still not well described. Understanding these phenomena is however essential to further develop pharmacological protective approaches. Here, we studied the effect of Cy at different doses and timing on follicular development as well as the potential gonadoprotective effect of the GnRH analogues (GnRHa) during chemotherapy.
Materials and methods
Cyclophosphamide at different doses was injected i.p. (100, 200 and 500 mg/kg). Mice were sacrified at nine different time points between 1 h and 7 days post-injection. In a second experiment, the mice were daily injected with various doses (2, 20, 200 and 500 μg/kg) of GnRHa s.c. or i.m. for 21 days with or without Cy at day14. The followed parameters were evaluated: fertility, estrous cycles by vaginal smears, ovarian reserve by follicular count, growing follicles ratio and immunohistology (TUNEL, caspase-3, Ki67 and AMH).
Results and discussion
The cyclophosphamide induced-follicular depletion was correlated to the dose with a mean follicular loss of 50% after one 200 mg injection. Chemotherapy affected both quiescent and growing follicles. No apoptosis has been observed on primordial and primary follicles despite the pool was already reduced after 24 h. Mechanisms of action of Cy on this pool should be further investigated. GnRHa had no effect on Cy induced-follicular depletion and did not inhibit the pituitary–gonadal axis in mice as effectively as in human.

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P173

Fibrotic remodeling and duct obstruction are major patho-physiological mechanisms in bacterial epididymitis in mice

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Introduction
Bacterial epididymitis is a common disorder in urological outpatient clinics and usually results from ascending infections originating in the urinary tract often involving uropathogenic *E. coli* (UPEC) as causative pathogen. In 40% of patients with acute epididymitis, impaired semen parameters persist even after antibiotic treatment. Members of the TGFβ cytokine superfamily, such as activin A, have recently been identified in the epididymis and are critical mediators of inflammation and fibrosis. Inhibition of activin action by follistatin has emerged as a potential therapy, because it modulates inflammation and fibrosis.
Materials and methods
Activin signaling molecules and fibrotic markers were investigated by immunohistochemistry and qRT-PCR in an acute epididymitis model by infecting mice with the uropathogenic isolate UPEC CFT073 for 3d and 7d.
Results and discussion
At 3d post-infection, epididymides showed a leukocytic infiltration and decreased sperm numbers in the lumen, but no or mild interstitial fibrosis. At 7d post-infection, ductal obstruction and massive fibrotic remodeling was visible especially in the cauda region. Concomitant elevation of epididymal smooth muscle actin (SMA) and collagen 1α mRNA expression accompanied the fibrosis. At 3d post-infection, a strong increase (>20-fold) in mRNA expression of inhibin subunit-α (Inha), activin receptor (ActvR)IIB and SMA were detected in the cauda, whilst no changes were observed in activin subunits (Inhba, Inhbb), ActvRI and follistatin. Of note, Inhba mRNA, SMA and collagen 1α were all increased considerably at 7d post-infection, raising the possibility of a causative link between the increase in activin and the fibrotic damage in acute epididymitis.

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P174

Model for differentiation of mouse embryonic stem cells into steroidogenic cells

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Introduction
Steroidogenic factor 1 (SF-1) is essential for the development and function of steroidogenic tissues. Stable incorporation of SF-1 into embryonic stem cells has been reported to prime the cells for steroidogenesis.
Materials and methods
In this study, we established SF1 transgenic mouse embryonic stem cell (SF1-mES cells) and analyzed expression of steroidogenesis-related genes and gonadal lineage-markers. We measured the secreted progesterone in the cell medium because progesterone is the first metabolite of sex steroid hormone. As well as, we differentiated mES cells into functional granulosa-like cells or Sertoli-like cells using various culture condition including growth factors or hormones. To test the phenotype for granulosa-like cell, we confirmed transcripts of specific forkhead transcription factor *FOXL2* and the FSH receptor (*FSHR*). In the other hand, we monitored some specific genes related with differentiation into testicular tissue.
Results and discussion
We observed the progress to primitive streak–mesendoderm by gene expression analyses. In addition, we observed that differentiated SF1-mES cells expressing the steroidogenic enzymes, such as 3β-hydroxysteroid dehydrogenase, cytochrome P450-containing enzyme (CYP)-11A1, and CYP19A1. Using the advanced approach, we explored culture conditions that optimize SF-1-mediated differentiation of ES cells into defined steroidogenic and gonadal lineages. We induced functional granulosa-like cells or Sertoli-like cells. We established the effective protocol to generate functional ovarian or testicular cells. The derivation of these cells explores new avenues for the further study and potential application of these cells in steroidogenesis.

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P175

Early life programming of adult Leydig cell function

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Introduction
Adult male testosterone levels are influenced by fetal events, but how is unknown, as adult Leydig cells (ALC) do not differentiate until puberty. We hypothesised that adult Leydig stem cells are present in the fetal testis, and are susceptible to programming by fetal androgen exposure. We have shown that ALC derive from interstitial cells that express chicken ovalbumin upstream promoter transcription-factor II, essential postnatally for ALC development, and androgen receptor (AR). These cells are androgen regulated and present in the fetal testis across species. As deficient fetal androgen action is implicated in the origin of male reproductive disorders, we investigated a potential mechanism to explain how fetal programming of adult Leydig stem cells occurs.
Materials and methods
AR knockout mice were generated by Cre/LoxP technology. Pregnant female rats were treated daily (e13.5–e21.5) with 500 mg/kg per day DBP in 1 ml/kg corn oil. Testes from DBP-exposed rats were evaluated in adulthood using RT-PCR, triple IHC and ChIP. Testosterone and LH were measured in blood plasma.
Results and discussion
Reduction in fetal androgen action via AR knockout (mice) or dibutyl phthalate (DBP)-induced reduction in intratesticular testosterone (rats), reduced adult Leydig stem cell number by ~40% at birth through to adulthood, and induced compensated ALC failure (low/normal testosterone and elevated LH). Reduced *Star* transcription in DBP-exposed adult rats may occur as a result of increased trimethylation of Lys27 at histone 3 (H3K27me3), in the proximal promoter of *Star*. These findings suggest that fetal androgen exposure can fundamentally reprogramme adult hormone production and thus overall male health.

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P176

Characterization of pituitary-derived cell lines, Tpit/E, Tpit/F1 and TtT/GF

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Introduction
The pituitary anterior lobe consists of five types of hormone-producing cells and non-hormonal cells. In non-hormonal cells, *Sox2*-expressing cells exist as stem/progenitor cells and play a role in the regeneration of hormonal cells. However, elucidation of the differentiation mechanism is yet uncovered. Established cell lines are frequently used as a model system not only for cellular physiology and gene expression but also for cell differentiation. In this study, we compared gene expression profiles of the pituitary-derived non-hormonal cell lines, Tpit/E, Tpit/F1 and TtT/GF.
Materials and methods
Comparison of gene expression profiles by using microarray analysis and real-time PCR for the three cell lines and immunocytochemistry were performed.
Results and discussion
Three lines showed an expression of *Sox2* at various degrees. Tpit/E showed the epithelial phenotype by expression of *E-cadherin, EpCAM, Krt8* and *Snail2*. Notably, expression of stem/progenitor marker *Sox2*, *Sox9* and *Cxadr,* indicate that it has highest stemness among three cell lines. Tpit/F1 expressed marker genes of epithelial and mesenchymal factors in addition to *Sox2*, suggesting that this cell line is in transiting state of the differentiation or MET. TtT/GF showed the mesenchymal phenotype expressing *Vimentin, Prrx1, Prrx2, Nestin* and *CD34* genes, and might have ability to differentiate into pituitary vascular endothelial cells or pericyte. These data suggest that these three cell lines are useful model to investigte of pituitary stem/progenitor cells as well as pituitary organogenesis.

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P177

Functional and molecular features of the ID4+ germline stem cell population in mouse testes

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Continual spermatogenesis relies on the actions of an undifferentiated spermatogonial population that is comprised of stem cell and progenitor fractions. Self-renewal by spermatogonial stem cells (SSCs) maintains a foundational pool from which progenitor spermatogonia arise that transiently amplify in number before committing to a pathway of terminal differentiation. At present, molecular features that distinguish the stem cell state in mammalian spermatogonia are undefined. In previous studies, we identified the transcriptional repressor inhibitor of DNA binding 4 (ID4) as a putative SSC-specific marker. To examine this population in more detail, we have generated a novel transgenic mouse line in which ID4 expressing cells are marked by GFP. We found that ID4-GFP+ cells exist primarily as a subset of the type A-single pool and frequency is greatest in neonatal development then decreases in proportion during establishment of the spermatogenic lineage, eventually comprising ~2% of the undifferentiated spermatogonial population in adulthood. Based on functional transplantation analyses with isolated ID4-GFP+ and ID4-GFP− spermatogonial fractions, we discovered that most, if not all, SSCs reside in the ID4-GFP+ spermatogonial population and the ID4-GFP− spermatogonia represent the progenitor spermatogonial population. RNA-sequencing analysis revealed that 11 and 25 genes are expressed differentially between ID4-GFP+/stem cell and ID4-GFP−/progenitor fractions respectively. Collectively, these findings provide the first definitive evidence that stem cells exist as a rare subset of the A-single pool and reveal transcriptome features distinguishing stem cell and progenitor states within the mammalian male germline. This research was supported by grant HD061665 awarded to J.M.O. from the National Institutes of Health.

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P178

Presence of the juxtacrine factor EphrinB2 in a rat pituitary stem/progenitor cell niche

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Introduction
The anterior pituitary lobe is a key endocrine tissue composed of five types of endocrine-cells and non-hormonal cells. Among non-endocrine-cells, *Sox2*-expressing cells exist as stem/progenitor cells and play a role in the regeneration of endocrine-cells in the adult pituitary. Recently, we have reported that transcription factors, *Prop1*, *Prrx1* and *Prrx2,* are expressed in the pituitary stem/progenitor cells and these cells contact via a tight-junction protein CAR to construct a stem/progenitor cell niche. However, the micro-environment of niche for maintenance of stemness and self-renewal in the pituitary is still obscure. In this study, we attempted to identify the expression of juxtacrine factor *Ephrin* (*Efn*) and its receptor (*Eph*) in the pituitary stem/progenitor cells.
Materials and methods
Real-time PCR for *Efn/Eph* was performed using cDNA libraries of the pituitary and pituitary derived cell lines. Immunohistochemistry was examined to determine localization of EFN/EPH in the pituitary.
Results and discussion
Real-time PCR demonstrated that some *Efns* and *Ephs* related to *EfnB*-signaling are expressed in the postnatal pituitary. Immunohistochemistry for EFNs detected the cells positive for EFN-B2 and SOX2 in the marginal cell layer. Recently, we identified a candidate cell line of pituitary stem/progenitor cells, Tpit/E, which maintains stemness and self-renewal. Notably, Tpit/E cells express not only *Efn-B2* but also its receptors, such as *EphA4*, *EphB3, EphB4* and *EphB6*, indicating that reciprocal EFN-B2 signaling within Tpit/E cells themselves might maintain their stemness and self-renewal. Thus, the environment of pituitary stem/progenitor cell niche might be preserved by EFN-B2 signaling.

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P179

Krüppel-like factor 6 (klf6) is expressed in rat pituitary stem/progenitor cells and regulates the PRRX2 gene

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Introduction
Paired-related transcription factors, PRRX1 and PRRX2, which are expressed in mesenchymal tissues and participate in mesenchymal cell differentiation, were recently found in stem/progenitor cells of the pituitary gland of ectodermal origin. The present study aimed to clarify the role of PRRX1 and PRRX2 in the pituitary gland by identifying factors that regulate the expression of both genes.
Materials and methods
Immunohistochemistry, promoter assay, EMSA and siRNA-knockdown analysis were performed.
Result and discussion
Immunohistochemistry showed different stage of appearance for PRRX1 at the embryonic period and PRRX2 at the postnatal one, indicating a presence of distinct regulatory system for each gene. Assay for *Prrx1*- and *Prrx2*-promoter activity by co-transfection of the expression vector of several transcription factors showed a cell-type dependent manner. Comprehensive comparison of transcriptional activity of several factors performed in CHO cells, which do not express *Prrx1* and *Prrx2*, revealed the presence of common and distinct regulatory factors between both genes. Among them, KLF6 showed specific and remarkable activation of the *Prrx2*-promoter. EMSA and siRNA interference analysis revealed a potential ability of KLF6 to regulate the *Prrx2*-expression. Notably, KLF6 is likely to be involved in cell proliferation through the activation of *Prrx2*-expression. Finally, immunohistochemistry confirmed the presence of KLF6 in SOX2/PRRX2-double positive stem/progenitor cells of the postnatal pituitary gland. Thus, the finding of KLF6 might provide a novel clue to clarify the maintenance of stem/progenitor cells of the postnatal pituitary gland.

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P180

Concentration levels of TSH evaluation with species-specific MABs due to the dog reproduction disorders assessment in the course of hypothyroidism

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Introduction
Hypothyroidism is the disorder influences on reproduction of many species, including dogs. However the results are different, even among the same species.
Materials and methods
The concentration of TSH with species-specific monoclonal anti-TSH in dogs hypothyroidism was evaluated. TSH concentration was measured with a chemiluminescent ELISA solid phase two-point (TSH canine Immulite, DPC, LA, CA). Coefficients of variation within one were 5.4 and 3.8% respectively, with TSH concentrations 0.2, 0.5 and 2.6 mg/l. The inter indicators of variation were 6.3 and 8.2% at the level of TSH respectively 0.16 and 2.8 mg/l.
Results
The average TSH concentration in all the dogs was found to be normal. Nevertheless the increased TSH concentration (0–0.6 ng/ml) was found only in 5% dogs especially in females suffer from galactorrhoea. The reproduction disorders were not clinically detected in hypothyroidism dogs.
Discussion
The possible cause of the galactorrhea coexists with arterial hypertension is lack of the negative feedback of the HT in the hypothalamus causes the increase of TRH furthermore stimulates the secretion of prolactin according to the experimental results The significant increase in IGF1 follows the hyperprolactinemia. In hypothyroid dogs the increased basic GH secretion is directly related with primary hypothyroidism s causes increased serum IGF1 concentrations. The presence of the biendocrine cells produce simultaneously GH as well as TSH was confirmed. The transdifferentiation of the pituitary somatotrope cells into the tyrosomatotropic cells causes the GH increase in hypothyroid dogs according to the experimental results.

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P181

Invasion of S100β-positive cells into pituitary gland during embryonic period

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Introduction
Pituitary cells positive for S100 protein are non-hormone-producing cells and are composed of heterogeneous populations, including folliculo-stellate cell. They are argued for having various functions and are detectable only after birth, but their origin is not yet clarified. Recently, we had detected *S100β*mRNA in the cDNA library of embryonic pituitary by real-time PCR and observed invasion of non-hormone-producing cells from surrounding mesenchyme into the anterior lobe participating in pituitary vasculogenesis. These data indicate that a possibility of invasion of extra-pituitary precursor of S100-positive cells. This study aimed to verify whether S100-positive cells exist during embryonic period by using the transgenic rat expressing GFP driven by *S100β*-promoter (S100*β*-TG).
Materials and methods
S100-TG rat embryonic pituitaries at E15.5 to E20.5 were subjected to immunohistochemistry with various antibodies.
Results and discussion
Immunohistochemistry of the embryonic pituitary at E15.5 demonstrated that GFP-positive cells existed in the mesenchymal cells surrounding the pituitary and at the Atwell's recess, the intraglandular fossa. Thereafter, they were observed in the parenchyma of the anterior lobe on E18.5. Immunohistochemistry for GFP, PRRX1 (mesenchyme/pituitary-progenitor cell marker) and SOX2 (stem/progenitor cell marker) showed that GFP-positive cells were negative for PRRX1 and SOX2 in the Atwell's recess on E15.5, followed by conversion to PRRX1-positive/SOX2-negative cells. Further immunostaining with other markers suggested that GFP-positive cells have characters of mesenchymal-, neural crest- and/or vascular-cells. These data indicated that some of S100-positive cells in the pituitary gland are originated from the extra-pituitary cells during embryonic period.

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P182

Neuronatin is first expressed in pituitary stem/progenitor cells

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Introduction
Neuronatin (NNAT) is first identified in the neonatal neural tissue and is assumed to be involved in embryonic neurogenesis. Although defect of PROP1, a pituitary specific transcription factor important for pituitary organogenesis, causes reduced expression of the Nnat, the cellular localization and the vital roles of NNAT in the pituitary are still unclear. The present study examined the population of the NNAT-positive cells as to understand the role of NNAT in the pituitary.
Materials and methods
Ontogeny of NNAT expression by real-time PCR and immunohistochemistry for embryonic and postnatal pituitaries were performed.
Results and discussion
Real-time PCR showed that *Nnat* is abundantly expressed in the prenatal period and markedly decreased after birth. Immunohistochemistry showed that NNAT-signals were detected in the SOX2-positive cells in the pituitary primordium at embryonic day (E) 11.5, followed by appearance in all of pituitary cells at E13.5. On E21.5, the number of NNAT-positive cells gradually decreased. At E13.5, they were positive for PROP1 and then both proteins independently faded from the developing pituitary cells. Staining with cocktail of antibodies for pituitary hormones showed a small number of NNAT/hormone-double positive cells on E21.5. In the adult pituitary, a small number SOX2-positive cells were present and a few of them were positive for NNAT. Notably, there were other NNAT-positive cells negative to PROP1 and hormones as well as SOX2 suggesting that NNAT plays a role in SOX2-positive stem/progenitor cells, which launch to differentiate, and disappears in the terminal stage.

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P183

Early aging of mesenchymal stem/stromal cells derived from the bone marrow of α-1,3-galactosyltransferase knockout pig

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Introduction
The α-1,3-galactosyltransferase knockout (GalT KO) pig is used as the major model for xenotransplantation studies since the knockout of the α-1,3-galactosyl epitope makes pig organ transplantation successful in primates. The homozygous GalT KO pig is produced by a combination of nuclear transfer technique and mating with a heterozygous GalT KO pig. Since most studies using GalT KO pigs have analyzed only the immunological features of their cells, we analyzed the physical and genetic features of ear fibroblasts and bone marrow mesenchymal stem cells (BM-MSCs) derived from GalT KO pig and compared them with those of the wild pig.
Material and methods
Cells were seeded at concentrations of 1×10^3 or 1×10^4 cells/well in a 96-well plate. Grow rate was analyzed by ELISA using BrdU incorporation. At ~80% cell confluence, cell size was analyzed using an automated cell counter. For immune tolerance test, activated human peripheral blood mononucleated cells and BM-MSCs were cocultured in six transwells. The expression levels of p53, Bax, Bcl2, and TERT mRNA were analyzed by real-time PCR.
Result and discussion
GalT KO cells showed lower growth rate than wild cells, regardless of the cell concentration. The cell size of GalT KO BM-MSCs was 15.2 ± 0.2 μm (size range of other cells: 13.6 ± 0.3–14 ± 0.4 μm). GalT KO BM-MSCs showed higher immune tolerance than wild BM-MSCs, which showed the highest telomerase activity. Thus, our study showed that GalT KO BM-MSCs showed early aging compared to wild BM-MSCs *in vitro*.
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Keywords: α-1, 3-galactosyltransferase knockout pig, aging, telomerase activity, immune tolerance

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P184

Derivation of naive-type induced pluripotent stem cells in cattle using piggyBac transposition of doxycycline-inducible transcription factors

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Pluripotent stem cells (PSCs) in large domestic animals including cattle are expected to be used as means for genetic improvement and production of disease-model animals. There are two types of PSCs: naive and primed. Ontogenetically, naive PSCs correspond to an earlier developmental state compared to other types of PSCs and have the capacity for producing chimeric offspring. Previously reported PSCs in large domestic animals are classified as primed type. In this study, putative naive-type bovine induced pluripotent stem cells (biPSCs) were generated. Bovine amnion cells were transduced with piggyBac transposon (PB) vectors expressing the reprogramming factors (*Oct3/4, Sox2, Klf4, c-Myc* and/or *Nanog*). Since the PB vectors shared with a doxycycline (Dox)-inducible system, the expression of reprogramming factors can control under the condition with or without Dox. Two weeks after the addition of Dox, two types of colonies were obtained in different culture conditions. Under a primed PSC culture condition, flattened colonies were obtained and were mechanically propagated over 60 passages. Under a naive PSC culture condition, colonies showed round morphology and could be passaged as a single cell over ten times. When the former were passaged and maintained under the latter condition, the naive-type colonies were appeared. Resultant naive-type biPSCs had alkaline phosphatase activity and expressed pluripotent-related genes (OCT3/4, NANOG, ESRRβ and REX1). They also showed a normal karyotype for over 60 passages, formed embryoid body and differentiated into all three germ layers *in vitro*. This study shows the generation of biPSCs having similar characteristics to those of naive PSCs.

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P185

Serum-free culture of mouse trophoblast stem cells

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Introduction
Trophoblast stem cells (TSC) maintain their undifferentiated status under the presence of fibroblast growth factor 4 (FGF4), heparin and feeder cell-conditioned medium (CM). It has been reported that activin A can replace CM. However, even in such stem cell condition, a portion of TSC spontaneously undergoes differentiation. In addition, when TSC are induced to differentiate, all trophoblast subtypes autonomously appear without the addition of any exogenous factors. In order to ask at what degree various unidentified factors contained in fetal bovine serum (FBS) affect differentiation of TSC, we sought to establish serum-free culture conditions (SFC) for TSC.
Methods
FBS in the conventional TSC medium was replaced by knockout serum replacement (KSR). Growth of TSC was assessed by counting number of viable cells during serial passages. Integrity of TSCs in SFC was examined by RNA-sequencing. Furthermore, differentiation potency of TSCs maintained under SFC was investigated *in vitro* by expression analysis of marker genes and *in vivo* by the chimera analysis.
Results and discussion
Fibronectin or laminin was necessary for adhesion of TSCs in SFC. Presence of FGF4, heparin and activin A was not sufficient to achieve proliferation rate comparable to FBS-containing conditions. Addition of a pan-retinoic acid receptor inverse agonist and a ROCK-inhibitor was found to promote proliferation in SFC. TSCs cultured in such SFC had a gene expression pattern characteristic to TSCs and exhibited a differentiation potential both *in vitro* and *in vivo*. Thus, we established SFC for maintenance of TSC, which should be useful for future study.

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P186

Incomplete reprogramming of bovine fetal fibroblasts by the stimulus of low pH*

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Stem cells can provide a useful tool for studying on mechanisms of embryonic development and production of transgenic animal. Although somatic cells are able to convert into reprogrammed cells by introduction of exogenous reprogramming factors, however, the efficiency of iPSC is still low. Murine somatic cells without transfection of exogenous transcription factors were easily able to be reprogrammed by stimulus of low pH. Here we attempted whether to convert bFFs into pluripotent cells by the stimulus of low pH. To examine the conversion of somatic cells into pluripotency, we prepared for various culture conditions (A:DMEM/F-1220%FBS, B:DMEM/F-1220%KOSR, C:DMEM/F-122%B27) supplemented with LIF, bFGF or LIFbFGF. After the cells were treated with pH 5.7 of HBSS for 25 min, they were plated in each culture medium. As results, colony formation was observed in only the cells derived from bFFs following the exposure of low pH. The colonies of bFFS stimulated bythe low pH in all culture conditions were positively reacted with AP staining. The number of positive AP colony significantly increased in B group when compared to those in A and C groups. Especially B group supplemented with LIF showed the highest AP positive staining (52.4%). Additionally we tested expression of pluripotent genes in the low pH stimulated cells using RT-PCR. Interestingly, *OCT4, SOX2, KLF4* were slightly expressed but *NANOG* in the cells. These results showed that bFFs by stimulus of low pH were induced to convert into incomplete reprogrammed cells.

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P187

Leukemia inhibitor factor (LIF) is essential for long term maintenance of pluripotency of porcine induced pluripotent stem cells*

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piPS cells are divided into naïve and primed states. These states may depend on culture conditions with/without cytokines. Only piPS cells of naïve state have the capacity for producing chimeric offspring and long term maintenance of pluripotency. The objective of this study was to generate piPS cells of naïve state under culture conditions with 2i of the Erk/2 and of GSK3. PFFs were transduced with SIX reprogramming factors. After the transduction piPS cells were plated in DMEM, LIF and bFGF. Colonies were individually picked by manual method, replaced in culture mediums containing LIF, bFGF or 2i. As results, piPS cells at passage ten grown by the culture medium supplemented with LIF and LIF 2i were presented morphology of 'naïve' stem cells and positively AP activity, whereas the piPS cells in bFGF and bFGF 2i culture conditions were shown as the morphology of 'prime' stem cells. The piPS cells cultured with LIF and LIF 2i induced expressions of pluripotent and naïve specific markers but primed specific markers. Exogenous reprogramming factors were also expressed in both LIF and bFGF culture conditions. piPS cells in culture conditions with LIF and LIF 2i were positively exhibited antibodies against *OCT4*, *NANOG*, *SOX2*. These results indicated that PFFs after the transduction of exogenous reprogramming factors could be converted into the pluripotent cells and retained long term maintenance of pluripotency in LIF and LIF 2i culture conditions.

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P188

Function of leukemia inhibitory factor in spermatogenesis, revealed by using medaka spermatogonial culture system

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Introduction
Spermatogenesis is a dynamic process. Its control mechanisms are very complex and many molecules are concerned. Our previous study showed that spermatogonia are abnormally proliferated when *p53* (a tumor-suppressor gene) was knocked out in medaka (*p53*-KO medaka). The *p53*-KO medaka provides a hint about the regulatory mechanisms of spermatogonial proliferation and differentiation.
Materials and Methods
Since some cytokines have been known as the growth factors of spermatogonia, we analyzed mRNA expression of several cytokines in the medaka testis by -quantitative PCR. Leukemia inhibitory factor (LIF)-expressing cells were examined in the testis by *in situ* hybridization and immunostaining.
To investigate whether LIF promotes spermatogonial proliferation, we produced recombinant LIF protein by baculovirus expression system, and added them directly to a culture system that reproduces all processes (from spermatogonia to spermatozoa) of spermatogenesis *in vitro*, the system we have previously established.
Results and discussion
We found that the mRNA expression levels of *LIF, IL-11b, MIF* in p53-KO medaka are higher than these in WT medaka. LIF mRNA and protein expression was detected in Sertoli cells, especially in those surrounding type-A spermatogonia

(undifferentiated spermatogonia). The expression was also detected in some of type-A spermatogonia but not in type-B spermatogonia (differentiated spermatogonia).
We also found that spermatogonial proliferation is significantly enhanced in a medium supplemented with the recombinant LIF proteins. These findings reveal that LIF promotes spermatogonial proliferation in the medaka testes, suggesting that LIF functions as a growth factor of spermatogonia in medaka.

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P189

RNA binding protein Lin28a interacts with various mRNA in the mouse trophoblast stem cells

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Introduction
Trophoblast stem (TS) cells are capable of differentiating into all subtypes of trophoblast cells. Lin28a, an RNA-binding protein involved in the post-transcriptional regulation of gene expression, is highly expressed in undifferentiated TS cells but is greatly reduced in its expression in differentiated trophoblast cells. Lin28a is known as a major factor controlling growth and metabolism through regulation of the maturation of *let-7* microRNA. Considering that Lin28a binds to mRNA as well, it was assumed that Lin28a also regulates gene expression independent of *let-7*. In this study, we aimed to identify mRNAs that interact with Lin28a in TS cells.
Material and methods
We performed RNA-immunoprecipitation sequencing analysis using anti-Lin28a antibody and TS cell extract. Expression levels of Lin28a-associated mRNA candidates were analyzed in a Lin28a-knockdown TS cell line.
Results and discussion
Anotated functions of the proteins encoded by Lin28a-associtated mRNAs were diverse. A transcription factor Cdx2 that is essential for the maintenance of TS cells was included in these mRNAs. Knockdown of Lin28a in TS cells caused decrease in the expression level of *Cdx2*. Thus, Lin28a interacts with various mRNAs, and possibly control many physiological functions in TS cells.

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P190

Derivation of porcine iPS-like cells from fibroblast of a translocated azoospermic boar

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Chromosomal rearrangements have a crucial impact on the proper proceedings of meiosis and can lead by several mechanisms to the production of unbalanced gametes or to the complete arrest of gametes production. To assess the impact of these rearrangement in the early development of pig germ cells, we proposed to generate a library of stem cells from an azoospermic boar carrying a reciprocal translocation t(Y:14), as a new tool for the development of an *in vitro* differentiation system from pluripotent stem cells to germ cells.
We report the reprogramming of translocated fibroblast by integrative or non-integrative viral overexpression of Oct4, Sox2, Klf4 and c-Myc. iPS cell lines were characterized for pluripotency, cell cycle and differentiation potential by conventional methods. Genomic stability was analyzed by G-banding karyotype, comparative genomic hybridization and FISH.
The porcine iPS-like cell lines harbored characteristics of ground and naïve pluripotency when cultured in specific media. They expressed several pluripotency genes and harbored an ES-like cell cycle. Nevertheless, contrary to mouse and human iPS, they did not silence the integrated exogenes, leading to a poor differentiation potential. Moreover, cytogenetic analysis revealed a high genomic instability upon passaging which suggest the development of population with an increased selective advantage. We characterized the selected duplications and compared them to those previously described in other species. In contrast, non-integrative reprogrammation system gives us promising results regarding differentiation potential and genomic stability and will bring new insights into the molecular factors controlling and maintaining pluripotency in the pig species.

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P191

Derivation of bovine induced pluripotent stem cells by a transposon approach

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Introduction
Induced pluripotent stem (iPS) cells are considered as a seminal breakthrough in stem cell research and are promising for the development of advanced regenerative therapies. Considering the potential of this technology for both basic and clinical research, it is tempting to extend this research to important livestock species, such as cattle, in which ES cell lines are yet not available.
Materials and methods
Here, we describe a non-viral method for the derivation of bovine iPS cells, employing the piggyBac (PB) transposon system. The reprogramming PB transposon encode the reprogramming factors OCT4, SOX2, KLF4, MYC, LIN28 and NANOG, each separated by self-cleaving peptide sequences and driven by the chimeric CAGGS promoter. One PB transposon-reprogrammed bovine iPS (biPS) cell line was established.
Results and discussion
One PB transposon-reprogrammed bovine iPS (biPS) cell line was established. The derived bovine iPS line expressed typical markers of embryonic stem cells (OCT4, SOX2, c-MYC, KLF4 and NANOG), showed long term proliferation under feeder-free culture conditions, differentiated into derivatives of the three germ layers *in vitro*, and formed teratomas after SUBCUTANEOUS injection into immune deficient nude mice. These results are a major step towards the derivation of authentic bovine iPS cells, in which the transposon transgenes can be eliminated after reprogramming.

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P192

Effect of melatonin supplementation during *in vitro* maturation on intracellular ROS levels in porcine oocytes

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Oocytes of domestic mammals present high concentrations of lipids, and this is particularly evident in porcine oocytes that exhibit higher levels than other species, becoming more susceptible to oxidative stress and lipid peroxidation. Considering that melatonin is an effective antioxidant for protecting macromolecules against oxidative stress caused by reactive oxygen species (ROS), the purpose of the present study was to examine the effect of melatonin supplementation during *in vitro* maturation on intracellular ROS levels in porcine oocytes. Oocytes were *in vitro* matured in TCM199 medium containing different concentrations (0, 10^{-6} and 10^{-9} M) of melatonin (Melatonin, Sigma–Aldrich). As a positive control (induction of oxidative stress), oocytes were incubated with 1 mM H_2O_2 for 10 min. Intracellular ROS levels were detected using the intracellular dye 2,7-dichlorofluorescein diacetate (DCF-DA, Sigma–Aldrich) and assessed by fluorescence intensity using the ImageJ software (NIH, USA). Melatonin supplementation contributed to reduce intracellular ROS ($P<0.01$), and values were 43.79 ± 14.9, 23.47 ± 12.2, 17.76 ± 7.5 in medium containing H_2O_2, 0 and 10^{-6} M melatonin, respectively. However, melatonin effectiveness was more expressive in the lowest concentration (12.83 ± 6.5 in 10^{-9} M; $P<0.05$). The substantial intracellular ROS decrease may be attributed to the powerful ability of melatonin to scavenge ROS and its indirect antioxidant properties, which stimulate anti-oxidative enzymes and inhibits pro-oxidative enzymes. Even though ROS play important roles as second messengers in cellular functions through activation of cell signaling cascade in the oocyte, high concentrations may promote imbalance between the oxidation–reduction reactions, negatively interfering on cell function. Thus, melatonin supplementation during *in vitro* maturation reduces intracellular ROS levels and may improve porcine oocyte viability.

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P193

X chromosome dosage affects completion of pluripotential reprogramming

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Direct reprogramming of somatic cells to pluripotent stem cells entails the obliteration of somatic cell memory and the reestablishment of epigenetic events. Induced pluripotent stem (iPS) cells have been created by reprogramming somatic cells through the transduction of reprogramming factors. During cell reprogramming, female somatic cells must overcome at least one more barrier to enter a pluripotent state compared to male somatic cells, that is, the reactivation of inactive X chromosome (Xi). To verify this phenomenon, we investigated whether the sex of somatic cells affects the efficiency of reprogramming, differentiation potential, and post-transcriptional process of *Xist* RNA after reprogramming. There were no differences between male and female iPS cells in the reprogramming efficiency, and in their differentiation potential *in-vivo*. However, the reactivation of Xi was determined to be a slower process than that of pluripotency-related genes; more than 90% of iPS cells did not complete the reactivation of Xi until 30 day post-infection. Next, we investigated the post-transcriptional processing of *Xist* RNA, and found that male embryonic stem (ES) cells expressed only the *Xist* long-isoform,while female ES cells expressed both the *Xist* long- and short-isoforms.This finding was also observed in iPS cells; male iPS cells only expressed the *Xist* long-isoform, and female iPS cells expressed the two different *Xist* isoforms. Based on these findings, we determined that the direct reprogramming of somatic cellsby the transduction of transcription factors results in slow reactivation of Xi, but leads to thecorrect post-transcriptional reprogramming.

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High-throughput screening revealed a clinically relevant drug to induce sperm motility

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Introduction
Sperm dysfunction is the commonest cause of infertility, yet there is currently no drug a man can take, or be added to his sperm *in-vitro*, to improve fertility. The rationale of drug discovery research is to find a drug which increases sperm motility and success of ART. Despite limitations in understanding of sperm physiology, it is acknowledged calcium is central to motility and function. Validated high-throughput screening of compounds from University of Dundee Drug Discovery Unit has identified Trequinsin, a potent phosphodiesterase III inhibitor, to induce significant intracellular calcium ($[Ca^2]i$) in human spermatozoa.
Experimental design
Semen from healthy volunteer donors and patients attending Ninewells Assisted Conception Unit (ethical approval 08/S1402/6) was prepared by percoll gradient. Effects of Trequinsin were evaluated *in-vitro* using CASA. Kremer testing was used to assess functional motility response.
Flow cytometry (FACS) determined the proportion of cells that responded to Trequinsin and evaluated acrosome reaction.
Results
Trequinsin significantly increased total and progressive motility in 40 and 80% sperm fractions from healthy donors under capacitating and non-capacitating conditions ($P < 0.05$). Motility was significantly increased in sperm from a patient affected by failed fertilisation following ICSI.
FACS demonstrated Trequinsin induced an increase in $[Ca^2]i$ in a higher proportion of 80% fraction cells (78%) compared to 40% (35%). Acrosome reaction was not significantly increased.
Discussion
Increase in $[Ca^2]i$ induced by Trequinsin causes an increase in motility parameters of spermatozoa, including patient samples, without significantly affecting acrosome reaction. Increasing data may provide evidence to justify its use in a clinical setting.

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P195

Steps forward canine primordial germ cell isolation and characterization

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Introduction
Primordial germ cells (PGCs) are specified during early mammalian post implantation development. They are precursors of adult animals gametes and are considered stem cells of germline. Most of PGCs studies were performed in rodents or human with limited studies in other mammals such as canine. We have recently demonstrated a model to derive embryonic germ cells (EGC) in canine species.
Materials and methods
We isolated canine embryos between 25 and 30 days old and cultured on a continuous cell line of canine embryonal fibroblasts as feeder-cells in the presence of leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF). The culture characterization was detect using alkaline phosphatase activity. Immunophenotype of PGCs was evaluated by immunohistochemical with specific markers (Oct3/4, c-kit and Vasa).
Results and discussion
PGC *in vitro* culture from both pooled and individual embryos resulted in successful derivation of putative EGC and showed colony formation in early passages. Canine PGCs in culture showed similar morphology pluripotent stem cells. The EGCs expressed alkaline phosphatase activity, this result suggests a characteristic of pluripotency and germ cells in canine EGCs. Immunohistochemical analysis revealed Oct4/3, c-kit and Vasa expressions PGCs in the hind gut epithelium, in the mesenchyme of dorsal mesentery and in gonadal ridge development PGCs are cells expressing Oct3/4 after posgastrulation, which has a role in the totipotent phenotype while c-kit and Vasa controls proliferation, survival and migration.

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P196

Effects of early embryo environment on embryonic stem cell derivation and behaviour

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The embryonic environment can induce permanent changes in metabolism and increase disease risk in adults. Rodent models show that modifications in maternal diet exclusively during pre-implantation development can induce metabolic disease in adults. Changes must therefore occur within the early embryo and be maintained throughout development. Determining adaptive mechanisms is challenging due to the size of the early embryo. We generated ES cells from inbred C57BL/6 mice as a model to overcome these problems.
Methods
ES cell lines were derived from blastocysts of C57BL/6 mice assigned to either an isocaloric low protein diet (LPD), or a control diet exclusively through pre-implantation development. Lines were characterised for karyotype, sex, gene expression, and functional characteristics including proliferation and death at standardised passages.
Results
LPD had no impact on blastocyst formation *in vivo*. Although LPD blastocyst outgrowth was comparable, there was a significant reduction in the capacity for ES cell derivation. While the ES cell lines retained similar levels of gene expression related to pluripotency, and developmental functions irrespective of diet, LPD cells showed increased basal apoptosis, and reduced phosphorylated ERK kinase.
Discussion
The reduced ES cell isolation efficiency may indicate an increased sensitivity of maternal LPD ES cells to apoptotic stimuli. ERK-mediated survival signalling may underlie this. Such adaptations in the early embryo may impact on lineage allocation as differentiation occurs. These ES cell lines may provide a model to investigate such mechanistic adaptations in post-implantation tissues providing insight into foetal responses to poor nutrition and the induction of adult onset disease.

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P197

Trophectoderm stem cells to model the effect of altered periconceptional diet on embryos

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Introduction
Poor maternal diet during development induces physiological, metabolic and anatomical alterations in offspring. These may increase immediate survival chances, but can enhance the risk of disease in later life. Maternal low protein diet (LPD) exclusively in pre-implantation development induces changes in endocytosis, postnatal hypertension, cardiovascular dysfunction and alters behaviour in our mouse model. We have previously shown changes in the embryonic stem cells isolated from pre-implantation blastocysts. Here isolate the less studied trophoblast stem cells (TSCs) from embryos, to establish characteristics of these cells as a model of development of the placenta.
Materials and methods
MF1 mice were mated and assigned a LPD or control diet (NPD) for the pre-implantation period. Blastocysts were collected and cultured on feeder cells. After embryo attachment and outgrowth, the outgrowth was disaggregated. TS cell colonies were expanded by passaging every 3–7 days, and frozen after 36 days. The established TS cell lines were studied in karyotyping, determination of the sex by PCR, and gene expression by qPCR and western bloting.
Results and discussion
We have produced a group of TS cell lines from E3.5 embryos of mothers which have received either NPD (18%) or LPD (9%) diets. The karyotyping showed TS cells have a tendency to spontaneous differentiation, with high tendency to show tetraploidy. Unlike ES cells from similarly treated embryos, there is no sex bias in TS lines, however TS cells demonstrate a far greater variability in their culture characteristics, growth rates and stability. This variation does not appear to depend upon the maternal pre-implantation diet.

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P198

Effect of storage time on Atlantic salmon (*Salmo salar*) sperm motility, viability and calcium levels, first communication

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Few studies have evaluated sperm function and intracellular calcium levels when storage of semen for a long period of time. We have assessed sperm viability, morphology, motility and intracellular calcium levels in fish semen after a prolonged storage period. Semen samples from Atlantic salmon were preserved at 4 °C for 25 days and then evaluated for viability. The motile sperm was determined using the Image J-CASA program. To measure the calcium content, the sperm were loaded with a calcium fluorescent probe and evaluated by confocal microscopy and microfluorimetry. Reduced sperm viability was observed after 10 days of storage. Motility remained high for the first 3 days. A decrease in the calcium content of more than 50% was observed after 20 days of storage. When sperm were activated, calcium levels increased over 200% of relative fluorescence units (RFU); this increase was lost when the samples were stored for extended periods and only partially manifested in a zero calcium solution. Our results suggest that *in vitro* storage of Atlantic salmon sperm at 4 °C for a period of 3 days preserves viability and motility at levels similar to those of fresh sperm and also maintains intracellular calcium stores, which are critical for sperm function.

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P199

Motility, viability and calcium in the sperm cells

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Sperm cells are complicated *in vitro* models. Its viability is limited, and its physiology is complex. The study of their properties is of great application in the animal production industry. Therefore, it is important to have viable and functional gametes. Consequently, it has been demonstrated that the decrease of sperm cells viability is related with the increase of the reactive oxygen species (ROS) and ROS is secondary to normal metabolic processes of the cell. One of the processes sperm cells, it is the flagellar movement. There is evidence of strategies that leads to reduce these ROS levels by using exogenous or endogenous antioxidant with the intention that seminal plasma protects the sperm cells and increases its viability, so it is feasible to suggest an increase in viability by reducing that flagellar movement which is regulated by calcium. The phenomenon has not been fully characterized, but it is established that in certain mammalian models, the entrance of calcium via specific channels such as CATsper or voltage-dependent channels, it is a signal for flagellar movement to occur. Previous reports have indicated that if changed the calcium concentration in solution or altered the conservation temperature (Althouse *et al.* 1998), the function of mammal sperm cells was reduced or blocked and the conservation was prolonged. It was observed that fish sperm remained immobile for several weeks and when are activated the number of mobile and viable sperm cells is reduced faster, but if the cells remain not mobile the semen can be preserved for long period (Cabrita *et al.* 2001, Alavi & Cosson 2005). We support the idea that modulating calcium channels in bovine and salmon sperm are able of reducing motility and increasing the viability of these cells in experimental conditions.

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P200

Reciprocity of bio-stimulation on testicular traits and subsequent fertility of South African Mukota sows

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Introduction
Bio-stimulation is the stimulus provoked by the presence of a male which induces fertility through genital stimulation or other external cues. In animals, courtship and foreplay which precede mating have reciprocal effects on sexual and reproductive milieu of both sexes. An increase in male exposure to females will also tend to evoke increases in reproductive capacity of both sexes. The study evaluated reciprocity of boar exposure on testicular characteristics and subsequent fertility of their female counterparts.
Materials and methods
Twenty-four South African Mukota boars were allowed 0, 30, 60, 90 or 120 min of fence-line exposure to 120 sows, twice daily during oestrus detection protocols, throughout the 120 days observation to evaluate reciprocal stimulatory effects of boar exposure on testicular characteristics and subsequent fertility of sows following artificial insemination (AI). Non-return rate (NRR) was evaluated as the proportion of females that did not return to service from the overall AI sows.
Results and discussion
Boar exposure for 120 min caused the weight of the testicles to hypertrophise by 96.5%, with the highest total daily sperm production ($22.3 \pm 2.1 \times 10^9$ per testis) and total Leydig mass (113.5 ± 8.2 g) and largest tubular values. Semen collected following 120 min boar exposure gave highest non-return rate ($93.5 \pm 2.9\%$), farrowing rate ($88.7 \pm 5.1\%$,), litter size (14 ± 0.02) and live piglets (12 ± 0.06). Results suggest that prolonged (120 min) fence line boar exposure has reciprocal effects on boar testicular characteristics leading to significant improvements in the fertility rates of breeding sows.
Keywords: *Sus scrofa mukota*, bio-stimulation, testicular hypertrophy, female fertility

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P201

Alternative splicing: a mechanism for spermatogonia differentiation, meiosis progression and spermatid maturation

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Introduction
The production of functional spermatozoa is a highly complex process that requires tight regulation of gene expression. Pre-mRNA splicing is an essential post-transcriptional process. Over 95% of multi-exon human genes undergo alternative splicing to create diverse arrays of mRNAs from a single gene. Disturbance of splicing regulation is associated with many human diseases.
Materials and methods
A genome-wide ENU mutagenesis screen was carried out to identify novel male fertility regulators. An array of gene functional analyses was carried out to define the function(s) of genes identified through the ENU mutagenesis approach.

Results and discussion
We identified the RNA binding protein RBM5 as an essential splicing regulator in male germ cells. Male mice carrying a missense mutation (R263P) in the second RNA recognition motif (RRM2) of RBM5 were sterile due to defects in spermatogonia differentiation, meiosis progression and spermatid maturation, which ultimately led to azoospermia. In round spermatids, RBM5 interacted with several splicing factors and regulated the splicing of several target pre-mRNAs, which have been implicated in germ cell adhesion, spermatid head shaping, and acrosome and tail formation. In addition, RBM5 dysfunction resulted in a significant reduction of spermatogonial progenitor cells and only 27% of those that entered meiosis successfully made the conversion to round spermatids. These findings suggest a role for RBM5 in spermatogonia differentiation and meiosis progression. In summary, our study demonstrates that RBM5 is a critical splicing regulator in male germ cells and is absolutely required for the correct progression of spermatogenesis and male fertility.

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P202

Different roads to fertility: distinct molecular and physiological strategies warrant the phenotype 'high fertility' in two outbred mouse models

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Animal models are valuable tools in fertility research. Worldwide >800 transgenic or knockout mouse models are available showing a reproductive phenotype; almost all of them exhibit an infertile or at least subfertile phenotype. In contrast, animal models revealing an improved fertility phenotype are barely described. Here, we present data on two outbred mouse models showing a 'high fertility' phenotype. These mouse lines have been generated via selection over a time period of >40 years and 161 generations. During this selection period the number of offspring per litter and the total birth weight of the entire litter nearly doubled. Concomitantly with the increased fertility phenotype several endocrine parameters (e.g. serum testosterone concentrations in male animals), physiological parameters (e.g. body weight, accelerated puberty, and life expectancy) and behavioral parameters (e.g. behavior in an open field, endurance fitness on a treadmill) have been altered. We demonstrate that the two independently bred high fertility mouse lines warranted their improved fertility phenotype using different molecular and physiological strategies. The fertility lines display female- as well as male-specific characteristics. These genetically heterogeneous mouse models provide new insights into molecular and cellular mechanisms that enhance fertility. In view of decreasing fertility in men it will therefore be a precious information source for human reproductive medicine.

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P203

Abstract withdrawn.

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P204

Recovery of porcine sperm from fetal testicular tissue xenografted into nude mice

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Porcine spermatogonia can develop to sperm in neonatal testicular tissue that were cryopreserved and grafted into nude mice. Live piglets were born from zygotes produced by intracytoplasmic sperm injection using these sperm (Kaneko *et al. PLoS ONE*, 2013). Utilization of fetal tissue will give us valuable chances for conservation of genetic resources and also for improvement of testicular xenografting. We aimed to examine whether porcine fetal testis can produce sperm after grafting into nude mice.

Testes obtained from 55- and 90-day-old crossbred fetuses (the date of artificial insemination was defined as day 0) were minced into fragments measuring 1.5×1.5×1.5 mm (F55 and F90 groups, respectively). Tissue fragments were incubated in vitrification solution (35% ethylene glycol, 5% polyvinylpyrrolidone and 0.3 M trehalose in a base solution) for 10 min at room temperature. They were then dropped with ~4 µl of vitrification solution into liquid nitrogen and were stored in liquid nitrogen. After storage, microdroplets containing tissue were transferred into warming solution (0.4 M trehalose in base solution) at 37 °C for 2 min then consecutively transferred for 2-min periods into 0.2, 0.1, and 0.05 M trehalose in base solution at room temperature. A total of 20 fragments were grafted subcutaneously into castrated nude mice.
Sperm recovery from the grafted tissue was confirmed in the F55 group at 180 days after grafting (1/5 mice). However, sperm recovery rate increased at 240 days (4/5 and 5/5 mice in the F55 and F90 groups, respectively) and at 300–420 days (7/10 and 13/14 mice, respectively).
These results demonstrate that fetal testis can produce sperm even after cryopreservation.

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P205

The impact of oxidative stress on chaperone-mediated human sperm–egg interaction

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Introduction
Defective sperm–egg recognition is recognised as one of the major causes of failed fertilization in IVF programs. Our recent findings have revealed that such defects may be linked to the molecular chaperone, heat shock protein A2 (HSPA2), due to its ability to facilitate the formation of multimeric zona pellucida (ZP)-receptor complexes on the surface of human spermatozoa. This study aimed to examine the impact of oxidative stress on the formation of these ZP receptor complexes.
Materials and method
For the purpose of this study, low levels of oxidative stress were induced in populations of human spermatozoa through treatment with 4-hydroxynonenal (4HNE) or hydrogen peroxide (H_2O_2).
Results and discussion
The surface expression of the predominant HSPA2 complex containing the putative ZP receptors sperm adhesion molecule 1 (SPAM1) and arylsulfatase A (ARSA) was found to be significantly disrupted by both 4HNE and H_2O_2 treatment. Furthermore, the ability of sperm to interact with ZP ($P<0.01$) and undergo an induced acrosome reaction ($P<0.05$) was also severely compromised. Upon investigating the mechanism of action it was demonstrated that oxidative stress targeted HSPA2 for covalent modification by 4HNE. From these data we infer that the adduction of HSPA2 by 4HNE may cause attenuation of its chaperone activity and a subsequent deregulation of the coordinated surface expression of SPAM1 and ARSA. The perturbation of this complex may thereby contribute to the observed loss of ZP binding ability and supports a key role for this complex in the initial tethering of human spermatozoa to the ZP.

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P206

Prostaglandin J_2 added to artificial insemination sperm dosage reduces the expression of the inflammatory genes in uterus sow

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Introduction
Billions of sperm are used during porcine artificial insemination (AI) but only a few thousand are able to reach ampullary-isthmic junction of the oviduct to facilitate fertilization. One of the mechanisms which reduce this sperm population is the inflammatory reaction that takes place within the uterus following insemination. Prostaglandin J_2, also known as 15-deoxy-Delta-12,14-prostaglandin J_2 (15d-PGJ_2) is a recently discovered prostaglandin which is recognized by the receptor peroxisome proliferator-activated receptor gamma (PPARγ). This property is responsible for many of the 15d-PGJ_2 anti-inflammatory functions

described in multiple organs and tissues. The aim of this study was to determinate if the addition of exogenous 15d-PGJ_2 to the insemination dosage could reduce the inflammatory response within the uterus.
Material and methods
Two groups of sows were inseminated using post-cervical IA: i) control group: 500 million sperm in 20 ml; ii) PGJ_2 group: 500 million sperm in 20 ml, 15d-PGJ_2 (10 μM). Twenty-four hours after AI, uterine samples were obtained by surgical laparotomy. Total RNA was extracted from samples and cDNA was synthesized. Different genes related with the inflammatory response (COX-2, TNF-α, IL8, ILIβ, and NFKβ1) were analyzed using real-time PCR.
Results and discussion
The expression of the five genes analyzed was reduced in the sows inseminated with 15d-PGJ_2 in comparison with the control group (NFKβ1: twofold decrease; COX-2 and TNF-α: threefold decrease; ILIβ and IL8: fourfold decrease). This could indicate a reduction in uterine immune reaction allowing more sperm to reach the oviduct. Supported by AGL2012-40180-C03-01-02, European Commission *(FEDER*/ERDF) and Fundación Séneca (0452/GERM/06).

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P207

Effect of extracellular long-chain fatty acids on mouse sperm motility

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Free fatty acids are not only energy fuels but also signaling molecules. In fact, long-chain fatty acids (LCFAs) have been identified as the ligands for G protein-coupled receptor 40 and 120 (GPR40 and 120). We have previously observed that GPR120 mRNA is expressed in the mouse sperm head. Based on the result, we hypothesized that the LCFAs act as signaling molecules to regulate sperm motility through GPR120. The aim of present study is to clarify whether extracellular LCFAs act as the regulator of flagellar motility of the mouse sperm. Sperm were obtained from the caudal epididymidis of the matured ICR mouse. Expression of GPR40 and 120 mRNA were observed by RT-PCR, and the localization of GPR120 were identified by the immunohistochemical method. Further, sperm motility was analyzed as the flagellar beat frequency and movement distance.
GPR120 mRNA expression was confirmed in sperm, but GPR40 mRNA expression was not observed. GPR120 protein was localized in the head and middle-piece of sperm. 20 min exposure of sperm to oleic acid, docosahexaenoic acid or GPR120 agonist GW9508 induced an increase in flagellar beat frequency and movement distance of sperm. On the other hand, the flagellar beat frequency and movement distance are decreased when sperm were exposed to palmitate. These results indicate that extracellular unsaturated LCFAs induce an increase in flagellar beat frequency and quicken movement speed, and extracellular saturated LCFAs decrease flagellar beat frequency and decelerate movement speed. In addition, the result of GW9508 suggests involvement of GPR120 to this sperm motility regulating mechanism.

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P208

Peroxisomes are essential for regular spermatogenesis

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Peroxisomes are cell organelles with important functions in the metabolism of lipids and reactive oxygen species. In germ cells, they have only recently been described by our groups. Their role for spermatogenesis has not been characterized in detail yet. We have established a mouse model with a conditional knockout of *Pex13* in pre-meiotic germ cells to analyse the functions of peroxisomes for development and differentiation of male germ cells. The peroxisomal membrane protein *Pex13* is part of the translocation machinery required for import of peroxisomal matrix proteins into the organelle. The inactivation of *Pex13* leads to a biogenesis defect of peroxisomes with loss of all metabolic functions. Based on the *Cre*-lox technique, floxed *Pex13* mice were crossed with transgenic mice expressing cre recombinase under control of the *Stra8* promoter for inactivation of *Pex13* in pre-meiotic germ cells. Histological analysis of knockout mice revealed a severe disturbance in germ cell differentiation with generation of multinucleated giant cells and post-meiotic arrest of spermatogenesis. Depending on their differentiation state, the multi-nucleated cells were TUNEL-positive. As a result of the peroxisomal dysfunction in germ cells we found a significant accumulation of lipid droplets within the germinal epithelium. On the ultrastructural level we could observe acrosome formation in multinucleated spermatids. However, several nuclei frequently shared one acrosome.

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P209

Effect of local treatment of seminal vesiculitis on the quality of equine frozen semen

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The seminal vesiculitis leads to contamination of the semen with inflammatory cells, causing agglutination, precipitation, decreased sperm motility and increased reactive oxygen species (ROS). This deleterious effect is most pronounced during the cryopreservation process. Therefore, the aim of this study was to evaluate the quality of frozen semen from stallions with seminal vesiculitis after local treatment. Five stallions with seminal vesiculitis were used and local treatment was performed by endoscopy for 10 consecutive days. This consisted of flushing with Ringer Lactate solution followed by infusion of the antibiotic of choice selected after antibiogram of bacterial culture of seminal vesicles flush. The kinetic parameters, plasma membrane integrity, lipid peroxidation index and the level of ROS in the frozen semen before (M0), after 1 week (M1) and after 1 month (M2) therapy were evaluated. The data were analyzed by (two-way ANOVA) followed by Tukey's test ($P<0.05$). The values (mean ± S.E.M.) of seminal parameters on M0, M1 and M2 were the following, respectively: sperm kinetics (total motility: 20.8 ± 2.12a; 44.8 ± 2.97b; 24.3 ± 3.02a; progressive motility: 7.5 ± 1.05a; 19.6 ± 2.55b; 9.9 ± 1.73a; rapid sperm: 10.7 ± 1.51a; 27.0 ± 1.80b; 14.3 ± 3.01a), plasma membrane integrity (26.6 ± 2.42a; 42.2 ± 2.09b; 28.3 ± 2.36a), lipid peroxidation index (1.2 ± 0.12a; 0.75 ± 0.09b; 0.98 ± 0.11a) and level of ROS (5.83 ± 2.06a; 1.72 ± 0.57b; 1.87 ± 0.81ab). The results demonstrate that local treatment after a week leads to an improvement on frozen sperm quality, however this is not maintained after 1 month of therapy, since the seminal parameters at this time are similar to the pre-treatment, which can be justified by the recurrence of disease.
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P210

Proteomic analysis of human testicular interstitial fluid reflects disordered spermatogenesis in Klinefelter's syndrome

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Introduction
Primary spermatogenic failure is the commonest cause of male infertility. Changes in Sertoli and germ cell function may be reflected in the testicular interstitial fluid (TIF) proteome and its analysis may provide insights into the pathophysiology. Microdissection testicular sperm extraction (microTESE) provides sperm for ICSI in ~50% of non-obstructive azoospermia (NOA) cases. As the first step in constructing a diagnostic approach for broader application in NOA, we chose to examine the TIF proteome of Klinefelter's men with severely impaired spermatogenesis.
Materials and methods
We pooled TIF from Klinefelter's syndrome (KS) men with a predominantly Sertoli cell-only histological phenotype, but in whom spermatids were found at microTESE, and compared their TIF proteomic profile with that of control (obstructed) cases ($n=3$/group). Low-abundant proteins were isolated from each TIF pool using BioRad Proteominer beads, and labelled using ICPL chemistry to quantify protein expression differences by mass spectrometry (MS). Samples were combined, digested with trypsin, and peptides pre-fractionated by nano-reversed phase HPLC, prior to identification using MALDI tandem MS and ESI-LCMS/MS.

Results and discussion
1248 proteins were characterized; 59 were down regulated greater than two-fold (including known Sertoli cell- or germ cell-specific proteins) and 70 up regulated greater than two-fold (these tended to reflect altered intracellular signalling or regulation of somatic cell origin). We conclude that Sertoli and germ cells proteins can readily be recognised in TIF, and that differences in their expression provides a promising basis upon which to identify sub-phenotypes of spermatogenic failure and a means to predict the recovery of sperm at TESE.

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P211

The role of the molecular chaperone HSPA2 in unexplained failure of IVF

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Introduction
A common defect encountered in the spermatozoa of male infertility patients is an idiopathic failure of sperm–egg recognition. The aim of this study was to investigate the molecular basis of this condition.
Materials and methods
For the purpose of this study we compared the proteomic profiles of spermatozoa from male infertility patients exhibiting an impaired capacity for sperm–egg recognition with that of sperm from males of proven fertility using label free mass spectrometry-based quantification. The dominant protein identified as being differentially expressed between these sperm samples was characterized to determine its role in fertilization.
Results and discussion
Our analysis indicated that impaired human sperm–zona pellucida binding was associated with reduced expression of the molecular chaperone, HSPA2, from the sperm proteome. This defect did not extend to other members of the HSP70 family. HSPA2 was present in the acrosomal domain of human spermatozoa as a major component of five large molecular mass complexes, the most dominant of which was found to contain just two other molecules, SPAM1 and ARSA, both of which that have previously been implicated in sperm–egg interaction. Furthermore, we demonstrated that HSPA2 regulates the expression of SPAM1 and ARSA on the surface of human spermatozoa. The close association between SPAM1, ARSA and HSPA2 in a multimeric complex mediating sperm–egg interaction, coupled with the complete failure of this process when HSPA2 is depleted in infertile patients, provides new insights into the mechanisms by which sperm function is impaired in cases of idiopathic male infertility.

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P212

Androgen receptor signalling in testicular Leydig cells is essential for Leydig cell maturation and survival

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Androgen receptor (AR) is expressed by testicular somatic cells including steroidogenic Leydig cells (LC). Its role in LC is ill defined, with hypotheses based on evidence from total androgen receptor knockout models implying that LC AR is responsible for final LC number, regulation of steroidogenic enzyme expression and LC maturation.
To better define the role of AR in LC, we generated a mouse line with a LC specific ablation of AR (LCARKO) using the Cre/lox system.
AR is ablated from 75% of LC in the LCARKO. Total LC number does not differ between LCARKO and controls. Despite reaching normal adult numbers, LC lacking AR fail to mature, as demonstrated by the significant reduction in adult LC-specific transcripts. Despite the failure of maturation, LCARKO mice have normal intratesticular testosterone levels at d80. Testicular histology is normal until d80 when focal degeneration of the seminiferous epithelium is noted, which becomes progressively worse with ageing. Testes at d180 were examined for evidence of apoptosis. None was noted in controls, however, age-matched LCARKO testes demonstrated interstital apoptosis not seen at earlier ages. Apoptosis was also noted in biopsies of post-pubertal human males with complete androgen insensitivity syndrome (CAIS).
Maturation of LC and their survival with ageing is dependent on autocrine AR signalling, but achievement of final LC number requires AR signalling in another cell type. Apoptosis of LC occurs in both LCARKO mice and CAIS humans, suggesting the underlying cause is common to both. The mechanisms of these observations are under investigation.

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P213

Semen collection and preservation in the salt water crocodile *Crocodylus porosus*

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Successful semen collection and preservation is vital to the development of a crocodile artificial insemination program for the purpose of intensive production or endangered species breeding. We describe a manual cloacal palpation method for the safe and repeatable recovery of semen from *Crocodylus porosus* along with corresponding seminal characteristics. Experiments were also conducted to investigate fundamental sperm physiology, including the effect of excessive dilution, rapid temperature change, exposure to anisotonic media, cryoprotectant toxicity and cryopreservation. Spermic samples were recovered from 30 of 31 collection attempts from a total of 24 sedated males ranging from 197 to 400 cm in body length. Mean ($\pm$S.E.M.) seminal volume, pH, osmolality, sperm concentration, percentage motile sperm and the percentage of sperm with an intact membrane measured 0.91 ± 0.16 ml, 7.3 ± 0.1, 335.5 ± 9.0 mOsm/kg, $2.29 \pm 0.26 \times 10^9$, $50.7 \pm 4.2\%$, and $79.9 \pm 3.6\%$, respectively. Sperm abnormalities included macro and microcephalic nuclei, teratoid spermatozoa, loose heads and a range of abnormal flagella. Crocodile spermatozoa were capable of being diluted 1:16 without any reduction of motility, were not affected by rapid temperature shock to 0 °C and the plasma membrane showed a remarkable tolerance to extremely hypo-osmotic media (25 mOsm/kg). Motility of crocodile sperm diluted in PBS with increasing molarities (0.068, 0.135 and 2.70 M) of glycerol, DMSO and DMA showed a corresponding decrease in sperm motility after 1 h incubation at 4 °C but there was no adverse effect on plasma membrane integrity; sperm cryopreserved in these same diluents showed only moderate levels of plasma membrane integrity post-thaw (18–26%) but no motility.

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P214

The association between spatial distribution patterns of SPACA1 in human ejaculated sperms and outcomes of conventional IVF

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Introduction
The aims of this study were to show the existence of individual differences in the spatial distribution patterns of sperm acrosome-associated 1 protein (SPACA1) in human ejaculated sperms and to reveal a correlation between distribution pattern of SPACA1 and outcome of conventional IVF.
Materials and methods
Informed consent was obtained from all the participants for this study. Viable sperms obtained by swim-up method were selected morphologically and incubated to induce capacitation. Then the sperm suspension was introduced

into the medium containing eggs. Fertilization was confirmed by the observation of two pronuclei at 19 h after co-incubation with sperms. The surplus sperms were used to detect SPACA1 by indirect immunofluorescence and the distribution patterns were classified. The association between distribution patterns of SPACA1 and fertilization rate or blastocyst development rate in IVF was examined.
Results and discussion
SPACA1 was localized definitely in the equatorial segment, but variedly in the acrosomal principal segment. The distribution patterns of SPACA1 in the acrosomal principal segment could be classified into three categories: i) strong, ii) intermediate or faint, and iii) almost no immunofluorescence. There were large differences in terms of the composition of the classified sperms among patients. Although fertilization rates were not influenced by the differences, the rates of blastocyst formation and high quality blastocyst formation were significantly associated with the rate of class-A sperms. These results suggest that SPACA1 of the acrosomal principal segment may be involved in early embryonic development *in vitro*.

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P215

Detection of potassium channel defects in ICSI patients by patch clamp electrophysiology

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Introduction
Potassium channels are essential for the physiological regulation of cell excitability through control of membrane hyperpolarisation. In human sperm slo1 and/or 3 have been proposed to mediate this function (Mannowetz *et al.* 2013, Mansell *et al.* 2014). However, to date there is no information on dysfunction in men and the consequence of this for their fertility.
Materials and methods
Men undergoing ICSI treatment or recalled as part of failed fertility clinic attended Ninewells Assisted Conception Unit, Dundee. Surplus semen samples at time of treatment, or produced specifically for research purposes were subjected to patch clamp electrophysiological analysis under quasiphysiological conditions (Mansell *et al.* 2014). Control currents were obtained from healthy donor cells.
Results and discussion
Control outward K^+ currents were robust and present in all cells recorded (reversal potential, Erev = −20 mV; 42 pA/pF at +68 mV). Infertile men were screened and two patients were identified to have different altered potassium conductances. Patient 1 had a depolarised membrane potential and was essentially devoid of outward K^+ current (Erev = 0 mV; 3 pA/pF at +68 mV). Patient 2 membrane potential was partially depolarised and exhibited an outward current that was notably less than control values but with a very similar burst pattern phenotype (Erev = −7 mV; 7 pA/pF at +68 mV) indicating a very low level of channel expression. A further 16 ICSI patients were found to have normal K^+ conductance. Updated screening numbers will be presented. Patch clamp electrophysiology reveals rare K^+ channel dysfunction in patient sperm and represents a powerful screening technique that permits uniquely quantifiable assessment of membrane potassium channel (dys)function in patient sperm.

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P216

Spermatogenesis in pubertal common eland (*Taurotragus oryx*, Pallas 1766)

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Introduction
Timing of sexual maturity is essential in order to establish when reproductive technologies can successfully be performed. To date, little is known about reproductive biology in the *Taurotragus* genus, which includes only two species: common eland (*Taurotragus oryx*) and giant eland (*Taurotragus derbianus*). Here, for the first time, we explored testicular development throughout puberty in common eland.
Materials and methods
Samples were collected from six young (15–33 months) slaughtered eland. Spermatogenesis was evaluated performing the fine needle aspiration cytology technique. Animals were categorised according to their age into two even groups: below and above 2 years old, respectively. The percentage of each cell type was determined on at least 200 spermatogenic and Sertoli cells. Indices of spermatogenic efficiency, Sertoli cell functionality and workload were determined. Animal biometrics and epididymal sperm parameters were assessed.
Results and discussion
Cytological analysis showed that full spermatogenesis was already established at 15 months. Spermatozoa were found both in testis and epididymal cauda indicating that at this age eland already reached puberty. An overall increase of spermatogenesis efficiency and Sertoli cell functionality were observed when comparing individuals below and above 2 years old, although differences were not statistically significant ($P > 0.05$). Sperm concentration was correlated with Sertoli cell functionality and workload ($r = 0.912$, $P = 0.011$ and $r = 0.956$, $P = 0.003$, respectively). In conclusion, our study provides the first insights on the onset of puberty in common eland, which can be used as a model even for the critically endangered western giant eland.

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P217

Evaluation of the binding capacity of fresh and frozen semen to the oviductal cells cultured *in vitro*

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The binding of sperm to the oviductal sperm reservoir appears to be an important tool to increase the population responsible for the fertilization of the oocyte cells. However, frozen sperm can lose this function due to the injuries resulting from the freezing process, therefore the aim of this study was to evaluate the binding capacity of fresh and frozen sperm to the oviduct epithelial cells (OEC), cultured *in vitro*. The semen from ten Andalusian stallions were used fresh and frozen using the extender Botucrio, and both semen were incubated with cells *in vitro* from istimo. After the incubation period, the sperm complex aggregates were transferred to a glass slide and covered with a cover slip. Two images were captured using a differential interference contrast microscope (DIC). The number of sperm bound per square millimeter of the aggregate area was calculated using an image-editing software. There was no statistical difference in the rate of the sperm to bind to the CEO using fresh and thawed semen (88.2 ± 13.5 and 72.8 ± 6.8 sperm/mm^2 of the aggregate area, respectively). This result confronts the findings of previous studies, which reported a decrease in capacity of frozen semen to bind to CEO *in vitro*. Probably, this occurred because the sperm frozen with Botucrio have higher structural integrity of the proteins responsible for the binding to OEC, which did not happen with other freezing diluents.
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P218

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P219

Antioxidant vitamins supplementation decreases motility in both fresh and refrigerated ram semen

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Introduction
Spermatozoa death is accelerated by reactive oxygen species in fresh, refrigerated or frozen/thawed semen. In sheep, antioxidant therapy may alleviate the effects of oxidative stress by favoring antioxidant status. Our aim was to compare the effect of antioxidant therapy on sperm motility in ram semen conserved at both ambient (AT) and refrigeration (RT) temperatures.
Materials and methods
Five rams were daily supplemented with vitamins C 600 mg and E 420 IU during 15 days, while other five were controls. Ejaculates obtained by electro-stimulation were immediately evaluated for volume and sperm concentration. Semen was diluted (Tris/citric acid/fructose medium; 100×10^6 sperms/ml) and evaluated for motility in a CASA system. Semen was divided and maintained at AT (21–26 °C) or RT (5–6 °C). Motility was subsequently evaluated at 1–3–6–12–24–36 and 48 h. Results were analyzed by ANOVA and Duncan test.
Results and discussion
No initial differences between vitamins-treated and control rams were observed in semen volume (1.7 ± 0.2 ml), concentration ($3.4\pm0.5\times10^9$ sperms/ml) or motility ($70.0\pm5.6\%$). After 1-h, motility decreased 50% in RT semen, remaining stable until 12-h. At AT, motility decreased slowly until 6-h ($56.5\pm6.3\%$); at 12-h it was similar to that refrigerated ($30.0\pm5.9\%$). At 24-h, motility was higher in RT than AT (18.0 ± 4.5 vs $6.2\pm3.8\%$, respectively; $P=0.05$), which continues decreasing rapidly after this time, being almost null at 48-h in both groups. Antioxidants supplementation decreased sperm motility from 1-h until 6-h, without effect in subsequent times. It is concluded that supplementation with antioxidant vitamins impaired semen quality in both AT and RT ram semen.
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P220

Crosstalk of TGFβ superfamily with Smad1 in testis

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The TGFβ superfamily consists of more than 30 ligands, which exert their multiple functions during early embryonic development and in adult tissues. During spermatogenesis, several TGFβ superfamily ligands are present simultaneously to regulate critical stages of development through shared receptors and overlapping transduction molecules. However, the understanding of this promiscuity of signalling pathways remains unfortunately incomplete. Our aim is to study the mechanisms and physiological outcomes of signalling interactions between several TGFβ superfamily ligands on testicular differentiation.

To study the relevance of TGFβ crosstalk in testes, our lab established a mechanical method to isolate and culture tubule fragments *in vitro*. Seminiferous tubule fragments were isolated from 6 to 11 weeks testes of rats. Testes were decapsulated and dissected into small fragments, which were further disaggregated with Medimachine. The tubule fragments were obtained by centrifugation for 5 min at 500 r.p.m. and plated into 24-well culture plates.

Our first attempt was to examine the interaction between TGFβ1/2 and BMP signalling pathways in testis. We found induction of Smad1 phosphorylation by TGFβ1/2 in a time-dependent manner in tubule fragments. Of note, induction of phosphorylation can be eliminated specifically by a BMP receptor inhibitor LDN193189. These results indicated that TGFβs can additionally phosphorylate Smad1 via BMP receptors. To further define which BMP-type receptors are possibly involved in induction of p-Smad1 by TGFβs, the tubule fragments were treated TGFβ1 with individual ALK2, ALK3, and ALK6 receptor inhibitors. We found that inhibition of the ALK2 receptor caused stronger effects compared to the ALK6 and ALK3 receptors. In summary, this is the prior report showing that TGFβ signal also via BMP receptors and Smad1 in testicular cells.

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P221

Meiotic and post-meiotic germ cells up-regulate blood–testis barrier function

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Introduction
The blood–testis barrier (BTB) remains semi-permeable to tracers of increasing molecular weight in an adult rat model of spermatogenic re-initiation. Complete closure of the BTB occurred when steps 2–7 round spermatids re-appeared in the epithelium, and coincided with the localisation of a new tight junction (TJ) protein claudin-12 (Cldn12) at the BTB. We hypothesise that meiotic and/or post-meiotic germ cells up-regulate Sertoli cell TJs, and aim to demonstrate isolated germ cells stimulate TJs in rat Sertoli cells *in vitro*.
Material and methods
Sertoli cells (d20) were cultured for 5 days, and then pachytene spermatocytes (PSC) or round spermatids (rST) were added in co-culture for 24 h. TJs were monitored by *trans*-epithelial electrical resistance (TER). Clnd11 and Cldn12 were examined by qPCR and immunofluorescence.
Results and discussion
TER increased two- to 2.7-fold with added PSC or rST to Sertoli cells, but only when cells were in direct contact. Tight junction protein Cldn11 mRNA expression decreased two- to 2.2-fold whilst Cldn12 expression was up-regulated 1.5- to 2.4-fold by germ cells. Cldn11 protein localisation was largely unaffected by germ cells, however Cldn12 localisation was intensely present at cell junctions when germ cells were added. This data provides strong evidence that an extra group of TJ proteins, in addition to the androgen-dependent claudins (Cldn11 and Cldn3) exist in rat Sertoli cells and are up-regulated by meiotic and post-meiotic germ cells. This data suggests a new mechanism by which BTB function may be regulated in normal spermatogenesis, of likely importance in diseases or conditions which impact these germ cell types.

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P222

The RNA-binding protein Musashi-2 (MSI2) controls mRNA processing and translational regulation via interactions with SFPQ and PIWIL1 during mammalian spermatogenesis

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Introduction
RNA-binding proteins (RBP) are important catalysts of post-transcriptional gene regulation. The RBP MSI2 directs stem cell function and division through established roles in cell cycle dynamics and cell fate determination. During the complex process of mammalian spermatogenesis, the directed uncoupling of transcription and translation is essential for the correct development of male gametes. We have previously established that dysregulation of MSI2 during germ cell maturation is detrimental to sperm development and fertility. In this study we characterise the molecular mechanisms via which MSI2 exercises control of protein expression during spermatogenesis.
Materials and methods
Differential gene (microarray) and protein (iTRAC) expression studies were undertaken to establish the predominant molecular pathways and biological processes affected by up-regulation of MSI2 in a testis-specific MSI2 over-expression mouse model. Immunoprecipitation assays were performed to identify specific MSI2 interactions in post-meiotic round spermatids.
Results and discussion
Of the differentially expressed (DE) genes, the key molecular networks identified were implicated in cell death, cell morphology, cellular growth and proliferation, and the cell cycle. The most relevant biological processes that characterised the DE proteins related to transcriptional control, the cell cycle and cellular proliferation, translation, apoptosis, RNA splicing/processing, and spermatogenesis. Protein-immunoprecipitation identified that MSI2 acts in complex with

slicing factor, SFPQ, and piRNA component, PIWIL1, within round spermatids. This data indicates a role for MSI2 in mRNA processing and translational regulation via direct interactions with SFPQ and PIWIL1. And firmly establishes MSI2 as a key regulator of post-meiotic germ cell development.

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P223

GATA4 serine 105 phosphorylation is required for testicular steroidogenesis in the mouse

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GATA4 is a transcription factor required for testicular differentiation and for male fertility. In spite of its crucial roles in male reproduction, how the activity of this factor is regulated has yet to be fully understood. The GATA4 protein is phosphorylated on at least two serine residues. This includes GATA4 S105, which has been reported to modulate GATA4 transcriptional activity in cell line studies. Its contribution to endogenous GATA4 activity in the testis, however, has not been investigated.

To gain insight into the physiological role of GATA4 S105 phosphorylation, we examined GATA4 S105A male mice, which carry a Ser to Ala mutation preventing phosphorylation at this site. A breeding trial and hormonal profiling were used to assess the fertility and endocrine status of GATA4 S105A mutant males. Gene expression and immunohistochemistry experiments were then used to characterize the testicular phenotype associated with the S105A mutation.

While the fertility of homozygous GATA4 S105A adult males was normal, the weights of several androgen-dependent organs (epididymis, seminal vesicle, and prostate) were noticeably smaller when compared to WT controls. This suggested a possible defect in androgen production and/or action. An examination of serum testosterone levels showed a significant 70% decrease in adult S105A males. This decrease was associated with a significant reduction in *Star*, *Cyp11a1*, *Hsd3b1/6*, *Cyp17a1*, and *Hsd17b3* mRNA and protein levels in GATA4 S105A mutant testis. These results provide the first demonstration that GATA4 via S105 phosphorylation is essential for testicular testosterone production via control of multiple genes in the steroidogenic pathway.

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P224

Maintenance and amplification of testicular tumors by subcutaneous grafting in zebrafish

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Introduction

Testicular tumors not only facilitate analyses of tumorigenesis in testes but also are a good resource for culturing spermatogonial stem cells (SSCs) that allow us to manipulate genetic modification. In zebrafish, a hypertrophied tumorous testis happens to be found occasionally, but no method for maintenance of the individual tumorous testis has prevented systematic analyses of the characters and the practical use. We describe here the method for maintenance of testicular tumors by subcutaneous grafting into an immunodeficient mutant zebrafish.

Materials and methods

Three independent tumorous testes were isolated from different lines. The testis was dissected into five to ten small fragments that contained outer layer of the testis, and then transplanted under the abdominal skin of mutant fish. Recipient fish were reared in fish water containing antibiotics for 4 days, and then maintained normally.

Results and discussion

Almost all recipient fish survived more than 1 month, and tumorous testes grew under the abdominal skin. Some of them survived more than 1 year. After growth of tumorous testes as same as the original size, we removed them and repeated dissection and transplantation. Histological analyses revealed that two of three tumorous testes maintained SSCs and the testicular structure while one testis lost them after successive transplantations. In *in vitro* cultures, SSCs from the two testes grew without any abnormality. These results suggest that testicular tumors can be maintained and amplified by subcutaneous grafting in the immunodeficient mutant when an appropriate testicular tumor is selected in each transplantation step.

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P225

Effect of aromatase on sperm penetration and embryo cleavage rate during IVF in pig

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Introduction

Marker genes for evaluating sperm fertility have been developed during a few decades in various species. The CYP19A1/Cyp19a1 has been considered to one of the most common factor related to sperm quality regardless to species. In our previous report, *CYP19A1* mRNA quantity was suggested to sperm fertility marker in pig. So, in this study, we examined whether CYP19A1 level and activity are related to sperm fertility to confirm importance of CYP19A1 in porcine sperm.

Materials and methods

First, to confirm the relation between CYP19A1 level and sperm fertility, one ejaculate was divided into two fractions by density and they independently assessed to IVF to measure the rate of sperm penetration and embryo cleavage, and western blot to evaluate CYP19A1 level respectively. Obtained results with those fractions were compared each other. Second, for the analysis of sperm fertility following CYP19A1 activity, CYP19A1 inhibitor, exemestane, was treated with concentrations that have no effect on porcine sperm viability. Inhibitor treated sperms were assessed to IVF for evaluation of their fertility.

Results and discussion

Separated sperm fractions showed that low density sperm population contained significantly high penetrate rate ($P<0.05$) but not in the embryo cleavage rate and CYP19A1 level than high density fraction. CYP19A1 inhibitor treatment significantly reduced sperm fertility ($P<0.05$). In conclusion, CYP19A1 contributes to penetration capacity and fertility of porcine sperm. Density of sperm has difference in penetration capacity of sperm, but not in CYP19A1 quantity.

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P226

Effects of different concentration of *Escherichia coli* on boar sperm quality and field fertility

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One of the most frequently isolated bacteria in porcine semen was *Escherichia coli*. Overgrowth by contaminant bacteria such as *E. coli* has a deleterious effect on semen quality and longevity. The objective of this study was to determine the effects of *E. coli* isolated from porcine semen on sperm motility, viability, semen pH, and field fertility in sow. Semen samples were prepared using BTS extender that did not contain antibiotics. *E. coli* was isolated from fresh porcine semen collected at dedicated boar studs. It was hemolytic and enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST-1)-positive. Three different levels of *E. coli* were artificially innoculated to semen with following concentrations; control (0), T1 (500 cfu/ml), T2 (5000 cfu/ml), and T3 (50 000 cfu/ml). Semen samples were preserved at 17 °C for 5 days in semen storage box until analyzed. Sperm motility were significantly decreased ($P<0.05$) on day 3 in T2 compared to control groups. The percentage of viable spermatozoa was significantly decreased ($P<0.05$) in all groups from day 3. Sample pH was maintained 7.0–7.2 in all groups during the experimental period. To evaluate the field fertility outcomes in bacteria contamination semen, we performed artificial insemination (AI) with 56 herds of weaned Duroc sows. Semen was stored at 17 °C for 3 days before AI. The pregnancy rate and litter size tend to decrease by increasing the concentration of *E. coli* in semen. In particular, the rate of pregnancy was lower in T3 (58.3%) compare to the other groups (81.8, 75.0, and 76.5%). These results suggest that *E. coli* contamination has a concentration-dependent detrimental effect on extended porcine semen quality.

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P227

Single-cell level dynamics of mouse spermatogenic stem cells after transplantation

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In mice, a technique was developed in which transplanted spermatogenic stem cells (SSCs) reconstitute complete spermatogenesis in the testes of infertile recipient. However, the dynamics of SSCs during the reconstitution process after transplantation is largely unknown. To dissect post-transplantation dynamics of SSCs, the behavioral process of donor spermatogonia was observed by *in vivo* live-imaging, and their fate was analyzed at a single-cell resolution. For live-imaging, singly dissociated testicular cells from UBI-EGFP mice (1×10^6 cells/testis) microinjected into the seminiferous tubules of were filmed according to the protocol of Yoshida *et al*. (2007). For fate analysis, spermatogonia expressing GFRα1, a SSCs marker, were pulse-labeled with persistent GFP expression following administration of 4OH-tamoxifen to GFRα1/CreER^{T2}; CAG-CAT-EGFP mice, and transplantation was conducted as same way described above. The constitute numbers of cells in individual clones were scored according to the GFRα1 expression by whole-mount immunostaining of recipient tubules. The live-imaging study has revealed that a single isolated spermatogonium actively migrated with extend lobopodia while interconnected spermatogonia actively divided adjacent to the vasculature. In addition, the death of donor spermatogonia was frequently observed. Fate analysis suggested that vast majority of GFRα1+ spermatogonia that first settled on the basement membrane disappeared (probably death) within 20 days post-transplantation, while a tiny part of surviving GFRα1+ spermatogonia reconstituted colonies. Interestingly, the fates of individual pulse-labeled GFRα1+ spermatogonia were found highly variable. The average numbers of GFRα1+ spermatogoina and GFRα1$^-$ spermatogenic cells in each clone were exponentially increased up to 20 days post-transplantation, and then remained constant. At present, we are trying to mathematically capture such of post-transplantation dynamics of SSCs.

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P228

Effect of ascorbic acid 2-glucoside supplementation on sperm characteristics during semen cryopreservation in Korean native pig

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Ascorbic acid 2-glucoside (AA-2G), a stable ascorbate derivative, is a prominent antioxidant characterized by its stability in an oxidative environment. The objective of this study was to evaluate the effect of AA-2G on sperm motility, viability and acrosome integrity during semen cryopreservation in Korean native pig. Semen samples were diluted into a freezing extender (LEY) containing lactose hen-egg yolk with glycerol and Orvus Es Paste (OEP) and loaded in 0.5 ml plastic straws. In this experiment, each ejaculated semen sample was split into five aliquots and extended with LEY that supplemented with 0, 100, 200, 300, or 400 μM AA-2G. For freezing the semen samples were cooled from 5 to −5 °C at 6 °C/min, held at −5 °C for 30 s while ice crystal formation was induced, then further cooled from −5 to −80 °C at 40 °C/min, and thereafter from −80 to −150 °C at 60 °C/min using programmable semen freezer. To evaluate the post-thaw sperm quality, semen was thawed at 38 °C for 20 s. Sperm quality in terms of motility, viability, and acrosome integrity was improved by addition of AA-2G to freezing extender during semen cryopreservation. In particular, the sperm motility and acrosome integrity at 1 and 2 h of incubation was higher ($P<0.05$) in the group treated with 300 μM AA-2G during freezing procedure when compared to the control group. The result of this study shows that the addition of AA-2G to the freezing extender (LEY) improves motility and acrosome integrity of frozen–thawed sperm in Korean native pig.

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P229

Trehalose in glycerol-free freezing extender preserves mitochondria membrane potential of post-thaw boar spermatozoa

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Introduction
Movement of spermatozoa and the penetration of zona-pellucida depend on the energy produced in mitochondria. Major steps of cryopreservation can exert stress on sperm mitochondria membrane. Better post-thaw motility, viability and acrosome integrity of spermatozoa was observed when extended in trehalose in a previous study. Objective was to evaluate the effect of trehalose on mitochondria integrity of post-thawed boar spermatozoa.
Materials and method
Spermatozoa were diluted in egg yolk extender containing 0.25% Equex STM and glycerol (100 mM) or trehalose (0, 50, 100, 150, 200, and 250 mM) and cryopreserved with straw freezing procedure. Frozen sperms were thawed at 39 °C for 30 s and analyzed for motility with CASA and mitochondrial membrane potential (MMP) with JC-1/PI staining. Data were analyzed with ANOVA and Pearson's product moment correlation (mean ± S.E.M).
Results and discussion
Motility in glycerol (100 mM) and trehalose (0, 50, 100, 150, 200, and 250 mM) was 21.2 ± 2.3^b, 9.5 ± 3.8^c, 24.5 ± 2.1^{ab}, 32.9 ± 2.9^a, 25.4 ± 0.6^{ab}, 23.5 ± 1.1^{ab}, and 20.8 ± 1.3^{ab} while the spermatozoa with high MMP was 38.4 ± 2.1^b, 18.6 ± 4.1^c, 41.0 ± 6.9^{ab}, 54.7 ± 3.6^a, 46.9 ± 4.4^{ab}, 53.2 ± 1.4^{ab}, and 45.9 ± 1.8^{ab} respectively. Motility and MMP was significantly high in 100 mM trehalose compared to 100 mM glycerol ($P<0.0001$, $n=6$). Significant negative correlation was observed between motility and trehalose concentration after 100 mM ($r=-0.95$, $P=0.049$) although the MMP was not significantly correlated ($r=-0.59$, $P=0.41$). Even though the high concentrations of trehalose slightly reduced the motility, probably due to the viscosity of the medium, it retained the MMP. Differences in motility were observed with previous study, possibly due to the seasonal and intra-ejaculate variations. In conclusion, along with previous data, glycerol-free trehalose extender preserved post-thaw survival including MMP in boar spermatozoa.

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P230

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Knocking-down detrimental seminal protein PDC-109 minimizes cryoinjury to bull spermatozoa

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Introduction
Seminal PDC-109, most abundant protein in bulls, destabilizes sperm membrane by causing efflux of cholesterol. This detrimental effect is minimized to some extent by addition of egg yolk (EY) in extender. Knocking-down of free floating PDC-109 protein by antibodies (Ab) can be another method not yet reported. Therefore, a study was designed to assess the effect of antibodies-mediated sequestration of PDC-109 protein as compared to EY on minimization of cryoinjury to bull sperm.
Materials and methods
Anti-PDC-109 raised in rabbits were isolated, purified, and added in ejaculates. Cholesterol content, viability, and cryoinjury as manifested by chlortetracycline assay at different stages of cryopreservation as progressive series, were evaluated ($n=6$). Three groups were: Gr I, control, ejaculates processed in EY+tris-glycerol (TG) -extender (EYTG); Gr II, ejaculates collected directly in EY+TG; efficacy of EY was tested from this group; Gr III, Ab+EY+TG, processed for cryopreservation.

Results and discussion
The pre-freeze and post-thaw cholesterol content (μg/100 million spermatozoa) and viability were significantly higher in antibodies treated group as compared to Gr I and II. The chlortetracycline assay revealed significantly higher non capacitated spermatozoa in Gr III and conversely, mean values for pre-freeze spermatozoa showing pattern B and AR were significantly lower in Gr III than other two groups. It can be concluded that sequestration of PDC-109 protein by antibodies and EY increases tolerance of spermatozoa to cryoinjury. This study underlines that cryoprotection of spermatozoa can be achieved much more efficiently by addition of antibodies at processing.

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Ultrastructural analysis of epididymis of agouti (*Dasyprocta* spp) during sexual development

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The agouti is a rodent found in the South America and one of the most predated species in Brazil, which has shown a significant decrease of population size. The principal function of epididymis is maturation of spermatozoa, in addition to conducting the same for the vas deferens. The study of sperm pathway, especially of epididymis, can promote morphophysiological information necessary for reproduction biotechnologies. The aim of this study was to describe the ultrastructure of the epididymis during sexual development of agoutis kept in captivity. The animals were kept at Center of Animal Wilds in northeastern, Mossoró, Brazil. The stages of testicular development (prepubescent, prepubertal, pubescent, and adult) were previously determined. Segments of the epididymis of agouti males at different stages of sexual development were performed to semithin cut and analyzed by transmission electron microscopy. This was a pioneer study in which it was observed that the epididymis of agoutis are composed of principal, basal, halogen, apical, and clean cells. During prepubescent and prepubertal stages were observed clean cells in addition to principal, basal, and halogen cells. On puberty apical cells were also present, and clean cells were not visible. We concluded that the pseudostratified stereociliated epithelium of the epididymis of agoutis undergo morphological and functional changes during sexual development. This range of information provides new approaches for future investigations involving the reproductive biology of agouti, which could be an important experimental model in reproductive biology research.

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Ultrastructural analysis of spermatozoa in agoutis during sexual development

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The study of the sperm is particularly relevant to reproduction of the species and can promote morphological information necessary for reproduction biotechnologies. The agouti is a rodent found in the South America and represents an excellent source of animal protein. In captivity, the establishment of pubescent occurs at 9 months of age. The aim of this study is to describe the ultrastructure of sperm during sexual development of agoutis kept in captivity. The testes of animals were collected by orchiectomy and weighed. Subsequently, the testes were fixed by immersion in a solution of 2.5% glutaraldehyde for 24 h. Segments of the testis of agouti males at different stages of sexual development (prepubertal, pubescent, and adult) were performed by transmission electron microscopy. During prepubertal stage occurs the formation of pre-acrosome vesicle and headline, in addition to organization of centrioles. Mitochondria migrate to the opposite pole of the cell and arrange themselves to form the flagellum. From the pubescent stage, the spermatozoa are already fully formed: head, neck, body, and tail. This study was the first about ultrastructure of the sperm in the agouti. The sperm is not fully formed during prepubertal stage. During the pubescent stage the sperm is characterized by a tapered head without distinction as of acrosome, unlike other wild species like the cavy, guinea pig, and armadillo.

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Characterisation of Gankyrin expression in testicular germ cell cancer

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Introduction
Testicular germ cell cancer (TGCC) has been increasing in incidence over recent decades. We have previously shown that the precursor lesion for TGCC, carcinoma *in situ* (CIS) cells, have different phenotypes which determine proliferation rate. CIS which express only OCT4, a well-established marker for CIS, proliferate more that those expressing OCT4 and MAGE-A4 (differentiated germ cells marker). Gankyrin is an oncogene that interacts with MAGE-A4 and supresses the degradation of OCT4. We hypothesize that Gankyrin would maintain OCT4 expression, whilst MAGE-A4 would reduce the oncogenic potential in CIS cells.
Materials and methods
We performed qPCR and immunofluorescence for Gankyrin expression using TGCC tissue obtained from patients undergoing orchidectomy. We assessed the expression in areas of normal spermatogenesis (NT), CIS adjacent to the tumour (AT) and tumour (TT). We also used siRNA to inhibit Gankyrin and study the effects of its down regulation on germ cell development.
Results and discussion
Gankyrin expression was localised to Sertoli cells cytoplasm in tubules with active spermatogenesis and in CIS containing tubules adjacent to the tumour, whilst no expression was observed in the tumour tissue. Overall relative mRNA amount of Gankyrin was 27% higher in TT (1.03×10^{17}) compared to AT (7.51×10^{16}) and AT was 29% higher compared to NT (4.52×10^{16}), although this did not reach significance. The immunohistochemistry has shown that MAGEA4, OCT4, and Gankyrin can be found together in an individual tubule however no relationship between Gankyrin expression and the phenotype of CIS could be observed. siRNA studies are ongoing and will also be presented.

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Tamoxifen inducible Cre systems: adverse effects of low dose tamoxifen treatments on the male reproductive tract

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Introduction
Tamoxifen inducible Cre systems have been used to study development specific roles of genes in the testis as they allow tight temporal control of genetic manipulation. However, tamoxifen is an anti-estrogen that competitively binds estrogen receptors. Despite the antagonistic properties of tamoxifen, it also acts as a weak estrogen agonist, hence exerting estrogenic effects in a tissue and cell specific manner. Given the duality of tamoxifen function and the importance of estrogen signalling in male reproduction, here we utilise an inducible nestin-Cre reporter to examine testicular impacts of tamoxifen in inducing transgene expression.

Materials and methods
Inducible nestin-Cre recombinase reporter mice were treated with tamoxifen at low and high doses. Standard techniques used to analyse testicular structure and function.
Results and discussion
A high dose of 3 mg tamoxifen at day 21 causes a significant impact on testis function and seminal vesicle atrophy. Surprisingly, even low doses of tamoxifen (250 μg, 500 μg, and 1 mg) administered at day 16 show a long-term effect on the testis in adulthood. At day 47, a significant decrease in testis and seminal vesicle weight is observed in all three low dose groups. This is partly due to a decline in spermatogenesis as indicated by a significant decrease in seminiferous tubule diameter. Additionally, Leydig cell maturation is also perturbed in all treatment groups. These findings demonstrate that tamoxifen administration during postnatal development cause persistent changes in adult testis function. Therefore, tamoxifen inducible gene targeting during postnatal development may not be an appropriate tool in the study of male reproduction.

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Involvement of peroxisomes in steroid synthesis in Leydig cells and their role for male fertility

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Introduction
Peroxisomes are ubiquitous organelles, which play an essential role in human physiology. Besides other metabolic pathways, peroxisomes are involved in β-oxidation of fatty acids, ROS-metabolism, and cholesterol synthesis, wherefore alterations of peroxisomal metabolism might affect steroid metabolism and the regulation of corresponding signaling pathways. Indeed male patients with peroxisomal dysfunction exhibit either cryptorchidism or a range of testicular pathologies leading to male infertility.
Materials and methods
We used the mouse tumor Leydig cell line (MLTC-1) and mouse primary Leydig cells to knockdown the *Pex13* gene by transient transfection using microporation. The resulting phenotype was characterized by immunofluorescence, ROS− and glutathione assays, western blots, qRT-PCR, and RIAs/ELISAs.
Results and discussion
Using *Pex13*-RNAi technology we show that peroxisomal dysfunction in Leydig cells, as monitored by mistargeting of peroxisomal enzymes to the cytoplasm, leads to an increase in ROS production and mitochondrial dysfunction. The hCG-mediated induction of the intramitochondrial 30 kDa mature form of StAR protein was blocked in Leydig cells with peroxisome deficiency. Moreover, we found an up-regulation of aromatase and estrogen receptor alpha expression. Finally, we show that peroxisomal knockdown leads to significantly reduced levels of progesterone, testosterone, and estrone synthesis. In contrast, an increased level of estradiol was noted. Our results suggest that peroxisome dysfunction induces oxidative stress, mitochondrial alterations, and androgen/estrogen imbalance, which may contribute to the pathogenesis of male infertility.

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Targeting high mobility group box protein 1 ameliorates experimental autoimmune orchitis

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High mobility group box protein 1 (HMGB1) plays an important role in onset and progression of autoimmune diseases. This nucleosomal protein is actively secreted during inflammation and acts as a late phase cytokine. Targeting HMGB1 by ethyl pyruvate (EP) significantly reduces its pro-inflammatory effects. Experimental autoimmune orchitis (EAO) serves as a rodent model to study autoimmune based chronic testicular inflammation characterized by elevated levels of pro-inflammatory cytokines, lymphocytic infiltrates in the interstitium, germ cell loss, and subsequent infertility.
EAO was induced in Wistar rats by immunization with testicular homogenate. To block HMGB1 action, EAO and control animals received EP (40 mg/kg, i.p.). HMGB1 localization in testis was analyzed by immunostaining. Levels of HMGB1, IL6, and TNFα were measured using ELISA and real-time qRT-PCR. Interaction of HMGB1 with its receptors RAGE and TLR4 was determined by *in situ* proximity ligation assay. Activation of MAPK kinases was investigated in isolated testicular macrophages (TM), peritubular (PTC), and Sertoli cells (SC) by western blot.
HMGB1 was translocated from the nuclei in EAO testis followed by significant elevation of HMGB1 levels during the chronic phase of the disease. HMGB1 receptors TLR4 and RAGE were differentially expressed in testicular somatic cells. Binding of HMGB1 to TLR4 was significantly higher in TM compared to SC and PTC with higher levels of HMGB1–RAGE interaction. In support, HMGB1 triggered RAGE-dependent ERK1/2 and CREB activation in SC and PTC. *In vivo* treatment of EAO animals with EP successfully reduced disease progression. Inhibiting HMGB1's action during the course of testicular inflammation could be a promising therapeutical approach to prevent loss of spermatogenesis.

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Effect of addition post-thaw of homologous seminal plasma on quality of donkey frozen semen

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A recent study showed increase of pregnancy rates in donkey frozen semen with addition of seminal plasma post-thaw. The aim of the present study was to evaluate the effect of addition post-thaw of seminal plasma on donkey frozen semen quality. Five ejaculates from three donkeys were used. Semen was diluted (1:1) with a milk-based extender (Botu-Semen). The semen was frozen with Botu-Cryo extender in isothermal box in straws containing 100×10^6 of total sperm. The samples were thawed at 46 °C for 20 s. After this, the straws of each stallion were divided in two groups: control group (CG) the semen was incubated at 37 °C for 5 min and plasma seminal group (PG) the semen was incubated at 37 °C for 5 min with 30% of homologous seminal plasma. Sperm kinetic analysis (CASA), plasma membrane integrity (MI) by epi-fluorescence microscopy (propidium iodide and 6-carboxyfluorescein diacetate) were evaluated. Comparison of sperm parameters was performed by *T* test. Total motility (%, CG = 75.4 ± 8.2^a vs PG = 57.5 ± 16.4^b), progressive motility (%, CG = 42.0 ± 8.7^a vs PG = 33.3 ± 13.2^b), progressive angular velocity (μ/s, CG = 95.8 ± 10.8^a vs PG = 88.9 ± 10.9^b), percentage of rapid sperm (%, CG = 58.4 ± 12.5^a vs PG = 41.0 ± 17.3^b), and MI (%, CG = 61.2 ± 10.5 vs PG = 40.7 ± 19.4) were higher in CG compare to PG. Straightness (%, CG = 82.0 ± 1.5^a vs PG = 87.2 ± 3.0^b) and linearity (%, CG = 48.5 ± 2.2^a vs PG = 58.9 ± 4.1^b) were higher ($P<0.05$) in PG group compare to CG. The results of this study showed that addition of homologous seminal plasma post-thaw decrease the sperm kinetic parameters and viability of donkey frozen semen.

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Difference between of homologous and heterologous seminal plasma addition on post-thawn donkey frozen semen

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One study showed increased fertilization in IVF when added seminal plasma of bulls with high fertility rates in semen of low fertile. The aim of the present study was to evaluate the difference of addition homologous and heterologous seminal on donkey frozen semen. Five ejaculates from two donkeys were used. Semen was diluted (1:1) with a milk-based extender (Botu-Semen). The semen was frozen with Botu-Cryo extender in isothermal box in straws containing 100×10^6 of total sperm. The samples were thawed at 46 °C for 20 s. After this, the straws of each donkey were divided in two groups: homologous group (G1) the semen was incubated at 37 °C for 5 min with 30% of homologous seminal plasma and heterologous group (G2) the semen was incubated at 37 °C for 5 min with 30% of heterologous seminal plasma. Sperm kinetic analysis (CASA), plasma membrane integrity (MI) by epi-fluorescence microscopy (propidium iodide and 6-carboxyfluorescein diacetate) were evaluated. Comparison of sperm parameters was performed by *T* test. No difference ($P > 0.05$) were observed in total motility (%, G1 = 38.0 ± 12.6 vs G2 = 40.0 ± 6.0), progressive motility (%, G1 = 18.8 ± 7.5 vs G2 = 21.5 ± 5.5), progressive angular velocity (μm/s, G1 = 86.7 ± 6.1 vs G2 = 88.8 ± 5.3), percentage of rapid sperm (%, G1 = 24.8 ± 10.5 vs G2 = 27.8 ± 5.4), and MI (%, G1 = 32.8 ± 14.3 vs G2 = 28.3 ± 7.6) between the groups. The results of this study showed have not difference in addition of homologous or heterologous seminal plasma post-thaw on quality of donkey frozen semen.

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Evaluation of donkey frozen semen quality in different freezing conditions

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There are few studies with donkey semen in the literature. In horses, is related that no difference are in freezing semen using automated systems or Styrofoam box. The aim of the present study was evaluate donkey frozen semen quality in different freezing conditions. Five ejaculates from there donkeys were used. Semen was diluted (1:1) with a milk-based extender. The pellet was resuspended with Botucrio and the straws were packaged. The straws were kept in a refrigerator at 5 °C for 20 min. After this, the samples were divided in: Styrofoam box group (SG) where the straws were placed 6 cm above liquid nitrogen for 20 min and automated system group (AG) where straws were placed in a freezing machine at rate temperature of −60 °C/min until −140 °C. After this, Straws of both groups were immersed in liquid nitrogen. Sperm kinetic analysis by CASA, plasma membrane integrity (MI) by epi-fluorescence microscopy were evaluated. Comparison of sperm parameters was performed by *T* test. No difference ($P > 0.05$) were observed in total motility (%, SG = 75.4 ± 8.2 vs AG = 78.0 ± 9.5), progressive motility (%, SG = 42.0 ± 8.8 vs AG = 45.5 ± 8.5) and MI (%, SG = 61.2 ± 10.5 vs AG = 63.5 ± 11.8) between the groups. AG had higher trend (0.05^a vs AG = 101.8 ± 9.7^b) and percentage of rapid sperm (%, SG = 58.4 ± 12.5^a vs AG = 64.6 ± 12.1^b). The results of this study showed have not difference in frozen donkey semen using Styrofoam box or automated system, however when automated system was using there is higher trend of velocity sperm.

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Acute reduction of Sertoli cell numbers during development leads to a subsequent reduction in sperm numbers in adulthood

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Sertoli cells (SC) are key initiators of testis development and have a fundamental role in spermatogenesis. However, it is uncertain how an acute reduction in SC number, in a hormone independent-manner at key points in development or adulthood, will impact testis function.

To induce acute SC-ablation at key points of testicular development, we used Cre-loxP recombinase technology and the expression of Diphtheria toxin and its receptor. We utilized these lines to determine i) the impact on testicular development and function arising from a reduction in SC numbers and ii) the capacity of the remaining SC population to compensate this reduction at different developmental stages.

When SC-ablation occurred during embryogenesis, tubular structure was absent at day 0, but recovered by day 9. However, daily sperm count and epididymal reserves were reduced in adulthood, consistent with previous studies showing a relationship between SC number and quantitative spermatogenesis.

When ablation was induced by > 10 ng DTX post-puberty, disruption remained apparent 30 days post-ablation. Conversely treatment with < 10 ng DTX failed to impact testicular architecture and function, suggesting a threshold of SC loss must be reached before testis function is affected. Throughout the study reduction in sperm number correlated to the extent of SC reduction.

We have demonstrated that an acute reduction in SC numbers negatively impacts adult sperm number and that testicular recovery appears to be age dependant. Further characterization will produce a unique model of subfertility, in which the hypothalamic–pituitary–gonadal axis is unaffected, allowing analysis of novel therapeutics for improving sperm production and male fertility.

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Differential expression of proteins from the human sperm head and its relation to infertility

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Introduction

Male infertility is one of the major challenges in reproduction. The nature of sub-fertility due to male is as complex as that of female and contributes to about 40–50% of infertility cases in human.

Objective

We propose to identify all the protein molecules that differ in the different region of the human spermatozoa, i.e. the head and the tail. The aim of the study was to identify the differentially expressed protein in the head fraction of the both motile and non-motile human spermatozoa samples, which might provide an insight into the cause of infertility.

Material and methods

Semen samples of normal (above 60% sperm motility) and with low sperm motility (below 15% motility) were taken and the sperm cells were separated. Heads were isolated by sucrose density gradient. The head proteins were trypsin digested and subjected to iTRAQ labelling followed by identification by LC/MS.

Results

The identification of these proteins provides us with information that the head of the sperm is a repertoire of binding proteins, catalytic and structural and molecular activity proteins. So the lower expression or absence of these proteins results in infertility due to head structural deformities or incapability release the necessary enzyme or to mediate effective binding with the oocyte.

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Study of spermatogonia stem cells from domestic animals: derivation and *in vitro* culture

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Introduction
It is necessary to establish and culture of spermatogonia stem cells (SSCs) *in vitro* to perform the meiotic differentiation study and to generate transgenic spermatozoa *in vitro* or *ex vivo*. This study was focused on *in vitro* derivation and establishment of SSCs from porcine and canine.
Materials and methods
Five day-old porcine and 2-month-old canine testes were used to isolated SSCs and testicular somatic cells. For the establishment of porcine and canine SSCs, SSCs and testicular cells were cultured in stempro-34 and DMEM with 5% FBS medium, respectively, supplemented with GDNF, EGF, FGF, and LIF in 31 and 37 °C respectively. To identify the SSCs in culture, OCT4, NANOG, THY1, PLZF, and PGP9.5 were analyzed. Characteristics of SSCs were analyzed with xeno-transplantation of *in vitro* cultured porcine SSCs into immune deficient mice testes.
Result and discussion
SSCs established from both porcine and canine showed the strong alkaline phosphatase staining, pluripotency markers, and undifferentiated germ cell markers gene expressions. Both SSCs were stably maintained undifferentiated germ cell and stem cell characteristics for over 100 days on GATA4 expressed feeder cells, and xenotransplantation of pSGCs to immune deficient mice demonstrated the successful colonization in the recipient testes. In addition, GFRα-1 and UTF1 were identified as a putative specific markers for pSSCs, and total transcriptome analysis of pSSCs revealed the expressions of CD14, CD209, KLF9, IGFBP3, and IGFBP5 genes. This finding may facilitate the study on spermatogenesis and further application of spermatogonial stem cells in large domestic animals.

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Proteomic approach of cryo-damage in bovine spermatozoa

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Cryopreservation is the most promising approach for storing spermatozoa. However, it could lead substantial damage to spermatozoa. We present here a couple of functional tests and 2DE PAGE proteome map of bovine spermatozoa in order to evaluate the effect of each cryopreservation procedure, e.g. fresh semen, after cooling, adding cryo-protectant (CP) and thawing on spermatozoa. Our result demonstrated that the cryopreservation procedure reduced the motility (%), viability (%) and mitochondrial activity (%) of spermatozoa whereas the acrosome reaction (%) increased significantly. The differentially expressed proteins (greater than threefolds) in fresh vs cooling, fresh vs CP, fresh vs thawing, cooling vs CP, cooling vs thawing, and CP vs thawing were 7, 3, 3, 2, 6, and 2 respectively. Among the proteins differed in abundance (total 23), F-actin-capping protein subunit beta (CAPZB) and outer dense fiber protein 2 (ODF2) were linked with actin-based cytoskeleton assembly, whereas glutathione *S*-transferase mu 3 (GSTM3) was related with glutathione metabolism. The significantly correlated proteins with notch pathway were CAPZB, triosephosphate isomerase (TPI), pyruvate dehydrogenase E1 component subunit beta (PDHB) and ODF2. TPI, PDHB and CAPZB, ODF2 were correlated with glucose metabolism and actin cytoskeleton regulation respectively. Additionally, CAPZB and NME7 were correlated with actin cytoskeleton assembly and pyrimidine metabolism, respectively. These marked differences in proteins and their related signaling pathways suggest a logical ground of sperm functional modifications during cryo-preservation in the proteomic viewpoint. Additionally, we anticipate that these finding might apply for development of novel approaches to evaluate sperm cryo-damage. This work was supported by the 'Cooperative Research Program for Agricultural Science and Technology Development' (PJ008415) of RDA, Korea.

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Effect of Sertoli cell ablation on testicular function in adult mouse

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The aging of western societies, and the associated increase in obesity, brings with it an increase in prevalence of disorders such as metabolic syndrome which are associated with reduced androgen levels in men. As Leydig cells are the source of androgens in the male, establishing the mechanisms which control Leydig cell development and function is crucial to our understanding of aging and male health. Sertoli cells regulate testicular fate in the differentiating gonad and are essential for development of the adult Leydig cell population but their role in regulating adult testis function, beyond spermatogenesis, is unclear. To examine the function of the Sertoli cells in adult testis biology we have used a recently described transgenic mouse model which allows controlled, cell-specific ablation of the Sertoli cell population in the adult animal. Results from this model show that the Sertoli cells are required in the adult testis for retention of the normal adult Leydig cell population. Following Sertoli cell ablation, adult Leydig cell numbers declined by 75% although there was a compensatory increase in activity. In the absence of Sertoli cells, PTMC activity was reduced but the cells retained an ability to exclude immune cells from the seminiferous tubules. This data shows that the Sertoli cells are critical for maintenance of the adult Leydig cell population which has significant implications for our understanding of both male reproductive disorders, and wider androgen-related conditions affecting male health.

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Comparision of DNA fragmentantion and plasma membrane integrity between chilled and frozen semen of bulls

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In fixed time artificial insemination (FTAI) is frozen semen is commonly used, however the use of cooled semen is a practice alternative to reduce costs. The aim of this study was compare the difference in DNA fragmentation and plasma membrane integrity between chilled and frozen semen of bulls. Two ejaculates from 20 bulls were collected. Semen was diluted in extender with egg-yolk and glycerol (Botu-Bov™) and divided in two groups: chilled group (CG) samples were cooled at 5 °C for 24 h and frozen group (FG) semen was frozen. Kinect sperm parameters were evaluated by CASA, plasma membrane integrite (PMI, propidium iodate, and FIT–PSA) DNA fragmentation (DNA, acridine orange) by flow cytometry (BD LSRFortessa). The data were compare using T Test. Total motility (%, CG = 89.7 ± 0.9^a vs FG = 76.3 ± 0.1^b), progressive motility (%, CG = 65.2 ± 1.7^a vs FG = 58.2 ± 1.2^b), percentage of rapid sperm (%, CG = 86.0 ± 1.3^a vs FG = 71.3 ± 1.7^b) and PMI (%, CG = 76.5 ± 2.2^a vs FG = 40.7 ± 1.9^b) were higher ($P < 0.05$) in CG compare to FG. DNA (%, CG = 1.1 ± 0.1^a vs FG = $1.6 \pm .1^b$) was lower ($P < 0.05$) in CG than FG. These results showing that chilled semen in bull provides significantly higher sperm kinect parameters and viability and lower DNA fragmentation in comparison with frozen semen. In conclusion, the use of chilled bovine semen is an important alternative in fixed time artificial insemination.

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Acceptability of New Zealand imported semen to the Japanese dairy farmers

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Introduction
The USA and the Canadian type of Holstein Friesian semens are widely utilized in Japan. However, imported semens from New Zealand (NZ) have started to utilize among some of Hokkaido dairy farmers gradually. The objective of this study is to clarify reasons and acceptability of NZ semen to the Japanese dairy farmers.
Materials and methods
Interviews are held to the NZ semen trading company and nine dairy farmers in Hokkaido, Japan, who are the customers of the firm.
Results and discussion
The main reason of start importing NZ semen by the company is due to the strong consuming request by the dairy farmers who have been to New Zealand. The imported semen is produced by the Livestock Improvement Corporation (LIC). The average herd size of those who wish to purchase NZ semen is 59.2 milking cows and 45.1 ha management land including 13.1 ha grazing land. All of the nine dairy farms graze their cows in paddock which is unique and uncommon in Japan. There are four reasons to introduce NZ semen into their management. i) Improving the fertility. ii) Improving conversion efficiency from grass to milk. iii) Improving cow legs suit for grazing and iv) improving cow body smaller for grazing. There is new movement in Japanese dairy farmers using NZ semen and adopting grazing system to produce low cost fresh milk.

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P250

Exome sequencing identified QRICH2 as a candidate gene responsible for stump-tailed sperm defects in Chinese consanguineous male offspring

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Introduction
Dysplasia of the fibrous sheath (DFS) and stump-tail sperm is a rare sperm abnormality often associated with parental consanguinity and found to be associated with recessive mode. However, the molecular pathogenesis remain elusive.
Material and methods
Whole-exome sequencing was performed in a typical pedygree with two affected brother. Multiple-tissue real time PCR was used to check the expression of QRICH2 in human multiple tissues. Sperm immunostaining was used to determine the localization of QRICH2 protein in human sperm. Zebrafish whole-mount *in situ* hybridization was used to check the expression of qrich2 gene during embryo development. Qrich2 Morphonilo was used to silencing qrich2 gene and immunostaining of acetylated tubulin was used to determine whether knock down qrich2 could affect cilia development.
Results and discussion
One testis specific expressed gene, QRICH2, was confirmed that mutated in two brothers with similar phenotypes. AA64 was identified to have a non-sense homozygous mutation of TGA. Multiple-tissue real time PCR showed that it specifically expressed in the tesits. Immunostaining of sperm confirmed that the gene is expressed in the mitochondrial of the sperm. Bioinformatic analysis showed that it interacts with PANK2, which can be confirmed in Co-IP experiment. Knockdown of zebrafish qrich2 gene affect cilia development in KV knob and pronepraph. It is concluded that qrich2 is essential for cilia development possibly through regulation of PANK2 mediated energy metabolism in the mitochondrial of sperm flagella. Supported by National Basic Research Program of China (2012CB944903).

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P251

Manipulating the germ stem cell 'niche' to elucidate the origins of testicular germ cell cancer

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Germ stem cells in the testis reside in a unique microenvironment, known as the 'niche'. In humans testicular germ cell cancer (TGCC) is thought to arise when the 'niche' fails to induce germ cell maturation. Gonocytes that fail to differentiate into spermatogonia during fetal life form pre-neoplastic carcinoma *in-situ* (CIS) cells. CIS cells then transform into an invasive tumour in adulthood. Despite the lack of an animal model of TGCC it may be possible to mimic the 'niche' by developing a testicular re-aggregation system. Such a system could be used to recapitulate 'niche' conditions which prevent gonocyte maturation. E17.5 Wistar rat testes were dissociated and grafted into castrate nude mice. After 2 weeks, grafts were retrieved and immunohistochemistry for markers of germ, Sertoli, Leydig, and peritubular myoid (PTM) cells was performed. Subsequent experiments used this approach to introduce GFP gonocytes from e15.5 rats into re-aggregated seminiferous cords of e19.5 rat testes. Morphological analysis of retrieved tissue showed seminiferous cord reformation with Sertoli, germ, and interstitial cells readily identifiable histologically. The cords were surrounded by a PTM cells which expressed SMA. AMH expression was not observed in SOX9+ cells suggesting Sertoli cell immaturity. Leydig cells were primarily in the interstitium although some intratubular 3βHSD positive cells were identified. E15.5 GFP gonocytes into e19.5 rat seminiferous cord mixing experiments are underway and will be presented. Testis dissociation and re-aggregation represents a useful model to investigate seminiferous cord formation and its disruption which may be useful for investigating the origins of TGCC.

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P252

Roles of testicular macrophage in Leydig cell proliferation in colony-stimulating factor treated mice

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Introduction
Leydig cells (LCs), the key producer of testosterone (T) in the testis, are affected by various factors such as hormone stimulation. There are various testicular cells that also affect LC function such as testicular interstitial macrophages (TIMs). TIMs are immune factor-producing cells, located adjacent to the LCs, sharing a close physiological and functional relationship. TIMs are associated with development and function of LCs. Ablation of TIMs have shown to impact testosterone production, proliferation and differentiation of LCs in adult rats, suggesting that TIMs are important for LC function. By injecting colony-stimulating factor (CSF; increasing the number of TIMs) at different LC developmental stages, we aimed to establish the role of TIM during LC development.
Materials and methods
Mice were divided into four groups (two CSF or PBS injected-groups were injected for 4 days at the LC proliferating stage (days 29–35)). Groups were culled either at the end of the injecting period (acute effect) or at adulthood (chronic effect). Samples were analysed by immunohistochemistry, stereology, and ELISA.
Results and discussions
Following CSF treatment we observed a significant increase in numbers of resident TIMs in the day 35 testis. There was a significant increase in circulating LH level, with no change in circulating testosterone. These preliminary data suggest that effect of increased TIMs during the LC developing stage by injecting CSF could be involved in modifying the hypothalamic–pituitary–gonadal axis at various points including the pituitary and directly in the testis. Further experiments are underway to elucidate these mechanisms.

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P253

Panel of MABs to sperm surface proteins as tool for monitoring of sperm–zona pellucida receptors localization and identification

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Introduction
Primary binding of the sperm to the zona pellucida (ZP), or its analogues, is one of the many steps necessary for successful fertilization in all sexually reproducing species. Sperm bind ZP by means of membrane receptors which recognize carbohydrate moieties on ZP glycoproteins according to a well-precised sequential process. Primary-binding receptors are localized throughout the acrosomal region of the sperm surface of which many have been disclosed in various mammals.
Materials and methods
Panel of MABs against proteins from the sperm surface was prepared. Antibodies were screened by imunoflourescence and western blotting for protein localizations and competence of antibodies respectively. Proteins localized on the sperm head and simultaneously detected by western blot were further studied in terms of imunolocalization in reproductive tissues and fluids, binding to ZP, imunoprecipitation and sequencing.
Results and discussion
Out of 17 prepared antibodies, eight antibodies were recognizing proteins localized on the sperm head and also detecting proteins of interest by western blotting. Further only three antibodies recognized proteins which also coincided in binding to ZP. These three antibodies were used for imunoprecipitation, and further protein sequencing of imunoprecipitates revealed that the antibodies distinguish acrosin precursor, RAB2A protein, and lactadherin P47. Acrosin and lactadherin P47 have been already detected on the sperm surface and their physiological functions in reproduction have been proposed. To our knowledge, this is the first time RAB2A has been found on the surface of sperm. This work was supported by GACR P503/12/1834, P502/14/05547S, UNCE204025/2012, RVO61388971, and by BIOCEV CZ.1.05/1.1.00/02.0109 from the ERDF.

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P254

Buffalo (*Bubalus bubalis*) spermatozoa based biosensor for the detection of Cereulide toxin from milk powder

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Introduction
Mammalian cell based biosensor consists of biological entity which can be whole mammalian cell, i.e. gametic or somatic cells that interacts with an analyte and produces a signal which can be measured fluorimetrically, colorimetrically, etc. Mammalian cell based biosensor not only helps in the detection but also detect physiological changes Spermatozoa based biosensor rapidly interpret the inherent interaction of spermatozoa with cereulide in a physiologically relevant manner. Use of ejaculated spermatozoa for studying the toxic effects of mitochondrial toxins provide several advantages over other *in vitro* systems due to their highly active and sensitive mitochondria. Cereulide, a dodecadepsipeptide produced by *B. cereus* which is an opportunistic pathogen belongs to hazardous group 2. Cereulide toxin is highly resistant to pH, temperature, radiations, etc. Milk powder is mainly contaminated with this toxin.
Material and methods
Different *B. cereus* cultures purchased from MTCC were screened for cereulide production. Certified milk powder was inoculated with viable counts of *B. cereus* (10^3–10^4) and cereulide was purified using HPLC using valinomycin as standard. Different concentrations of cereulide was prepared and examined by spermatozoa motility assay. EC_{50} value was calculated which was also validated with five different milk powders.
Results and discussion
This bioassay is non-laborious and can be executed with equipments present in most of the laboratories. Cereulide has potassium ionophoric action which inhibit transmembrane potential and mitochondrial activity and easily detected through spermatozoa. EC_{50} value calculated was 5 ng/ml, i.e. the lowest concentration of cereulide extracted from milk powder which inhibit the motility of 50% of buffalo spermatozoa.

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P255

Canine spermatogonial stem cells transfected with GFP reporter gene transplanted in recipient teste mice

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Introduction
Spermatogonial stem cells (SSCs) characterized by ability to self-renew and proliferated, differentiated, and transmitted genetic information. In canine the first attempt (1999) of xenotransplantation into mice did not successfully produce spermatozoa. However, there are evidence that xenogeneic transplant of testis cells can engraft in host testis, and generate donor derived sperm; it suggests the SSCs transplantation may offer a similarity to transgenesis in the canine model. Our aims were to characterize canine SSCs *in vitro* and development a xenotransplant assay for SSCs transfected with GFP reporter gene into mice.
Materials and methods
Testicular germ cells were isolated and cultured from prepubertal canines and methods for enrichment culture systems were established. The SSCs were transduced with a GFP reporter gene by a lentiviral vector to target the SSCs in recipient testes. Then GFP-transduced SSCs was transplanted into eight testes of C57BL/6 mice treated with busulfan. At 25 and 90 days after xenotransplantation, four recipient animals were euthanized and number of GFP-expressing in testis was analyzed.
Results and discussion
SSCs canine maintained the expression of CD49f and C-kit for 10 days in culture using our enrichment culture system. For the xenotransplant, a total of 10^5 cells were injected and 20–43% of these cells engraft the testes. Moreover, the clump-forming canine germ cells GFP+ cells colonized membrane basal of seminiferous tubules of mice. This research was conducted in accordance with the Committee of Ethics of the Faculty of Animal Sciences and Food Engineering FZEA-(USP).

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P256

Mass-specific metabolic rate influences sperm performance through energy production in mammals

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Introduction
Mass-specific metabolic rate, the rate at which an organism consumes energy per gram of body weight, is negatively associated with body size in metazoans. Since the basal metabolic rate of a whole organism represents the sum of its cellular metabolic rates, small species have higher cellular metabolic rates and are capable of processing resources at a faster rate than large species. Recent studies have shown that mass-specific metabolic rate positively influences the size of sperm among mammals. Most of the cellular metabolic rate is represented by ATP production, which is essential for sperm flagellar beating, hence motility. We hypothesized that mass-specific metabolic rate could influence sperm energetic metabolism at the cellular level if sperm cells 'inherit' the metabolic rate of organisms that produce them and, in turn, influence sperm function.
Materials and methods
Data on body mass, testes mass, total sperm length, sperm swimming velocity, mteabolic rate, and sperm ATP content were obtained from the literature for 41 mammalian species and analyzed using phylogenetic generalized least-squares method.
Results and discussion
We found that the mass-specific metabolic rate positively influences sperm swimming velocity independently of its effect on sperm size. Moreover, our analyses show that species with a higher mass-specific metabolic rate have a higher ATP content per sperm and a higher length-adjusted ATP concentration, which are possitively associated with sperm velocity. In conclusion, independently of its effect on the production of larger sperm, the mass-specific metabolic rate is able to influence sperm velocity by increasing sperm ATP content in mammals.

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P257

The role of estrogen and the effect of age on porcine testicular extracellular matrix

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Introduction
Extracellular matrix (ECM) has both structural and regulatory roles in tissues and influences the activity of local growth factors. In testis, ECM plays an integral role in the differentiation of germ cells and the function of support cells. It is becoming clear that sex steroids influence ECM and growth factors. In swine, suppression of aromatase activity resulted in an estrogen receptor-mediated increase in Sertoli cells (Berger *et al.* 2013), with significantly reduced *WISP2* mRNA expression up to 5 weeks of age.
Materials and methods
To determine the action of estrogens on ECM in porcine testes, male pigs were treated weekly for 1–6 weeks with vehicle or an enzymatic inhibitor of estrogen synthesis (letrozole, 0.1 mg/kg body weight) and killed at 6, 11, 20, or 40 weeks of age and testes collected for histology. Image analysis of silver-stained sections was performed to determine the proportion of collagen, and other ECM components were evaluated by immunohistochemistry.
Results
Although there were no differences attributable to treatment, there was a significant reduction in the proportion of collagen with age: from $24.8 \pm 2.8\%$ at 6 weeks to $16.1 \pm 4.1\%$ at weeks of age ($P < 0.001$). Collagen types I and III, and versican, fibronectin and fibrillin 2 all decreased in expression with age. Collagen types I and III were localized to the peritubular region, fibronectin was localized to the interstitial space and versican and fibrillin 2 localised to the perivascular connective tissue.
Conclusion
Significant changes in ECM occur during testis development with age, and may be independent of estradiol synthesis during the developmental window examined.

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P258

Testicular chemical pollutants alter sperm vitality, motility, and morphology: comparative studies in the human and dog

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Introduction
Declining human and canine male fertility has been associated with exposure to environmental chemicals (ECs). We investigated the effects of ECs on canine and human sperm vitality, motility, and morphology.
Methods
Mean canine testicular levels (MTL) of PCB153 and DEHP were 0.9 ± 0.14 μg/kg ($n = 8$) and 0.31 ± 0.03 μg/g ($n = 5$) respectively. Spermatozoa from dogs ($n = 7$) and human donations ($n = 9$, four donors: approved by Nottingham Medical School Ethics Committee) were cultured with 0, 2×, 10×, 100× MTL of PCB153, DEHP, or mix (PCB153 + DEHP). Sperm vitality and morphology were measured (nigrosin eosin/VitalStain: dog/human) at 0 and 240 min. Motility was measured using computer-assisted sperm analysis.
Results and discussion
Dog: PCB153 (2× MTL) and DEHP (independent of concentration) reduced sperm vitality ($P = 0.049$ and $P = 0.002$ respectively). PCB153 increased percent linearity ($P = 0.036$) and straight line velocity ($P = 0.05$) whereas DEHP decreased these parameters ($P = 0.031$ and $P = 0.0037$ respectively). PCB153 increased percent motility ($P = 0.031$) and DEHP decreased percent straightness ($P = 0.032$). Morphology: no effects. *Human:* Effects on motility inconclusive: number of Cat B sperm increased with DEHP ($P < 0.001$), not Cat A. The proportion of morphologically normal sperm was decreased ($P = 0.007$) and this was confirmed by an increased proportion with abnormal morphology ($P = 0.007$). Treatment effects were primarily due to PCB153. In conclusion, exposure of ejaculated sperm to ECs effects sperm vitality and motility in the dog and sperm morphology in the human. Although the mechanisms underlying these effects and species differences are unknown, they may be associated with declining male fertility.

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P259

SSP411 a sperm factor involved in oocyte cleavage

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Introduction
Encoding an 88 kDa protein with a thioredoxin-like domain at the N-terminal region, *ssp411* is predominantly expressed at several specific stages from round spermatids to spermatozoa in the rat testis at both mRNA and protein levels. SSP411 is conserved in many species, and can be localized in the testis and mature sperm of human, mouse, and rat.
Materials and methods
A line of *ssp411* knockout mice ($ssp411^{-/-}$) was introduced. The fecundity of $ssp411^{-/-}$ mice was evaluated by mating test, testis was histologically analyzed, motility, and morphology of sperm was examined, and fertilization capability of *ssp411*-defect sperms was assessed by ICSI.
Results and discussion
Only $ssp411^{-/-}$ males are sterile. Compared with WT, testicular weights of $ssp411^{-/-}$ males are significantly lower, and many vacuoles exist in seminiferous tubules. The number of caudal sperm of mature $ssp411^{-/-}$ males is significantly decreased, none of sperms exhibited rapid progressive linear motility, and the morphology of most sperm is abnormal. *ssp411*-defect sperms could fertilize mouse eggs via ICSI normally, but subsequent cleavage of zygote was delayed by 10–20 h, which does not affect development of morula and blastocyst. Combined with reports that mRNA of *ssp411* exist in zygotes but absent in the oocyte, our data suggests that *ssp411* not only involves in male reproduction but also acts as a sperm factor that may activate oocyte cleavage. We are carrying out studies to uncover the mechanism of male sterility caused by *ssp411* defect and its role in activation of first cleavage.

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P260

In vitro effects of hydrogen peroxide on ALF expression in male mouse germ cells

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Introduction
ALF is a germ cell-specific counterpart of the large (α/β) subunit of general transcription factor TFIIA and has a vital role in the meiotic and post-meiotic development of male germ cells. We have found *in vitro* a variety of effects of hydrogen peroxide (H_2O_2) on different types of germ cells, including the induction of DNA strand breaks and apoptosis. We therefore examined its effect on the expression of genes such as ALF in order to investigate the mechanisms responsible.
Methods
Following STAPUT separation, the fractions containing only spermatocytes or spermatids were cultured for 16 h before the addition of H_2O_2 at final concentrations of 0, 10, 50, or 100 μM. After 2 h the cells were harvested and the total protein or RNA was extracted. Western blotting and reverse-transcripted quantitative PCR were performed to determine the effects on ALF expression levels.
Results
QRT-PCR and western blotting showed that both ALF mRNA and protein levels were reduced at the higher levels (50 and 100 μM) of H_2O_2 exposure but not at a lower dose (10 μM). The effect was relatively more pronounced in spermatocytes than spermatids.
Discussion
H_2O_2 exposure results in the specific down-regulation of a transcription factor in developing male germ cells. Whether this is a cause or consequence of the known effects of H_2O_2 on such cells remains to be shown. The relatively greater effect on spermatocytes than spermatids correlates with their greater sensitivity to the induction of DNA damage and apoptosis we have found recently.

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P261

Time-lapse imaging as a tool to evaluate contractile cell function and sperm transport

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Introduction
Testicular peritubular cells and the smooth muscle cell layer surrounding the epididymal duct are responsible for the transport of spermatozoa and thus contribute to maintaining male fertility. The intracellular second messenger cGMP mediates smooth muscle cell relaxation and components of cGMP signaling were found in smooth muscle cells of the testis and epididymis. A time-lapse video approach was used to study contractility of seminiferous tubules and the epididymal duct in a near-physiological setting and effects of cGMP-related signaling.
Material and methods
Seminiferous tubules from rat and man as well as rat epidiymal duct segments from caput, corpus and cauda were isolated, embedded in collagen for immobilization and exposed to cGMP-elevating agents. Time-lapse imaging allowed to visualize wall contractions and sperm transport and to assess cGMP-related effects. Fourier analysis was used to characterize irregular contractile activity of rat seminiferous tubules.
Results and discussion
In rat epididymis, regular phasic contractions inducing movements of intraluminal contents were observed. Contractile frequency was reduced when cGMP was elevated. In contrast, the wall of seminiferous tubules showed an irregular and undulating contraction pattern. Fourier analysis allowed us to identify a characteristic spectrum of contraction frequencies with a shift towards lower frequencies upon cGMP elevation.
In human seminiferous tubules, time-lapse imaging could reveal peristaltic contractions and sperm transport.
Time-lapse imaging is a promising tool to evaluate contractile function and sperm transport in seminiferous tubules and epididymal duct segments and allowed us to study effects of various signaling systems.

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P262

Abstract withdrawn.

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P263

Comparison between egg yolk-based and coconut water-based extenders to freeze buffalo semen

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The aim of this experiment was compare two egg yolk-based (Tris and botu-Bov) and a coconut water-based (ACP-111) extenders to freeze buffalo semen. Four ejaculates of four buffalo bull were collected. Semen was divided in three groups: EY1 group, the semen was diluted with Tris-Yolk extender; EY2 group, the semen was diluted with Botu-Bov ™; CW group, the semen was diluted with ACP-111™. The samples were packed in 0.5 ml straws containing 30×10^6 of total sperm and were freezing in isothermal box. The sperm kinetics was evaluated by CASA method (HTM-IVOS, IMV, USA) and the plasma membrane integrity and acrosomal (probes propidium iodide and FITC-PSA) were analyzed by flow cytometer (BD LSR Fortessa, BD, Mountain, USA). The evaluations were performed post-thaw. Comparison of sperm parameters was performed by ANOVA. The values were expressed as mean and standard error. CW was smaller ($P<0.05$) than EY1 and EY2 in total motility (%, EY1 = 52.64 ± 3.15^a; EY2 = 58.59 ± 3.56^a; CW = 36.94 ± 4.05^b), progressive motility (%, EY1 = 40.39 ± 2.58^a; EY2 = 47.56 ± 2.82^a; CW = 29.59 ± 3.34^b), and percentage of rapid sperm (%, EY1 = 48.29 ± 3.17^a; EY2 = 55.47 ± 3.49^a; CW = 34.06 ± 3.94^b). No difference ($P>0.05$) was observed in IMP (%, EY1 = 25.90 ± 2.14^a; EY2 = 29.72 ± 2.39^a; CW = 24.37 ± 2.34^a), The results of this study allow us to conclude that egg yolk-based extenders showed to be more efficient than coconut water-based extender to freeze buffalo semen.

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P264

Placental phenotype of Turner mouse model: differences between XmO and XpO

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Background
Turner syndrome in human arises from the loss of genetic material from a sex chromosome, resulting in the 45,XO genotype, with the remaining X chromosome being maternally-inherited (Xm) or paternally-inherited (Xp). As the gene dosage and expressions of Xp and Xm chromosomes are epigenetically different, this study aims to investigate the different influences of Xm and Xp on placental phenotype, using a mouse model.
Methods
14 placentas from pre-term MF1 mice (3XX, 3XY, 5XmO, and 3XpO) at 18.5 days post-coitum were isolated, formalin-fixed, paraffin-embedded, and sectioned. To examine the placental morphological differences, sections were stained histologically (H&E, PAS, and Azan) and immunohistochemically (CDX2, CK19, fibronectin, laminin, and pecam-1). Student's *t*-test was used to analyse various comparison parameters, including the size of the placenta and its constituent layers; as well as the estimates of cell density, and CDX2-positive and glycogen cell numbers in the junctional zone (Jz).
Results
WT (XX and XY) placentas are quite similar to each other. XpO placentas are significantly different from the other three genotypes, especially in the Jz, where there is pronounced lateral Jz hyperplasia ($P=0.038$), with increased numbers of CDX2-positive ($P=0.0092$) and glycogen cells ($P=0.017$). XmO placentas exhibit large morphological variations, with mixed characteristics between WT and XpO placentas.
Discussion
Our findings show that XmO placentas are less phenotypically abnormal, suggesting that they may function better than XpO placentas. This may help explain the findings from previous literature documenting that XmO mice foetuses are more viable than XpO foetuses, and that most of the human 45,XO foetuses surviving to birth have Xm.

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P265

The tight junction molecules expression and localization in mouse placenta

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Introduction
Tight junctions (TJs) are composed of a branching network of sealing strands. TJs regulate paracellular conductance and ionic selectivity. The established paracellular transport barriers that control transportation of molecules within intercellular space.
Methods
We examined expression of mouse placental claudin family. Pregnant C57/BL6 mice were used in this study and TJ proteins including Claudin-1 to Claudin-24 expressions. The pregnant mice were divided into three groups depending on pregnant day (on days 12.5, 16.5, and 20.5 of gestation).
Results and discussion
In the transcription levels, Claudin-1, Claudin-2, Claudin-4, and Claudin-5 expression levels were relatively high expression when compared to other claudin family in all the periods of pregnant. The Claudin-4 expression, which reduces permeability of ions, was increased over a period of time. However, Claudin-5 expression that is the responsive protein for a decrease in paracellular conductance, were decreased. In previous study using the siRNA, Claudin-1 and Claudin-4 has been known as a responsive gene for a decrease in paracellular conductance. On the other hand, Claudin-2 and Claudin-5 has been known as

increase paracellular conductance. In addition, immunohistochemistry was performed to identify their localization for inferring permeability in placenta. This study will provide the claudin expressions and their localization in the mouse placenta, which may contribute to assuming the roles of these tight junction genes regarding the maternal–fetal ion transportation in the placenta.

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P266

The acyl-CoA dehydrogenase, very long chain (ACADVL) expression under hypoxic condition in human placental cell line (BeWo)

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Introduction
Pre-eclampsia (PE) is a medical condition characterized by high blood pressure and significant amounts of protein in the urine of a pregnant woman. Hypoxia can result from a failure at any stage in the delivery of oxygen to cells. In peripheral tissues, oxygen again diffuses down a pressure gradient into cells and their mitochondria, where it is used to produce energy in conjunction with the breakdown of glucose, fats, and some amino acids.
Materials and methods
We induced hypoxic stress in BeWo cell cultured under 1% O_2, 5% CO_2, and balanced with N_2. The expression of β-oxidation related genes (*ACADVL*, *EHHADH*, *HADH*, and *ACAA*) were observed under hypoxic condition in BeWo cells by using real-time PCR.
Results and discussion
The expression of genes known as biomarkers for hypoxia, HIF1α, was increased in BeWo cells induced preeclampsia. The elevated level of HIF1α is indicative that our experimental conditions closely mimicked ones that are associated with preeclampsia. The β-oxidation related genes, *ACADVL*, *EHHADH*, and *HADH* expressions were significantly increased by hypoxic stress in BeWo compare with normoxic control. Further study is being done on the release of HIF1α and its affect on the metabolism of β-oxidation in BeWo cells. Taken together, these results indicate that changes of β-oxidation related genes observed under hypoxic BeWo cells are similar to ones associated with preeclampsia, and the expression of β-oxidation related genes were up-regulated by hypoxic stress. It may be involved in pathogenesis of preeclampsia during gestation.

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P267

The regulation of calcium transport channels, TRPV5, TRPV6, PMCA1, NCKX3, and CaBP-9k in ro410960-treated mice of pregnancy

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Introduction
Preeclampsia is a pregnancy-specific disease characterized by concurrent development of hypertension, proteinuria, and oxidative stress in the placenta. Preeclampsia-like genetic models were also developed by modification of preeclampsia-related genes, such as catechol-*O*-methyltranferase (COMT).
Materials and methods
In this study, we induced COMT inhibition in mice during pregnancy to reproduce physiological conditions associated with preeclampsia. The expression of gene known as hypoxia biomarker, HIF1α, was highly induced in placenta of this model. The over-expression of HIF1α demonstrates that our experimental conditions closely were similar with preeclampsia. We measured the expression of several calcium transporters (TRPV5, TRPV6, PMCA1, and CaBP-9k) in the placenta, duodenum, and kidney after COMT inhibition on gestation day 17.5 (GD17.5). In addition, we evaluated the calcium transporters in the kidney, duodenum of non-pregnant female mice.
Results and discussion
Placental TRPV5, TRPV6, and PMCA1 expressions were down-regulated by COMT inhibitor (ro41-0960). In addition, the reduced PMCA1 expression in the placenta was reversed by calcium supplementation. Duodenal expressions of TRPV5, TRPV6, and PMCA1 were decreased in the COMT-inhibited mice, and slightly recovered after calcium supplementation. Renal expression of TRPV5, TRPV6, and PMCA1 was also decreased by COMT inhibition, while it was reversed by calcium supplementation to the level of control. Duodenal- and renal calcium transporting genes, *TRPV5*, *TPRV6*, *PMCA1*, and *CaBP-9k*, were down-regulated by COMT treatment in female mice. Taken together, these results indicate that physiological changes observed in COMT inhibition were showed similar symptom to preeclampsia, which may be related with disturbance of calcium metabolism during pregnancy.

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P268

Expression and regulation of N-myc downregulated gene 2 in mouse uterus during the peri-implantation period

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Introduction
A sucessful implantation depends on the exquisite coordination between embryo and maternal uterus. N-myc downregulated gene 2 (NDRG2) belongs to the NDRG gene family, a new class of Myc-repressed genes, and possesses tumor suppression function. Given the similarities between embryo implantation and the growth of cancer cell, the present study was undertaken to examine the expression and regulation of NDRG2 during peri-implantation period.
Materials and methods
The expression pattern of NDRG protein and mRNA during peri-implantation period in mice were determined by immmunohistochemical and RT-PCR analyses. The delayed implantation and artificial decidualization mouse models, as well as the treatment of E_2 or/and P4, were performed to observe the regulation of uterine NDRG2 expression.
Results and discussion
No detectable NDRG2 protein signal was found in uterus on days 1–3 of pregnancy, and a faint possitive signal could be observed in stroma on day 4 of pregnancy. NDRG2 protein was detected in the secondary decidual zone on days 5 and 8 of pregnancy, and the expression level in decidual zone was markedly increased. The expression of *NDRG2* mRNA was detected from day 1–8 of pregnancy, and its expression level at the implantation site was significantly higher compared to the inter-implantation site. Under *in vivo* artificial decidualization, NDRG2 protein expression was significantly increased compared with control. Compared with the progesterone-primed delayed implantation uterus, an up-regulated expression of NDRG2 protein was detected in estrogen-activated implantation uterus. In ovariectomized mouse uterus, estrogen and progesterone could induce the expression of NDRG2 protein. These results suggest that NDRG2 might play an important role during mouse embryo implantation, and estrogen and progesterone could regulate the expression of NDRG2 gene.

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P269

The influence of aldrin and dieldrin on the function of bovine uterus and ovary *in vitro*

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Introduction
Aldrin and its derivative dieldrin belong to the group of chloroorganic pesticides, which due to their lipophilic properties and resistance to biodegradation are accumulated in tissues of animals and humans, and they can mimic the action of endogenous hormones.
Materials and methods
Myometrial strips and endometrial, myometrial, granulosa, and luteal cells obtained from cows on days 8–12 of the estrous cycle, were incubated with aldrin or dieldrin (0.1, 1, or 10 ng/ml) for 24 or 72 h. Next viability of cells, contractions of strips, synthesis and secretion of prostaglandins (PGF2α and PGE2) from uterine cells and oxytocin (OT), progesterone, estradiol, and testosterone secretion from ovarian cells, were determined.
Results and discussion
None of used xenobiotics affected ($P > 0.05$) the viability of studied cells. Therefore the observed, in further experiments, dysfunction of uterine and ovarian cells, were not evoked by cytotoxic effect of aldrin and dieldrin. Both pesticides decreased ($P < 0.05$) the basal and OT-stimulated myometrial contractions. In spite of these, they did not affect ($P > 0.05$) the synthesis and

secretion of prostaglandins from both uterine cells ($P>0.05$) but xenobiotics abolished the stimulatory effect of OT ($P<0.05$) on prostaglandin secretion from endometrium. While used pesticides stimulated ($P<0.05$) the secretion of OT and steroids from ovarian cells. The data show that aldrin and dieldrin, impaired the myometrial contractions in cows. It can be a result of their effect on secretory function of uterus and ovaries. This study was supported by the National Science Centre (project no. N311 082140) and the Polish Academy of Sciences.

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P270

PGRMC1 protein expression in the canine endometrium

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Introduction
The sex steroid hormone progesterone (P) is the most important hormone in the canine estrous cycle for initiation and preservation of pregnancy. The responsiveness of the endometrial cells to P depends on progesterone receptors (PGRs). In recent studies alternatives to the classical progesterone receptor were identified as membrane-associated progesterone receptors (MAPGRs). Progesterone receptor membrane component-1 (PGRMC-1) is a representative of the MAPGRs which was identified in human, monkey, bovine and murine endometrium so far. The distinct function of PGRMC-1 is not yet clarified but it is supposed that PGRMC-1 is important in cell-cycle regulation and stromal cell decidualization. Furthermore, in the murine endometrium PGRMC-1 deficiency leads to development of cystic endometrial hyperplasia (CEH). In the canine endometrium CEH is a common pathological alteration leading to infertility and inflammatory processes which predispose the endometrium to a secondary, potential life-threatening bacterial infection (pyometra).
Material and methods
In the present study PGRMC-1 expression in the healthy ($n=20$), CEH-affected ($n=8$) and pyometra-affected ($n=8$) canine endometrium was demonstrated by means of immunohistochemistry. Additionally PGR-B expression and proliferative activity were determined to demonstrate distinct expression patterns of PGRMC-1 from PR-B and the involvement of PGRMC-1 in endometrial cell-cycle regulation.
Results and discussion
Five of the CEH-affected endometria lacked of PGRMC-1 whereas in the healthy uteri PGRMC-1 protein was identified in cycle-dependent expression patterns in endothelial, epithelial, stromal and myometrial cells. This study is the first to determine PGRMC-1 in the canine endometrium and suggests a role of PGRMC-1 in the development of canine CEH.

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P271

Do maternal obesity and mood disorders correlate with changes in placental glucocorticoid transporter gene expression?

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Introduction
Both obesity and mood disorders associate with altered glucocorticoid (GC) secretion and action. During pregnancy placenta regulates foetal exposure to maternal GC through enzymatic clearance (HSD-2) and retrograde transfer (ABC transporters). We and others have shown that maternal obesity and/or mood disorders alter placental HSD-2 mRNA level. We therefore hypothesised that placental ABC transporter gene expression is also altered, and investigated this using samples from obese and lean women with mood assessments in pregnancy.
Materials and methods
We administered General Health Questionnaire (GHQ) twice during pregnancy to evaluate mood in 135 lean and 222 severely obese (SO) women (BMI $22.77\pm$S.D. 1.65 vs 44.20 ± 4.10 kg/m^2, $P\leq0.0001$). Placental ABC transporters (ABCB1, ABCC1, and ABCG2) mRNA levels were measured by QPCR against housekeeping genes (YWHAZ, TBP) in a subset of placental samples (lean $n=19$, SO $n=24$).
Results and discussion
SO mothers had higher GHQ scores (indicating more depressive symptoms) than lean (mean difference (S.D.) $+1.613$ (0.302), $P\leq0.0001$). ABC transporters mRNA levels were similar in SO and lean. ABCC1 gene expression, but not ABCB1 or ABCG2, correlated negatively with GHQ scores in early ($\rho=-0.442$, $P=0.016$) and late pregnancy ($\rho=-0.369$, $P=0.038$). Therefore placental GC transporters do not seem to regulate foetal GC exposure in obese pregnancies. However as ABCC1 pumps GC to foetal circulation whilst ABCB1 and ABCG2 pumps GC back to maternal circulation, these findings suggest a placental protective mechanism from maternal GC in women with depressive symptoms during pregnancy.

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P273

Estradiol promotes differentiation of uterine natural killer cells during establishment of pregnancy

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Background
Uterine natural killer (uNK) cells are a phenotypically distinct population of tissue-resident immune cells that are reported to regulate vascular remodelling within the endometrium. The mechanisms that control accumulation of uNK cells in the endometrium remain unresolved although there is evidence to suggest that homing and differentiation of peripheral blood NK (pbNK) cells may contribute to the rapid increase in cell numbers during the secretory phase of the menstrual cycle. NK cell phenotype is characterised by expression of the surface proteins CD56 and CD16; the majority of pbNK cells are $CD56^+CD16^+$ whereas uNK cells are $CD56^+CD16^-$.
We have previously reported that during the secretory phase, decidualisation is associated with increased biosynthesis of estradiol (E_2) resulting in an estrogen-dominated microenvironment. Given that uNK cells express estrogen receptor (ER)β we hypothesised that E_2 might promote differentiation of pbNK cells recruited to the uterus during preparation for pregnancy.
Methods
PbNK cells were isolated from healthy pre-menopausal female donors by Percoll gradient separation and magnetic sorting for $CD56^+$ cells. Purified pbNK cells were treated with E_2 and the impact on expression of NK cell markers was assessed using flow cytometery.
Results and discussion
Analysis of pbNK cells revealed that E_2 altered expression of surface proteins consistent with differentiation towards a uNK cell phenotype. Specifically, the number of CD16 positive NK cells was decreased and the proportion of $CD56^+CD16^-$ cells was increased by treatment with E_2. These data offer novel insight into the regulation of uNK cell differentiation during establishment of pregnancy.

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P274

A role for androgens in the regulation of endometrial breakdown and repair in a mouse model of menstruation

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Background
The human endometrium is a dynamic, multi-cellular sex steroid-dependent tissue subject to cyclical episodes of breakdown (menses), repair and regeneration. The mechanisms responsible for initiation of menses are well characterised; the molecular and cellular changes responsible for rapid repair are poorly understood. Re-epithelialisation of the tissue is thought to involve contributions from both the stromal and epithelial compartments. A role for androgens in endometrial repair has not been elucidated.

Methods
Artificial 'menstrual' cycles were induced in mice: ovariectomised mice were treated with oestradiol (E_2) and progesterone (P) and decidualisation was induced on day 8. Endometrial breakdown was initiated by removing the P pellet, 90 h after oil injection. Mice were given a single injection of DHT at the time of P withdrawal. A PCR array was utilised to investigate the effect of DHT on genes known to regulate mesenchymal–epithelial transition (MET), chromatin immunoprecipitation (chIP) allowed investigation of direct regulation by the androgen receptor.
Results and discussion
Evidence for a delayed onset of 'menses' was observed in DHT-treated animals. Treatment with DHT was associated with reduced concentrations of mRNAs encoded by genes involved in cellular remodelling, cell adhesion and cell matrix. Preliminary chIP analysis has identified a candidate set of MET-related genes that contain an androgen response element and therefore may be directly regulated by DHT. These studies implicate androgens in regulating genes involved in endometrial remodelling and repair which suggests that disturbances to peripheral androgen levels may contribute to the aetiology of the endometrial disorder, heavy menstrual bleeding.

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P275

Apoptosis and neutrophil infiltration in the human endometrium at menstruation, recapitulated in mouse model of induced menstruation

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Introduction
Menstruation is an inflammatory process characterised by tissue breakdown, bleeding and recruitment of leukocytes. Resolution of this inflammation at menses is critical both to limiting tissue damage and to efficient repair. Apoptosis and clearance of apoptotic cells are necessary for this process, and are thought to involve neutrophils.
Our aims were to (i) delineate neutrophil numbers and identify apoptosis in peri-menstrual endometrium and (ii) examine a mouse model of induced menstruation to determine if similar processes are present.
Materials and methods
Late secretory to early proliferative phase endometrial biopsies were collected with ethical approval from consenting women with regular cycles and no overt pathology. Cycle stage was determined by serum hormones, day of cycle and histology.
C57Bl/6J mice underwent induced menstruation using sequential oestrogen and progesterone exposure and uterine oil injections to effect decidualisation. Progesterone withdrawal induced bleeding.
Tissue sections were immunohistochemically-stained for cleaved caspase-3 (an apoptosis marker), and elastase, MPO and Gr-1 (human and mouse neutrophil markers). Caspase-3 activation was assessed by semi-quantitative histoscoring, while neutrophil abundance was determined by stereology microscopy and ImageJ.
Results and discussion
Immunostaining revealed increasing epithelial and stromal caspase-3 activation prior to menstrual bleeding, followed by a precipitous influx of neutrophils at menses. Apoptosis preceded leukocyte recruitment in both human and mouse endometrium, and is likely, as in other systems, to be crucial for resolution of inflammation.
We have demonstrated that this induced mouse menstruation model shows changes analogous to the human endometrium and is a useful model for future mechanistic studies.

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P276

Glucocorticoids trigger macrophage migration inhibitory factor (MIF) secretion by decidualized human endometrial stromal cells *in vitro*: the modulatory effect of Bisphenol A

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Introduction
Pro-inflammatory cytokines are important mediators of the early phases of gestation. Among all these cytokines, the macrophage migration inhibitory factor (MIF) plays significant roles in pregnancy establishment. In many cell types, low concentrations of glucocorticoids (GCs) are known to enhance MIF secretion, but the effect of GCs on endometrial cells has not been elucidated. Bisphenol A (BPA) is an environmental chemical that can alter reproductive physiology by mimicking the action of the endogenous hormones, including GCs. The aim of study was to investigate the effect of GCs on endometrial cells, and then to discover whether BPA could act on endometrial cells through/by modulating GCs' pathway.
Materials and methods
Primary cultures of human endometrial stromal cells ($n=8$) were used as an *in vitro* model of the decidua at the beginning of gestation. Cells were exposed to BPA (1 nM) alone or in combination with cortisone and hydrocortisone (1 μM–0.1 nM). MIF and hydrocortisone secretion were monitored by ELISA assay. The effects of BPA on the GCs' pathway were monitored by real-time PCR and SDS–PAGE immunoblotting on selected markers (GCs receptor; 11β-HSD enzymes).
Results and discussion
Hydrocortisone stimulates MIF secretion by endometrial stromal cells. BPA triggered an exaggerated pro-inflammatory response in decidualized endometrial cells, acting in a glucocorticoid-like fashion. Surprisingly, BPA suppressed the GC-driven release of MIF when administered together with these hormones. These results raise concern on the ability of BPA to modulate the inflammatory response in the maternal decidua at the time of pregnancy establishment and development.
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P278

Bovine placental lactogen is cleaved by matrix metalloproteinases and resulted 25k N-terminal fragments inhibit the proliferation of vascular endothelial cells

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Bovine placental lactogen (bPL) is a classical member of a prolactin (PRL) gene family expressed in the placenta. bPL exerts lactogenic activity similar to pituitary PRL, however, detailed information about the role of bPL during bovine gestation is still limited. The 16 k N-terminal fragments of PRL, generated by enzymatic cleavage, have angiostatic activities in human and rodents. In the present study, we examined the feasibility of N-terminal fragments of bPL following cleavage by placental enzymes. bPL (32 kDa) was cleaved by matrix metalloproteinase (MMP)-8, -9 and -13 resulting in the 25 kDa N-terminal fragments. Gelatinase and collagenase activities in placental explant culture media were confirmed by gelatin and casein zymographies, respectively. The expression of Mmp13 in the trophoblast was detected by *in situ* hybridization. Cleaved form of bPL was generated by recombinant expression and purified truncated protein was used for following studies. The cleaved N-terminal fragments of bPL did not stimulate the proliferation of Nb2 cells indicating the loss of lactogenic activity. Angiogenic or angiostatic activity of cleaved bPL was examined by using cultured bovine brain microvascular endothelial cells (BBMC). Cleaved bPL inhibited the proliferation of BBMC *in vitro* while intact bPL had no effect for BBMC growth. Results in the present study show that bPL is cleaved by placental MMPs and resulted N-terminal fragments inhibit the proliferation of BBMC in vitro. Angiostatic activity of cleaved bPL could be shared in specific mechanisms in the regulation of placental angiogenesis. (Supported by JSPS Grants-in-Aid for Scientific Research 2013-13351115.)

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P279

An estradiol metabolite 2-methoxyestradiol exhibits estrogen-like effect *in vitro* and *in vivo*

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Introduction
2-Methoxyestradiol (2-ME) is an endogenous metabolite of 17-estradiol (E_2) that interacts with estrogen receptors (ERs) and microtubules, and it has a low affinity for ERs. It has been identified as a potential novel antitumor agent combining anti-proliferative activity on a wide range of tumor cell types with anti-angiogenic actions. Also, 2-ME attracted considerable interest as a potential anti-cancer therapeutic.
Materials and methods
To performed *in vitro* experiment to evaluate estrogenic effect of 2-ME using GH3 cells, we measured mRNA expression of CaBP-9k and progesterone receptor (PR), the indicators of estrogenic effects using real-time PCR. *In vivo* experiment also investigated how 2-ME has an estrogenic effect on immature mice uterus by measured mRNA expression of lactoferrin and ER, the indicators of estrogenic effects using real-time PCR.
Results and discussion
In vitro CaBP-9k mRNA expression was increased in 2-ME (10^{-7} M) treatment group in parallel with response to E2 (10^{-9} M). *In vivo* lactoferrin mRNA expression was also increased in 2-ME (40 mg/kg BW) group to similar response with E_2 (40 μg/kg BW). As a blocker for ER activity, ICI 182 780 reversed the both E2-mediated and 2-ME-mediated increase of CaBP-9k and lactoferrin mRNA. We also investigated how 2-ME is associated with ERs and PR. *In vitro,* 2-ME did not significantly induce ER transcripts but PR transcripts. *In vivo,* 2-ME did not significantly induce PR transcripts unlike ER. Based on the study, we demonstrated that 2-ME has an estrogenic effect on *in vitro* and *in vivo* condition.

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P280

Prominin-1 and -2 in uterine epithelial cells during early pregnancy

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Introduction
In preparation for blastocyst implantation, the microvilli of uterine epithelial cells (UECs) are lost and the apical plasma membrane flattens. Prominin-1 is a membrane glycoprotein restricted to the apical domain of epithelial cells, while the structurally related prominin-2 is distributed throughout the plasma membrane of epithelial cells in a non-polarised way. This study aimed to examine the expression of prominin-1 and -2 in UECs to examine their potential role in blastocyst attachment and implantation.
Materials and methods
Female virgin wistar rats were mated and tissue was collected at the time of fertilisation and the time of receptivity. Western blotting was performed on isolated UECs and immunofluorescence was performed on cryosectioned tissue. Ishikawa cells, a human uterine epithelial cell line, were also examined by confocal microscopy and western blotting. Deglycosylation analysis was also performed *in vitro* and *in vivo*.
Results and discussion
Prominin-1 and -2 are glycosylated in UECs and Ishikawa cells. At both the time of implantation and the time of receptivity, prominin-1 was found to be concentrated in the apical portion of UECs, while prominin-2 was restricted to the basolateral plasma membrane. Prominin-1 expression increased at the time of implantation, whereas prominin-2 expression decreased. These results suggest that prominin-1 may be actively involved in uterine receptivity for blastocyst attachment, while prominin-2 may be lost to allow disassociation of the UECs and blastocyst penetration.

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P281

Trophoblast-decidual crosstalk optimises trophoblast invasion

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Introduction
During the establishment of pregnancy, extravillous trophoblast (EVT) must invade through the decidua to form a functional placenta. Impaired decidualization is associated with placental insufficiency, including preeclampsia. The mechanisms by which decidual cells interact with EVT remain largely unknown. We hypothesised that decidual–EVT interactions are critical for appropriate invasion.
Methods
Primary human endometrial stromal cells (HESC) were decidualized with estradiol 17β-methoxyprogesterone acetate cAMP or EVT conditioned media (CM) ($n=3$). Prolactin (PRL; decidual marker) was measured by ELISA. CM was collected from non-decidualized (nd) and decidualized (d) HESC. Cytotrophoblast isolated from 1st trimester placenta were cultured on Matrigel to differentiate to EVT and subjected to outgrowth and invasion assays ($n=6$). Proteins in EVT CM were identified by mass spectrometry.
Results and discussion
Decidualized CM significantly enhanced outgrowth (compared to nd CM; nd:100; d:161.6+28.4%; $P<0.05$) and invasion (nd:100; d:126.4+10.4%; $P<0.05$) of EVTs isolated from weeks 6 to 8 of gestation, whereas decidualized CM significantly suppressed outgrowth (nd:100; d:77.3+8.4%; $P<0.05$) and had no effect on invasion of EVTs isolated from weeks 10 to 12. Decidual CM induced EVT expression of proteins which promote cell migration/invasion (profilin-1). EVT CM induced HESC decidualization (control:10.7+3.8 mIU/l; EVT:82.0+19.9 mIU/l PRL; $P<0.05$). EVT CM contained progesterone (194.1+73.3 nmol/l; $n=6$), however HESC treatment with progesterone (50–300 nmol/l) could not stimulate decidualization to the same extent as EVT CM (31.5+6.5 mIU/l PRL). Proteomics revealed EVT CM also contained other proteins (nidogen, fibulin) known to stimulate decidualization.
Conclusion
Decidualization promoted EVT invasion and EVT CM induced HESC decidualization showing that decidual–EVT crosstalk is critical for appropriate EVT invasion and therefore placental formation.

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P282

Caveolins redistribute in uterine epithelial cells during early pregnancy: role in epithelial polarisation

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Introduction
Uterine epithelial cells (UECs) undergo a number of changes to become receptive to blastocyst implantation including the loss of integrin based focal adhesions (FA), and increase in basolateral plasma membrane (PM) curvature; despite these changes UECs maintain a polarised state. Caveolae are lipid rich invaginations of the PM which contribute to membrane curvature. Major caveolae proteins (caveolin 1, and 2, PTRF, and SDPR) were investigated to explore the possible roles they play in remodelling the basolateral PM of UECs during early pregnancy in the rat.
Methods
Western blotting, immunofluorescence (IFM) and transmission electron microscopy (TEM) was performed on rat uterus during early pregnancy; and in hormone treated ovariectomised rats.
Results and discussion
Cytoplasmic caveolin 1, 2 and SDPR was seen at time of fertilisation (TOF). Caveolins 1 and 2 shifted to the basal PM while SDPR was absent at time of implantation (TOI) and under progesterone influence. This corresponded to a 3× increase in morphological caveolae basally at the TOI. Protein analyses showed an increase in caveolin 1 and a decrease in caveolin 2 at this time. PTRF protein abundance did not change, while SDPR was present at the TOF only. Co-immunoprecipitation experiments confirmed associated between caveolin1 and PTRF in UECs at the TOI. While at the TOF caveolin1 demonstrated an association with integrin β1, indicating an association between FAs and caveolae. The increased caveolar formation and the accompanying increased turnover at the basal PM may be a mechanism to compensate for the loss of membrane junctional structures and maintain UEC polarity.

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P283

Interleukin-11 is critical for placentation in mice

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Introduction
Placentation involves trophoblast cell invasion into the decidua to remodel maternal arteries. Interleukin (IL)-11 is critical for human trophoblast cell migration/invasion *in vitro*, however its role in placentation *in vivo* has not been investigated. We hypothesised that IL11 plays a critical role in trophoblast function during placentation. The aim of this study was to examine the effect of IL11 inhibition on placentation in mice.
Methods
IL11 and IL11-receptor(R)-α were immunolocalised in mouse implantation sites throughout gestation ($n=4$/time-point). To determine the role of IL11 during placentation, C57BL/6J mice were administered with a unique PEGylated IL11 antagonist (PEGIL11A; $n=4$/group, 600 μg/application PEGIL11A or PEG control) twice daily at days (D)10–13, or 10–17 of pregnancy (D0: day of plug). Implantation sites collected at D13 or 17 were stained with haematoxylin and eosin, cytokeratin (trophoblast marker), isolectin-B4 and α-SMA (vascular markers).
Results
IL11 and IL11Rα localised to the maternal decidua and highly vascularized placental labyrinth, mid-gestation of pregnancy in mice. IL11Rα localised to fetal endothelial and trophoblast cells. IL11 was produced by trophoblast cells. IL11 inhibition resulted in dysregulated placental labyrinth structure at D13, with a significant reduction in trophoblast staining area (25.25% ± 1.98 vs control 37.58% ± 2.13; $P<0.01$) and morphologically altered vascular spaces. PEGIL11A treatment from D10–17 lead to altered placentas and an edematous fetal phenotype compared to controls.
Conclusion
Our data showed that blocking IL11 during placentation altered placental trophoblasts and vasculature, demonstrating that placental derived IL11 is required for normal placentation. This study highlights the important role of IL11 in placentation *in vivo*.

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Distinct sex-specific gene expression changes in the human placenta in association with childhood allergy at 2 years

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The prevalence of childhood allergic disease has increased dramatically in developed countries. Modern environmental changes are hypothesised to be causing deviations in fetal programming, in which the placenta plays a central role, increasing the prevalence of disease. We hypothesised that susceptibility to childhood allergy is determined by changes in placental function that programs fetal immune function. In this study we aimed to identify candidate genes and pathways in human placental tissue that may contribute to the development of childhood allergy. Human placental tissue was obtained after delivery at the Lyell McEwin Hospital, Adelaide, and global gene expression was examined via microarray analysis. Placentae from pregnancies that gave rise to children with allergy by 4 years of age ($n=45$) were compared to placentae from children with no allergy ($n=17$). Differentially expressed genes and pathways associated with allergy were identified using Ingenuity Pathway Analysis software and validated by qPCR. Microarray analysis identified placental gene changes in dermatological and respiratory disease networks in males, and immunological disease and immune cell trafficking networks of female offspring who developed an allergy. qPCR validation showed altered expression of interleukin 13 receptor alpha 1 (IL13RA1), ORM (yeast)-like protein isoforms 3 (ORMDL3) and matrix metalloproteinase 9 (MMP9) ($P<0.05$). These immune genes were also expressed in a sex-specific manner. This study has identified immune genes that are differentially regulated in male and female placentae. The roles of each gene in placental function and fetal development are unknown, and may have sex-specific functions in the programming of fetal immune function and allergy susceptibility.

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P286

The EpCAM-claudin 7 protein complex decreases during early pregnancy in the rat

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Introduction
During implantation, uterine luminal epithelial cells (UECs) disassemble cell–cell and cell–matrix adhesions that contribute to the formation of an epithelial barrier, which is refractory to blastocyst implantation. These changes are critical for successful implantation. We have shown previously that claudin 7 is present in UECs and is lost prior to implantation in the rat. Epithelial cell adhesion molecule (EpCAM) is another protein frequently associated with claudin 7. This study investigated whether EpCAM is present and whether EpCAM is associated with claudin 7 in UECs during early pregnancy. This study also investigated factors that regulate the expression of EpCAM to elucidate its role in UECs during early pregnancy.
Methods
Immunofluorescence and western blotting was performed on uterine tissue from pregnant (day 1 and 6), pseudopregnant (day 6), and cytochalasin D-treated (day 1) rats and ovariectomised rats subjected to ovarian hormone regimes. Co-immunoprecipitation was performed on isolated UECs from day 1 pregnant rats.
Results and discussion
EpCAM localized to the basolateral surfaces of UECs during normal pregnancy and decreased at the time of implantation (day 6) ($P<0.05$, $n=5$). At the time of fertilisation (day 1), EpCAM co-localised and co-immunoprecipitated with claudin 7, CD9 and α-actinin. EpCAM was not decreased with ovarian hormone replacement ($P>0.05$, $n=5$) or in pseudopregnancy ($P>0.05$, $n=5$) but decreased with cytochalasin D treatment ($P<0.05$, $n=5$). These results suggest that prior to implantation, EpCAM exists in a protein complex with claudin 7 to mediate intercellular adhesion but during implantation, decreases in response to factors other than ovarian hormones and the blastocyst.

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P287

Short-term administration of ulipristal acetate modulates endometrial sex steroid receptor expression and cell proliferation markers

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Introduction
Uterine fibroids cause pain, pressure symptoms and heavy menstrual bleeding (HMB). Fibroid growth is sex steroid dependent. The selective progesterone receptor (PR) modulator (SPRM), ulipristal acetate (UPA) reduces fibroid size and alleviates HMB in 90% of women. UPA has both agonist and antagonist properties and induces (S)PRM-associated endometrial changes (PAEC). The mechanism of UPA action and aetiology of PAEC are unknown. We propose that modulation of steroid receptor signalling is involved.
Materials and methods
Endometrium from women with fibroids was collected with ethical approval/-consent after UPA treatment (5 mg od, up to 12 weeks) and from non-treated controls ($n=9/18$). Oestrogen receptor alpha (ERα), androgen receptor (AR) and progesterone receptors (PR(AB), PR(B)) mRNAs and protein were examined by q-rtPCR and immunohistochemistry. Steroid responsive genes, phosphatase and tensin homolog (PTEN), Indian hedgehog (IHH) and COUP transcription factor 2 (COUP-TFII) mRNA were examined by q-rtPCR. Ki67 immunohistochemistry was used to assess cell proliferation.
Results and discussion
AR, ERα, PR(AB) and PR(B) mRNA concentrations were significantly higher in UPA-treated endometrium compared to controls ($P<0.5$). UPA treatment reversed the normal pattern of glandular and stromal protein expression for ERα and both PR isoforms. UPA induced immunoexpression of AR in glandular

epithelium and elevated IHH mRNA over controls ($P<0.01$). PTEN and COUP-TFII mRNAs were unaffected and Ki67 immunostaining was similar in UPA-treated and control endometrium.
In conclusion, UPA treatment altered steroid receptor expression and resulted in up-regulation of PR responsive genes. No effects on cell proliferation were demonstrated.
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P288

Periconceptional alcohol exposure programs sex specific hyperinsulinemia possibly through dysregulation of placental *O*-linked glycosylation

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Introduction
Exposure to an adverse environment around the time of conception can program adult onset disease although mechanisms involved are unclear. Recently we showed that periconceptional alcohol (PC:EtOH) consumption causes placental stress demonstrated by elevated levels of 11βHSD-2, and sex-specific increases in placental glucose transporters and glycogen accumulation. *O*-linked-*N*-acetylglucosamine transferase (OGT), an X-linked gene product, responsible for *O*-linked glycosylation (*O*-GlcNAcylation), has also been identified as a placental biomarker of maternal stress. Indeed, the ability to respond to stress via *O*-GlcNAcylation is linked with cellular survival. Moreover, dysregulation of *O*-GlcNAcylation is also implicated in metabolic disease. We examined OGT levels in placentas following PC:EtOH-exposure and determined postnatal metabolic outcomes.
Materials and methods
Sprague–Dawley rats were exposed to an EtOH containing diet (12.5%, $n=12$/group) during the PC period (E-4 to E4). Placentas were collected from a subset of dams on E20 and levels of OGT were assessed via western blotting. Fasting glucose and insulin concentrations were measured in six month offspring of a different subset of dams and the HOMA-IR-index calculated.
Results and discussion
PC:EtOH-exposure resulted in fetal growth restriction. OGT levels were specifically elevated in the spongiotrophoblast in placentas from female ($P<0.05$), but not male fetuses following PC:EtOH-exposure, while OGT levels in the labyrinth were unchanged in placentas from both sexes. Interestingly, insulin levels ($P<0.05$) and HOMA-IR-index ($P<0.05$) were elevated in male offspring following PC:EtOH-exposure. Our results suggest that sex-specific alterations in placental OGT levels in response to PC:EtOH-exposure may act to protect the female fetus from hyperinsulinemia in later life.
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P289

Analysis of EMG signal linear synchronization in porcine uterus

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Introduction
Understanding the direction and quantity of information flowing in a complex system of myometrium is a fundamental task in signal processing. The new perception of such related functioning systems can be given by the evaluation of synchronization between signals.
Materials and methods
Spontaneous uterine activity was recorded in ten mature Polish Landrace sows directly by commercial implants TL10M3-D70-EEE (DSI, USA) surgically positioned between the abdominal muscles and three silver bipolar needle electrodes sutured on the uterine horns and corpus uteri. We used the linear measures: the cross-correlation function ($f_{x,y}(l)$, $f_{y,z}(l)$) and the cross-coherence function ($C_{xy}(f)$, $C_{yz}(f)$) to assess synchronization between three simultaneously, telemetrically (PowerLab (AD Instruments, Australia) Chart v.4.1), recorded time series of a diestral uterine EMG bursts in sows.
Results and discussion
The aim was to plot the directional map of signal flow between the channels. The results show a slight superiority of the cross-coherence function. We observed the synchronization between all simultaneously recorded signals. The EMG bursts migrated along the uterine horn in both cervico-tubal and (predominantly) tubo-cervical direction. Results are encouraging for the future use of analysis methods for resolving of the directionality of uterine EMG flow under the effect of various medicaments administration and may provide a way of finding pacemaker localization in the uterus.
Keywords: EMG, uterus, sow, bursts migration
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P290

Sex specific effects of maternal dietary protein upon uterine blood flow and fetal growth

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Introduction
Neonatal and fetal mortality is increased in male fetuses. Evidence is emerging of sex specific differences in fetal and placental perfusion[1]. This study investigates effects of dietary intervention at specific time points upon blood flow and fetal growth trajectory in cattle.
Materials and methods
Primiparous heifers were either individually- (Australia, Au; $n=360$) or group-fed (UK; $n=188$) diets containing different levels of crude protein (Au, 14 and 7% CP; and UK, 18 or 10%CP) from 60 d pre-conception (fixed timed artificial insemination, FTAI) until 98 days post-conception (dpc) in a 2×2 design with diet changing at FTAI (UK) and 23 dpc (Au). Fetal development was assessed at 36 (crown rump length, CRL) and 60 dpc (biparietal distance, BPD) and Doppler-scanning of mid-uterine artery at 150, 180 and 210 dpc. Data was transformed and analysed using ANOVA for repeated measures.
Results and discussion
Diet change after implantation (23 dpc), rather than at FTAI, had a longer lasting effect on fetal growth (CRL and BPD, $P<0.05$). Preconception diet at 7% CP increased uterine blood flow ($P<0.05$), with the former reducing CRL ($P<0.05$) and BPD ($P<0.05$). Interestingly, post-conception diet also reduced CRL at lowest (7%; $P<0.05$) and highest levels (18%; $P<0.05$), irrespective of diet change time. Sex specific effects in preconception diets were observed, with male-carrying heifers showing an increase in uterine blood flow ($P<0.05$).
These results suggest that periconception diet influences oocyte and embryo development which may have long term and sex specific effects for the offspring.
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Reference
1. Prior T, *et al. PLoS ONE* 2013 **8** (2) e56933.
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P291

Effect of pentraxin 3 on inflammation-induced fetal loss

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Pentraxin 3 (PTX3) is an essential component of the innate immunity with a non-redundant role in conferring resistance to pathogens and in modulating inflammatory reactions in several diseases. Pregnancy is characterized by mild systemic immunosuppression and inflammation. An uncontrolled immune activation at the fetal–maternal interface caused by bacterial infections or inflammatory stimuli is associated with high risk of miscarriage both in mice and in women. In this study, we investigated the effect of the administration of recombinant human-PTX3 (rhPTX3) on a lipopolysaccharide (LPS)-caused early fetal loss model.
Pregnant 129/sv mice were treated at 9.5 dpc with LPS $\pm$ full length rhPTX3, or the rhN-terminal or rhC-terminal domain PTX3 and sacrificed at 13.5 dpc to assess the embryonic resorption rate. Circulating levels of TNF-α and IL10 and decidual levels of rhPTX3 were measured at 1.5 h post-treatment by ELISA.
We found that the administration of rhPTX3 significantly improved pregnancy success reducing the percentage of LPS-induced fetal loss from 70 to 35%.

The rhC-terminal domain, but not the rhN-terminal domain, mimicked the action of the full length protein, indicating that the protective role played by PTX3 is exclusively exerted by its pentraxin domain. The circulating levels of TNF-α and IL10, key players in mediating and inhibiting the LPS-induced abortion, respectively, were not altered by rhPTX3. Noteworthy, the administered rhPTX3 efficiently penetrated into the decidua. These results suggest that PTX3 is able to rescue embryos from the adverse effects of LPS by acting at the local level.

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P292

Glycoforms of α-2-macroglobulin in uterine fluid, are altered during the menstrual cycle, and differ between fertile and infertile women

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Introduction
Our previous 2D-DIGE studies of uterine fluid, identified differing isoform distributions of the serine protease inhibitor α-2-macroglobulin (A2M) resulting from post-translational modification (PTM's), among fertile and infertile women. Carbohydrate side chains are a key PTM, often conferring recognition between protein partners, e.g. enzyme and inhibitor. Altered PTM's may potentially change the interactions of A2M with proteases, effectively altering the proteolytic environment of the uterine cavity and impacting embryo-endometrium interaction.
We hypothesised that specific A2M glycoforms may be expressed during the phases of the menstrual cycle and serve as markers of endometrial receptivity. Our aim was to identify A2M glycoforms that may discriminate between fertile and infertile, receptive and non-receptive endometrium.
Materials and methods
A2M glycoform distribution was determined in uterine lavage from proliferative and secretory phases of fertile and infertile women using 21 lectins in arrays with an A2M specific antibody. For the most promising lectins, assays were developed on Luminex and a cohort of fertile and infertile women assayed.
Results and discussion
DBA and GSLI binding glycoforms were significantly elevated in the secretory phase compared to proliferative phase of fertile women; however infertile women showed significantly lower secretory phase concentrations. LEL binding forms were significantly reduced among the infertile cohort during the proliferative phase.
Thus, specific glycoforms of A2M may be useful as markers of endometrial receptivity since changes with cycle phase and with fertility status were demonstrated.

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P293

Non-cytopathic BVDV suppressed innate immune response in bovine endometrial cells exposed to bacterial lipopolysaccharide

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Introduction
Infection with non-cytopathic BVDV (ncpBVDV) is associated with uterine disease and infertility in dairy cows although the underlying mechanisms are poorly understood. This project investigated the influence of ncpBVDV on innate immunity of the bovine endometrium.
Materials and methods
Primary bovine endometrial cells from each of six cows were divided into four groups (Control; LPS; BVDV; BVDV LPS). Cells were infected with ncpBVDV for 4 days followed by treatment with LPS for 6 h respectively. Whole-transcriptome gene expression was measured by Affymetrix Bovine Gene 1.1 Array containing the probes for 23000 transcripts.
Results and discussion
Analysis focusing on the comparison between BVDV LPS vs LPS groups showed 218 differentially expressed genes ($P<0.05$; ANOVA and paired *t*-test with BH adjustment). Of these, 192 genes were mapped and analysed with Ingenuity Pathway Analysis. The top two associated network functions were 'Antimicrobial Response, Inflammatory Response, Infectious Disease', score 33; and 'Infectious Disease, Post-Translational Modification, Protein Folding', score 31. The top Canonical Pathways were 'Activation of IRF by Cytosolic Pattern Recognition Receptors' and 'Interferon Signalling'; $P<0.001$. Top down-regulated molecules (*RSAD2, ISG15, BST2, MX2, USP18, IFIT3, IFI27, SAMD9, IFIT1, DDX58, IRF3* and *MX1*) are genes involved in innate immune signalling and the interferon pathway. Analysis of upstream regulators predicted the inhibition of *IRF7, TLR3, IFNL1, IFNA2* and *IFNB1*.The results indicate that ncpBVDV suppressed a type 1 interferon/dsRNA recognition response in infected bovine endometrial cells exposed to bacterial LPS. The compromised immune state may predispose dairy cows to postpartum endometritis and a decrease in fertility.

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P294

Remodelling-associated processes during *postpartum* uterine involution in mice

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Introduction
Following labour, the uterus rapidly returns to its pre-pregnancy state. Exact mechanisms underlying labour and *postpartum* involution are not fully understood but inflammation is understood to play a key role. It has been hypothesised that rather than initiating or propagating labour, inflammation may prime the uterus for extensive *postpartum* tissue repair and remodelling, in a manner akin to repair in exercising skeletal muscle. This study aims to investigate the expression of mRNA for heat shock proteins (HSPs), matrix metalloproteinases (MMPs), as well as quantification of mast cells (MCs), all of which have previously been associated with repair and remodelling mechanisms in other models of wound healing.
Methods
Uterine tissues of C57BL/6 mice were collected at Day 1 ($n=5$), 4($n=6$) and 7($n=5$) *postpartum*. Virgin females (proestrous stage) were used as non-pregnant controls ($n=5$). mRNA expression was determined by qRT-PCR. MCs were visualised by toluidine blue staining and quantified. Data were analysed by Kruskal-Wallis test, followed by Dunn's Multiple Comparison Test.
Results and discussion
A three fold increase in expression of *Hspb1* (HSP27) at Day 7 *postpartum* ($P=0.04$) and *Mmp8* at Day 4 *postpartum* ($P=0.03$) compared to non-pregnant samples was found, suggesting that HSP27 and MMP8 may have a role in *postpartum* uterine involution. MC numbers did not change throughout the post-partum period, however, clustering at the endometrium/myometrium border was observed. Future studies will determine the activation status of these cells. This study provides a foundation for further examination of the physiological process of uterine involution and remodelling processes.

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P295

Investigating the role of androgens in endometriosis

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Introduction
Androgen receptors (AR) have been detected in the human endometrium throughout the menstrual cycle and are reported to regulate expression of genes associated with cellular proliferation and cell-cycle regulation. Endometriosis is defined by establishment and growth of endometrial tissue in ectopic locations (typically on the peritoneal wall); patients often experience pain and infertility. In the current study we have used human tissue and a new mouse model of endometriosis to investigate the expression of AR and the potential impact of androgens on cellular proliferation and gene expression.
Materials and methods
Tissue samples (eutopic endometrium, peritoneum and endometriotic lesions) were obtained from women attending a pelvic pain clinic who gave informed consent. Equivalent tissue samples were collected from a recently established mouse model of endometriosis. Some mice were injected with dihydrotestosterone (DHT). Messenger RNA concentrations and proteins were investigated by RT-PCR and immunofluorescence.
Results and discussion
Immunoexpression of AR in normal mouse uterus was localised to stromal fibroblasts consistent with that reported for human endometrium. In contrast to

normal uterus, in endometriotic lesions recovered from both women and mice AR was localised to stromal and epithelial compartments. There was minimal co-expression of AR and proliferation markers in endometriotic cells. DHT treatment altered the expression of putative AR-regulated genes in the mouse uterus. These results demonstrate that AR expression in a mouse model of endometriosis parallels human disease and provides a platform for investigating the impact of androgens/selective AR ligands on endometriosis.

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P296

Evaluation of protein profile in uterine secretion on early cyclic and gestational diestrus in bitches

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Introduction
During peri-implantation period the canines conceptus are nourished by secretions in uterine lumen, a phenomenon known as histotrophic nutrition, in which several proteins are present. The aim of this study was to compare the protein profile in uterine secretion in early pregnancy and early diestrus cyclic using two-dimensional electrophoresis technique.
Material and methods
For that, ten bitches were submitted to OHE on the 12th day after the preovulatory LH surge. Five females were in early diestrus cyclic (Group IA) and the others were in gestational diestrus (group IB). The samples were obtained by uterine flushing using PBS solution and resulting wash was storage at −20 °C. We prepared pools with those samples, each one containing five samples of the respective groups. Two-dimensional electrophoresis was performed with 138.40 μg /μl of protein per strip (7 cm) and four replicates per group. The gels were stained using Coomassie G-250, scanned and interpreted using an specific software (Image Master version 7.0, GE HealthCare, Upsalla, SE).
Results and discussion
Thirty three spots were expressed in group A and 29 spots in group IB. Three proteins were expressed exclusively in non pregnant bitches (group IA) and eight proteins were exclusively expressed during early pregnancy (group IB), this fact proves the existence of a distinct protein secretion in canine uterus on early pregnancy. Those exclusive proteins in gestation may have an important potential in precoce diagnosis of pregnancy in bitches, as well as being involved in embryo implantation process.

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P297

Does decidual macrophage polarisation affect trophoblast behaviour in co-culture studies?

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Introduction
Decidual macrophages constitute the second largest leukocyte cell type in the decidua during early pregnancy, and have been implicated in the regulation of extravillous trophoblast (EVT) invasion. Macrophage cells in the decidua are thought to have an anti-inflammatory M2 phenotype and the presence of macrophages with a more pro-inflammatory M1 phenotype has been associated with preeclampsia. The aim of this study was to determine whether decidual macrophage polarisation state has an impact upon EVT behaviour.
Materials and methods
The human acute monocytic leukemia cell line (THP-1) was differentiated with phorbol 12-myristate 13-acetate (PMA) into macrophages. Once differentiated, the cells were polarised using LPS and IFN-γ to generate M1 macrophages, or IL4 and IL13 to generate M2 macrophages. Culture supernatant from the polarised cells was used to treat SGHPL-4 cells, a cell line derived from first-trimester EVT, and SGHPL-4 cell death, proliferation, motility and invasion were analysed.
Results and discussion
Culture supernatant from the M1 macrophages was found to significantly reduce SGHPL-4 motility when compared with the M2 macrophages and the media control.The culture supernatant from both M1 and M2 macrophages was found to inhibit SGHPL-4 proliferation but increase SGHPL-4 invasion, when compared with the media control. No effect upon SGHPL-4 cell death was observed with any of the treatments. Thus, the polarisation state of macrophage-like THP-1 cells impacts upon their ability to regulate extravillous trophoblast motility *in-vitro* indicating that decidual macrophage polarisation state may have an important role in the regulation of placental development.

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P298

Decidual stromal cell regulation of trophoblast in first trimester pregnancies with normal and impaired spiral artery remodelling

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Introduction
Decidualisation of the endometrium is the differentiation of uterine stromal cells into specialised secretory decidual stromal cells (DSC) primed for pregnancy. If pregnancy occurs, DSC control and regulate fetal trophoblast invasion. Pre-eclampsia is a disorder associated with inadequate trophoblast invasion and spiral artery (SA) remodelling. Uterine artery Doppler resistance index (RI) in the first trimester of pregnancy measures the extent of SA remodelling; pregnancies either have a high (high RI) or normal (normal RI) risk of poor SA remodelling. The phenotype of DSC with a high or normal uterine artery RI and their effect on trophoblast function was investigated.
Materials and methods
DSC were isolated from first trimester terminations of pregnancy screened by uterine artery Doppler ultrasound. DSC were decidualised *in vitro* using cAMP and medroxyprogesterone 17-acetate (MPA); secretion of IGFBP1, PRL and FOXO1 were measured by ELISA and western blot. The trophoblast cell line SGHPL-4 was incubated with DSC conditioned media (CM) and chemotaxis and motility were measured.
Results and discussion
Decidualisation of DSC increased secretion of IGFBP1, PRL and FOXO1 ($P<0.05$). Decidualised DSC CM from pregnancies <10 weeks gestation that have a normal RI significantly stimulated trophoblast chemotaxis ($P<0.05$) compared to pregnancies >10 weeks gestation. DSC CM from pregnancies with high RI had no effect on trophoblast chemotaxis. DSC CM had no effect on trophoblast motility.

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P299

Effect of maternal diet on the amino acid composition of human uterine fluid

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Introduction
Preimplantation embryos depend on nutrients provided in the reproductive tract for development. Early human embryos have an increasing requirement for amino acids as they develop from the 1-cell through to the blastocyst stage. *In vivo*, the uterus provides the final environment for preimplantation development, but surprisingly, the nutritional composition of human uterine fluid has been little investigated. This study aims to examine the amino acid composition of human uterine fluid and how this alters with maternal diet.

Materials and method
The gynaecological history and a validated, food frequency questionnaire was completed by all women recruited to this study. Uterine fluid was obtained from 56 women aged 18–45, undergoing Hystero-Salpingo-Contrast-Sonography with ethical approval (08/H0502/162). Reverse phase high pressure liquid chromatography (HPLC) was used to analyse the amino acid concentrations within the uterine fluid.
Results and discussion
Human uterine fluid contained an amino acid concentration of 4.92 ± 0.53 mM. Glutamate was present in the highest concentration in uterine fluid followed by glycine and alanine. In contrast, methionine and tryptophan were present in the lowest concentration. Several amino acids including the branched chain amino acids ($P < 0.05$) were found to be increased in those women with a negative compared to a positive prudent diet score. These data suggest that maternal diet has a significant impact on the amino acid composition of the uterine fluid. Further research is required to determine the impact of maternal diet during the periconceptional period and how this may affect both the *in utero* environment and embryo development.

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P300

Reproductive performance, oxidative enzyme and gonadotropins status of rabbit does fed organic selenium as dietary supplement

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Introduction
Selenium deficiency is known to affect negatively the reproductive performance of farm animals including rabbits. This study evaluated the effect of feeding organic selenium (OSe) as dietary supplement on the reproductive performance, oxidative enzyme and gonadotropins status of rabbits does.
Materials and methods
Sixteen female rabbit does of 6 to 7 months of age and weighing between 1.95 to 2.03 kg were assigned to four treatment groups receiving 0.00, 0.15, 0.30 and 0.45 mg/kg of OSe as dietary supplement from mating to end of lactation in a completely randomised design (CRD). Rabbit does in each treatment were house individually in cages. The reproductive and *post-partum* performance of kits were observed. Oxidative enzyme and gonadotropins status of the does were determined at end of 28 days lactation using standard methods. Data was analysed using ANOVA.
Results and discussion
Gestation length, body weights (BW) of litters and kits at birth were similar ($P > 0.05$). Conception rate, weight gain of does during gestation, litter size at birth and number of stillbirth of rabbit doe were better ($P < 0.05$) for rabbits fed 0.15 and 0.30 mg/kg OSe. Litter size, litter BWs, kits BW and kits BW gain at weaning were higher ($P < 0.05$) for rabbit kits of does fed 0.15 mg/kg OSe. Selenium, malondialdehyde, superoxide dismutase, and glutathione peroxidase status among treatments were different ($P < 0.05$). While FSH levels of does fed Ose differed from the control group, LH levels remained constant ($P > 0.05$). Supplemental feeding of Ose to rabbit does improved the reproductive performances of the dam by enhancing the oxidative enzyme and FSH status of the does.

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P301

Secreted phosphoprotein 1 (*SPP1*), vascular endothelial growth factor A (*VEGFA*), heparinase (*HPSE*), hypoxia-inducible factor (*HIF1A*) and uteroferrin (*ACP5*) gene expression in uterine and placental tissues from Large White-Landrace (LW-LD), hyperprolific Large White (hLW) and Meishan (MS) pigs

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Introduction
This study compared uterine and placental expression of genes implicated in placental attachment and angiogenesis in two prolific (hLW and MS) and one control (LW-LD) pig breeds.
Methods
Placental and endometrial tissues supplying the smallest and a normal-sized foetus within the same litter were collected from five LW-LD, five hLW and four MS gilts on days 40–45 of pregnancy. RNA was isolated and qPCR for *SPP1*, *VEGFA*, *HPSE*, *HIF1A* and *ACP5* performed. Normalised relative expression data were analysed by a mixed model in SAS, accounting for the common maternal environment.
Results and discussion
SPP1, *VEGFA*, *HPSE*, *HIF1A* and *ACP5* mRNA was present in all tissues studied but, with the exception of *ACP5*, expression levels varied with breed and foetal size. Endometrial expression of *VEGFA*, *HPSE* and *HIF1A* was higher in LW-LD compared to either MS ($P = 0.002$; $P = 0.05$ and $P = 0.001$, respectively) or hLW ($P = 0.0011$; $P = 0.0001$ and $P < 0.0001$, respectively). Placental *SPP1* expression was higher in hLW than in LW-LD ($P = 0.03$) and, *VEGFA* expression was higher in placentas supplying normal foetuses in hLW than in MS ($P = 0.04$). Expression of *SPP1*, *VEGFA*, *HPSE* and *HIF1A* in LW-LD and MS and of *HPSE* and *HIF1A* in hLW was higher in endometrium supplying the smallest compared to normal foetuses. Placental gene expression mirrored differences in endometrial gene expression with the exception of *SPP1*, which was higher in placentas supplying normal foetuses of all breeds and *HPSE* which was higher in placental tissue supplying normal hLW foetuses. Breed and foetal size differences in gene expression support their role in foetal growth and prenatal survival.
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P302

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P303

Effect of progesterone supplementation on endometrial receptivity for implantation in the pig

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Progesterone (P_4) produced by the corpus luteum (CL) is required for the attainment of endometrial receptivity and further conceptus implantation. Induction of puberty with PMSG/hCG in gilts results in lower P_4 concentration in blood serum, that is accompanied by decreased endometrial expression of genes involved in conceptus-maternal interactions. Therefore, the present study aimed to determine the effect of P_4 supplementation in pregnant gilts on endometrial preparation for implantation. Prepubertal gilts were induced to ovulate with 750 IU PMSG / 500 IU hCG, and inseminated. On days 3 to 10 of pregnancy, gilts received i.m. injections of corn oil vehicle ($n = 4$) or P_4 (50 mg/100 kg; $n = 4$). Blood samples were collected each day beginning form day 1 of pregnancy to analyze the profile of P_4 concentration using RIA method. Animals were slaughtered on day 12 of gestation. Uterine luminal flushing (ULF) was examined for E_2, PGE_2 and IL6 concentrations with RIA/EIA. Uterine horns were weighted and endometrial expression of PTGS2, HOXA10, LIF, TGFβ1 and IL6 mRNA was determined using qPCR. P_4-treated gilts demonstrated greater concentrations of P_4 in peripheral blood serum on days 8 to 10 and had heavier uteri after slaughter ($P < 0.05$). The concentration of E_2 in ULF increased, but PGE_2 decreased, after P_4 treatment ($P < 0.05$). Neither IL6 content in ULF, nor endometrial IL6 mRNA expression differ between groups. Similarly, PTGS2, HOXA10 and TGFβ1 mRNA expression was not affected by P_4 injections. Summarizing, P_4 supplementation had no significant impact on the expression of markers of endometrial receptivity in the pig. Supported by NSC grant 2011/01/B/NZ9/07069.

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P304

The relaxing effect of dihydrotestosterone on myometrial smooth muscle contraction is mediated via blockage of intracellular calcium increase and inhibition of myosin light chain phosphorylation

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Background
The impact of androgens on myometrial contraction has received less scrutiny, compared to progesterone and estrogens. We sought to i) examine the effect of dihydrotestosterone (DHT; a non-aromatizable androgen) on stretch-induced contraction of human myometrium and ii) gain mechanistic insights by studying changes in intracellular calcium [Ca^{2+}] and phosphorylation of myosin light chain (MLC).
Methods
Myometrial strips were recovered from biopsies obtained from women undergoing elective caesarean delivery at term prior initiation of labour. Strips were placed under tension in a tissue-bath system and allowed to develop spontaneous contractions. Contractions were quantified before and after addition of DHT (10–100 μM, 30 min). A myometrial smooth muscle cell line (PHM1-41s) was used to measure [Ca^{2+}] concentrations (Fluo-4 calcium assays) and phosphorylation of MLC (in-cell western); oxytocin (OXT) was used as a positive control.
Results
Addition of DHT relaxed spontaneous myometrial contractions in a dose dependent fashion: amplitude of contractions was 5% ± 3% of controls following addition of 100 μM ($n=7$ women, $P<0.001$). Treatment of PHM1-41s with OXT (10 nM) increased [Ca^{2+}] ($P<0.001$, $n=7$) and significantly increased phosphorylation of MLC (OXT 100 nM, $P<0.0001$, $n=6$). Pre-treatment with DHT for 15 min blunted the OXT effect on [Ca^{2+}] (DHT: 800 nM; $P<0.05$, $n=6$) and phosphorylation of MLC (DHT: 50 μM; $P<0.05$, $n=6$).
Conclusions
DHT inhibits myometrial smooth muscle contraction. The mechanism of this effect may involve the blockage of increases in intracellular Ca^{2+} and phosphorylation of MLC.

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P305

Detection of circulating miRNAs in the maternal serum during early pregnancy in the pig by qRT-PCR and digital RT-PCR

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Exosomes are recognized as new mediators of intercellular cell signaling between neighboring cells and distant tissues, acting independently but synergistically with growth factors and hormones. Trophoblast and stromal cells of the villi were identified as a source of exosomal miRNAs released into the maternal circulation. In this study we hypothesized that miRNAs participating in the embryo–maternal dialog are released into the maternal blood stream during early pregnancy in pigs. From our previous global transcriptomic experiments we selected 11 miRNAs (miR-1, miR-23b, miR-26a, miR-27a, miR-34a, miR-125b, miR-199a-5p, miR-199a-3p, miR-203b, miR-205, miR-302), showing differential expression in embryos/trophoblasts or endometrium, and tested them in maternal serum collected on day 16 of the estrous cycle and pregnancy ($n=6$/status) using either qRT-PCR and digital RT-PCR. Data were analyzed using PCR Miner and GraphPad Prism.
Among tested miRNAs only three (miR-1, miR-199a-5p, miR-302) were not detected in the maternal serum. qRT-PCR indicated different levels of miR-26a and miR-125b in pregnant and cyclic animals, showing elevated levels during pregnancy ($P=0.003$ and $P=0.006$, respectively). Digital RT-PCR analysis allowed detection of 766 ± 159 vs 2057 ± 494 copies/μl of miR-26a ($P=0.002$) and 2675 ± 556 vs 6068 ± 729 copies/μl of miR-125b ($P=0.007$) in cyclic and pregnant animals, respectively. Additionally, miR-23b showed increased levels in serum samples of pregnant animals ($P=0.006$; 151 ± 33 vs 314 ± 32 copies/μl). This data revealed altered profiles of miRNAs in the material circulation on day 16 of pregnancy, suggesting status-dependent release of miRNA into the maternal circulation of the pig. It seems likely that unique set of miRNAs are released into maternal circulation as a consequence of early embryo–maternal communication.

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P306

Inhibition of the polyamine pathway induces an embryonic diapause-like state in the mouse embryo

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Introduction
Embryonic diapause is a period of developmental arrest in which the embryo is maintained in a dormant state for an extended period of time. Over 130 species of mammals undergo embryonic diapause, but the molecular control mechanisms remain unknown. In this study, we investigated the potential role of polyamines during embryonic diapause in the mouse. Polyamines play essential roles in cell growth and proliferation, and the regulation of their expression is controlled by the rate limiting enzyme, ornithine decarboxylase (ODC1). In the mink, inhibition of ODC1 with difluormethylornithine (DFMO) induces a reversible arrest in embryonic development.
Materials and methods
To characterise the effect of ODC1 inhibition on mouse embryo development, pregnant mice were treated with DFMO from dpc 4 to 7 and autopsied on dpc 8. The uteri were flushed and the non-implanted blastocysts cultured *in vitro*. Expression of the polyamine pathway and implantation-related factors were examined in the uterus.
Results and discussion
In 78% of females, we observed no signs of implantation whilst the remaining females exhibited disrupted placental formation and degenerate embryos. From the non-implanted females we obtained viable blastocysts that had limited cell proliferation, but which were able to reactivate when cultured *in vitro*. Hence, ODC1 inhibition induces a diapause-like state in mouse embryos. Previously, SAMDC has been shown to be able to compensate for a lack of ODC1. To date, we have observed no evidence for compensatory effects. Hence, these results suggest that embryonic diapause is caused by the paucity of polyamines necessary for activation of the embryo.

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P307

Effect of flax seed (*Linum usitatissimum*) extract on modulation of prostaglandin E_2 production by buffalo endometrial stromal cells cultured *in vitro*

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Different hormones have been tried to increase embryonic survivability with inconsistent results. Plant based treatment plays positive role in enhancing reproductive functions, acting as precursors for different signalling molecules and influencing cell processes in humans as well as animals, but lacks scientific validation. Effect of aqueous and ethanolic extracts of flax seed on PGE_2 modulation by buffalo endometrial stromal cells was studied. Buffalo stromal cells were isolated by double enzymatic digestion and cultured till confluence. Cytotoxic effect was studied using MTT assay. Stromal cells were treated with different dose of aqueous (78.1, 39.0, 19.5, 9.7, 4.8 and 2.4 μg/ml) and ethanolic extract (19.5, 9.7, 4.8, 2.4 and 1.2 μg/ml), keeping positive and normal control. Mean PGE_2 concentration with different aqueous extracts concentration was significantly higher ($P<0.01$) as 573.02 ± 3.24, 598.41 ± 2.60, 633.98 ± 3.27, 639.97 ± 2.88, 687.36 ± 1.76, 698.19 ± 1.63 pg/μg protein, respectively than control (503.83 ± 1.79) and positive control (511.82 ± 5.20). PGE_2 concentration was also significantly higher ($P<0.01$) with different ethanolic extract concentration as observed 511.23 ± 0.80, 594.24 ± 3.01, 623.74 ± 2.84, 612.81 ± 1.38, 639.7 ± 2.90 pg/μg protein, respectively than control. Findings revealed maximum PGE_2 production with 2.4 μg/ml aqueous extracts, exhibited 1.39-fold increase followed by 1.2 μg/ml concentration of ethanolic extracts, shown 1.27-fold increase. Both extracts significantly increased PGE_2 production by buffalo stromal cells *in vitro* might be due to PUFA rich fraction of flax seed, hold promise to enhance embryonic survival thus fertilty in future.

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P308

Detection of SPAM1 in the bovine oviductal fluid

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Introduction
The sperm adhesion molecule 1 (SPAM1) is a glycoprotein present in the sperm membrane and it is involved in degradation of cumulus matrix and ZP binding. In the mouse, SPAM1 is secreted by the oviduct and the uterus and is acquired by the sperm during its transit through the genital tract. Our aim was to determine the presence of SPAM1 in the bovine oviduct.
Materials and methods
Ovaries and oviducts were obtained from slaughterhouse cows at the preovulatory stage. PCR analyses were performed using isolated cells from the ampullary mucosa. The oviductal fluids (OF) were obtained by aspiration and analyzed by electrophoresis, immunoprecipitation and western blot (WB). To identify if the SPAM1 is present in the oviductal exosome the Exoquic kit was used. For proteomic analysis, different samples were digested with trypsin and analyzed with Agilent Ion-Trap XCT-Plus mass spectrometer equipped with an electrospray interface. SPAM1 isolated by affinity chromatography was incubated with immature ovarian oocytes and analyzed by confocal microscopy.
Results and discussion
An amplicon of 210 pb that corresponds to the SPAM1 RNAm was obtained and confirmed by automatic sequencing. Four different bands of 75, 60, 35 and 25 kDa were identified by WB. Moreover, proteomic evidences indicates that the SPAM1 protein is a component of the oviductal exosomes. Isolated SPAM1 bound specifically to the ZP of ovarian oocyte. Future research will be necessary to clarify the role of SPAM1 during *in vivo* fertilization. This work is supported by MINECO (AGL2012-40180-C03-01-02) and the European Commission (FED-ER/ERDF).

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P309

Comparison between the effect of hCG and GnRH analogue (buserelin) treatment post laparoscopic insemination on reproductive performance of ewes

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The present study aimed to compare between the effect of hCG and GnRH analogue treatment post laparoscopic insemination on reproductive performance of ewes. Oestrus synchronization was performed for 120 ewes (Najdi = 60 and Naeimi = 60) using intra-vaginal progesterone sponge and equine chorionic gonadotrophin (eCG). Semen was collected from six proven fertile rams by artificial vagina. Ewes were laparoscopic inseminated 48–50 h after sponge withdrawal,and divided randomly into three groups. Group A: ewes were not given any treatment (control). Group B and C: ewes were injected with 500 IU hCG or GnRH (8 μg buserelin), respectively. Reproductive performance traits including pregnancy rate, fecundity, prolificacy, type of birth, litter weight, lamb's birth weight, gender ratio, lamb mortality rate and gestation length were calculated. The results revealed that the pregnancy rate, fecundity, prolificacy, triplet percentage, litter weight and lamb's birth weight were significantly ($P < 0.05$) higher in hCG treated ewes (65.7, 64.9, 134.8, 204.2, 22.0, 7.26, 3.65, respectively) in comparison with other groups, whereas GnRHewes had higher male percentage and longer gestation length ($P < 0.05$) than the other groups. Najdi and Naeimi ewes did not differ ($P > 0.05$) in all evaluated reproductive performance traits except for gestation length and birth weight; Najdi ewes had longer ($P < 0.05$) gestation length and lighter ($P < 0.05$) birth weight than Naeimi ewes. In conclusion, hCG treatment post insemination improved most of reproductive performances of Najdi and Naeimi ewes, while GnRH treatment increased male percentage and gestation length.

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P310

Novel sequence variations in exon 25 of JHDM2A among overweight and obese sub/infertile males in South India

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Objectives
To analyze three exons of *JHDM2A* gene in obese males associated with sub/infertility and to compare with non-obese infertile and fertile males as controls in Karnataka, South India.
Materials and methods
For the candidate gene analysis, 200 cases with obesity/ and infertility and 50 controls (non-obese fertile males) were selected. After 3–5 days of ejaculatory abstinence the semen samples were collected in a sterile plastic container by the process of masturbation from the subjects (WHO, 1999). The collected samples were allowed to liquefy at 37 °C for 30 min and analyzed for semen volume, sperm count, motility, viability, etc. within 1 h after collection. The blood samples of the cases and controls were analyzed following the genomic DNA extraction and RT-PCR amplification. The qPCR products then immediately were subjected to High Resolution Melting analysis in order to find out the variation in the mentioned exons. The suspected samples with variation were sequenced for confirmation, using Applied Biosystem 3130 genetic analyzer, and the sequences were subjected for multiples nucleotide sequence alignment using CLC Main Workbench 6.0 to analyze the changes in nucleotide as well as amino acid sequences.
Results
Variations including single or double nucleotide insertion as well as substitution was found in exon 25 of the candidate gene in 11 subjects. No variation was observed in either exon 23 and 24. Semen parameters were found to be more impaired in obese infertile males compared to other groups in the study.
Conclusion
Obesity was found to be linked with male fertility problems. Although the JHDM2A variants were found in obese males to be associated with subfertility or infertility, this study encourages further comprehensive multidisciplinary research taking diverse large human populations to understand the relationship between obesity and male infertility.

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P311

Incerement of proinflammatory cytokine (IL6) and lipid peroxidation in obese infertile males may lead to more impaired seminogram compared to non-obese infertile males

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Objectives
The present study measures the level of interleukin-6 (IL6) and the lipid peroxidation (LPO) marker, malondialdehyde (MDA), in the semen ofobese and non-obese infertile males vs obese and healthy fertile males and to correlate their level with the semen characteristics.
Materials and methods
A total of 100 infertile participants, consisting of equal number of obese infertile and non-obese infertile males ($n = 50$) were selectedas cases. To include age matched controls 40 obese fertile and 50 non-obese fertile males were randomly selected from Mediwave IVF and fertility research hospital and different obesity clinics in Mysore. Semen analysis was performed for all of the participants according to WHO guidelines within 1 h after collecting the samples. Seminal IL6 levels were measured using ELISA.
Results
LPO levels were determined by measuring malondiadehyde (MDA) in seminal plasma. All tests were performed in duplicates and mean values were recorded. The effect of BMI on seminal level of IL6 and LPO were compared with some lifestyle factors such as smoking, alcohol consumption, and mobile and laptop usage. Obese infertile males had higher level of seminal IL6 in comparison to the non-obese infertile and both control groups. Seminal MDA levels were significantly higher in obese infertile group than other groups. Moreover, most semen parameters such as count, motility, viability, morphology, hypo osmotic swelling test (HOS), nuclear chromatin decondensation test (NCD) and acrosomal intactness test (AIT) showed negative correlation with BMI and the levels of seminal IL6 and MDA. Besides, BMI and smoking showed significant effect on IL6 and LPO levels.

Conclusion
We demonstratedelevated levels of seminal IL6 and LPO in obese infertile males. This increase may contribute to other biological pathway which lead to the decreased sperm quality andpossibly give rise toinfertility in obese infertile males and not obese fertile males.

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P312

Metallothionein gene expression in green mussels (*Perna viridis*) as a bioindicator for heavy metal on the North Coast of Java and its impact on the development of reproductive organs

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Jakarta Bay with 13 rivers that flow into, have experienced contamination and is the most polluted bays in Asia as a result of industrial and household waste. At this time, there are approximately five million kinds of chemicals that have been identified and have been known to 60 000 types of them have been used, even 1000 of other kinds and new chemicals freely traded every year. One of the B3 waste is heavy metals. Various ways have been conducted to measure the effects of environmental damage, one of them by measuring the level of contaminants in the environment or by the accumulation of toxic compounds in the tissues of organisms. Biological response, a biomarker is a sensitive indicator but need a relevant prediction. Protein metalotionin is a special bio-indicators of the heavy metals in the tissues. The nature of the toxic and hazardous heavy metals, indicated by the physical and chemical properties of materials, both quality and quantity. The entry of these wastes into the ocean waters has caused water pollution. In the cytoplasm of metal ions will bind to the protein metalotionin, so it accumulates in the cell. Thus metalotionin protein serves as a means for stockpiling metal detoxification. Shellfish can accumulate more metals than other aquatic animals and have a high tolerance to certain metals, so that these marine species can be used as an indicator to study the level of pollution in aquatic ecosystems. The ability to bind metal ions depends on metalotionin protein expression and regulation of its expression depending on the metal ions into the body. Result indicate that The pollution has an impact on reproductive organs mussels female and male, can even damage the organism on the cellular level and may affect the ecological balance.

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P313

Allopregnanolone promotes angiogenesis and inhibits apoptosis in the corpora lutea in rat ovarian cycle

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We report the effect of allopregnanolone (ALLO, 3α-hydroxy-5α-pregnan-20-one), on morphological changes and on angiogenesis in the rat ovary. ALLO, one of the best characterized neurosteroids, has effects on the reproductive female biology. First, we previously demonstrated that ALLO inhibited ovulation, secretion of LH and progesterone, increased the prolactin levels, inhibited the female receptivity and apoptosis in rat corpus luteum (CL). Now, we show the effect of ALLO on ovarian structures and angiogenesis 24 h after i.c.v. administration in cycling rats. Angiogenesis is regulated by several protein factors that are heavily regulated and proper vascularization of a tissue is dependent on a relationship between them.
Material and methods
ALLO 6 μM was injected intracerebroventricularly (i.c.v.) during proestrus. Morphometrical analysis and inmunohistochemistry of Von Willebrand (VW) and α-actin were performed on the estrous morning.
Results and discussion
By morphometrical studies of the ovaries, we observed an increase in the number of luteinized unrupted follicles in treated animals compared to controls ($P<0.001$). ALLO caused an increase in the VW factor and in α-actin protein ($P<0.01$ and $P<0.05$, respectively) promoting development and stability of blood vessels in the ovary. These results suggest that ALLO acts as an angiogenic factor and also as a survival factor in CL and follicles. This is the first report that shows the effect of ALLO on the vascular development and stability in the ovary. ALLO could be used as a molecule controlling inappropriate development of ovarian structures and highlighting their role in ovarian physiology.

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P314

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P315

Protein hormone proteolysis in target cell endosomes and lysosomes and release of previously unrecognized signaling information

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Organisms use protein hormones even when smaller molecules can trigger identical transduction paths. Why? Could residual peptides from hormone proteolysis extend the mechanisms of action of these hormones? Protein hormones are endocytosed by target cells and digested by cathepsins (A, B, C, D, F, H, L, O, X) acting sequentially as endosomes/lysosomes move centripetally. *In silico* prediction of multiple cathepsin action on each of 92 hormones usually leaves 3–24 residual peptides of 6–25 amino acids; post-translational modification maps suggest some predicted cleavages may be blocked *in vivo* so residual peptides may be even longer (the 30-residue hCGβ C-terminus may remain nearly intact). Stepwise application of cathepsins to hormones known to contain independently active peptides also suggests etiologies (cathepsins S and K release obestatin from GHrelin). Complete endosomal digestion of most protein hormones is unlikely so more secondary hormones (vasoinhibin from prolactin, preptin from IGFII) or peptides active in metabolically modulating cytoplasmic proteins in target cells are expected. To find transducer or modulator peptides and their cytoplasmic targets (peptide-motif-matched-proteins, PMMPs) BLASTp (1D matches) and LabelHash (3D matches) are applied; 1D matches with $E<0.05$ and 3D matches with $P<0.01$ for LRMSD for non-parent homologs are common with many located on PMMP surfaces. PMMPs used to seed network software (CytoScape, STRING) identify PMMP partners; peptide motif involvement in PMMP-partner binding is ascertained from known complex structures or docking software. Complexes that use peptide motifs dictate future bench tests for peptide actions. Present results suggest proteolytic peptides do extend protein hormone action mechanisms.

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P316

Deficiency of monoclonal nonspecific suppressor factor beta (MNSFβ) gene leaded to the early loss of pregancy in mice

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Introduction
Immune tolerance at the feto–maternal interface must be established during the processes of implantation and pregnancy. Monoclonal nonspecific suppressor factor beta (MNSFβ) is a secreted protein that possesses antigen-nonspecific immune suppressive function. The objective of this study was to establish the MNSFβ gene knockout mouse model, observe and analyze its reproductive phenotypes to further explore the roles of MNSFβ played in mouse embryo implantation and pregnancy.
Materials and methods
The MNSFβ gene knockout mice were constructed by the Cre/loxP system. The mice phenotypes were identified by PCR, western blot and immunohistochemical methods.
Results and discussion
The MNSFβ heterozygous mice ($MNSF\beta^{-/-}$), but not the MNSFβ homozygous mice ($MNSF\beta^{-/-}$), were obtained through $MNSF^{flox/flox}$ mice mated with EIIa-cre transgenic mice. $MNSF\beta^{-/-}$ murinereproductivity reduced and sex imbalance occurred possibly resulted from preferential fetal lethality in female offsprings. Expression of MNSFβ protein was barely detected in the $MNSF\beta^{-/-}$ murine thymus tissues. Further flow Cytometry analysis showed that, the distribution pattern of several immune cells and immune cells secreting four cytokines could

not be affected by the MNSFβ gene excalation. In an *in vitro* co-culture model of ICR mouse blastulas and MNSFβ$^{\pm}$ mouse endometrial stromal cells, it was found that the MNSFβ gene excalation could delay the time and process of the mouse blastulas implantation. So, it was proposed that MNSFβ gene was required for normal fertility and fecundity.

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P317

Analysis of marsupial ZP: molecular and phylogenetic approach

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Introduction
The zona pellucida (ZP) is an extracellular matrix that surrounds mammalian oocytes. Marsupial ZP has been considered formed by three proteins. However, the recent description of four glycoproteins (ZP1, ZP2, ZP3, and ZP4) in the ZP of some species of eutherian (rat, human, hamster, rabbit) suggests the need for a reanalysis of the ZP composition in marsupials.
Material and methods
Complementary DNA was obtained from ovaries from one Australasian marsupial (Bennett's wallaby) and genomic DNA was obtained from one Australasian marsupial (koala) and two South American marsupials (common opossum and Pinheiro's slender opossum). Specific primers for ZP1, ZP2, ZP3, and ZP4 were designed for PCR analysis. Different amplicons were obtained and automatically sequenced.
Results and discussion
In wallaby, PCR amplifications resulted in the complete amplification of the open reading frame (ORF) of ZP1 and ZP4 and partial amplifications of ZP2 and ZP3. In opossums the analysis of the amplicons obtained indicated that ZP4 present stop codons along its sequence. In Koala, ZP4 is free of stop codons. Wallaby ZP expresses four genes. Koala ZP expresses a functional ZP4, while in common opossum and Pinheiro's slender opossum ZP4 is not functional. These results strongly suggest the presence of a ZP4 pseudogene in the Didelphimorphia order, demonstrating that ZP composition is different in Australasian and South American marsupials.
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Transmission of lethal phenotype in a Mendelian fashion by genetically modified pigs that underwent blastocyst complementation

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In our previous study, we demonstrated that pigs expressing neonatally lethal phenotype induced by genetic modification could be rescued by induction of chimerism by using blastocyst complementation. Here, we show that the obtained chimeric pigs can faithfully transmit the genotype, which is the cause of the lethal traits, to progenies.
Cloned embryos (male) of pigs showing the phenotype of pancreatogenesis deficiency by expression of the *Pdx1-Hes1* gene were complemented with cloned embryos derived from three types of female pigs (humanized Kusabira-Orange transgenic, Pdx1-Venus transgenic, and coat colored WT) to produce six chimeric boars.
Except for one, all chimeric boars grew normally and reached sexual maturity. A total of 120 fetuses and piglets were produced by mating the five chimeric boars with 12 WT females. Analysis of 82 fetuses (day 47–109) and 38 piglets showed that 61 (50.8%) were Pdx1-Hes1 transgenic, which inherited the phenotype of pancreatogenesis deficiency. The average birth weight of the transgenic piglets was significantly lighter than that of the non-transgenic piglets (906.5 ± 40.8 g vs 1247.1 ± 41.7 g; $P < 0.01$). All transgenic piglets showed a high glucose level of 400 mg/dl or more, and died by several days. Our results demonstrated that the chimeric boars produced by blastocysts complementation of *Pdx1-Hes1* transgenic-cloned embryos sired fetuses/piglets with the apancreatic phenotype in a Mendelian fashion. Thus, fetuses/piglets expressing lethal traits such as organogenesis deficiency are potential and powerful tools for research on regenerative medicine. This study was supported by JST, ERATO, Nakauchi Stem Cell and Organ Regeneration Project, and Meiji University International Institute for Bio-Resource Research (MUIIBR).

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P319

Reproductive and toxic effects of methanol extract of *Alchornea cordifolia* leaves

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Alchornea cordifolia is commonly used as a medicinal plant throughout its area of distribution in Africa including Nigeria and Democratic Republic of Congo. The leaves are traditionally used for the treatment of veneral diseases. Using 36 male albino rats, randomly divided into six groups of six rats each, the effects of oral administration of the methanol extract of the leaves of *A. cordifolia* on some reproductive and haematological parameters of male rats were investigated at 0, 100, 200, 400, 800, and 1600 mg/kg. The values of total and differential white blood cell in the control and test animals were similar at all treatment doses. The erythrocyte count, packed cell volume, haemoglobin concentration and haematmetric indices except mean corpuscular volume decreased significantly ($P < 0.05$) at the 1600 mg/kg dose compared with the control. Markers of hepatic damage (alanine and aspartate transferases) and renal damage (urea and creatinine) were significantly elevated ($P < 0.05$) at 800 and 1600 mg/kg. Although, significant increases were observed in testicular weight, spermatozoa count and motility, and serum testosterone levels, at all treatment levels, severe deleterious effects were not recorded on the liver, kidney and testis at doses below 400 mg/kg. The study concludes that, the extract of *A. cordifolia* leaves, though apparently toxic on haematological parameters, the liver and the kidney in high doses may have beneficial effects on male reproduction when used at maximum dose of 400 mg/kg.

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P320

Pinacidil, a K_{ATP} channel opening drug, protects human oocytes against metabolic stress

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Introduction
The primary treatment of infertility is assisted reproductive technology (ART). However, removal of an oocyte from its natural environment and exposure to *in vitro* conditions diminishes oocyte quality during the process of ART. In our preliminary studies we have shown that oocytes express cytoprotective ATP-sensitive K^+ (K_{ATP}) channels. Therefore, the aim of our study was to elucidate whether activation of these channels protects human oocytes against metabolic stress.
Materials and methods
Supernumerary human oocytes in different stages of maturation were obtained from patients undergoing IVF and ICSI procedures. Cells were stained with Ca^{2+}-sensitive dye Fluo-3 and continuously monitored for 120 min using laser confocal microscopy in the presence and absence of pinacidil, a K_{ATP} channel opener, and 2,4 dinitrophenole (DNP), an inhibitor of oxidative phosphorylation.
Results and discussion
We have found that intracellular Ca^{2+}, an indicator of the metabolic state of cells, increases over time when oocytes are left incubating *in vitro* showing that *in vitro* conditions are stressful for oocytes. Pinacidil (100 μM) significantly decreased the rate of Ca^{2+} increase. To induce more severe metabolic stress, we exposed oocytes to DNP (100 μM). DNP induced significant increase in intracellular Ca^{2+}. This effect of DNP was inhibited by pinacidil (100 μM) suggesting that the activation of K_{ATP} channels protects oocytes against stress. Consequently, a K_{ATP} channels-based strategy that would reduce cellular stress could have a potential to significantly improve the success rate of assisted conception by improving the quality of oocytes used for this purpose.

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P321

Fetal overexposure to androgens, not estrogens, leaves a permanent legacy in terms of adrenal function in offspring

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Introduction
Overexposure to androgens during fetal life creates offspring with a polycystic ovary syndrome (PCOS)-like phenotype. Whether this phenotype is attributable to androgens during fetal life, or estrogens via maternal/placental metabolism, remains uncertain. The potential for *in utero* steroidal disruption of adrenal development and function is also unclear. We have examined the adrenal during development and adulthood in response to *in utero* androgenic and estrogenic over-exposure.
Methods
Ovine female fetuses were injected with 20 mg of testosterone propionate (TP) or diethylstilbesterol (DES) at days 62 and 82 of gestation, then sacrificed at day 90. A subset were carried to term and conventionally reared until 1 year old. Steroidogenic gene expression (qRT-PCR) was assessed in fetal and postnatal adrenals and cortisol and testosterone secretion in response to a Synacthen challenge was determined by ELISA and RIA respectively.
Results and discussion
During fetal life DES increased *CYP11B1* mRNA; $P<0.05$. During adulthood, prenatal TP was associated with elevated mRNA concentrations of *STAR*, *CYP11B1*, *CYP21A*, *HSD3B1*, and *HSD17B1* ($P<0.05$), whereas fetal DES treatment was associated with decreased ACTH-receptor, *CYP11A1* and *HSD17B1* mRNA expression ($P<0.05$). Cortisol secretion in response to Synacthen stimulation was unaffected by either prenatal treatment, and DES had no effect on testosterone secretion. However, adrenal testosterone secretion was elevated 30 min post stimulation in animals prenatally exposed to TP (untreated: 0.46 ± 0.18 vs prenatal TP: 1.538 ± 0.18 ng/ml; $P<0.0001$). Female fetal androgen over-exposure is associated with increased adrenal androgen secretion in offspring, supporting fetally programmed adrenal hyperandrogenism in PCOS.

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P322

Inhibition of the TLR4 signalling pathway protects from intrauterine heat-killed *Escherichia coli*-induced pre-term delivery

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The pathophysiology of preterm labour is poorly understood and the causal factors uncertain, but inflammatory mechanisms are clearly implicated. *Escherichia coli* contain lipopolysaccharide, a pathogen-associated molecular pattern (PAMP) which binds Toll-like receptor 4 (TLR4) and related TLR-associated receptors to activate inflammation and induce preterm delivery (PTD). This project seeks to investigate whether inhibition of the TLR4 signalling pathway using small molecule inhibitors of TLR4 signalling may prevent the parturition cascade caused by heat-killed *E. coli*-induced inflammation. The effect of administration of TLR4 inhibitors in preventing heat-killed *E. coli*-induced PTD was investigated in C57Bl/6 (B6) mice.
Pregnant B6 females were administered intrauterine heat-killed *E. coli* or PBS, with or without co-administration of TLR4 inhibitor, on gestational day (GD) 16.5 and were either killed 4 h later for RT-PCR analysis on the gestational tissues, or allowed to deliver pups.
Heat-killed *E. coli*-induced PTD was successfully alleviated using TLR4 inhibitors in B6 mice, preventing fetal loss associated with death *in utero* and/or early delivery, resulting in on-time birth with normal perinatal characteristics and survival rates in pups. These results indicate that TLR4 is required for the precocious activation of the inflammatory response induced by heat-killed *E. coli* and implicates TLR4 as a key trigger for infection-associated preterm labour. Early intervention with TLR4 inhibitors can inhibit progression of the LPS-induced inflammatory cascade and prevent preterm birth without adverse perinatal or postnatal consequences in mice. The TLR pathway warrants further investigation as a potential target for new prevention or treatment options in women with infection-associated, threatened PTD.

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P323

Effect of doses and time post-injection on hormonal induction of spermiation in *Bufo calamita* (Anura: Bufonidae)

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Introduction
Currently, one-third of amphibians species are threatened with extinction and assisted reproductive technologies (ART) play an important role in their genetic management. The first step in developing ART is to create protocols for obtaining gametes. The aim of this study was to develop a protocol for hormonal induction of spermiation in the Natterjack toad (*Bufo calamita*).
Materials and methods
Males were divided into three groups ($n=5$/group) and each group was given an i.p. injection of 5, 10, or 15 IU hCG/g bodyweight. Spermic urine was collected from all males before the treatment and every hour post-treatment (up to 9 h) and sperm quality was assessed.
Results and discussion
None of the 15 males produced sperm before the hormone treatment. Results showed that all males treated with 10 and 15 IU/g of hCG released sperm, while only 40% of the males responded to 5 IU/g. There was no effect of hormone dose on sperm parameters, while time post-treatment did affect spermatozoa. The total percentage of moving sperm, percentage of sperm moving forward and quality of motility was higher from 1 to 4 h post-treatment with 10 IU hCG and from 1 to 6 h post-injection with 15 IU hCG ($P\leq0.044$). Concentration of spermatozoa was lower in the first collection. Therefore, 15 IU of hCG should be used when sperm is going to be collected over a longer period of time for artificial fertilization. Here we describe for the first time a non-invasive protocol for spermatozoa collection from *Bufo calamita,* which may be important for future conservation efforts in this species.

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P324

Effects of early exposure to bisphenol A, indole-3-carbinol, and/or genistein and the prostatic development in male rats offspring

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Bisphenol A (BPA) has been investigated for its potential carcinogenic activity for prostate. Studies have shown that indole-3-carbinol (I3C) and genistein (GEN) can activate antiproliferative and proapoptotic signaling pathways. The aim of this study was evaluate the influence of I3C (20 mg/kg) and GEN (5 mg/kg) on the development of prostate of offspring rats exposed during gestation and lactation to BPA (10 mg/kg). Pregnant female Sprague–Dawley rats were divided into five groups: G1: control; G2: BPA; G3: BPA + GEN; G4: BPA + I3C + GEN; and G5: BPA + I3C. All treatments were performed by gavage from gestational day 17 until postnatal day 21. After weaning, ten male rats/group were euthanized by decapitation. Histological sections of the ventral prostate were stained with H&E, picrossírius, reticulin, and the collagen content was quantified by LeicaWin Software. The prostate histological analysis showed numerous small acini with open and regular lumen and less crimped in G1 and G2. The groups G3 to G5 exhibited acinar epithelium more folded than G1 and G2. The acini epithelium showed to be predominantly high single cylindrical cells and delicate fibromuscular stroma. Reticular fibers were thin and continuous and surround the prostatic acini, delimiting basically the basal membrane of epithelial cells. The collagen content was significantly higher in G2 when compared to the other groups, while the G5 group showed reduction when compared to G1, as well as G4 in relation to G3 respectively. The proportion of immunoreactive epithelial cells to androgen receptor (AR) decreased significantly in G5 compared to G1 and G2. BPA exposure influenced the normal prostate development, and I3C appears to reduce partially their effects.

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P325

Chemical fingerprinting of embryotoxic mineral oil

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Introduction
Mineral oil is widely used to culture embryos in biomedicine, agriculture, and embryo/stem cell research, yet is poorly defined with significant batch variation causing detrimental effects. The highly hydrophobic nature of this petroleum product attracts polycyclic aromatic hydrocarbons (PAH) and other potentially harmful compounds. Industry requirements for screening oil utilize methods that lack the sensitivity necessary to avoid oil that is overtly embryotoxic or covertly affects embryo development or stem cell derivation. The objective of these experiments was to determine the chemical fingerprints of embryotoxic mineral oil using advanced analytical methods. GC×GC TOFMS is an emerging analytical technique for determining a wide range of environmental contaminants from a single sample. This separation and analysis technique is able to detect sub-pg/l concentrations of ecotoxic contaminants such as PCBs and PAHs.
Materials and methods
Mineral oil with known embryotoxicity identified with time-lapse imaging of mouse embryos was studied. A GC×GC-TOFMS installed with a Rtx-PCB (60 m×0.18 mm×0.18 μm) in the first dimension and Rxi- 17 (1.5 m×0.1 mm×0.1 μm) column in the second dimension was used to separate unknowns. Principal component analysis of data was performed to differentiate the two samples.
Results and discussion
Thousands of unique compounds were identified and quantified in each sample from many classes of chemicals including known environmental toxins. Fingerprinting showed clear differences in the signature of the two oils. Further studies are underway to determine embryotoxicity of individual contaminants as well as development of methods for routine screening of mineral oil quality.

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P326

Abstract withdrawn.

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P327

Establishment of immortalized neuronal cell lines derived from fetal goat hypothalamus

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Neurons essential for reproductive functions are distributed in hypothalamus. The precise functions of these neurons are not fully analyzed at a cellular level, especially in domestic animals. To analyze cellular mechanisms that control ruminant reproduction, we generated neuronal cell lines by immortalizing fetal goat hypothalamus.
The hypothalamic arcuate nucleus was collected from a female Shiba goat at E118. The tissue was dispersed and plated. Twenty-four hours after the primary culture, lentiviral vector containing SV40 large T antigen gene (*T-Ag*) was added, then cells were selected by G418. We performed cell cloning from the obtained cell population, and analyzed gene expressions of each clone by RT-PCR. The expression of neuronal marker (neuron specific enolase (NSE)), glial marker (glial fibrillary acidic protein (GFAP)), *T-Ag*, and *GAPDH* were examined.
The primary-cultured cells acquired strong proliferative activity after the viral infection. All clones expressed *T-Ag*, suggesting that these cells were immortalized by the insertion of *T-Ag*. We obtained 61 clones from the immortalized population. Thirty-six out of 61 clones were *NSE*-positive and *GFAP*-negative, which were defined to be neuron-derived cell lines.
In summary, we established a number of neuronal cell lines derived from goat hypothalamus. Further analysis of expression of neuropeptides or receptors related to reproduction is required to determine neuronal cell lines appropriate for examining central mechanism regulating reproduction *in vitro*.

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P328

Development of novel neurokinin-3 receptor agonists that potently stimulate the GnRH pulse generator

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Introduction
Pulsatile GnRH secretion is a key determinant for normal gonadal activities. Recent emerging evidence suggests that neurokinin B (NKB) plays a pivotal role in generating GnRH pulse. Here, we aimed to develop novel compounds with potent NKB receptor (NK3R) agonistic activity for future therapeutic applications to improve gonadal functions.
Materials and methods
On the basis of the structure-activity relationship study of NKB and tachykinin peptides, we designed and synthesized 72 compounds. Their binding affinity and agonistic activity for G_q-coupled NK3 receptor were evaluated by an *in vitro*-binding inhibition assay and an intracellular Ca^{2+} flux assay respectively. Actions of the peptides on the GnRH pulse generator were evaluated by the multiple-unit activity (MUA) recording method in ovariectomized goats, in which the electrophysiological manifestations of the GnRH pulse generator activity can be represented as characteristic increases in MUA (MUA volleys). Thirty nanomoles of each peptide was i.v. administered at the mid-point of two successive endogenous MUA volleys occurring with a constant interval of ~25 min. The given peptide was assessed to stimulate the GnRH pulse generator when the next MUA volley occurred within 240 s after administration.
Results and discussion
Eight compounds were found to exhibit superior *in vitro* activity than senktide, a classical NK3R agonist, and induce an MUA volley after administration. Because those compounds were capable of stimulating the GnRH pulse generator by peripheral administration, they might be potential candidates for novel agents to improve reproduction acting centrally.

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P329

Changes in gene expressions induced by perinatal estrogen related to the brain sexual differentiation in rodents

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Brain mechanism responsible for LH surge generation shows sexual dimorphism in rodents. The dimorphism is considered to be due to the perinatal estrogen converted from testicular androgen during developing period. The present paper aimed to clarify the mechanisms mediating the estrogen-induced defeminization of LH surge-generating system. The microarray analysis is an attempt to obtain the candidate gene(s) mediating the perinatal estrogen action causing the brain sexual differentiation. Female mice were injected with estradiol benzoate (EB) or vehicle on the day of birth, and the hypothalamus was collected at either 1, 3, 6, 12, or 24 h after the EB injection. More than 100 genes down-regulated by the EB treatment in a biphasic manner peaked at 3 h and 12–24 h after the EB treatment, while 40–70 genes were constantly up-regulated after the treatment. *Ptgds,* encoding prostaglandin D2 (PGD2) synthase, was chosen for further examination by semiquantitative RT-PCR in the anterior hypothalamus of neonatal male and female rats. *Ptgds* expression was significantly lower in the anterior hypothalamus of male rats than females at the day of birth. PGD2 have been reported to play a role in neuroprotection, suggesting that *Ptgds* could be one of the possible candidate genes, which may mediate the effect of perinatal estrogen responsible for sexual differentiation of the LH surge-generating system.

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P330

Metabolic reprogramming may underpin the aetiology of endometriosis

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Transforming growth factor beta (TGFβ) can induce The Warburg-effect, or aerobic glycolysis, in tumorigenesis. Energy-rich lactate, a by-product of glycolysis, 'feeds' tumour cells and increases apoptosis resistance, angiogenesis, and invasion, fuelling tumour progression and metastasis. As TGFβ is increased in the peritoneal fluid of women with endometriosis, we asked, is there a Warburg-like effect at play in endometriosis.

Peritoneal fluid (PF) (n = 16), peritoneal mesothelial cells (PMC) (n = 6), peritoneum, endometriosis lesions, and endometrium biopsies were collected from women with/without endometriosis. TGFβ1 and lactate were assayed in PF. Glycolic-pathway enzyme expression in tissue biopsies was determined by qRT-PCR and immunohistochemistry. PMC were exposed to physiological concentrations of TGFβ1.

TGFβ1, HIF1α and its target genes; *LDHA* and *PDK1* were significantly increased in ectopic compared to eutopic endometrium ($P < 0.05$). Peritoneum adjacent to endometriosis lesions expressed significantly higher levels of TGFβ1, HIF1α, and GLUT1 ($P < 0.05$) and these proteins were localized to the PMC. PF of women with endometriosis had significantly higher TGFβ1 ($P < 0.05$) and lactate ($P < 0.05$) (positive correlation). Exposure of PMC to TGFβ1 increased lactate levels ($P < 0.05$) and HIF1α protein. GLUT1 LDHA and PDK1 were all significantly increased on exposure to TGFβ1 in PMC ($P < 0.05$).

We have shown that ectopic endometrium may have an altered metabolism induced by TGFβ1 which may explain endometriosis lesion development. Increasing lactate within the PF and by ajacent peritoneum may 'feed' lesions, establishing an integrated endometrosis niche. Therapies which inhibit glycolosis may provide potential treatments for endometriosis.

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P331

Deletion of the C-terminal region of OVGP1 affects porcine IVF

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Introduction

The female reproductive tract provides the optimal environment for gamete interaction and embryo development. Understanding the oviductal environment is important for reproducing the *in vivo* conditions *in vitro*. OVGP1 is the major non-serum oviductal protein with a variable protein activity depending on its regions. To investigate the potential role of the OVGP1 regions on its activity, full-length (prOVGP1, 527aa) and C-terminal truncated (prOVGP1TR, 481aa) recombinant histidine-tagged porcine OVGP1 proteins were expressed in mammalian cells.

Material and methods

Secreted proteins were purified by immobilized-metal affinity chromatography followed by size exclusion chromatography. Media from transfected cells were incubated with *in vitro*-matured (IVM) porcine oocytes. After 1 h, oocytes were used to measure the resistance of zona pellucida (ZP) to pronase digestion and imaged by confocal microscopy using anti-His antibody. In a second experiment, purified prOVGP1TR protein was incubated with IVM oocytes for 1 h. Oocytes were washed and transferred to fresh IVF medium for insemination.

Results and discussion

Recombinant proteins were detected by fluorescent signal bound to ZP. The ZP resistance to proteolytic digestion increased in the oocytes incubated with prOVGP1TR protein compared with prOVGP1. The oocytes exposed to prOVGP1TR showed a significant increase in the percentage of monospermy and a decrease in the percentage of penetration compared with control oocytes. These results reveal that preincubation of oocytes with truncated OVGP1 protein not only increases ZP resistance to proteolysis but also affects sperm-penetration ability suggesting that C-terminal region of OVGP1 modifies protein function.

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P332

Androgen receptor-signalling in the male pituitary is dispensable for normal LH secretion but suppresses prolactin production

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Production of testosterone by testicular Leydig cells is under tight regulation by the hypothalamic – pituitary – gonadal (HPG) axis. GnRH, secreted by the hypothalamus, stimulates secretion of LH from the pituitary. LH will then stimulate testosterone production in the Leydig cells, which will feedback to the hypothalamic-pituitary element to inhibit LH secretion.

Previously it was accepted that testosterone exerted its negative feedback actions on the pituitary by binding to androgen receptor. However, several recent clinical and experimental studies have suggested that androgen-AR signalling in the pituitary may be redundant for the control of LH secretion, and that feedback may act via testosterone conversion to estradiol.

To investigate the possible role(s) of AR in the male pituitary, we have developed a unique transgenic mouse line where the androgen receptor is conditionally ablated in the pituitary (PARKO). Initial analysis demonstrated that LH transcript levels, as well as LH and testosterone circulating levels remain unchanged in PARKO males. Aromatase transcript was not found in the pituitaries of either control or PARKO males, however surprisingly both pituitary prolactin transcript and circulating prolactin were significantly increased.

Results suggest that inhibition of LH by testosteroneis not acting at the level of the pituitary in the mouse. If pituitary feedback is via estrogens, then production must be at a site other than the pituitary. In contrast these results suggest that a role of pituitary AR-signalling is to negatively regulate prolactin production, however further study is necessary to elucidate this mechanism.

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P333

Selective expression of two DAZL isoforms in human fetal ovary but not testis

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Introduction

Deleted in azoospermia-like (DAZL) is an RNA-binding protein essential for germ cell entry into meiosis and later stages of germ cell maturation, and its absence is associated with infertility in vertebrates. Efforts to identify *in vivo* mRNA targets of DAZL have mainly been restricted to mouse, thus human-specific investigations are required.

Materials and methods

RT-qPCR and western blotting were used to profile DAZL isoform expression in human fetal ovary and testis. Human DAZL protein was immunoprecipitated from 17-week ovarian lysate; bound RNAs were analysed by RT-qPCR.

Results and discussion

Two isoforms of DAZL differing in the 5′UTR and N-terminus coding sequence are reported in NCBI. mRNA for DAZL isoform 2 (the original isoform) was predominant over DAZL isoform 1 in fetal gonads of both sexes, and its expression increased across gestation in the ovary but not testis. DAZL isoform 1 protein was detected in both fetal gonads, whilst isoform 2 was absent in the testis. RT-qPCR of DAZL-immunoprecipitated ovarian RNA demonstrated enrichment of murine DAZL targets *VASA*, *TEX14*, and *SOX17*.

We have identified the expression of a DAZL isoform not previously reported in human gonads. The selective expression of DAZL isoform 2 in fetal ovary may suggest it is this isoform that is involved in meiotic control of ovarian gametogenesis. Specific functional roles of DAZL isoform 1 remain unknown. Preliminary data confirm *VASA*, *TEX14*, and *SOX17* mRNAs as targets of human DAZL, as in mouse.

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P334

Development of knock-in somatic cells to produce human FGF2 protein on the bovine β-casein gene locus using F2A self-processing peptide

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Introduction
The production of recombinant protein in transgenic domestic animal is one of the major successes of biotechnology. Knock-in system is a more powerful method to produce mammary gland bioreactor. This study is conducted to development of knock-in somatic cells using bovine β-casein genome for produce of biological substance.
Material and methods
The knock-in vector was constructed by using 5.9 kb fragment of upstream of bovine β-casein genome containing exons 1, 2, and 3 as the left arm and using ~2.08 kb of 3′ region containing bovine β-casein exons 4, 5, and 6 as the right arm. Human *FGF2* gene was subcloned in the bovine β-casein gene exon 3 and the gene was linked with F2A self-processing sequence. Neo gene was used as positive selection marker and diphtheria toxin A (DT-A) gene used as negative selection marker. For identification of human FGF2 expression in the knock-in vector, human FGF2 knock-in vector was transfected into HC11 cell line and the mRNA of bovine β-casein-hFGF2 fusion gene was analyzed by RT-PCR. For transfection, linearzed knock-in vectors were introduced into bovine ear fibroblasts by electroporation. After 72 h, the cells were selected with G418 during 11 days. The G418-resistant colonies were picked and analyzed by PCR.
Result and discussion
The *hFGF2* mRNA was detected in the HC11 cell transfected with knock-in vector by RT-PCR. The 504 G418 resistant colonies were screened by PCR and four colonies were occurred homologous recombination. These cells may be used to production of knock-in transgenic bovine by somatic cell nuclear transfer.

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P335

Neurokinin B activates synchronized intracellular Ca^{2+} oscillations in KNDy neurons obtained from the hypothalamic arcuate nucleus of *Kiss1*–GFP transgenic mice

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Pulsatile secretion of GnRH/LH is indispensable for puberty onset and normal reproductive functions in mammalian species. A cohort of neurons expressing three neuropeptides, kisspeptin, neurokinin B (NKB), and dynorphin (KNDy neurons), localized in the hypothalamic arcuate nucleus (ARC), are considered to be a source of GnRH pulse generator. A synchronous discharge of KNDy neurons might be obligatory for pulsatile GnRH secretion. The present study aimed to determine whether NKB–NK3R signaling is required for synchronized activities in KNDy neurons.KNDy–GFP cells taken from the ARC of the *Kiss1*–GFP transgenic mice embryos at days 17–18 of embryonic age were cultured on a glass-base dish for 3–6 weeks. Intracellular Ca^{2+} concentrations were measured in individual KNDy–GFP cells, which were superfused with/without 1 μM senktide, a NKB receptor agonist, using the fluorescent Ca^{2+} indicator Fura-PE3. Frequent and synchronized intracellular Ca^{2+} oscillations were observed in cultured KNDy–GFP cells with senktide, whereas few Ca^{2+} oscillations were found without senktide. The senktide-induced Ca^{2+} oscillations were synchronized in the neighboring KNDy–GFP cells. Such Ca^{2+}oscillations were abolished by chelating extracellular Ca^{2+} with EGTA, suggesting that Ca^{2+} oscillations are caused by an influx of extracellular Ca^{2+} through the calcium channels in KNDy–GFP cells. These results indicate that NKB–NK3R signaling facilitates synchronized activities in neighboring KNDy neurons. This work was supported in part by the Research Program on Innovative Technologies for Animal Breeding, Reproduction, and Vaccine Development.

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Autophagy supports the survival of the conceptus obtained by assisted reproductive technologies

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Assisted reproductive technologies (ARTs) are generally considered to be safe, but recent studies suggest an excess of negative pregnancy outcomes. The mechanisms responsible for adverse events share similar condition like abnormal placentation, which finally leads to impaired embryo growth due to limited nutrient uptake. High autophagy activation was reported in placentae obtained from normal and compromised pregnancies. Given the evidence of placental abnormalities in pregnancies obtained by *in vitro*-protocols, the aim of this work was to investigate if autophagy influences the development of *in vitro*-produced (IVP) sheep foetuses. We have compared early sheep placentae collected after natural mating (CTR) with placentae obtained following transfer of IVP embryo. Our ultrastructural (TEM) and molecular (qPCR) analysis revealed a high proportion of autophagic cells and an upregulation of autophagic markers in early IVP placentae. The increased autophagy activation may be attributed to a compensatory mechanism resulting from placental self-adaptation to the limited nutrient uptake associated with the application of ART.

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Gonadotropin-independent follicle development in the *Kiss1*$^{-/-}$ female rats

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Introduction
Kisspeptin, encoded by *Kiss1*, is a neuropeptide that directly regulates GnRH secretion from the hypothalamus. The present study aimed to examine a relationship between the circulating gonadotropin levels and follicle development using the *Kiss1* knockout (*Kiss1* KO) adult female rats.
Materials and methods
Kiss1 KO and WT animals were obtained by mating of *Kiss1*$^{+/-}$ males and females. The plasma was daily collected to determine gonadotropin levels for 4 days. The follicle development was examined by morphometric analysis for serial ovarian sections, and reproduction-related gene expressions were determined by *in situ* hybridization and real-time PCR.
Results and discussion
While plasma LH and FSH levels of the WT rats were fluctuated with a peak at proestrous afternoon, those of *Kiss1* KO rats were undetectable throughout the consecutive 4 days. *Kiss1* KO ovaries were smaller than WT ovaries, and secondary interstitium were predominantly observed in the sections. Although some antral follicles attained the maximum diameter nearly 300 μm, most of follicles sized more than 200 μm showed signs of atresia in *Kiss1* KO ovaries. The secondary interstitium and theca cells expressed LH receptor (LHR) mRNA in *Kiss1* KO ovaries. The granulosa cells in the antral follicles expressed *FSHR* mRNA, however, no follicles attained developmental stages where the granulosa cells expressed *LHR* mRNA. Since gene quantification suggested that *Kiss1* KO ovaries expressed more mRNAs encoding inhibin α and inhibin/activin βA than WT ovaries, such paracrine regulators could develop follicles to antral follicle stage without gonadotropins.

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Hypothalamic arcuate nuleus-specific enhancer for kisspeptin expression of female mice

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Two populations of kisspeptin neurons, located in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV), are considered to be involved in generating GnRH pulse and surge respectively. The present study aimed to determine the region-specific enhancer for ARC *Kiss1* gene expression by *in vivo* reporter assay using transgenic (Tg) mice. Three GFP reporter constructs (long-, middle-, and short-length) were generated by insertion of GFP cDNA in the translational start site of *Kiss1* gene in the mouse BAC clone. The DNA constructs were microinjected into the pronucleus of fertilized one-cell stage embryos of BDF1 mice. GFP expressions in the hypothalamus were validated in ovariectomized (OVX) Tg female mice in the presence or absence of estradiol-17β. Tg lines bearing long- and 5′-truncated middle-length constructs showed GFP-immunoreactivities in both AVPV and ARC in female mice. On the other hand, Tg mice bearing 5′-truncated short-length construct showed few GFP signals in the ARC kisspeptin neurons, while the Tg line showed GFP-immunoreactivities in the AVPV kisspeptin neurons. Chromosome conformation capture assay showed an interaction between the promoter and 5′ region of *Kiss1* locus in the ARC, but not in the AVPV. Taken together, these results suggest that the upstream region of *Kiss1* locus is essential for ARC *Kiss1* gene expression in mice via formation of chromatin loop with the proximal promoter of *Kiss1* gene. This work was supported in part by the Research Program on Innovative Technologies for Animal Breeding, Reproduction, and Vaccine Development.

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P340

Proteome-wide changes in liver function by continuous exposure to sewage sludge

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Introduction
A complex cocktail of poorly biodegradable chemicals is ubiquitous in the modern environment. Exposure to such chemicals contributes to diseases such as metabolic syndrome and infertility. Since the liver is the primary defence organ against xenotoxicants, we analysed the liver proteome of sheep continuously exposed (from pre-conception until early adulthood) to sewage sludge. Our aim was to identify dysregulated pathways and understand how a chemical cocktail can predispose for disease.
Materials and methods
Liver protein extracts from adult sheep that grazed on control or sewage sludge-fertilised pastures throughout their lives (gestation and lactation via the mother and post-weaning grazing) were divided in four groups ($n=10$–12/group) according sex and treatment. Proteins were resolved using 2D differential in gel electrophoresis and compared using SameSpots Software. Differentially expressed protein spots were identified by tandem liquid chromatography/mass spectroscopy (LC–MS/MS).
Results and discussion
Out of 445 gel spots, 229 were significantly different among the four groups. Sewage sludge exposure grossly affected the liver proteome by altering 193 spot volumes in male and/or female sheep compared to controls. 134 spots were differentially expressed by sex in controls, while exposure induced or abolished sex differences in 102 spots. We identified 20 proteins primarily involved in metabolic pathways relating to fatty acid metabolism (ACAA2, and ECHS1), steroid hormone synthesis/metabolism (APOA1, SCP2, and HSD17β10), and iron biology (TF, FLT, and HBA1/HBB). Our results show that life-long exposure to complex cocktails of everyday chemicals alters liver function, potentially laying the foundations for modern diseases.
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Determination of expression of MFGE8 in porcine oviduct and uterus

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Introduction
Billions of sperm are deposited in female tract during porcine insemination but only a few thousands are able to reach the oviduct to facilitate fertilization. It has been reported that some of the sperm membrane proteins involved in fertilization are secreted by epididymis (i.e. SPAM-1, CRISP, and MFGE8). It was observed that the mouse oviduct is able to secrete some of these proteins. Some of them are implicated in fertilization since sperm fertility of KO mice (CRISP) is rescued during the IVF. The aim of this study was to analyze the expression of MFGE8 in porcine oviduct and uterus.
Material and methods
RNA was isolated from the ampullary region of the porcine oviduct ($n=7$) and uterus ($n=3$). cDNA was synthesized with oligo-dT as primer and was used as template for PCR amplifications using primers based on GenBank sequence of MFGE8 in *Sus scrofa* (NM_001076439).
Results and discussion
PCR amplifications resulted in the obtention of one amplicon in oviduct (145 bp) and other one in uterus (252 bp) corresponding to the MFGE8 transcript as corroborated by automatic sequencing.This study demonstrates for the first time the existence of mRNA codifying for MFGE8 in porcine oviduct and confirms its presence in the uterus. Future studies are necessary to investigate the implication of this protein in the sperm maturation in the female genital tract and its contribution to the sperm–ZP interaction in the oviductal environment. This work was supported from the MINECO (AGL2012-40180-C03-02), FEDER/ERDF, and Fundación Séneca (04542/GERM/06).

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Mechanisms underlying pituitary microvascular remodelling in thoroughbred horses during the annual reproductive cycle

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In photoperiodic species, remodelling of pituitary microvasculature appears to play a key role in the regulation of the annual reproductive cycle. Recent studies have revealed seasonal changes in the vascular loops of the ovine pituitary stalk. Microvasculature remodelling is under the control of pro-angiogenic ($VEGF_{165}$a) and anti-angiogenic ($VEGF_{165}$b) isoforms of vascular endothelial growth factor (VEGF). Here, we examined the differential expression of these VEGF isoforms in the pars tuberalis (PT) and pars distalis (PD) of the pituitary gland of gonadal intact (stallions) and orchidectomised (geldings) thoroughbred horses, during the breeding season (BS) and non-BS (NBS). VEGF isoform expression was investigated by double immunohistochemistry. Statistically significant effects of season, gonadal status, and the interaction between them were observed in both regions of the pituitary. In stallions, an increase in $VEGF_{165}$a was detected in the PD during the NBS ($P<0.01$). Conversely, the expression of $VEGF_{165}$b in this region was increased during the BS ($P<0.01$). Similar changes were observed in the PT. Critically, robust seasonal effects on VEGF isoform expression were detected in the PD of geldings, with significant changes in $VEGF_{165}$a opposite to those recorded in intact animals, i.e. increased expression in the BS ($P<0.01$). In these animals, alterations in VEGF isoform in the PT were also apparent, with increased expression of $VEGF_{165}$a in the BS ($P<0.01$). The results show that a mechanism for vascular remodelling in the equine pituitary is highly sensitive to seasonal regulation, and that this mechanism is modulated by gonadal feedback.

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Lack of gonadotropin release in *Kiss1* knockout male rats

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Kisspeptin, encoded by *Kiss1* gene, has attracted attention as a key neuropeptide in controlling reproduction in mammals. Earlier studies with *Kiss1* or its cognate receptor *Gpr54* knockout (KO) mice showed the indispensable role of kisspeptin–GPR54 signaling on reproduction, but detailed analysis of gonadotropin release were still limited. Here we have generated *Kiss1* KO rats to evaluate a role of kisspeptin on pulsatile gonadotropin secretion in males. The targeting vector was constructed by replacement of the last two exons (exon 2 and 3) of *Kiss1* gene with tdTomato reporter gene. *Kiss1* KO male rats showed no kisspeptin-immunoreactivity in the hypothalamic arcuate nucleus, while they showed tdTomato fluorescences in the nucleus. *Kiss1* KO male rats showed normal growth, but they failed to show testicular descent and balano-preputial separation as external signs of puberty onset. Plasma FSH, LH, and testosterone concentrations were almost undetectable in testis-intact *Kiss1* KO rats. The plasma FSH and LH levels were also undetectable in *Kiss1* KO rats even after gonadectomy. The testis was significantly smaller in *Kiss1* KO rats compared to the WT littermates. Frequent blood sampling definitely exhibited no pulsatile LH secretion in gonadectomized *Kiss1* KO rats. Thus, the present results indicate that kisspeptin is indispensable for pulsatile gonadotropin release to control reproduction in male rats. This study was supported in part by a grant from Research Program on Innovative Technologies for Animal Breeding, Reproduction, and Vaccine Development.

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The gender-specific hormone INSL3 demonstrates inter-fetal transport of hormones between male and female fetuses

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Introduction
The peptide hormone insulin-like peptide 3 (INSL3) is a unique product of the developing fetal testis that can be detected at a very early stage of pregnancy in fetal blood and also in the surrounding amniotic fluid from where bidirectional exchange of components can occur as the fetal skin is still not keratinized. To explore inter-fetal transport processes, the pig, as a multiparous species, provides an excellent model.
Materials and methods
Using our modified time-resolved fluorescence immunoassay (ruminant), we assessed INSL3 concentration in blood, amniotic or allantoic fluids of male and female pig fetuses from GD30 onwards and in maternal blood across gestation.
Results and discussion
INSL3 was detected in large amounts uniquely in male fetuses in all fluid compartments from GD45 onwards. Importantly, INSL3 of male fetal origin was also detected in significant amounts in allantoic fluids from female fetuses at GD45, which were directly adjacent to male fetuses *in utero*. Like amniotic fluid in human and ruminants, allantoic fluid is believed to be largely of fetal origin in pigs. Inter-fetal transport of this male hormone was not evident at later gestational ages, implying that by mid-gestation mechanisms are in place, which support the endocrine autonomy of the pig fetus. Together with our earlier results demonstrating transport of INSL3 between the male fetal calf and its mother, these results show that the early mammalian fetus is vulnerable to endocrine agents from the uterine or maternal environments, and hence also to potential endocrine disrupting substances of relevance for DOHaD.

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Does xenotoxicant-disrupted fetal sheep thyroid development persist into adulthood?

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Introduction
A complex cocktail of poorly biodegradable chemicals is ubiquitous in the modern environment and exposure to such chemicals contributes to diseases including thyroid dysfunction. The thyroid gland regulates growth and function of multiple organs and we previously showed that exposure to xenotoxicants (via maternal grazing on sewage sludge fertilised pastures) disturbed fetal sheep thyroid development. Our aim here was to assess whether the effects seen in fetal sheep persist into adulthood.
Materials and methods
Thyroids from sheep that grazed on control or sewage sludge-fertilised pastures throughout their lives (gestation and lactation via the mother and post-weaning grazing) were divided in four groups ($n=6$–10/group) according sex and treatment. Thyroid morphology was assessed using H&E staining. Transcript levels and expression of proteins important for thyroid function were measured using real-time PCR, western blot, and immunohistochemistry respectively in control and treated animal thyroids and livers.
Results and discussion
No gross histological differences between control and treated groups were observed. Most thyroids examined contained benign non-neoplastic cysts that were significantly larger in males ($P<0.005$, irrespective of treatment). No difference in thyroid-specific *TTF1*, *PAX8*, *TG*, *DIO2*, *CD31*, *ESR1*, *TSHR*, *SLC5A5*, *TPO*, and *THRA* or liver-specific *THRB* and *SERPINA7* transcript levels were found among groups indicating normal thyroid function overall. Consistent with these findings, plasma free T_3 and free T_4 were not altered by sewage sludge exposure. The results here show that even though exposure to complex cocktails of everyday chemicals affects the ovine thyroid during prenatal development, no obvious abnormalities are seen in adulthood.

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The role of kisspeptin for defeminization and masculinization of sexual behaviors in rats

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Kisspeptin, encoded by *Kiss1* gene, is considered to be a key regulator for GnRH secretion. *Kiss1* knockout (KO) rats are infertile due to the absence of gonadotropin secretion in both sexes. *Kiss1* KO male rats receiving testosterone during their adult age showed no male sexual behaviors (mount, intromission, and ejaculation), but showed female sexual behavior (lordosis) in *Kiss1* KO males. To examine whether kisspeptin is also needed to induce male sexual behaviors in adulthood, *Kiss1* KO males received central injection of kisspeptin (1 nmol). Kisspeptin treatment did not recover male sexual behaviors of testosterone-primed *Kiss1* KO males. It is plausible that kisspeptin acts in the processes of masculinization and defeminization of brain during perinatal period. In order to clarify perinatal kisspeptin involvement in remodeling the brain controlling sexual behaviors, plasma testosterone levels, and exogenous estradiol effects were investigated in *Kiss1* KO rats. *Kiss1* KO males showed a high plasma testosterone level at embryonic day 18 and postnatal day 0 similar to WT males. Administration of 150 ng estradiol benzoate at postnatal day 0 suppressed female sexual behavior in *Kiss1* KO adult male rats. The present study showed that kisspeptin is needed for masculinization and defeminization of sexual behavior in male rats. Taken together, kisspeptin is most likely to have a role in a conversion of estradiol from testosterone in a rat brain during perinatal period.

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Mating decreases the sulfated HNK1 carbohydrate motif over a 130 kDa glycoprotein in the rat endosalpynx

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Mating is a source of signals to the female reproductive tract to ensure reproductive success. Previously, we reported that mating induces the expression of carbohydrate sulfotransferase 10 (CHST10) in the rat endosalpynx. This enzyme participates in the sulfation of the carbohydrate motif named human natural killer 1 (HNK1), synthesized over membrane proteins and lipids. Both molecules, CHST10 and HNK1, are involved in cell-to-cell interactions. Herein, we determined the effect of mating on the localization and level of the sulfated HNK1 (sHNK1) in the rat endosalpynx. Sprague–Dawley female rats were caged with fertile males at 22 h of pro-oestrous and 30 min later we verified the presence of spermatozoa in the vaginal smear. Mated rats were isolated and 3 h later oviduct or endosalpynx was removed and processed by immunofluorescence or western blotting respectively. The control group were non-mated rats isolated at 22 h of pro-oestrous and sacrificed 3.5 h later. We detected that sHNK1 carbohydrate motifs were localized on the luminal face of epithelial cells forming patches. The quantity of patches varied between oviductal segments: 75% in the Fimbria, 22% in the Ampulla, and 3% in the Isthmus in both conditions. On the other hand, we found six glycoprotein bands with the sHNK1 carbohydrate motif, having molecular weights of 25, 42, 45, 70, 90, and 130 kDa. The 130 kDa band was more abundant in non-mated rats than in mated rats. In conclusion, mating decreases the synthesis of sHNK1 over a 130 kDa glycoprotein in the rat endosalpynx. It suggests that mating regulates cell-to-cell interactions that take place on the luminal face of the rat endosalpynx.

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Effects of cooling and cryopreservation on sperm mitochondrial membrane and sperm motility

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The aim of this study was to analyze the effects of refrigeration and cryopreservation on dog sperm special on sperm motility and mitochondrial membrane potential (MMP). A total of 15 ejaculates, first and second fractions, were collected from five dogs. Semen was diluted (80×10^6 sptz/ml) on Tris-egg-yolk medium with 8% of glycerol (one step), filled into 0.5 ml French straw and refrigerated at 5 °C for 1 h. After straws were suspended 6 cm above liquid nitrogen for 20 min and plunged. At each stage (after collection (FS), cooling (CS), and cryopreservation (FTS)) sperm total motility (TM), progressive motility (PM), and % of rapids (RAP) were accessed by CASA and the MMP by flow cytometerusing 1 µl of 1.53 mM JC-1 stain in DMSO (incubated for 30 min at 37 °C).

Statistics

Kolmogorov–Smirnov, variance and Dunn's tests. Results: **FS,** TM 85%±7, PM 67%±9, RAP 80%±9; high MMP (HMMP)85.1%±1.0; low MMP (LMMP) 14.8±1.0. CS: TM 86%±5, PM 68%±9, and RAP 78%±8; HMMP 60.5±2.4; LMMP 39.5±2.4. FTS: TM 72%±12, PM 55%±11, and RAP 63%±13; HMMP 38.1±1.6; LMMP 62.0±1.6. There were significant ($P<0.0001$) differences between all groups, showing that refrigeration and cryopreservation chances sperm structures. We conclude that despite acceptable motility refrigeration and cryopreservation cause damage to sperm mitochondrial membrane and this could impair spermatozoa life spam.

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Defining the role of sry-related HMG box 4 in male differentiation *in vivo*

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Mammalian gonads arise in both sexes from bilateral urogenital ridges that have the potential to develop as ovaries or testes. In mice, the genital ridges are first evident at embryonic day (E) 9.5. At E10.5, a critical switch in gonad development occurs in which XY gonads express the testis-determining gene (**s**ex-determining **r**egion **Y** chromosome) *Sry*. Sry belongs to the SOX family of nuclear transcription factors. Male sex determination is governed by Sry-dependent activation of another SOX protein, namely, Sox9, leading to differentiation of Sertoli cells and other testicular cell lineages, and to testis development. In contrast, the absence of *Sry* expression in XX bipotential gonads leads to ovary development. We have recently discovered that another *SOX* family member, *Sox4,* is strongly and specifically expressed in the testis cords of XY embryos at E11.5, the earliest stage of testis development. Remarkably, our preliminary data indicates that XY *Sox4*-deficient embryos develop ovaries or ambiguous 'ovostestis'. At E14.5, when the testis should be rounding in shape and migrating caudally towards the bladder, XY *Sox4*-deficient embryos exhibit an elongated gonad morphologically indistinguishable from an ovary ($n=4$), which remains rostrally located and axiolateral to the kidneys, similar to what is observed in normal XX embryos. Given the established role of other members of the Sox family in controlling male differentiation, our exciting preliminary findings strongly support a role for Sox4 in XY testis development *in vivo.*

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Granulosa cells from human small antral follicles changes gene expression during culture

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Background

As a consequence of cryopreservation of human ovaries preformed in our laboratory we have access to human small antral follicles.

Material

Human granulosa cells (GC) from individual small antral follicles (4–6 mm in diameter) were spilt in two and either cultured or snap-frozen immediately after isolation from the follicular fluid. Microarray analysis was used on both the cultured and the non-cultured sample, to examine their gene expression profiles. Ten individual follicles, from six women were used in the present study. The results of the microarray analysis were validated on qPCR.

Results

Combining culture studies, microarray-and qPCR-analysis major changes in the gene expression profiles were observed between cultured and non-cultured GC samples.

The non-cultured samples showed a gene expression profile with high expression of FSH receptor (FSHR), anti-Müllerian hormone (AMH) and androgen receptor (AR), and low expression levels of the CYP19a1 and LH receptor (LHR), corresponding with what is expected in GC from human small antral follicles. Controversially, the GC from the cultured samples showed a gene expression profile with significantly lower FSHR, AMH, and AR expression and significantly higher expression of CYP19a1 and LHR, corresponding with expression profiles of GC from preovulatory follicles. Further, several other genes characteristic for GC from preovulatory follicles were up-regulated in the cultured cells.

Conclusion

Granulosa cells from human small antral follicles differentiated during culture into cells with a gene expression profile similar to GC from preovulatory follicles.

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Effects of common FSH receptor single-nucleotide polymorphisms on the follicular fluid hormone profile and the granulosa cell gene expression in human small antral follicles

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Background
FSH is one of the key hormones of mammalian reproduction. FSH signaling may be affected by single-nucleotide polymorphisms residing in the FSH receptor (FSHR) gene. The most pronounced effects of these polymorphisms are likely to be displayed in the follicle fluid, which acts as a reservoir for granulosa cell secreted hormones induced by FSH.
Methods
Genetic polymorphisms of *FSHR* in position -29 (FSHR -29) and in codon 307 and codon 680 (FSHR307/680) were correlated to measurements of intrafollicular levels of AMH, estradiol, progesterone, testosterone, and androstenedione. Furthermore, the genotypes of FSHR307/680 were correlated to granulosa cell gene expression of *FSHR, LHR, CYP19a1, AR, AMH*, and *AMHR2*.
Results
For FSHR307/680, significant genotype specific differences were observed for AMH, estradiol, and testosterone. The GG genotype had significantly higher AMH levels than the AG genotype, especially in the group of follicles with a diameter of 3–6 mm. In follicles exceeding 6 mm, AMH levels were highest in the GG group in parallel with levels of estradiol, progesterone, and testosterone. The gene expression profiles showed significantly elevated levels of *LHR* for the GG genotype, with similar patterns observed for *FSHR* and *CYP19a1*. For FSHR -29, the concentration of AMH and testosterone was higher for the AA genotype, while an inverse profile was observed for estradiol. The greatest effects were exerted by FSHR -29 in comparison to FSHR307/680.
Conclusion
Significant changes in the granulosa cell function of human small antral follicles can be observed as effects of the FSHR polymorphisms FSHR −29 and FSHR307/680.

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Telomerase reverse transcriptase is an epithelial progenitor marker in the mouse endometrium

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Rare epithelial and stromal stem/progenitor cells (SPC) have been identified in the mouse endometrium. However the role of these SPC in endometrial regeneration is unclear due to the lack of a traceable marker. We used transgenic mice with reporter constructs for the stem cell marker telomerase reverse transcriptase (mTert) to identify and characterize mouse endometrial SPC. Endometrial mTert expression was examined by microscopy and flow cytometry in mice expressing GFP under the control of the mTert promoter (mTert-GFP). The fate of the endometrial mTert lineage was determined in mTert-CreER::R26R mice. A rare population of intrinsic (CD45 −ve) endometrial stromal cells expressed mTert-GFP during development and in adult mTert reporter mice. Rare focal regions of epithelial mTert-GFP were observed in both the glandular and luminal epithelium in adult mice. These mTert-GFP cells were distinct from stromal and epithelial label-retaining cells previously identified in mouse endometrium. Stromal mTert-GFP cells did not express ERα but epithelial mTert-GFP cells were ERα positive. Epithelial mTert-GFP cells were not slow cycling and displayed a level of proliferation comparable to mTert −ve epithelium. Lineage tracing showed that mTert lineage cells were largely confined to the stroma of prepubertal endometrium, but contributed extensively to glandular and luminal epithelium in the endometrium of adult cycling mice. In adult mice, removal of endogenous ovarian hormones by ovariectomy blocked expansion of the epithelial mTert lineage. Our data suggest that epithelial structures of the adult cycling endometrium originate from an mTert positive stromal SPC that undergoes mesenchymal-to-epithelial transition during cyclic endometrial regeneration.

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Cdh1- and Cdc20-independent APC activity in mouse oocytes

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Introduction
The anaphase-promoting complex (APC) is an E3-ubiquitin ligase responsible for regulated destruction of substrates at specific stages of the cell cycle. Two APC co-activators, Cdc20 and Cdh1, mediate the timing and selectivity of substrates recognition. Progression through meiosis in oocytes utilises the same molecular players, although the start–stop nature of the female meiosis invokes additional levels of regulation. Recently, we reported that Cdk1 and MAPK play compensatory roles in regulating the APC by supressing its activity early in prometapahse I, thereby allowing accumulation of the APC substrates essential for meiosis I. Here, we investigate which co-activator is responsible for prematurely activating the APC when Cdk1 and MAPK are inhibited during prometaphase I.
Materials and methods
To deplete Cdc20 and Cdh1, we used antisense morpholino oligonucleotides, which were microinjected in prophase-arrested mouse oocytes 24 h before use. Cdk1 and MAPK were inhibited using Roscovitine and UO126 respectively. Western blotting and time-lapse imaging of oocytes expressing the APC substrates tagged to GFP were used to examine their stability.
Results and discussion
To date, the accumulated evidence suggests that the APC is only active in the presence of its known co-activators. Here, we report that depletion of Cdh1 and Cdc20 fails to inhibit the APC-mediated destruction of substrates that happens when Cdk1 and MAPK are inhibited during prometaphase I. Furthermore, a slow component of the APC-mediated destruction persists after depletion of Cdh1 and Cdc20 in prophase-arrested oocytes. These data suggest that alternative mechanisms for regulating APC activity may exist in mouse oocytes.

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P356

***In vitro* differentiation of spermatogonial stem cells to functional sperm in zebrafish**

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Introduction
Spermatogenesis is a complex process in which spermatogonial stem cells (SSCs) self-renew, develop to differentiated spermatogonia, and then give rise to meiotic spermatocytes. Functional sperm are ultimately differentiated from postmeiotic haploid spermatids. Cell culture methods representing this process will facilitate analyzing molecular function and imaging molecules. Here we describe consecutive two culture methods by which zebrafish SSCs enable to differentiate to functional sperm *in vitro*.
Materials and methods
We used *sox17* promoter–*EGFP* transgenic zebrafish that express GFP in the early stage of spermatogonia. Cells of the hypertrophied testis were cultured under effective long-term culture conditions for propagating zebrafish SSCs. After SSCs were maintained for 1 month, they were transferred and cultured on feeder layers of the Sertoli cell line in the medium that induced differentiation of spermatogonia.
Results and discussion
After 1 month of culture for SSCs, most germ cells became SSCs in morphology and expressed GFP. After plating SSCs on the feeders, meiotic spermatocytes appeared for 10 days. Expression of a meiotic marker, Sycp3, was also detected in spermatocytes. After 24 days of plating on the feeders, fertilized embryos were obtained by artificial insemination with differentiated sperm *in vitro*. They grew up normally, suggesting that normal haploid sperm were produced under this culture condition. Interestingly, sperm production continued for 1 month and fertilized embryos were obtained every week. These results indicate that a whole spermatogenic process in zebrafish is represented in our culture conditions.

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Effects of maternal peri-conception and first trimester protein supplementation on placental development

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Introduction
This study investigates the effect of dietary crude protein on the cellular composition of the 98 days bovine placenta.
Materials and methods
Primiparous 14-month-old *Bos. indicus* cross heifers in Australia ($n=350$) were individually fed isocalorific high (H; 14%) or low (L; 7%) CP from day −60 prior to conception to 23 days post conception (dpc). At 23 days post-conception the two groups were further split into high or low % CP creating four treatment groups: i) high/high (HH), ii) low/high (LH), iii) high/low (HL), and iv) low/low (LL). At 98 dpc ($n=4$/group per sex), placentae were weighed, fixed, serial sectioned, immunostained for binucleate cells or vasculature, and analysed using systematic random sampling and ANOVA. QPCR indicated placental vascular endothelial growth factor A (VEGFA) and placental growth factor (PGF) expression.
Results and discussion
Our recent study showed no effect between sex or dietary treatments on binucleate cell proportion or number, however a number of significant differences were observed in placental vasculature (proportion and number) in both sexes and across the different protein groups. In addition, VEGFA expression was lower in male HL vs female HH ($P<0.05$) and male vs female pre-conceptual high ($P<0.05$). PGF expression was lower in male HH than female LL ($P<0.05$), male vs female pre-conceptual high ($P<0.05$) and male post-conceptual high in comparison to female post-conceptual low ($P<0.05$). Dietary protein influenced placental weight, vasculature, and protein expression, without affecting trophoblast binucleate cell proportions.

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High testicular size is related with low intramale variation of sperm design in red deer

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Introduction
Testes investment plays a major role in male reproductive success because larger testes are expected to produce higher sperm number and quality. In birds, comparative studies have shown that testis size – a proxy measure of postcopulatory sexual selection – is negatively related with the intraspecific variation of sperm design. By contrast, links between the intramale variation of sperm morphometry and testicular size in mammals are unknown. Here, for the first time, we test the hypothesis that intramale variation in sperm morphometry is negatively associated with testes investment in red deer.
Materials and methods
Samples were collected from 17 adult red deers during the breeding season. Testicular mass was calculated averaging the values from the right and left testes. Spermatozoa were collected from the epididymal cauda and fixed in glutaraldehyde solution. Sperm head and flagellum morphometry were assessed by phase contrast microscopy on 25 spermatozoa/male using the Software ImageJ.
Results and discussion
Testicular mass showed a high variability between males ranging from 23.74 to 82.75 g. Overall, high testis mass was associated with low intramale variation of sperm design. Indeed, we found that testicular mass was negatively related with intramale coefficient of variation of total sperm length ($P=0.015$), head width and area ($P=0.001$ and $P=0.041$ respectively). The same trend of correlations was also found for the remaining morphometric parameters, although not significant. In conclusion, our results suggest that a low intramale variation in sperm morphometry indicates a high efficiency of sperm machinery.

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Improvement of cloned blastocyst quality upon CHIR treatment of donor cells

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Somatic cell nuclear transfer (SCNT) has a broad spectrum of many potential applications but the efficiency is still disappointingly low. It is known that major cause of it, is incorrect or incomplete reprogramming of the transferred donor nuclei.
Many report indicates that Wnt/β-catenin signaling pathway plays a pivotal role in the process of somatic cell reprogramming. As CHIR is known as one of the activators of Wnt pathway by inhibiting glycogen synthase kinase 3β (Gsk 3β), this helps to maintain the undifferentiated state of human and mouse embryonic stem cells, preserving the expression of main pluripotent genes.
Here we verify whether CHIR-treated donor cells are better reprogrammable upon nuclear transfer in sheep and mouse models.
CHIR-treated cells were used as donors for SCNT, and then the subsequent *in vitro* and *in vivo* quality and development of the reconstructed embryos were assessed.
Results showed significant differences in the cleavage rate (63% – CTRL to 72% CHIR – mouse; 60.3% CTRL to 79% CHIR – sheep) and blastocyst formation (18.8 vs 29.6% – sheep). Additionally, CHIR cloned blastocysts exhibited very robust, better-expanded morphologies than CTRL ones. Moreover, significant improvement of the total and inner cell mass (ICM) cells numbers in CHIR blastocysts have been observed. Those results can lead to better development of live offspring (experiments on going).
Donor cells, treated with Gsk 3β inhibitor, are easier reprogrammed upon nuclear transfer and help to improve quality of preimplantation cloned embryos. Future optimization of SCNT protocol may allow this approach to be effective on higher efficiency of live cloned offspring.

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The capacity to reprogramme is an intrinsic property of adult somatic cells

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Oct4 and Nanog are the transcription factors responsible for pluripotency maintenance in embryonic stem cells (ESCs), and activation of these genes in differentiated cells is considered the earliest signal of nuclear reprogramming. To date, nuclear reprogramming in somatic cells can be induced by nuclear transfer, induced expression of defined factors (Oct4, c-myc, Sox-2, and Kfl4) and, more recently, by a short exposure to a low pH. However, work in salamander as well as in plants, demonstrated that somatic cell show an intrinsic reprogramming capacity during tissues regeneration. In this work we monitored Oct4 and Nanog expression (by qPCR and immunofluorescence) in sheep/mouse tissues (ear/tail biopsy) and fibroblast primary culture derived from them. Here we report that the simple expansion of fibroblasts in culture dish, without any exogenous stimuli, switches on pluripotency markers. In fact, while Oct4 and Nanog expression was not detected in tissue biopsy, it was widely expressed in cultured fibroblasts. Furthermore, Oct4 and Nanog expression was further increased in response to exposure of GSK3β inhibitor (CHIR99021), a small reprogramming molecule used for ESC self-renewal. From these observations, we believe that reprogramming should be an automatic cell response, triggered when the cells lose the constraints imposed by a multicellular environment. Probably, a complete loss of cell-to-cell and cell-to-matrix communication activates a default gene network that, under certain culture conditions, can leads to pluripotency.

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P361

The role of cell adhesion molecules in primordial follicle formation

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Introduction
Oocytes develop in cysts of interconnected cells that undergo breakdown and become surrounded individually by granulosa cells to form primordial follicles. Mechanisms controlling cyst breakdown and primordial follicle formation are not well understood but cell adhesion molecules (CAMs) may a play role. Our model is that CAMs keep oocytes in cysts and must be down regulated for primordial follicles to form. A second set of CAMs would then be necessary for interactions between oocytes and granulosa cells during follicle formation. This model is supported by work in a hamster model (Wang & Roy 2010).
Materials and methods
Western blotting and immunocytochemistry were performed on mouse ovaries from 17.5 dpc to PND5 using antibodies against E-cadherin and N-cadherin to test for expression and cellular localization. To determine the role of each CAM, PND1 ovaries were cultured for 5 days in the presence function blocking antibodies and examined using confocal microscopy.
Results and discussion
Both E-cadherin and N-cadherin were detected in mouse ovaries from 17.5 dpc to PND5. E-cadherin was localized to oocytes in germ cell cysts and primordial follicles while N-cadherin was expressed in both oocytes and granulosa cells. CAM function blocking antibodies in ovary organ culture had no effect on cyst breakdown, oocyte number, or follicle development. This may be because PND1 ovaries were used for culture but cyst breakdown begins 2 days earlier at 17.5 dpc and PND1 may be too late to alter cyst breakdown. Organ culture experiments with CAM blocking antibodies are now being repeated using 17.5 dpc ovaries.

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P362

Nuclear distribution of genotoxic damage in rainbow trout (*Oncorhynchus mykiss*) sperm after cryopreservation

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Introduction
Sperm DNA is prone to suffer damage that could have a negative impact on the progeny development. Moreover genes packaged in different chromatin blocks or located in different nuclear areas, could have differential sensitivity to genotoxic agents. Our group has demonstrated that in rainbow trout, with the nucleus homogeneously compacted with protamines – lacking histones –, the distribution of damage is dependent on the genotoxic agent. The present work is aimed to analyze the potential differential susceptibility to DNA cryodamage.
Material and methods
Sperm was cryopreserved following two different protocols: using LDL or egg yolk in the extender and loading in straws or cryovials respectively. The number of lesion per 10 kb in nine different genes, with different relevance for developmental decisions, was analyzed with a qPCR approach. The presence of 8-hydroxyguanosine, DNA strand breaks and their co-localization with nuclear matrix proteins was analyzed by immunofluorescence, TUNEL, and confocal microscopy.
Results and discussion
The two freezing protocols promoted similar level of DNA lesions but significant differences were observed between genes. The immunodetection of specific lesions demonstrated a heterogeneous distribution of 8-hydroxyguanosine into the nucleus, located both in the peripheral and the inner areas of the nucleus. Also some lesions better co-localize with nuclear matrix proteins. The differential susceptibility and location of lesions did not match with the damage promoted by oxidative stress with H_2O_2, suggesting that other factors, apart from the generation of ROS, could render specific nuclear territories more sensitive to cryopreservation practices.
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Conceptus-derived proteins in bovine uterine luminal fluid at the time of pregnancy recognition in cattle

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Maternal recognition of pregnancy (MRP) in cattle requires secretion of sufficient interferon tau (IFNT) by the conceptus to prevent luteolysis. However, recent evidence suggests that additional conceptus-derived products may be involved. The aim of this experiment was to identify conceptus-derived proteins in uterine luminal fluid (ULF) during MRP. Following estrous synchronization, the protein content of ULF from cyclic ($n=4$) and confirmed pregnant ($n=4$) heifers was analyzed by nano LC MS/MS. In addition, the protein composition of spent culture medium from *in vivo* derived day 16 conceptuses cultured *in vitro* for 6 or 24 h (and blank controls) were also analyzed. 305 proteins were identified in the ULF of pregnant heifers while 299 were detected in the ULF of cyclic heifers; 85 proteins were common to both. Analysis of media following culture of day 16 conceptuses for 6 or 24 h (vs blanks) revealed the presence of 1043 and 1020 proteins respectively. Comparison of these proteins to those identified in the ULF of pregnant and cyclic heifers revealed 31 proteins unique to pregnant ULF (i.e. not detected in cyclic heifers) which were also produced *in vitro* by day 16 conceptus. The transcript abundance of 25 of these 31 proteins was significantly higher in the conceptus ($P<0.05$, measured by RNA sequencing) compared to endometrial expression and included *HSP7C*, *EF2*, *CH10*, *PDIA1*, and *CYTB*. In conclusion, this study provides evidence that the conceptus produces proteins, other than IFNT on day 16 which likely contribute to the process of MRP.

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Live imaging reveals novel dynamic events in transitioning mouse gonocytes

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Introduction
Early developmental events in the testis set the scene for future spermatogenesis. After birth, gonocytes, the precursors to type-A spermatogonia reproliferate and become transiently motile in a poorly understood process. Aberrations are associated with altered fertility or pre-malignant states. Gonocytes exist as overlapping subpopulations at varying developmental stages, which makes them difficult to study.
Materials and methods
Transgenic mice carrying enhanced green fluorescent protein under the control of the Oct4 promoter (Oct4:GFP) and a ubiquitous mTd Tomato gene were used. Isolated seminiferous tubules were explanted from fetal and neonatal testes with gonocyte development visualised using 4D time-lapse videomicroscopy. Using immunohistochemistry, qRT-PCR, and FACS analysis, markers associated with differentiation, apoptosis, and proliferation confirmed imaging events.
Results and discussion
An *ex vivo* testicular model in mice was used to follow gonocyte development in real time. After a period of quiescence, gonocytes proliferated and migrated to the basement membrane making extensive cell:cell contacts via lamellopodia which extended and retracted from their surface (days 1–4 *postpartum*). A novel process of cytoplasmic shedding was captured dynamically with a concomitant decrease in gonocyte size in a subpopulation found at the basement membrane. Additionally, a peak of apoptosis was seen at day 4 *postpartum* and confirmed with TUNEL staining. An understanding of gonocyte subpopulations behaviour is limited. This novel approach circumvents difficulties in studying testicular development by facilitating a dynamic study of gonocytes topographically organised within their microenvironment. For first time, cytoplasmic shedding is described in gonocytes making the transition to type A spermatogonia.

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The impact of DNA damage induced by sperm cryopreservation in *Xenopus*

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Introduction
This project aims to investigate the nature of cryo-induced DNA damage in the spermatozoa of the model species *Xenopus tropicalis*. We have turned to *Xenopus* as a tool to achieve this because the genome is available and we aim to identify specific genomic regions that are most affected during sperm cryopreservation.
Materials and methods
The sperm chromatin dispersion (SCD) test was used to show DNA damage and the potent DNA repair inhibitor 3-aminobenzamide (3-AB) was used to reveal the effects of sperm DNA damage in whole embryos produced by *IVF*. Morphological assessment and *in situ* hybridization was used to analyse the phenotypes. Biochemical techniques were used to validate genes, particularly gastrula markers which were identified as potential hotspots for DNA damage.
Results and discussion
Single and double stranded DNA breakage was induced by sperm cryopreservation. In fresh sperm samples 6.06% showed fragmentation compared to 17.65% in cryopreserved sperm samples. Furthermore, the dynamics of DNA damage revealed the chromatin is less stable following the freeze–thaw process. This is demonstrated *in vivo* by treating embryos derived from cryopreserved sperm with 3-AB. Over 65% of these embryos exhibit developmental abnormalities, mostly grastrula defects, compared to 17% abnormal embryos without 3-AB. Based on the observed phenotypes fibroblast growth factor 8 (Fgf8), a gene necessary for proper gastrulation, was identified as one of the genes affected most by cryopreservation. By better understanding cryo-induced damage we will be able to develop the practical applications of genetic resource banking and improve its generic success across a wider range of species.

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Sperm total motility and membranes integrity after selection by centrifugation in colloid solutions of ram cryopreserved semen

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Introduction
Frozen and thawed ovine semen undergo morphological and functional changes that prevent or decrease the efficiency of fertilization. Sperm selection methods seek to improve the quality and viability of the fertilizing materials.
Material and methods
Four sperm selection methods by centrifugation were employed, using two silica colloidal solutions coated with silane (Ovipure™– Nidacom, Gothenburg, Swedis) or by polyvinylpyrrolidone 45% and 90% (Percoll – Nutricell, Campinas, Brazil), and varying the volume of colloidal solution (4 or 1 ml). Sperm total motility (TM) were evaluated by means ± S.E.M. of CASA. In order to measure the integrity of the plasmatic and acrosomal membrane (integrity), an association of probes FIT-PSA/IP were used.
Results and discussion
Sperm samples TM before selection on 4 and 1 ml protocols were 27.0 ± 3.1% and 24.3 ± 1.9%, integrity were 19.9 ± 2.1% and 27.1 ± 1.6%. Protocols using silica colloidal-silane showed higher ($P < 0.05$) TM (4 ml: 62.0 ± 4.1%; 1 ml: 64.6 ± 3.8%) and integrity (4 ml: 52.8 ± 3.7%; 1 ml: 63.4 ± 4.1%) compared to the methods employing silica colloidal-PVP (TM 4 ml: 30.0 ± 3.6%; 1 ml: 45.9 ± 4.3% and integrity 4 ml: 37.1 ± 4.1%; 1 ml: 46.8 ± 4.2%) and compared to the samples prior to sperm selection. Only the method using 4 ml of silica colloidal-PVP was not efficient ($P > 0.05$) in selecting samples with better quality compared to the samples analyzed prior to sperm selection. The method using 1 ml of silica colloidal-silane was equally efficient to the method using 4 ml, presenting itself an alternative to process samples with lower sperm concentration.

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