IS-PS

Fertilization Studies and Assisted Fertilization in Mammals: Their Development and Future

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Scientific studies of fertilization began when it was recognized that spermatozoa and eggs, large or small, are single cells (von Koelliker, 1840; Gegenbaur, 1861) and their fusion, in particular the fusion of their nuclei, is the essence of fertilization and the beginning of life of a new individual (Hertwig, 1876; Fol 1879). Early studies of fertilization were done using invertebrates (such as sea urchins and annelids) because their gametes could be collected readily and fertilization and embryo development could be examined readily in a dish of sea water. Before 1960 mammalian fertilization studies were difficult. Collection of a large number of fully mature eggs at a time was difficult in many species, and no one knew what kinds of media they should use to handle gametes, zygotes and preimplantation embryos. Thanks to many researchers' endeavor, fertilization research in a variety of mammalian species has become far easier. Discovery that mammalian spermatozoa, unlike sea urchin spermatozoa, require "capacitation" to become fertilization-competent (Chang, 1951; Austin, 1951, 1952) triggered active research of mammalian fertilization. Today, papers dealing with mammalian fertilization published annually by far exceed those dealing with fertilization in non-mammalian vertebrates and invertebrates combined.

Progress in structural, biochemical and molecular studies of mammalian fertilization during the last 50 years is impressive (for reviews, see Austin, 1961; Schatten, 1994; Yanagimachi, 1994; Wassermann and Florman, 1997; Moro and Okabe, 2011). Progress, however, never ends. Even a long-standing dogma that mammalian spermatozoa undergo the physiological acrosome reaction after contact with the zona pellucida of eggs has been challenged this year by Jin *et al*. (2011) who demonstrated that most fertilizing (mouse) spermatozoa are acrosome-reacted already before they contact with zona surfaces. The concept that egg's zona pellucida is the sole biological molecule that triggers the physiological sperm acrosome reaction must be abandoned. Such a simple question as "how does mammalian spermatozoon pass through the zona pellucida?" has not been settled (Bedford, 2008). Molecular mechanisms by which spermatozoa become competent to fuse with egg plasma membrane (Kiawah *et al*., 2008) are yet to be clarified.

Among major technical breakthrough in the last century are: (1) cry preservation of gamete and embryos, (2) *in vitro* maturation of eggs, (3) assisted fertilization such as IVF and ICSI, (4) sperm-sexing, (5) non-surgical embryo transfer, and (6) preimplantation genomic analysis of embryos.

Subjects of future investigations related, directly or indirectly, to fertilization and the improvement of animal and human reproduction include: (1) permanent preservation of spermatozoa at ambient temperature, (2) sex selection of individual spermatozoa, (3) mass-production of mature gametes *in vitro*, (4) selection of genomically normal gametes prior to assisted fertilization, (5) conversion of diploid (somatic) cells to haploid (germ) cells, and (6) genomic manipulation of gametes for the production of superior animals.

IS1-1

A Historical Perspective on IVF, *In Vitro* Embryo Culture and ET in Japan

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In Japan, a pioneering research on mammalian *in vitro* fertilization (IVF) was performed by Jinshin Yamane (1889–1972) during the early 1930s. He observed the extrusion of the second polar body in the rabbit eggs following 'insemination' and concluded that the activation was caused by sperm penetration into the egg. Evidence of fertilization itself was not confirmed, however. Gregory Pincus (1903–1967), another pioneer in the U.S.A., was confronted with similar difficulties. Unequivocal cytological evidence of IVF was first obtained in 1954 by Charles Thibault and his colleagues in France, by using 'uterine spermatozoa' recovered from mated females, after the discovery of capacitation by C.R.Austin and M.C.Chang in 1951. Chang (1959) then obtained live offspring by transferring the IVF rabbit embryos into pseudopregnant recipients, as the definitive proof of normal developmental capability.

Since the first success in IVF-ET by Chang, the progress has been accelerated and the research fields expanded rapidly. Two major technical advances are considered most critical; that is (1) induction of *in vitro* capacitation, and (2) improvement of culture medium for preimplantation embryonic development.

The possibility of *in vitro* capacitation was first demonstrated by Yanagimachi and Chang (1963) who showed that hamster eggs can be fertilized *in vitro* by epididymal spermatozoa almost as readily as by uterine spermatozoa. Inspired by this landmark paper, young Japanese scientists challenged new subjects of IVF research in Chang's lab at the Worcester Foundation in Shrewsbury, U.S.A., in rapid succession during the next 15 years, resulting in a large amount of scientific contributions, including the first mouse IVF by epididymal sperm (Iwamatsu & Chang, 1969), the first rat IVF by uterine sperm (Miyamoto & Chang, 1973), and the first IVF-ET in the rat (Toyoda & Chang, 1974).

After coming back from Chang's lab to Japan, A. Hanada led the national team to put the IVF-ET to practical use for cattle production and finally succeeded in delivering a newborn calf in 1985, by non-surgical transfer of IVF bovine embryos derived from *in vitro* matured eggs. Fortunately non-surgical ET in cattle had already been established in this country by pioneering work of T. Sugie (1965). Moreover, Oguri & Tsutsumi (1974) had successfully applied the Sugie's procedure to equine blastocysts developed *in vivo*, as the first non-surgical ET in the horse.

In parallel to the progress in laboratory and farm animals, basic as well as clinical aspects for human IVF were extensively studied in several medical institutions in Japan, and the first IVF baby was born at Tohoku University Hospital, Sendai (M.Suzuki et al, 1983). It was five years later than the birth of the world's first IVF baby, Louise Brown, in Cambridge, U.K. (Steptoe & Edwards, 1978).

Current methodologies for *in vitro* culture of mammalian embryos are largely dependent on early studies on mouse preimplantation embryos, notably by J.D. Biggers, R.L. Brinster, W.K. Whitten and D.G. Whittingham. Defined culture media developed by them in the late 1960s to early 1970s have provided the bases of nearly all the IVF media developed thereafter, including those for humans. At about the same time, we could establish a repeatable procedure for mouse IVF (Toyoda, Yokoyama & Hosi, 1971) and our medium (TYH) has become widely used, although it is limited to the IVF, not for embryo culture beyond the 2-cell stage.

Efforts to improve the IVF medium have been continuing to date and will be continued in the future. Every effort to minimize the differences between *in vivo* and *in vitro* will provide an insight into the mechanisms of mammalian reproduction and development, thus leading to the refinement of assisted reproductive technologies (ART).

IS1-2

Frontiers of IVF in Livestock Species: Factors Affecting Efficiency

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Embryo *in vitro* production (IVP) systems in sheep, cattle and pigs have been established for over twenty years, yet the goals to fully understand the interaction of the gametes and achieve the highest fertilization rates possible remain fundamental to researchers. The embryo IVP efficiency equation is simple; *in vitro* fertilization (IVF) of the best quality oocytes with the best quality sperm under the optimal *in vitro* conditions will produce the best quality embryos.

Our understanding of oocyte maturation processes has progressed substantially over the past decade. It is now well accepted that communication between the maturing oocyte and its associated cumulus cells is a complex two-way interaction and that the intact follicle regulates meiotic progression at a level not yet achieved *in vitro*. Examination of the properties of cumulus-oocyte complexes classified according to antral follicle diameter, long known to be an indicator of oocyte quality, is still providing useful insight into the molecular and cellular events taking place during maturation. The development of embryo IVP systems in which oocytes are matured and fertilized individually, and the resulting embryos are cultured individually, are anticipated to accelerate our understanding of how developmental competence is acquired by the oocyte. Applying this knowledge in IVP embryo transfer programs may increase the number of transferrable embryos obtained per oocyte donor, which has remained static for many years.

For a multitude of reasons, sperm used in IVF systems of livestock species are almost always cryopreserved beforehand. Also, IVF incorporating sex-sorted sperm is regarded as an effective way to overcome the limitations of the sex-sorting technology, particularly in species that require large artificial insemination doses. Both the cryopreservation and sex-sorting processes expose sperm to stressors that reduce their fertility. The addition of seminal plasma or its components to processing media provides some benefit; seminal plasma improved the post-thaw quality of cryopreserved ram sperm and PSP-I/II spermadhesin reduced the capacitation-like changes in sex-sorted boar sperm. However, negative effects of seminal plasma addition have also been reported, and inconsistencies between studies highlight the biological variability of its composition and the differences in sperm membrane responsiveness. Proteomic analysis is being increasingly used to identify proteins of seminal plasma and sperm membranes that are associated with sperm function and male fertility. Understanding the role of these proteins during *in vivo* and *in vitro* fertilization remains a key challenge.

The methods used to prepare and select sperm for IVF and the *in vitro* conditions used during the co-incubation of sperm and oocytes also affect the efficiency of fertilization. While swim-up incubation and density gradient centrifugation are commonly implemented to select motile, viable sperm for IVF, the procedures often need to be refined depending on the quality of the sperm sample or the type of processing the sperm have been subjected to. Some novel approaches to selecting high quality sperm for IVF have potential to improve fertilization efficiencies. Finally, limiting the gamete co-incubation period is an effective strategy for reducing the incidence of polyspermy in porcine IVF, and may improve embryo development by decreasing oxidative damage associated with the production of reactive oxygen species. The continued refinement of IVF conditions to increase the rate of normal sperm penetration will undoubtedly benefit from an increased understanding of the differences in sperm properties between species, between males of the same species, and between ejaculates from the same male.

IS1-3

Puppies from Frozen Embryos

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The assisted reproductive techniques (ARTs) such as in vitro fertilization, embryo transfer and cryopreservation of gametes have considerably contributed to the development of biomedical sciences in addition to improved breeding in domestic animals and infertility treatment in humans. However, ARTs used in canine species have strictly limited utility when compared with other mammalian species including humans. Although successful somatic cell cloning has been reported, artificial insemination by frozen semen is only available for the improved breeding and reproduction for companion and working dogs as well as guide dogs for the blind to date. Cryopreservation for mammalian oocytes and embryos has been used for several purposes such as to preserve the genes of elite livestock, increase the efficiency of animal breeding, preserve valuable genetically modified animals and endangered species, and use germ cells effectively in ARTs in animals and humans. The first mammalian species to have embryos successfully cryopreserved was the mouse in the early 1970s, and over the next three decades this has been extended to embryos of most species including humans. However, cryopreservation of canine embryos has not progressed far enough. I describe here successful cryopreservation of embryos and subsequent embryo transfer in dogs. Embryos were collected from excised reproductive organs of Labrador Retrievers on day 2–10 after artificial insemination (on day 7–15 after LH surge) and subsequently cryopreserved by ethylene glycol based vitrification solution with a cryotop sheet. The embryos were examined under ultraviolet light using epifluorescent microscopy and the plasma membrane integrity of embryos was assessed with PI staining. The embryos that had blasotomeres negative for PI staining accounted for more than 75% were assessed as normal. For embryo transfer, the embryos were not stained with the PI. The localization of developing embryos in the reproductive tracts of Labrador Retrievers was clarified in this study. Embryos at morula stage migrated from the oviduct into uterus on day 10 after the LH surge. This transport might be completed within 24 hrs. By day 12 after the LH surge, all of developing embryos moved and localized in uterus. Embryos developed to morula by day 11–12 and to blastocyst by day 12–13 after LH surge, respectively. To assess membrane integrity of the cryopreserved embryos, when vitrified-warmed embryos were stained with PI, the viability of those embryos at 4– to 16–cell stage, morula and blastocyst was 90–100%, 50% and 40%, respectively. When vitirified-warmed canine embryos were non-surgically transferred with a cystoscope to recipients, normal pups were delivered reproducibly. Namely, of nine embryo transfer experiments five recipients became pregnant and four of them delivered seven pups in total. Percentages of development to newborn after the transfer of cryopreserved canine embryos were 9.1%. The microsatellite genotypes clearly demonstrated that those delivered pus were derived from donor embryos but not from the recipient animal. These results clearly indicate that cryopreserved canine embryos are able to develop to term, even if developmental rates after the transfer were lower. The suitable combination of stages between transferring embryo and estrus of recipient might be when 8-cell to 16-cell stage of embryos were transferred to recipients on day 8–9 post LH surge. The cryopreservation of embryo in canine species will make available for transportation, storage of genetic materials, and elimination of vertically transmitting diseases. And this technique will contribute to improved breeding of companion and working dogs such as guide dog, drug detecting dog or quarantine dog.

IS1-4

Sexing Mammalian Sperm

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The only procedure that has proven efficacious for sexing sperm is flow cytometry/cell sorting while measuring fluorescence of Hoechst 33342, which binds to DNA quantitatively. Since X-chromosome-bearing sperm of most mammals have about 4% more DNA than Y-chromosome-bearing sperm, this difference is the basis for sexing sperm. Refinements in sperm sorters enable sexing over 10 million sperm of each sex per hour per sorting nozzle with 90 % accuracy. This procedure for sexing sperm has been used for many mammalian species. However, sexing sperm on an every day, commercial basis is only being done with two species, humans and cattle, the latter at several million insemination doses per year, mostly with dairy cattle. There have been many research applications of sexing sperm, and some use has occurred in wildlife. Commercialization is just beginning with horses, deer, and sheep.

Sexed sperm usually are cryopreserved using standard procedures, and usually are used via artificial insemination, but *in vitro* fertilization followed by culturing and then transferring resulting embryos is another option. The limited number of sperm that that can be sorted per unit time economically has resulted in using about 2 million sexed sperm per insemination dose for cattle rather than typical doses of unsexed sperm of 10 million or more sperm. With good management of cattle, fertility of this low dose sexed sperm product usually is 70 to 90% of fertility of controls of 10 million unsexed sperm per insemination dose. About one-third of this decreased fertility is due to using fewer sperm, but the majority of the decrease is due to mechanical damage of the sperm during the sorting process. The exact cause and nature of this damage is not known. Thus, pregnancy rates, even with 10 million sexed sperm per insemination dose, are still low under most conditions.

Studies of thousands of calves produced via sexed sperm have not revealed any differences in incidences of abnormalities compared to control calves produced with unsexed sperm. While there are many applications for sexed sperm, the main one in cattle is to produce more females for breeding purposes or sale, primarily by breeding heifers to have heifer calves. An important human application is to avoid sex-linked genetic diseases by having female offspring. Sperm sexing technology is protected by numerous patents, and licenses for use in animals are controlled by the company Sexing Technologies, located in Navasota, Texas. For human applications, the intellectual property is controlled by the company Genetics and IVF Institute, in Fairfax, Virginia. These companies have made huge investments in developing and refining sperm sexing technology.

IS1-5

From a Functional to a Molecular Assessment of Totipotency

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Totipotency refers to the ability of a single cell to give rise to all of the differentiated cells that contribute to the development of an organism including its germline, and in mammals the extraembryonic tissues. Totipotency is a transitory nuclear state since restriction in term of cell developmental potentialities leads to pluripotency, a property defined as the ability of a (single) cell to differentiate *in vitro* and/or *in vivo* into the three primitive embryonic layers and to contribute to the germ line of the organism but in chimera experiments. The reprogramming of nuclear functions to totipotency after fertilization is thus the first crucial event of development.

Reprogramming the nucleus of a somatic cell to totipotency can be obtained following the introduction of its nucleus into an enucleated oocyte, a procedure known as cloning. In the lab, we are using cattle clones as a pertinent model to characterize totipotency, this for two main reasons: first, fertile adults can be at obtained from cattle cloned embryos at a relatively high rate and second, the three-weeks long preimplantation period of development allows an *in vivo* embryo sampling which encompasses the initiation of gastrulation when the three embryonic lineages are already differentiated.

Measuring the 5-methylcytosine levels of DNA from circulating leukocytes, we evidenced a high variability among healthy adult clones, a result which we confirmed at the tissular level by hybridization of immunoprecipitated methylated DNA on a cattle tiling array (MeDIP-chip) dedicated to 21416 promoter regions (UMD3.1 genome assembly). This provides support to the view that the dynamic process of reprogrammation to totipotency is intimately bound to a long lasting epigenetic adaptation of the genome. Ongoing statistical analysis should allow identifying the genomic regions with a high epigenetic variability in clones

To characterize this epigenetic adaptation at earlier developmental stages, when the functional reprogramming to totipotency can by definition not be determined, we used donor nuclei with different full reprogramming abilities but similar implantation rates to generate cloned embryos. We found that the intra-and inter-variability of gene expression between these donor nuclei is unexpectedly low even for the extra-embryonic tissues of late pre-implanting embryos (D18). At that stage, the embryonic lineages are properly individualised revealing reprogramming to pluripotency. This indicates that the subsequent gene deregulations associated to abnormal placentation, the most frequently reported reprogramming failure after nuclear transfer, have not yet occurred or are still not detectable at the onset of embryo patterning.

Molecular reprogramming of somatic nuclear activities to pluripotency after nuclear transfer is thus apparently very close to the reprogramming of gamete genomes after fertilisation, but their reprogramming to totipotency is not. Totipotency needs obviously additional and still uncharacterised epigenetic requirements. By studying the nuclear distribution of the heterochromatin protein CBX1 together with that of histone H3K9me3 and centromeric proteins CENPA/B just prior to embryonic genome activation, we have identified a subtype of nuclear organisation with condensed heterochromatin blocks that could correspond to poorly reprogrammed cloned embryos in comparison to fertilised controls. However, these embryos could to some extend be converted to a normal (totipotent) type by increasing the initial exposure of the donor chromatin to the oocyte cytoplasm.

In conclusion our data indicate that totipotency could be made accessible to a structural and molecular *pre ante* and not only to a functional ex *post* analysis as it is still mostly the case today.

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IS1-6

Abnormality and the Restoration of *In Vitro*-produced Porcine Embryos

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In vitro production (IVP) of embryos including *in vitro* maturation (IVM), fertilization (IVF) and embryo culture (IVC) is now an important procedure not only for basic study in reproductive biology but also for obtaining live offspring as an application technology. The *in vitro* developmental competence or viability of porcine IVM-IVF oocytes to the blastocyst stage was first confirmed about 20 years ago [Mattioli *et al*. 1989]. Since then, live-born piglets have been obtained from IVM-IVF embryos after IVC to the 2- to 4-cell stages [Mattioli *et al*. 1989; Yoshida *et al*. 1993; Funahashi *et al*. 1996; Funahashi & Day 1997] and to the blastocyst stage [Marchal *et al*. 2001; Kikuchi *et al*. 2002].

IVP procedures to generate porcine embryos have been improved, but the systems have still problems resulting in poor developmental ability, and low quantity and quality of the IVP embryos. This leads to the failure of pregnancy or embryo loss even after their transfer to recipients. One of the major causes of this problem is abnormal ploidy of IVP embryos causing by 1) fertilization of oocytes arrested at the immature stage [Kikuchi *et al*. 1999; Somfai *et al*. 2005] and 2) polyspermy during IVF [Somfai *et al*., 2008].

It is important to make every effort to achieve normality in IVP embryos after IVM and IVF. However, it is basically impossible to prevent immature meiotic arrest or polyspermy in all cultured oocytes even using currently established technologies. Another approach for obtaining a good result under these conditions is to select fertilized oocytes or embryos that guarantee the developmental competence to piglets when they are transferred to recipient females. It is generally accepted that only monospermic fertilized oocytes that have matured to the metaphase-II stage can be guaranteed to show normal embryonic development. On the other hand, in comparison with sperm, oocyte sources are limited, especially those from rare genetic resources, genetically modified and cloned animals. To apply *in vitro* reproductive technologies in such animals, it is important to utilize as many oocytes as possible from a limited number of females. For this propose, assuming some ability to develop to term, abnormally generated embryos may be used to generate offspring.

Aneuploidy during oocyte maturation has been noticed as a cause of embryo abnormality in pigs [Lechniak *et al*. 2007]. Mammalian embryos with abnormal ploidy have been known for long years to be capable of surviving even into the post-implantation period [Piko & Bomsel-Helmreich 1960; Bomsel-Helmreich 1971; Han *et al*. 1999]. However, the details of the mechanism involved have remained unclear. In our previous study, some IVM-IVF oocytes with abnormal ploidy after polyspermy found to be able to develop into diploid embryos [Somfai *et al*. 2008]. The mechanism by which such polyploid oocytes after IVF develop to a normal state during embryo development is still not well understood. Attempts to clarify this mechanism would give an increased efficacy in IVP system including other species, and also basic knowledge for human reproductive medicine.

In the present symposium, as well as in the previous review article [Kikuchi *et al*., 2009], we summarize the status and consequences of generating abnormal porcine IVP embryos obtained as a result of polyspermy and also by fertilization before completion of meiotic maturation. We also discuss the possibility of restoration of the abnormally generated embryos for improvement of porcine IVP efficacy.-

IS2-1

Critical Involvement of Aberrant X Chromosome Inactivation in the Defective Postimplantation Development of Cloned Embryos

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Cloning mammals by somatic cell nuclear transfer (SCNT) is highly inefficient because of erroneous reprogramming of the donor genome in the reconstructed cloned embryos. Reprogramming errors appear to arise randomly, but the presence of many SCNT-specific phenotypes (e.g.; placental abnormalities, obesity, immunodeficiency, and perinatal death) suggests that some reprogramming errors may also arise in specific genes or regions within the donor genome. We sought to identify the upstream dysfunction leading to impaired development of clones by DNA microarray analysis using single cloned embryos.

When we compared the global gene expression patterns of SCNT blastocysts with those of IVF embryos, genes on the X chromosome were specifically downregulated. This phenomenon was sex- and genotype-independent because the average X:autosome (X:A) expression ratio in the four types of cloned embryos (two males and two females) was consistently lower than in the corresponding control embryos. The chromosome-wide gene downregulation on the X chromosome in cloned embryos was reminiscent of X chromosome inactivation (XCI). As XCI is established by *Xist* RNA coating in cis, we next examined whether *Xist* was expressed excessively in cloned embryos. As expected, the *Xist* expression level was significantly higher in male and female cloned embryos than in corresponding IVF embryos. RNA fluorescent in situ hybridization (RNA FISH) revealed ectopic expression of *Xist* from the active X chromosome (Xa) in cloned embryos of the both sexes. Interestingly, deletion of *Xist* on Xa resulted in normalization of the expression levels of the majority of downregulated genes, not just on this chromosome but also on autosomes. This resulted in a 10- to 12-fold increase in the average cloning efficiency (producing birth rates of 5–18%), which clearly suggests that the ectopic expression of *Xist* impaired the development of SCNT embryos. Furthermore, transient downregulation of *Xist* by a RNA interference (RNAi) technique also lead to 10 times more survival of the *Xist*-siRNA embryos compared with controls as early as day 5.5 and this high survival persisted until term. This clearly indicates that SCNT-specific XCI dysregulation can be confined to a narrow stage before implantation and that *Xist* knockout exerts its greatest effect in rescuing cloned embryos during this very short period.

A detailed analysis of gene expression patters of *Xist*-knockout cloned blastocysts suggested the presence of another *Xist*-independent mechanism that specifically inactivated the *Magea* and *Xlr* family of genes on the X chromosome. These comprise gene clusters overlapping with regions enriched for histone H3 lysine 9 dimethylation (H3K9me2), which are highly conserved in somatic cells. We infer that they must have been in a constitutively repressed state, inherited from the donor genome. In contrast to the successful conversion of ectopic *Xist* expression by specific RNAi, this H3K9me2-induced epigenetic aberration was not rescued by RNAi against the *G9a* or *Glp* genes, which are responsible for the establishment of the H3K9me2 modification.

Thus, our studies provide the clear evidence that SCNT induces epigenetic errors specifically on Xa, which are likely beyond the ability of putative ooplasmic factors to remedy. Contrary to the general assumption, our results suggest that the fate of cloned embryos may be determined predominantly before implantation by their XCI status.

IS2-3

Difference in the DNA Methylation Status Arises after Segregation of Trophoblast and Embryonic Cell Lineages

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The mammalian genome contains tissue-dependent and differentially methylated regions (T-DMRs). Cell type-specific DNA methylation status of T-DMRs constitutes DNA methylation profile of the cell, which ought to be reconstructed as cells differentiate. The first cell differentiation in the mammalian development separates the trophoblast and embryonic cell lineages resulting in the formation of trophectoderm (TE) and inner cell mass (ICM) in blastocyst. Although lower level of global DNA methylation in TE compared to ICM has been suggested by immunostaining, dynamics of DNA methylation profile during the TE/ICM differentiation has not been carefully studied.

We aimed to identify T-DMRs between trophoblast and embryonic cell lineages and analyzed their DNA methylation status in pre- and post-implantation embryos. *Oct4* and *Nanog* are differentially methylated between trophoblast stem (TS) and embryonic stem (ES) cell lines (TS-ES T-DMRs). These two loci are hyper-methylated in TS cells and hypo-methylated in ES cells. *Elf5* is another known TS-ES T-DMR but exhibits the opposite methylation pattern. A number of other TS-ES T-DMRs have been also found so far in our laboratory. Bisulfite sequencing analysis of 11 TS-ES T-DMRs revealed that the DNA methylation status of them in E6.5 extraembryonic ectoderm and epiblast coincide with those in TS and ES cells, respectively, showing that the TS-ES T-DMRs are surely cell lineage-based T-DMRs. We then collected TE and ICM cells separately from E3.5 blastocysts and subjected to bisulfite sequencing. Interestingly, genomes of TE and ICM exhibited no difference in the methylation status of all T-DMRs examined. They were in extremely hypomethylated status. Thus we identified valuable T-DMRs for delineation of trophoblast/embryonic cell lineages. DNA methylation analysis of these T-DMRs suggested that the DNA methylation profiles specific to each cell lineage are established after the morphological delineation of trophoblast and embryonic cell lineages. DNA methylation may thus contribute to stabilize or fix the cell fate and the differentiation capacity.

IS2-4

Regulatory Pathways Controlling Uterine Spiral Artery Remodeling

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The maternal-fetal interface is a dynamic site where uterine and placental structures cooperate to promote development of the fetus. The rat, mouse, and human each possess a hemochorial placenta. This type of placentation is characterized by erosion of the maternal uterine epithelium and vasculature permitting the direct flow of maternal nutrients to trophoblast. Remodeling uterine vasculature is critical for the success of pregnancy. As gestation progresses, uterine spiral arteries supplying the placenta are modified creating flaccid, low resistance blood vessels. These vessels are the conduit required to meet the nutrient demands of the fetus. Defective hemochorial placentation, including impairments in uterine spiral artery remodeling, leads to pregnancy related disorders (preeclampsia, intrauterine growth restriction, preterm birth. The extravillous/invasive trophoblast lineage is of fundamental importance to development of the maternal-fetal interface and understanding diseases of placentation and pregnancy. Based on *in vitro* analyses several "candidate" signaling pathways regulating the extravillous/invasive trophoblast lineage have been identified; however, the significance of most of these pathways during *in vivo* placentation are unknown. Organization of rat and human placentation sites show significant conservation, especially regarding trophoblast-directed remodeling of the uterine spiral arteries. Both species exhibit deep trophoblast invasion. We have established relevant *in vitro* and *in vivo* research methodologies using the rat as an animal model to investigate the extravillous/invasive trophoblast lineage. Two pathways controlling the extravillous/invasive trophoblast lineage have been identified: i) hypoxia/hypoxia inducible factor and ii) phosphatidylinositol 3-kinase/AKT/FOSL1. We have established the importance of these pathways using both *in vitro* and *in vivo* experimentation. Dissection of these pathways has facilitated identification of fundamental regulators of the extravillous/invasive trophoblast lineage. Understanding molecular mechanisms underlying development of the extravillous/invasive trophoblast lineage is key to identifying developmentally sensitive events that are potentially susceptible to dysregulation; and represent opportunities to discover and evaluate approaches for the early detection and treatment of diseases of placentation. (Supported by NIH-HD20676)

IS2-5

Novel Proteins Affecting Cell Proliferation and Angiogenesis of Bovine Placenta

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Placenta is temporal organ that plays a role as an interface for feto-maternal dialogue. A variety of molecules is produced and utilized for the manifestation of placental function. Ruminants form unique cotyledonous placenta characterized by a large number of placentomes. Two separate types of trophoblasts, trophoblast mononucleate cells (MNCs) and trophoblast giant/binucleate cells (BNCs), play a principal role on the placental function in ruminants.

MNCs produce interferon tau, trophoblast kunitz domain proteins, pregnancy-associated glycoprotein-2 and other MNC-specific proteins. Recently authors have identified a novel intronless gene uniquely expressed in ruminant MNCs. Since open reading frame of this gene was flanked by reversed *Alu* sequences, we concluded this gene was one of the *Alu* integrated retrotransposon. This gene was composed of 22-a.a.-long signal peptide and 78-a.a.-long single Ly-6 domain, therefore we named this gene "Secreted protein Of Ly-6 Domain 1" (*SOLD1*). *SOLD1* mRNA is exclusively transcribed in MNCs, however SOLD1 protein is localized in the interstitial tissue of placenta. This apparent paradox is attributable to the anchoring of SOLD1 to extracellular matrix through interaction with type I collagen. SOLD1 protein regulates gene expression in cotyledonary fibroblasts *in vitro* and appears to participate in the construction of ruminant placenta. Our results suggest that the target site of collagen-anchored SOLD1 is restricted to near the storage site and biological signal of SOLD1 is triggered by their liberation during the degradation of extracellular matrices. Except in ruminants, neither placental expression nor intronless structure of Ly-6 family gene, have not been reported. The uniqueness of *SOLD1* gene implies that *SOLD1* is specific for ruminants at least in cattle, sheep and goats. Since it has been proposed that mammals have evolved by the insertion of exogenous elements such as retrotransposons and retroviruses, retroposed *SOLD1* might have impacts on ruminant implantation and placentation.

Ruminant placenta produces an array of proteins structurally and functionally similar to prolactin (PRL). Of these only the placental lactogen is known as a "classical" member because of functional similarity to PRL. The rest is called as "non-classical" member without lactogenic activity. Biological activities of non-classical members in ruminants remain unclear. The authors have cloned novel ovine *PRL* paralogue, named ovine prolactin-related protein (*oPRP1*). *oPRP1* had a typical *PRP* nucleotide sequence similar to *bPRP1* and was exclusively transcribed in BNCs as well as *bPRP1*. However translational frame shifting around the C-terminus region of *oPRP1* resulted in the premature termination, translated oPRP1 was anticipated to have a short polypeptide sequence. Recent studies revealed that rat and human PRLs could be cleaved by cathepsin and resulting fragments acquired a new biological activity quite different from that of parental molecules. Since biological activities of oPRP1 was appeared to be comparable with that of bPRP1, the authors hypothesized that bPRP1 expressed proper biological activity following C-terminal cleavage by some enzymes. Short-form (23–28 kDa) bPRP1 fragments co-existed with intact molecules in bovine cotyledonary conditioned medium (BCCM). Recombinant bPRP1 was cleaved by cathepsins and matrix metalloproteinases (MMPs) resulting in N-terminal fragments ranging 24–28 kDa. BCCM itself was capable of bPRP1 cleavage and contained MMP activity. Cleaved bPRP1 stimulated the proliferation of bovine brain microvascular endothelial cells *in vitro*. Our results suggest that bPRP1 is potential substrate for placental enzymes including cathepsins and MMPs, and resulted N-terminal fragments stimulate placental angiogenesis.

Collectively, ruminant placenta produces many kinds of molecules those act harmoniously, particularly placental proteins those responsible for tissue remodeling and angiogenesis play crucial roles for the maintenance of gestation and fetal development. (Supported by grants from the JSPS Japan, 20380159 and 22580327)

IS2-6

Select Nutrients in the Uterine Lumen of Sheep and Pigs Affect Conceptus Development

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Interferon tau (IFNT), the pregnancy recognition signal from trophectoderm (Tr) cells of ruminant conceptuses (embryo and its extra-embryonic membranes), abrogate the uterine luteolytic mechanism to ensure maintenance of functional corpora lutea for production of progesterone (P4). Additionally, IFNT acts in concert with P4 to induce expression of genes for production of histotroph. Histotroph includes nutrients, such as glucose and arginine (Arg), that activate mechanistic mammalian target of rapamycin (MTOR) nutrient sensing cell signaling to stimulate proliferation, migration, differentiation and translation of mRNAs by conceptus Tr.

In sheep, Arg and glucose increase 16- and 12-fold, respectively in uterine histotroph between Days 10 and 15 of pregnancy due to expression of transporters for glucose (*SLC2A1* and *SLC5A1*) and Arg (S*LC7A2B*) in uterine luminal (LE) and superficial glandular (sGE) epithelia induced by P4 and stimulated by IFNT. *In vitro*, 0.2 mM Arg and 4 mM glucose (physiological concentrations in plasma) stimulate phosphatidylinositol 3-kinase (PI3K) cell signaling to increase 3-phosphoinositide-dependent protein kinase 1 (PDPK1), V-akt murine thymoma viral oncogene homolog 1 (AKT1), cAMP response element-binding protein 1 (CREB1), glycogen synthase kinase 3-beta (GSK3B), MTORC1, eukaryotic translation initiation factor 4e-binding protein 1 (EIF4EBP1) and ribosomal protein S6 kinase (RPS6KB1). Further, in Day 16 ovine conceptus explants cultures, Arg increases GTP cyclohydrolase 1 (*GCH1*) mRNA while both Arg and glucose increase ornithine decarboxylase (ODC), nitric oxide synthase 2 (NOS2), and GCH1 proteins, and Arg increases IFNT. GCH1 is the rate-limiting enzyme for synthesis of tetrahydrobiopterin, an essential cofactor for all NOS isoforms. Arg can be metabolized to nitric oxide (NO), by NOS, and to polyamines, by ODC1, via arginase. Both NO donors (SNAP and DETA) and putrescine (polyamine) increase proliferation of oTr cells, and their effects are partially inhibited by L-NAME and Nor-NOHA that inhibit NOS and arginase, respectively. Secreted phosphoprotein 1 (SPP1) in ovine uterine histotroph increases migration, focal adhesion assembly, adhesion, and perhaps Tr cell proliferation.

In pigs, total recoverable glucose, Arg, Leu and Gln increase in histotroph with advancing days of the estrous cycle and pregnancy; the greatest increase being between Days 12 and 15 of pregnancy. The glucose transporter *SLC2A1* mRNA is highly expressed in uterine LE of cyclic and pregnant gilts, but less so in conceptus Tr. Expression of *SLC2A4* mRNA is similar to *SLC2A1*, but expression is lower. *SLC2A2* mRNA is abundant in conceptuses from Days 12 to 85 of pregnancy. *SLC5A1* mRNA is expressed specifically in uterine LE on Days 12 and 13 of pregnancy. *SLC2A3* mRNA is expressed very weakly in pig conceptus and uterine tissues. The most abundantly expressed amino acid transporters are those for glutamate and neutral amino acids (*SLC1A1*, *SLC1A4*) and cationic amino acids (S*LC7A1*, *SLC7A2*, *SLC7A7*, *SLC7A9*). *SLC7A3* mRNA is highly expressed in conceptuses from Days 25 through 85 of gestation. Treatment of porcine Tr cells with either Arg (0.4 mM) or Leu (0.4 mM) increases phosphorylated RPS6K, RPS6 and EIF4EBP1 in the nucleus or cytoplasm, and proliferation of Tr cells by 8.2- ($P<0.01$) and 11.5-fold ($P<0.01$), respectively. Glucose and fructose also stimulate proliferation of pig Tr cells 3.4- ($P<0.01$) and 5.2-fold ($P<0.01$), while Azaserine, an inhibitor of hexosamine biosynthesis inhibits their effects on Tr cell proliferation.

These results indicate mechanisms whereby select nutrients act differentially to affect translation of of mRNAs for cell signaling molecules that affect conceptus growth, development, and survival during the peri-implantation period of pregnancy.

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IS2-7

Gene Expression in the Bovine Endometrium: The Effects of Altered Progesterone Concentrations and the Presence of the Conceptus

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In cattle, the majority of pregnancy loss can be attributed to early embryonic loss which occurs prior maternal recognition of pregnancy on Day 16 (Day 0 = ovulation). During this time, two significant processes are required to establish and maintain pregnancy. Firstly, the developing conceptus (embryo and associated extra embryonic membranes) must undergo significant morphological changes from a blastocyst enclosed in a zona pellucida to a rapidly elongating conceptus. This elongation process is entirely dependent on maternally derived secretions (termed histotroph) from the uterine endometrium as, in sheep, ablation of uterine glands renders the uterus incapable of initiating this elongation process. Secondly the uterine endometrium must prepare for attachment and implantation of the conceptus which involves carefully orchestrated spatio-temporal alterations in the transcriptomic profile of the endometrium. The two main modulators of the endometrial transcriptome and by corollary maternally derived secretions are the hormone progesterone (P4) and the pregnancy recognition signal interferon tau (IFNT).

The important role of P4 in the establishment and maintenance of pregnancy have been well documented. Elevated concentrations of circulating P4, either endogenous or by exogenous supplementation, in the immediate post-conception period are associated with advanced conceptus elongation, increased IFNT production and higher pregnancy rates in both beef and dairy cattle. Conversely, low P4 concentrations have been implicated as a causative factor in the low pregnancy rates observed in dairy cows with recent data from demonstrating that post partum dairy cows have lower circulating concentrations of P4 associated with an impaired ability of the oviduct/uterus to support embryo development, compared to their dairy heifer counterparts. The mechanism through with P4 impacts on conceptus development is mediated through effects on the endometrium, rather than a direct effect on the embryo itself. Indeed, transfer of *in vitro* cultured embryos on Day 7 into recipient heifers that have had their P4 concentrations manipulated have resulted in altered conceptus elongation. Transcriptomic analysis of the uterine endometrium has shown that modulation of circulating P4 concentrations alters endometrial gene expression which is beneficial (when P4 is supplemented) or detrimental (when P4 is reduced) to the developing conceptus. These changes in the endometrium involve alterations to genes that can contribute to histotroph composition with demonstrated consequences for conceptus elongation. In addition to these P4-induced alterations in endometrial gene expression, P4 is required to down-regulate its own nuclear receptor in uterine luminal epithelium (LE) and superficial glandular epithelium (sGE) to establish uterine receptivity to implantation and to allow the expression of genes that encode for secreted proteins and transporters that drive conceptus elongation to be switched on.

In addition to P4 altering endometrial gene expression, the pregnancy recognition signal IFNT also has a significant impact on the endometrial transcriptome. IFNT, a type 1 interferon, includes the expression of classical interferon stimulated genes, which are predominantly maintained in the deep glandular epithelium and stromal cells, for protection of the developing semi-allograph conceptus from the maternal immune system. However, IFNT can also stimulate the expression of non-classical genes in the LE and GE which contributes to histotroph composition and drive conceptus elongation. In summary, the successful establishment of pregnancy in cattle requires a sequence of key events to ensure appropriate maternally derived secretions, establish uterine receptivity to implantation as well as an adequate endometrial response to IFNT production.

IS2-8

Development of the Endometrial Spheroids as a Model for Implantation *In Vitro*

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The endometrium is one of the most complex tissues; it undergoes dynamic changes in response to the process of implantation and the stages of pregnancy. An *in vitro* model may provide a tool for clarifying the complex implantation process. The spheroid has been utilized in cell biology research, because it appears to mimic the morphology and physiology of living tissues and organs are unlikely to conventional monolayer culture. The cells in multicellular spheroids are in close contact with each other and have considered useful in the investigation of intercellular interactions. Recently, we have developed multicellular spheroids composed of endometrial cells derived from bovine or rat endometrium. In this study, interaction of endometrial spheroids and embryos was analyzed in a co-cultured system in order to develop a model to clarify the mechanism of implantation *in vitro*.

Bovine endometrial spheroids were hetero-spheroids, composed of epithelial and stromal cells, generated using L-ascorbic acid phosphate magnesium salt *n*-hydrate which stimulated collagen synthesis in stromal cells. Histological observation revealed that hetero-spheroids were covered by epithelial cells on the outer layer. The spheroids expressed receptor genes for steroid hormones (*ERα*, *PR*) and INFτ (*INFR1*, *INFR2)*. Moreover, the spheroids expressed *Mx1* as a response to INF. The spheroids were co-cultured with the trophoblast tissues collected from bovine elongated embryos (5 mm) at d 18 of pregnancy. P4 (1 μM) and IFNτ (100 ng/ml) were added to the medium during co-culture. At the starting of co-culture, spheroids and trophoblast tissues were partially contacted. The spheroids gradually adhered to the trophoblast tissues, and most part of the spheroids were embedded in to the tissue at 7 days of co-culture. Further we observed that vesicles were developed from the trophoblast tissue during the co-culture. Expression of *MMP-9* was significantly increased in co-cultures, compared to single culture of spheroids or trophoblast tissues ($P<0.05$).

Rat endometrial spheroids were homo-spheroids, composed of only endometrial stromal cells derived from d 5 of pregnancy, generated using salmon aterocollagen (SAC) gels. Diameters of the spheroids were about 500 μm at d 5 after detachment from SAC gels. The results of gelatin zymography showed that both MMP-2 and -9 were produced in monolayer culture. However, after the detachment of the cell sheet, the production of both MMPs decreased immediately and could not be detected until 15 days after detachment. *In vitro* decidualization of the spheroids were induced by arachidonic acid (AA) treatment. RT-PCR analysis showed that typical marker genes for the decidualization, desmin and d/tPRP, were expressed in the spheroids after 2 days of AA treatment, but not in the control groups without the treatment. When the spheroids were co-cultured with hatched blastocyst rat embryos, they started to adhere at 24 h and embryos embedded in to the spheroids at 96 h after the beginning of co-culture. Furthermore, activities of MMP-2 and -9 were detected in the co-cultures, whose activities were not detected in single culture of the spheroids or the embryos.

The present study demonstrated that the endometrial spheroids express receptors for factors important in the process of implantation, such as steroid hormones and INFτ. Furthermore, the spheroids and the embryos adhered and interacted with each other for the occurrence of accompanying tissue remodeling which indicated by MMP expression. These results indicate that the endometrial spheroids provide a new insight into the study of endometrial functions and implantation, as a model for implantation *in vitro*.

IS2-9

Endogenous Retroviruses: A Model System for Understanding Physiological Adaptation to a Rapidly Evolving Ruminant Genome

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Endogenous retroviruses (ERVs) are present in the genome of all vertebrates and are remnants of ancient exogenous retroviral infections of the host germline transmitted vertically from generation to generation. Sheep betaretroviruses offer a unique model system to study the complex interaction between retroviruses and their host[1]. The sheep genome contains 27 JSRV-related endogenous betaretroviruses (enJSRVs) related to the pathogenic Jaagsiekte sheep retrovirus (JSRV). The enJSRVs have been integrating in the host genome for the last 5-7 million years. By using enJSRVs as genetic markers and morphological traits[2], it was determined that sheep populations originating from early domestication process in the Middle East (approximately 9,000 years ago) dispersed across Eurasia and Africa via two separate migratory episodes. The later migration, involving sheep with improved production traits, shaped the great majority of present-day breeds. The ability to differentiate genetically primitive sheep from more modern breeds provides valuable insights into the history of sheep domestication.

The exogenous JSRV is a causative agent of a transmissible lung cancer in sheep. Interestingly, enJSRVs are able to protect their host against JSRV infection by blocking different steps of the viral replication cycle. Sixteen of the 27 enJSRV loci contain an env gene with an intact open reading frame, and those env can block entry of the exogenous JSRV via receptor interference. Further, two enJSRV proviruses, which entered the host genome within the last 3 million years (before and during speciation within the genus Ovis), acquired in two temporally distinct events a defective Gag polyprotein resulting in a transdominant phenotype able to block late replication steps of JSRV and related exogenous retroviruses. Both transdominant proviruses became fixed in the host genome before or around sheep domestication. Indeed, endogenization and positive selection of ERVs acting as restriction factors is a mechanism used by the host to fight exogenous retroviral infections.

The enJSRVs are most abundantly expressed in the uterine luminal and glandular epithelia as well as in the conceptus (embryo and associated extraembryonic membranes) trophectoderm, and *in utero* loss-of-function experiments found the enJSRVs envelope (env) to be essential for conceptus elongation and trophoblast growth and development[3]. Recently, 5 of the 27 enJSRV loci were found to contain a complete and intact genomic structure and are able to produce viral particles *in vitro*. New evidence supports the idea that those intact enJSRV loci expressed in the uterine endometrial epithelia are shed into the uterine lumen and then transduce the conceptus trophectoderm. Infection of the conceptus with endometrial-derived enJSRVs is hypothesized to influence development and differentiation of the trophectoderm.

Collectively, available evidence in sheep and other mammals strongly supports the ideas that ERVs coevolved with their hosts for millions of years and were positively selected for biological roles in genome plasticity, protection of the host against infection of related pathogenic and exogenous retroviruses, and a convergent physiological role in placental morphogenesis and mammalian reproduction.

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IS3-1

DNA Methylation Profiling by Tissue-dependent and Differentially Methylated Regions (T-DMRs) in Pluripotent Stem Cells: Insights into Genome Organization

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Every cell type and tissue has a distinctive DNA methylation profile (an entity of epigenome) that consists of thousands of tissue-dependent and differentially methylated regions (T-DMRs) exhibiting differentially methylation status in a tissue-dependent manner. T-DMRs have been identified at promoter regions of genes encoding key molecules for cell phenotypes, such as *Pou5f1* and *Nanog* genes involved in pluripotency of embryonic stem cells. Genome-wide DNA methylation profile, which was investigated using a microarray-based analysis (D-REAM), illustrates the tissue/ cell-type specific function by illuminating the key transcription factors and their target genes, suggesting DNA methylation profile is fundamentals of genome function.

Uniqueness of DNA methylation profile to a cell type allows us to distinguish the kind of cell type from the others exhibiting similar phenotypes. For example, iPS cells shows distinct methylation profiles from the other pluripotent stem cells established from embryo, and incomplete methylation is observed at T-DMRs located in somatic tissue specific genes, which are methylated in ES cells.

When we focus the genomic features associated with T-DMRs, quite unique characteristics were associated with T-DMRs identified in ES cells in compared with somatic tissues/ cells. T-DMRs hypomethylated in the both human and mouse pluripotent cells were significantly associated with CpG islands (CGIs), and these T-DMRs were predominantly existed in short interspersed elements (SINEs) rich/ long interspersed elements (LINEs) poor genomic loci, spreading continuously over hundreds kb to 1 Mb containing multiple hypomethylated T-DMRs, which constituted epigenetic clusters. These epigenetic clusters were found within the gene clusters, however, are not always. By using mouse genome full-tiling array, T-DMRs were identified not only in genic but also in intergenic regions, and correlations between genomic activities and enrichment of transposable elements were observed around T-DMRs hypomethylated in the pluripotent cells. These genomic features around T-DMRs were conserved between human and mouse, suggesting that epigenome has an impact on genomic organization.

Gene clusters, generated by multipletimes of gene duplication, often show species or lineage specific patterns. Expansion of prolactin gene families had occurred in rodent genomes, and members in these gene families are expressed in tissue-specific manners. We identified T-DMRs at promoter regions of all members in prolactin gene family in mouse, which exhibit hypomethylation in tissues expressing the corresponding genes. Of our interest, we observed the correlation between genomic organization, DNA methylation patterns and probable ancestral genomic sequences. Considering the effects of DNA methylation in C->T conversion occurred in mammalian genomes, DNA methylation profile provides us insights into involvement of epigenetic system in genome organization.

IS3-3

Exposure to a Maternal High Fat Milieu Alters the Hepatic Epigenome in Mice

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Type2 diabetes mellitus (T2DM) and metabolic syndrome are modern day plagues in industrialized and developing nations alike. The incidence of these diseases has reached epidemic proportions. Exposure to a sub-optimal maternal environment such as, low protein diet, global caloric restriction, prenatal stress and high fat (HF) diet are known to be associated with increased incidence of cardiovascular disease, T2DM and metabolic syndrome later in life. However, the molecular basis of this enhanced susceptibility for metabolic disease is poorly understood. Dysregulation of the epigenome may play an important role in this process. Pathologic changes in gene expression observed prior to development of overt disease support this hypothesis. To address this hypothesis, we performed DNA microarray and genome-wide DNA CpG methylation analyses to measure mRNA expression and DNA methylation patterns in liver of 5 wk old male mice exposed to either a control or HF diet in utero.

Exposure to a HF diet in utero resulted in decreased fetal body weight, but accelerated weight gain, increased adiposity and large adipocytes early in postnatal life. Serum glucose was increased and adipokines including PAI-I and adiponectin were pathologically modulated in HF offspring. Furthermore, this is associated with the early life manifestation of impaired insulin and glucose tolerance. Exposure to a maternal HF milieu activated genes of immune response, inflammation and hepatic dysfunction. Conversely, genes of lipid metabolism and biogenesis were down regulated especially in the cholesterol biosynthesis pathway. DNA methylation was assessed by the HELP assay (HpaII tiny fragment Enrichment by Ligation-mediated PCR). This analysis revealed 3360 differentially methylated loci, the majority of which (76%) were hypermethylated in HF liver. DNA hypermethylation was accompanied by increased mRNA expression of DNMT1 and 3B in fetal liver. DNMT activity was also increased in fetal but not 5wk old liver. The distribution of DNA hypermethylation was similar in promoters, gene bodies and intergenic regions (75%, 82% and 72%, respectively). Less hypermethylation was noted at CpG islands particularly those present in promoters (51% hypermethylation). This pattern of promoter methylation is consistent with the results of the gene expression microarray whereby the majority of genes were upregulated in HF vs. Control liver. Interestingly, the distribution of hypermethylated loci on Chromosomes 4 and 18 displayed clustered patterns whereas the other chromosomes had a random distribution of hypermethylated loci. On chromosome 4, half of the hypermethylated loci (170 out of 347) mapped to the atherosclerosis QTL and 80% of the hypermethylated loci (67 out of 138) mapped to the Insulin dependent diabetes susceptibility 21 QTL on chromosome 18. Most of the hypermethylated genes in these hot spots are associated with cardiovascular system development and function. These results suggest that hypermethylation of loci in the QTLs on Chromosome 4 and 18 may be strongly involved in the increased susceptibility to metabolic syndrome seen in offspring exposed to a maternal HF diet. Of these, Rho Guanine Nucleotide Exchange Factor 19 (ARHGEF19), which plays a key role in adipocyte differentiation, was hypermethylated in the promoter region and this was associated with a 60% reduction in mRNA in HF exposed offspring. In summary, exposure to a maternal HF diet is associated with: 1) alterations in expression of hepatic genes in pathways of inflammation, fibrosis and cholesterol, 2) CpG DNA hypermethylation throughout the genome with clustered hypermethylation of loci in QTLs associated with atherosclerosis and diabetes, 3) promoter hypermethylation and reduced expression of ARHGEF19, a negative regulator of adipogenesis. Our data strongly suggests exposure to maternal HF diet programs increased susceptibility to insulin resistance, obesity and metabolic disease, in part, through increased DNMT activity and alterations in hepatic DNA CpG methylation.

IS3-4

Genome-wide DNA Methylation Profiling of Male and Female Mouse Embryonic Stem Cell Differentiation Using Massively Parallel Sequencing

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DNA methylation is a chemical modification of genomic DNA and occurs predominantly at the cytosine residue of CpG dinucleotides (CpGs) in mammals. The majority of CpGs in the mammalian genome are methylated, with only a minority of CpGs remaining unmethylated. CpG-dense regions are referred to as CpG islands, and are often located in close proximity to gene promoters, where they are normally unmethylated. Aberrant DNA methylation patterns are implicated in the pathogenesis of an increasing number of diseases, particularly tumorigenesis. More specifically, the acquisition of DNA methylation within the promoter regions of tumor suppressor genes is frequently observed in numerous tumor types. DNA methylation plays an important role in transcriptional regulation. When a CpG island adjacent to a transcription start site (TSS) becomes methylated, the corresponding gene tends to be down-regulated. Interestingly, recent research showed that methylation occurring within gene bodies is also correlated with gene expression, notably DNA hypermethylation in the gene body is associated with active transcription. This is opposite to the association in promoter regions. These findings indicate the importance of analyzing DNA methylation patterns genome-wide.

It is well known that DNA methylation is essential for normal development. Each tissue and cell type has a unique DNA methylation profile and the acquisition of the appropriate tissue-specific DNA methylation profile is important for having normal function of tissues. Inner cell mass (ICM) derived embryonic stem (ES) cells are pluripotent and are known to be hypermethylated cell lines when maintained in an undifferentiated state. Interestingly, this hypermethylation is only found in male (XY) ES cells, whereas DNA methylation in female (XX) ES cells is globally reduced with hypomethylation reported for repetitive elements and several unique sequences such as parentally imprinted genes. However, little is known about the differences in genome-wide DNA methylation profiles between XY and XX ES cells. We tested the patterns of DNA methylation during *in vitro* differentiation of both XX and XY ES cells to understand the epigenetic differences between male and female stem cells.

To test the genome-wide DNA methylation profile, we employed our novel protocol, HELP (HpaII tiny fragment Enrichment by Ligation-mediated PCR)–Tagging. HELP-tagging is a massively parallel sequencing (MPS) based technique. MPS is a remarkable new technology that has provided the foundation for many new genome-wide assays, with several protocols developed to test DNA methylation using MPS. The HELP-tagging assay is a rapid and relatively low-cost method for the quantitative, genome-wide assessment of cytosine methylation that can examine the methylation status of greater than 98% of CpG islands. The HELP assay uses the methylation sensitive enzyme HpaII and its methylation insensitive isoschizomer MspI to interrogate the methylation status of the CpG within the context of the CCGG sequence recognized by these enzymes. Tagging is accomplished through the ligation of adaptors within which resides the sequence for the type III restriction enzyme EcoP15I, which can generate 27 bp of flanking sequence. This tagging procedure facilitates the unambiguous identification and location of about 80% of MspI sites. Since MspI sites distribute evenly in unique and repetitive elements, genic and non-genic, and CpG dense or CpG depleted regions, HELP-tagging can assess DNA methylation status not only in promoters of genes or CpG islands, but also in gene bodies and CpG depleted regions. Using this technique, we confirmed the hypomethylated status in XX ES cells and we found the DNA methylation level of XX ES cells to increase during differentiation, consistent with prior observations. Furthermore we found that not only parentally imprinted genes but also non-imprinted genes and intergenic regions were hypomethylated in these female ES cells. In this talk, we are going to discuss the genome-wide DNA methylation profiling of male and female ES cells and the changes observed during *in vitro* differentiation.

IS3-5

Epigenetics of Human Reproduction and Developmental Abnormality

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Epigenetic gene regulation is essential in mammalian reproduction. Especially, it is well known that genomic imprinting, an epigenetic phenomenon whereby genes are differentially expressed according to their parental origin, is known to be crucial for placental development and fetal growth in mammals. Most imprinted loci contain a differentially methylated region (DMR) harboring allelic DNA methylation inherited from the male or the female gamete (germline DMR). Mutations/epimutations affecting DMRs cause rare imprinting disorders such as Silver-Russell syndrome (SRS) and Beckwith-Wiedemann syndrome (BWS). Whether imprinted genes also play critical roles in the more common abnormal pregnancy cases such as preeclampsia and intrauterine growth restriction (IUGR) is an important question yet to be fully evaluated. With the aim of addressing this question, we established quantitative COBRA (Combined Bisulfite Restriction Analysis) assay conditions for 30 DMRs from 22 loci that include the majority of known human imprinted DMRs. Using this COBRA assay system, we initially measured DNA methylation levels at these 30 DMRs in twenty blood samples from healthy individuals and twenty placental tissues from normal pregnancies, and determined the extent of tissue specificity and individual varieties of methylation levels. Subsequently, we have screened for epimutations at these DMRs in the placental tissues from approximately 100 IUGR cases. In two cases, among the DMRs examined, only H19- and IGF2-DMRs were found to be hypomethylated compared to the normal controls. Several other cases showed hyper- or hypo-methylation at the DMRs other than those in the H19/IGF2 locus, and are being further characterized. The genetic etiologies of IUGR are largely unknown and considered to be diverse. Our results suggest that placental epimutation at DMRs may account for the growth restriction phenotype of a subset of IUGR cases.

We also try to make whole genome DMR mapping with very rare human cases, a parthenogenetic mosaicism and an androgenetic mosaicism.

IS3-6

Role for PAD Enzymes in Chromatin Structure, Gene Regulation, and Disease

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The peptidylarginine deiminase (PAD) family of enzymes post-translationally convert positively charged arginine residues in substrate proteins to the neutral, non-standard residue citrulline. Over the last few years we have been investigating the role of PADs in the regulation of chromatin structure and disease. We have found that, in neutrophils for example, induction of PAD activity by TNFa leads to global histone hypercitrullination and subsequent release of chromatin from the nucleus into the extracellular space. This citrullinated chromatin appears to form the structural "backbone" for Neutrophil Extracellular Traps (NETS) which represent part of the innate immune response to stimuli such as pathogens and inflammation. More recently, we have investigated the role of PADs in gene regulation in the mammary epithelium. We have found that, in this context, PADs appear to primarily function as transcriptional co-regulators. For example, we found that EGF treatment of breast cancer cells induces citrullination of the oncogene Elk1 by PAD4 at promoters such as *c-fos*, leading to histone acetylation and transcriptional activation. On the other hand, we found that treatment of the same cell line with estrogen (E2) leads to PAD2-mediated activation of specific subsets of ER target genes. Interestingly, the mechanism of target gene activation here appears to be via citrullination of histone H3, which in turn, induces promoter-specific chromatin decondensation. Regarding possible links between PADs and breast cancer, we have found that PAD expression and activity is strongly upregulated in models of early stage breast cancer. Importantly, we have been testing several new PAD-specific inhibitors and found that these drugs block tumor progression in cell lines and also in mouse xenograft models of human breast cancer. Taken together, these findings suggest that, in some cases, PAD activity appears to play a positive role in disease resolution, while in other instances, induction of PAD activity appears to promote disease progression.

ISP1 Trophoblast-specific DNA Methylation Occurs after the Segregation of Trophectoderm and Inner Cell Mass in Mouse Periimplantation Embryo

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Cell type-specific DNA methylation status of tissue-dependent and differentially methylated regions (T-DMRs) constitutes DNA methylation profile of cell, which ought to be reconstructed as cells differentiate. The first cell differentiation in the mammalian development separates the trophoblast and embryonic cell lineages resulting in the formation of trophectoderm (TE) and inner cell mass (ICM) in blastocyst. Although lower level of global DNA methylation in TE genome compared to ICM has been suggested, dynamics of DNA methylation profile during the TE/ICM differentiation has not been elucidated. To address this issue, we first focused on the TS-ES T-DMRs that have been previously identified by comparison of trophoblast stem (TS) and embryonic stem (ES) cells. Bisulfite sequencing analysis verified that these TS-ES T-DMRs are differentially methylated between trophoblast and embryonic cell lineages not only in the *in vitro* cell line but also in tissues of embryonic day (E) 6.5 mouse embryos. We also found that the human genomic regions homologous to mouse TS-ES T-DMRs are differentially methylated between human placental tissue and ES cells. Collectively, we defined them as cell lineage-based T-DMRs between trophoblast and embryonic cell lineages (T-E T-DMRs). We then examined TE and ICM cells isolated from mouse E3.5 blastocysts. Interestingly, at all T-DMRs examined, both TE and ICM were in nearly unmethylated status and exhibited no difference, suggesting that the DNA methylation profiles specific to each cell lineage are established after the morphological specification. The T-E T-DMRs identified will serve as valuable epigenetic markers for study of trophoblast/embryonic cell lineages.

ISP2 ATP Content in Bovine Oocytes May not Determine the Timing of First Cleavage and Further Developmental Competence after IVF

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In bovine oocytes early timing of the first mitotic cleavage after fertilization is known to be associated with high developmental competence to the blastocyst stage (Lonergan *et al*., 1999). Also, in matured bovine oocytes ATP levels have been found to correlate with developmental competence (Stojkovic *et al*., 2001). This study was conducted to test the hypotheses that ATP level in bovine oocytes directly affects the timing of first cleavage and blastocyst development after IVF. In the first experiment early cleaving 2-cell stage embryos were selected 27 h after IVF of IVM oocytes (early cleaving group), and ATP levels and subsequent *in vitro* development were compared with those cleaving between 27–30 h of IVF (late cleaving group). In the second experiment oocytes were treated with 0.6 mg/ml L-carnitine during IVM to increase ATP levels and the effect of this treatment on timing of first cleavage and *in vitro* development after IVF was assessed. In early cleaving group the blastocyst formation rate was significantly ($P<0.05$) higher than that in late cleaving group (50.3% vs. 37.6%, respectively); however, ATP levels at the first cleavage did not differ significantly between the two groups (1.83 pmol/embryo and 1.62 pmol/embryo, respectively). L-carnitine increased ATP levels from 2.26 pmol to 3.16 pmol in MII stage oocytes ($P<0.05$), however, timing of the first cleavage and blastocyst formation rates after IVF were not affected. The results suggest that ATP level in IVM bovine oocytes may not determine fundamentally their cleavage speed and competence to develop to the blastocyst stage after IVF. This study was supported by RGJ-PhD grant and by NARO (N36G2Gr1).

ISP3 Excessive Pre-treatment of Mouse Spermatozoa with DNA Non-interacting Agents Causes Zygotic Chromosomal Damage

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[Background] Much time and effort has been invested to improve *in vitro* production (IVP) of embryos, and techniques such as sperm pre-treatment have been useful for obtaining fertilized eggs in IVP. However, the genetic risks to the produced embryos have yet to be fully elucidated. The aim of this study was to investigate the genetic safety of sperm pre-treatment, which is commonly used in IVP, and to elucidate the relationship between zygotic chromosomal integrity and embryonic development using a mouse model. [Methods] Spermatozoa were treated with methyl-β-cyclodextrin (0.75 mM, 90 min), lysolecithin (0.02%, 1 min), Triton X-100 (0.02%, 1 min) and dithiothreitol (DTT; 5 mM, 10–60 min), followed by microinjection into oocytes. Injected eggs were used to examine chromosomal integrity at 1-cell metaphase, and to monitor pre- and post-implantation development. [Results] Chromosomal integrity of spermatozoa was impaired by *in vitro* incubation and chemical antagonism. Particularly in the DTT 60-min group, severe chromosomal damage was observed. Despite this chromosomal damage, however, the resultant embryos frequently developed to the blastocyst stage. Embryos in the DTT 60-min group had significantly lower developmental competence to live fetuses. These results indicate that sperm pre-treatment such as DTT for 60 min generates severe chromosomal damage in ICSI oocytes, and fetal development is not in line with blastocyst formation. These findings provide evidence that paternal chromosomal integrity in zygotes is fully predictive of the fate of embryos in IVP. Given these issues, an innovative method of sperm genetic screening during the pre-fertilization period is urgently required.

ISP4 Epithelial-mesenchymal Transition of Trophectoderm Cells during Embryo Implantation

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At the early stage of embryo implantation to the maternal endometrium, trophoblast and uterine epithelium initiate their first contact via apical cell membranes. This epithelial-epithelial adhesion is recognized as one of the paradoxes in cell biology. In humans and rodents, trophoblast cells invade maternal decidua during placentation. For the acquisition of this invasion competence, it has been postulated that the trophoblast cells undergo an epithelial-mesenchymal transition (EMT). It has also been expected that the EMT participate in the trophectoderm acquisition of adhesion competence during implantation. In this study, we investigated the hypothesis that stimuli from the receptive uterus induce EMT in the trophectoderm.Expressions of epithelial and mesenchymal makers were immunohistochemically examined on tissue sections from gestation day 22 bovine uteri. Expression of genes implicated in EMT process was assessed by RT-PCR analyses of RNAs from days 17, 20 and 22 bovine conceptuses. To evaluate the effects of receptive uterus on the blastocyst, pregnant mice were injected with estrogen (E2) receptor antagonist Tamoxifen (TAM, 10 μg/animal) on gestation day 2.5. Blastocysts from TAM treated or intact mice were collected and subjected further analyses.Our observations revealed that the bovine trophectoderm modified its epithelial property during implantation process, including loss of cytokeratin, expression of vimentin, switch from E- to N-cadherin and upregulation of MMP2 and MMP9. Mouse E2 receptor inhibition model indicated that the down-regulation of blastocyst E-cadherin is regulated by E2 primed receptive uterus. We also observed that the expression of trophoblast stem cell marker Cdx2 was down-regulated during the implantation window. Collectively, our observations indicated that the EMT of trophectoderm was regulated by E2 induced secretion from the uterus, and the stimuli from the receptive uterus might also induce trophoblast differentiation.

ISP5 Identification of Endogenous Retroviruses Derived-gene Possessing a Zinc Finger Like Sequence during Bovine Peri-attachment Period

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[Objective] The endogenous retroviruses (ERVs) are thought to be derived from ancient viral infections of germ cells. Previous studies suggest that ERV genes play an important role in the development of the placenta and the trophoblast cell lineage in mammalian species. The exact evolutionary pathways and the extent to which ERVs function are still unclear. For the bovine species, the genes functioning in placental development are unknown despite their importance to the cattle industry. In this study, we analyze bovine ERV-like genes that are expressed during bovine placentation. [Materials and Methods] We collected bovine conceptuses of days 17, 20, and 22 (day 0 = day of estrus) from super-ovulated Japanese black cattle, and sequenced the expressed RNAs using the SOLiD3 System. We then searched for bovine ERV-like genes on the bovine genome by computational analysis and found candidates sequences. [Results]We used SOLiD3 System and computational analysis and found five ERV-like candidate sequences. Using PCR analysis, the expression of candidate genes were examined and found three candidates sequence's mRNA in days 17, 20, and 22 bovine conceptuses. In particular, expression of one candidate gene on Chromosome 7 (Chr7-539) increased on day 22. The motif search for Chr7-539 revealed that Chr7-539 possessed zinc finger like structure. These results suggest that this gene might function as a transcription factor.

ISP6 Effect of Naloxone, a Mu-opioid Receptor Antagonist, on Oocyte Maturation and Embryonic Development in Pigs

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Studies on horses, cattle and dogs have shown that when added to a maturation medium for oocytes naloxone (Nx), a mu-opioid receptor antagonist, reduces metaphase II (M-II) rate. However, at low concentration, Nx increase the rate of M-II oocytes. The purposes of this study are to investigate the expression of mu-opiod receptor (MOR) gene in oocytes and embryos at different stages, to examine the effect of Nx on nuclear and cytoplasmic maturation of oocytes, and on embryonic development in pigs. Also the combined effect of cAMP and Nx on maturation of oocytes was studied. MOR gene was expressed in oocytes at germinal vesicle and M-II stages, as well as 1-, 4-cell embryos and blastocysts. At concentration of 10^{-8}M Nx increased the M-II rate ($P<0.05$), but at concentration of 10^{-4}M it reduced ($P<0.05$) the rate. However, Nx had no effects on fertilization status and embryonic development ($P>0.05$). The supplementation of Nx into culture medium did not improve the blastocyst formation rate and cell number in blastocysts ($P>0.05$). At concentration of 10^{-4}M, Nx decreased the blastocyst formation rate ($P<0.05$). The addition of both cAMP and Nx into the maturation medium improved the M-II rate ($P<0.05$) compared with the addition of either cAMP or Nx individually. In conclusion, MOR gene was expressed in porcine oocytes and embryos during the maturation and developmental culture, respectively. Nx improved M-II rate at low concentration, but reduced the rate at high concentration. However, Nx did not promote cytoplasmic maturation and improve embryonic development. The addition of Nx and cAMP into maturation medium had a synergistic effect on oocyte maturation.