The 4th JSAR-KSAR Joint Symposium

on "Recent Advances in Animal Reproductive Biotechnology"

Sponsored by Korean Society of Animal Reproduction
and Japanese Society of Animal Reproduction

Organizing committee

Korea: Yong-Mahn Han (KAIST), Gi-Sun Im (NIAS),
Deog-Bon Koo (KRIBB), Bo-Suk Yang (NIAS)

Japan: Yuji Hirao (AFFRC), TAKU (AFFRC)

Friday, June 20, 2008

08:30-09:20 Registration
09:20-09:30 Opening ceremony

Plenary Session **Chairman: Takashi Nagai/Yong-Mahn Han**

09:30-10:10 **Hoon Taek Lee**, Ph.D., Konkuk University
Recent Progress in Animal Reproduction in Korea 3

10:10-10:50 **Eimei Sato**, Ph.D., Tohoku University
Recent Progress in Animal Reproduction in Japan 9

10:50-11:10 Coffee break

11:00-13:00 Poster Session I (odd number)

12:00-13:00 Lunch

Session I : Embryo & Development **Chairman: Won-Kyong Chang/Masashi Takahashi**

13:00 -13:25 **Jeong Mook Lim**, DVM & Ph.D., Seoul National University
Immune-Specific Stem Cell Establishment without Undertaking Nuclear Transfer 12

13:25-13:50 **Masashi Takahashi**, Ph.D., National Agricultural Research Center for Kyushu Okinawa Region
Heat Stress on Development of Bovine Embryos in Relation to Intra-and Extracellular Redox Status 13

13:50-14:15 **Kweon Yu**, Ph.D., KRIBB
Study of Metabolic Syndrome with the Drosophila Model System: sNPF Regulates Insulin Signaling 16

14:15-14:40 **Támas Somfai**, Ph.D., National Livestock Breeding Center
Development of Polyspermic Embryos in IVP Systems of Farm Animals 17

IS 001

Recent Progress in Animal Reproduction in Korea

Mukesh Kumar Gupta and Hoon Taek Lee

Department of Animal Biotechnology, Bio-Organ Research Center, Konkuk University, Seoul 143 701, South Korea

Reproduction technology and biotechnology have been interacting closely in Korea and constitute a rising industrial and scientific powerhouse of the country. The country's biotechnology industry comprises 600-odd companies, spread across four regional bio-clusters and 25 centers. Of these, ~71 percent companies are working in the area of biomedicine followed by bio-processing and equipment. Behind this phenomenal growth is not only the overheated vying for technology and the interests of scientists and entrepreneurs, but also is the continual support of the Korean government. The government is firmly committed to the development of biotechnology sector that US$1.35 billion would be invested. This is additional funds to state-based R&D projects valued at US$9.2 billion and raising another US$22 billion through issuing state based bonds to foster the commercialization of next generation technologies. Most recently, the "biomedicine and organ project"has been included among the 10 next generation growth engines, for which the government support and funding has been stepped up.

Embryo Technology:

Since the first tube-baby was born in 1985, more than a hundred infertility clinics have sprawled throughout the country and above 20% of the world's newborns that went through IVF or ICSI are born in Korea. And production of animal offspring through ART has become almost a routine technology in several institutes and live offspring have been produced by IVF, ICSI or SCNT for their commercial application. Transgenic mouse and cow producing Lectoferrin, pig producing erythropoietin and goat producing the human GCSF and growth hormone were produced in 1997 ~ 1998. Scientists have also been pioneering the cloned offspring production in pets such as dogs and cats (Lee et al. 2005; Yin et al. 2008). More recently, extensive research is also underway in the field of interspecies and intergenus SCNT (iSCNT) that has potential to save endangered

species (Uhm et al. 2007).

Epigenetics and phenotypic abnormalities in cloned animals

SCNT clones exhibit high levels of phenotypic abnormalities that are associated with incomplete or faulty epigenetic reprogramming and/or genomic damage of the transplanted nucleus. Scientists in Korea have long been investigating the causes and solution for these errors of SCNT. The research scientists at KRIBB had earlier shown the epigenetic error due to aberrant methylation pattern in satellite DNAsequences in cloned bovine (Kang et al. 2001a) but not porcine (Kang et al. 2001b) embryos. More recently, we found aberrant DNA methylation of imprinted genes in cloned porcine embryos and offspring (Han et al. 2008). An abnormality in histone acetylation has also been shown to be the cause of epigenetic errors in cloned embryos. Cho et al. (2007) produced three generations of transgenic pigs through SCNT of fibroblasts harboring the truncated human thrombopoietin gene and tested whether serial cloning could restore the phenotypic normality. This clearly showed that abnormal phenotype were due to errors in epigenetic reprogrammingand not due to damage to genome per se.

In the study the morphology and molecular nature of embryonic and extra-embryonic tissue of cloned animals, Chae et al. (2006) observed that extraembryonic tissue from SCNT pig embryos were abnormally small and had 39 differentially regulated proteins. They showed that abnormal apoptosis in the extraembryonic tissue during early pregnancy was a major reason for the low birth rate of cloned piglets. In another study, Lee et al. (2007) showed that SCNT pigs have placentas with severe villous hypoplasia. Proteomic analysis of term placentas from these animals identified 43 differentially expressed proteins that were mainly associated with apoptosis and oxidative stress. They team also found that hypoplastic umbilical arteries (HUAs) were present in nine of 65 cloned piglets. Proteome analysis of the HUAs revealed significant down-regulation of proteins preventing oxidative stress and controlling glycolysis and cell motility, while molecules involved in apoptosis were significantly up-regulated. Abnormalities in proteome of embryonic tissue has also been shown (Chae et al. 2008).

Embryo culture system:

Research in South Korea have also been focusing on improving the *in vitro* embryo culture system and understanding the mechanism underlying embryo fragmentation, abnormal cell division and chromosomal aberration *in vitro* produced embryos. Towards this goal, we have found that lipid peroxidation (LPO) is a key culprit of blastomeric fragmentation of porcine embryos and inhibition of LPO by novel compounds can improve their development potential. We also found that counting the number of blastomeres on Day 2 of *in vitro* culture could be used as a valuable non-invasive tool for morphological selection of good quality embryos. Abnormal cell division was positively correlated with chromosomal aberration, as revealed by fluorescent *in situ* hybridization technique.

Environment of *in vitro* embryo culture is also a vital factor in the development of SCNT embryos. Thus we have been analyzing the role and possible mechanism of action of mitogens, growth factors, amino acids, hormones and selenium on developmental ability, embryo quality and gene expression of cloned embryos and published it in journals. Recently, Roh et al. (2008) described a novel oil-free microtubule culture (MTC) system for culture of mouse embryos an alternative to conventional micro-drops, without the deleterious effects of oil. Embryos in MTC had a higher blastocyst formation rate and larger population of cells in the blastocysts compared with drop culture.

Cryopreservation of gametes and embryos:

Cryopreservation of games and embryos has met with limited practical success in animal system, especially with porcine oocytes. We recently showed for the first time in the world that vitrified pig oocytes can develop to blastocyst stage following IVF (Gupta et al. 2007). We and others (Yang et al. 2008) have also developed a method for cryopreservation of bovine oocytes while maintaining the developmental potential following subsequent IVF or SCNT. Vitrified-thawed bovine oocytes could support full term development into the subsequent stages after IVF and SCNT.

Spermatogenesis and spermatogonial stem cells:

Scientists in Korea have been trying not only to understand the regulatory mechanism of spermatogenesis but also to isolate and culture SSC that would provide a uniquely

valuable approach for the studying spermatogenesis and produced transgenic animals in species wherein embryonic stem cells are not available and somatic cell nuclear transfer and reprogramming pose several problems. Choi et al. (2007a) analyzed the mouse spermatocyte UniGene library containing 2155 gene-oriented transcript clusters and predicted that 11% of these genes were testis-specific. They also systematically identified 24 authentic genes specifically and abundantly expressed in the testis via in silico and *in vitro* approaches. These findings establish a new basis for future investigation into molecular mechanisms underlying male reproduction. Works are also underway to identify the mechanisms underlying sperm capacitation. Choi et al. (2008) undertook a high-resolution differential proteomic analysis of pig sperm cells and showed that a gradual increase of cytochrome c during incubation to induce capacitation determines sperm cell fate, i.e., apoptosis or further development for fertilization.

The SSC canestablish donor-derived spermatogenesis, when transplanted into the seminiferous tubules of an infertile male. In addition, when cultured in the appropriate conditions, they can acquire pluripotency and differentiate into derivatives of the three embryonic germ layers. Several laboratories in Korea have been attempting and showed success in isolation and *in vitro* culture of mouse, ratand pig SSC. We have recently established lines of mice SSC that could not only be maintained in undifferentiated state for long period of time but can also be differentiated into cells of three germ layer upon transplantation under kidney capsule of nudemouse. They can also be specifically differentiated to neuronal and cardiac cells. We have also established a method for isolation and enrichment of pig SSC that can be maintained in culture for long period of time and express markers of pluripotency. Choi et al. (2007b) have developed a technique for detecting transplanted pig testicular cells into recipient mouse testes using a combination of PKH staining and slide PCR methods. This would provide a valuable tool for studying SSC transplantation.

Reproductive biotechnology for stem cell:

Generation of pluripotent stem cells by therapeutic cloning has potential biomedical applications in the treatment of infertility and degenerative diseases in humans. We have recently shown the successful generation of autologous stem cells in mouse by SCNTas

well as by parthenogenesis (Ju et al., 2008). Being autologous in nature, these stem cells are likely to escape immnorejection upon transplantion. Scientists of Korea have also demonstrated that generation of induced pluripotent stem (iPS) cells in mouse and human using defined factors delivered through lentivirus system, unlike retrovirus vector system. They have been successful in deriving human iPS cells (hiPS) by infecting the human fetal lung fibroblasts with self-inactivating human immunodeficiency virus type-1 based lentiviral vectors containing four transcription factors (Oct4/Sox2/Nanog/Lin28). These hiPS cells showed typical hES cell morphology and growth pattern and expressed hES cell markers. In addition, these hiPs cells have normal karyotype (46, XX) and could formed embryoid bodies. These cells also had the potential to generate specific neuron differentiation, especially TH+ neuron. Similarly, mouse iPS cells have been obtained by infecting the Day 13.5 mouse embryonic fibroblasts with lentiviral vectors containing four transcription factors (Oct4/Sox2/C-myc/ Klf4). Genome-wide microarray analysis showed that there was a close correlation between mouse iPS cells and D3-ES cells.

References:

Chae JI, et al. 2006. Mol Cell Proteomics 5:1559-1566.

Chae JI, et al. 2008. Biochem Biophys Res Commun 366:379-387.

Cho SK, et al. 2007. Dev Dyn 236:3369-3382.

Choi E, et al. 2007a. BMC Genomics 8:256.

Choi YJ, et al. 2007b. Zygote 15:325-335.

Choi YJ, et al. 2008. J Reprod Dev 54:68-83.

Gupta MK, et al. 2007. Theriogenology 67:238-248.

Han DW, et al. 2008. Mol Reprod Dev 75:777-784.

Ju JY, et al. 2008. Fert Steril 89:1314-1323.

Kang YK, et al. 2001a. Nat Genet 28:173-177.

Kang YK, et al. 2001b. J Biol Chem 276:39980-39984.

Lee BC, et al. 2005. Nature 436:641.

Lee SY, et al. 2007. Proteomics 7:1303-1315.

Roh S, et al. 2008. Theriogenology 69:262-267.

Uhm SJ, et al. 2007. Mol Reprod Dev 74:1538-1547.

Yang BC, et al. 2008. Anim Reprod Sci 103:25-37.
Yin XJ, et al. 2008. 78:425-431.

IS 002

Recent Progress of Animal Reproduction in Japan

Eimei Sato
Laboratory of Animal Reproduction, Tohoku University, Sendai, Japan

The development and application of reproductive technology in Japan in recent years has been remarkable. New technologies have been developed based on innovative ideas and a number of Japanese scientists are now world leaders in the field of animal biotechnology.

Remarkable reproductive technologies in Japan

In the history of animal reproductive technology in Japan, it is worthnoting the methods of sexing day-old chicks. This technique was developed by Japanese scientists in the 1930s. The sexual organs of these birds are located within the body and are not easily distinguishable. However, Japanese scientists developed a technique and now the copulatory organ of chickens can be identified as either male or female based on shape. The school was established for education of young students under Livestock Technology Association and a number of technicians has been born in Japan and a certain number of the educated technician is working in the world.

It is noteworthy that the techniques of artificial insemination using frozen semen have spread throughout Japan and today more than 90% of cows become pregnant after artificial insemination. Approximately 8000 technicians, working both on animal farms and with veterinarians, have obtained licences to practice artificial insemination. A non-surgical technique of embryo transfer in cattle, *in vitro* matured, fertilized and cultured (IVMFC), which uses immature oocytes obtained from slaughterhouse-derived ovaries, was also developed in Japan. Based on these achievements, the Biotechnology Center (BC) was established by the corporation LIAJ Inc. A number of embryos are now being produced by (IVMFC) techniques at the BC-LIAJ and these are being sent to farms to produce calves by embryo transfer. Currently, more than 650 technicians, as well as veterinarians, are working on farms and are producing cattle by embryo transfer. In 2004, 16,127 and 2,129 calves have been produced by embryo transfer using *in vivo*-produced embryos and IVMFC embryos, respectively.

Techniques of embryo sexing, cryopreservation of embryos and oocytes, and somatic nuclear transfer techniques (SCNT) have all been developed by scientists in the field of animal reproduction in Japan. The accuracy of embryos sexing is now 100% and the pregnancy rate of cryopreserved embryos is now almost the same as that of fresh embryos. We have obtained offspring derived from cryopreserved immature oocytes[1].

SCNT techniques are now being applied in developing gene-modified domestic animals such as disease-model animals and animals for xenotransplantation. Recently, the Transgenic Domestic Animal Research Center (TDARC) was established at the National Institute of Agrobiological Resources, and we hope that the TDARC will be the primary center for this field in Japan.

On the other hand, animal reproductive technology is now influencing assisted reproductive technology (ART) in medicine. In Japan, more than 600 ART clinics are active and 1 in 65 babies are now being born as a result of *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). More than 1500 technicians are currently working as embryologists and an ART-licence for embryologists is now issued by the Japanese Society of Mammalian Ova Research (JSMOR). Up to 2007, 568 technicians were authorized by JSMOR and have obtained the licence. In 2007, 7 ART embryologists-supervisors were also authorized by JSMOR. A trainingcourse was established under the direction of the Japan Society of Fertilization and Implantation in 2006, and a number of scientists in the field of animal reproduction have been involved in this training course for embryologists, including ART-licence holders.

Reproductive biology in animal reproduction

We are currently discussing the name of the Japanese Society of Animal Reproduction (JSAR). At the meeting of the board of trustees held on 12 May 2008, the president of JSAR, Dr. M. Nishihara, proposed a new name for the society, the Society of Reproduction and Development, because JSAR is now publishing the ISI Journal and the Journal of Reproduction and Development, and is also working for the World Congress of Reproductive Biology (WCRB) in collaboration with SSR in the USA, SRF in the UK and SRB in Australia. The first meeting of WCRB is scheduled to be held in Hawaii on 2425 May 2008. There are a number of societies in the field of animal reproduction and reproductive medicine in Japan. JSAR has been appointed to be the official organization that works in collaboration with SSR, SRF and SRB. This emphasizes the fact that JSAR is active in reproductive biology as well as in reproductive technology.

Protein kinase B and oocyte maturation in mice

Related to reproductive biology, we are also working on oocyte maturation in mammals [2]. Accordingly, a study carried out in my laboratory is introduced [3]. Akt, also known as protein kinase B, is implicated in many cellular processes. Akt is phosphorylated at 2 residues: Thr308 and Ser473. Thr308-phosphorylated Akt is present in pericentriolar materials, whereas the localization of Ser473-phosphorylated Akt is similar to that of

microtubules in metaphase oocytes. Spindles are shorter and aberrant in oocytes injected with Thr308- or Ser473-phosphorylated Akt antibodies. Specifically, Thr308- and Ser473-phosphorylated Akts function independently, although both are necessary for the assembly of metaphase II (MII) spindles. Moreover, the functions of Thr308- and Ser473-phosphorylated Akts differ in MII oocytes. Although oocytes exhibit second polar body (PB2) emission after the injection of a peptide for Thr308, the chromosomal alignment and microtubular organization are aberrant. In contrast, the injection ofa peptide for Ser473 causes a failure of PB2 emission. These results suggest that both Thr308- and Ser473-phosphorylated Akts are involved in the completion of meiosis during fertilization, but that they play different roles; i.e., Ser473-phosphorylated Akts are involved in PB2 emission, whereas Thr308-phosphorylated Akts regulate the organization of microtubules.

1. **Abe, Y., Hara, K., Matsumoto, H., Kobayashi, J., Sasada, H., Ekwall, H., Rodriguez-Martinez, H., Sato,** E. Feasibility of a nylon mesh holder for vitrification of bovine germinal vesicle oocytes in subsequent production of viable blastocysts. *Biol.Reprod.*, 2005; 72:1416-1420
2. **Yokoo M, Sato** E. Cumulus-oocyte complex interactions during oocyte maturation. *Int Rev Cytol* 2004; 235: 251-291.
3. **Hoshino Y, Sato E.** Protein kinase B (PKB/Akt) is required for the completion of meiosis in mouse oocytes. *Dev Biol* 2008; 314: 215-223.

Key words) ***Reproductive technology, reproductive biology, oocyte maturation]***

IS 003

Immune-Specific Stem Cell Establishment without undertaking Nuclear Transfer

Jeong Mook Lim

Gamete and Stem Cell Biotechnology Laboratory, Seoul National University

We have long sought to replace somatic cell nuclear transfer (SCNT) with otheralternatives for deriving patient-specific embryonic stem cells (ESCs). We made our best efforts to develop autologous stem cell therapy and using of animal models, have suggested number of novel strategies that can be applied for human cell therapeutic technologies. First, we attempted to develop interspecies somatic cell nuclear transfer using human cord cells and bovine enucleated oocytes and subsequently succeeded to produce interspecies blastocysts in 2003. Second, we have developed a technique to derive autologous stem cells by parthenogenetic activation of unfertilized oocytes, and cellular and genetic properties of various stem cell lines derived from parthenogenesis were monitored by a microarrary gene expression analysis. Third, we succeeded to generate autologous stem cells by *in vitro*-culture of preantral follicles and parthenogenetic activation of intrafollicular oocytes that were matured in the follicles. We further applied the developed technology for culturing of primary follicles or the follicles retrieved from aged female. Finally, we established immune-specific stem cells derived from various somatic tissues without genetic modification and relevant results would be reported in the near future. We hope that the information derived from this model study contributes to developing an effective strategy for establishing patient-specific, autologous stem cells.

Key words) ***Autologous stem cell, interspecies nuclear transfer, parthenogenesis, preantral follicle culture, somatic cell-derivation***

IS 004

Heat Stress on Development of Bovine Embryos in Relation to Intra-and Extracellular Redox Status

Masashi Takahashi, Ken-ichi Yamanaka and Miki Sakatani

National Agricultural Research Center for Kyushu Okinawa Region, Suya 2421, Koshi, Kumamoto 861-1192, Japan

In vitro production of mammalian embryos has highly contributed to the progress of developmental abilities, basic research for differentiation and gene expression as well as efficient livestock production. In domestic animals, *in vitro* maturation, fertilization and culture techniques have strongly advantaged the production of offspring in a short period with embryo transfer techniques. However, there are still many points that have to be solved for improving the efficiency of livestock production. One of the factors that strongly affect the establishment of pregnancy including embryo development is a heat stressed condition. In recent years, the potential direct effects of possible global warming on summer season production and reproduction were evaluated for the many areas such as United States, Europe and Asian or more countries. In most mammalian species including cattle, heat stress in hot season causesdeleterious effects on physiological functions. Exposure the cattle to a hot environment causes the increase in the body temperature, decreasesthe length and intensity of estrus by disturbing ovarian function, fertilization failure, early embryonic death. Therefore, it is important to study heat stress mechanisms for embryo development not only for the side of physiological but also from molecular side so that to improve the effective production of cattle. We previously showed the stage-specific sensitivity of embryo development by heat stress associated with the increase of intracellular ROS levels using *in vitro* evaluation model. Six hours of heat stress exposure caused the significant decrease in the rates of development and number of cell number in the blastocyst. Especially, early stage embryos were more sensitive to heat stress. These results were similar to the report of *in vivo*, that the exposure to AI-treated cows to heat stress in earlier embryo-developmental stage caused the significant low number of recovered blastocysts on day7. Therefore *in vitro* model was effective for precise analysis of heat stress on embryo development. In addition, exposure the early stage embryos to heat stress also caused the increase in generation

of intracellular ROS levels. From the results, reducing the inhibitory effects oxidative stress itself or heat stress-derived oxidative stress is important to increase the efficiency of embryo production both *in vitro* and *in vivo*.

One of the ways for decreasing the toxic effect is to decrease of oxidative stress and keep the redox balance inside or outside of embryos. For such a purpose, antioxidants such as thiols, vitamins or polyphenols are candidates to reduce oxidative stress. Many investigators have found several types of antioxidants from leaf, bark, root and fruit of various plant species. Antioxidative polyphenols are widely known as safe and non-toxic antioxidants and used for human health to reduce the risk of heart and vascular diseases as well as cell growth or induction of apoptosis.

We reported the addition of antioxidants such as thiols or polyphonols derived from plants/seaweed effectively support embryo development under oxidative stress in relation to improve intracellular redox status. Anthocyanins are vacuolar flavonoids and widely contained in vegetables and fruits as natural pigments. Also anthocyanins have been reported to have various biological activities including antioxidant activities. Addition of b-mercaptpethanol or anthocyanins strongly improved the embryo development under heat stressed condition associated with reducing the intracellular reactiveoxygen species. This study shows that reducing oxidative stress protects embryo development under heat stressed condition. Heat stress also affects genital tracts such as oviduct that is an important tissue for fertilization and early development. We observed the oviductal antioxidative environments such as SOD activity and GSH levels weredecreased during hot season. These results highly suggest that heat stress during a hot season decrease the intraoviductal environments and these oxidative environments decrease sperm activity, fertilization and embryo development. Therefore, administration of antioxidants to cells, tissues and cattle itself as a feed for cattle canbe one of the ways to improve oxidative environment for embryo development under heat stress condition. Plant- or seaweed- related polyphenols can also be usefulfor animal feed as antioxidative supplements with direct feeding or biomass residue after processing. Mechanisms of digestion and absorption of plant-related polyphenols in cattleare still unknown. However, sufficient supplementation of such antioxidative polyphenols to cattle may alter oxidative status in reproductive systems exposed to oxidative- and heat-stressed condition.

In the symposium, the effects of heat and oxidative stress on intra- and extra- embryonic development are discussed.

Key words) ***Heat stress, oxidative stress, embryo development, cow***

IS 005

Study of Metabolic Syndrome with the Drosophila Model System: sNPF Regulates Insulin Signaling

Kweon Yu, Ph.D.
Center for Regenerative Medicine, KRIBB

Insulin and insulin growth factor play central roles in growth, metabolism, and aging of animals including *Drosophila melanogaster.* In *Drosophila*, insulin-like peptides (Dilps) are produced by specialized neurons of the brain. Here we show that *Drosophila* short Neuropeptide F (sNPF), an ortholog of mammalian Neuropeptide Y (NPY), and sNPF receptor sNPFR1 regulate expression of Dilps. Body size was increased by over-expression of sNPF or sNPFR1. Fat body of sNPF mutant animals had down-regulated Akt, nuclear localized FOXO, up-regulated translational inhibitor 4E-BP, and reduced cell size. Circulating levels of glucose were elevated and lifespan was extended in sNPFmutants. We show that these effects are mediated through activation of extracellular signal-related kinases (ERK) in insulin producing cells of larvae and adults. Insulin expression was also increased in an ERK dependent manner in cultured Drosophila CNS cells and in rat pancreatic cells treated respectively with sNPF or NPY peptide. *Drosophila* sNPF and the evolutionary conserved mammalian NPY appear to regulate ERK mediated insulin expression and to thus systemically modulate growth, metabolism, and lifespan.

IS 006

Development of Polyspermic Embryos in IVP Systems of Farm Animals

Tamás Somfai[1] , Kazuhiro Kikuchi[2], Takashi Nagai[3] and Kei Imai[1]

[1]Embryo Transfer Group, National Livestock Breeding Center, Nishigo, Fukushima, Japan

[2]Division of Animal Sciences, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan

[3]National Institute of Livestock and Grassland Science, Tsukuba, Ibaraki, 305-0901, Japan

Polyspermy (the penetration of an oocyte by two or more spermatozoa) is a fertilization-anomaly that causes an abnormally high ploidy of the resultant zygote. This undesirable phenomenon is present in *in vitro* fertilization (IVF) systems in human and also in animals. In large animals such as pigs and cows, the main reason of polyspermy seems to be caused by a large number of spermatozoa at the site of IVF along with the lack of regulatory effect of the female reproductive tract to control the quantity and quality of spermatozoa, which is prerequisite for the normal or monospermic fertilization *in vivo*. Although several strategies had been developed to prevent this problem, it has not been eliminated completely yet; we still have to deal with a relatively high rate of polyspermy especially in pig *in vitro* embryo production (IVP) systems. The developmental characteristics of polyspermic embryos are reported limitedly. They were shown to be able to develop to the blastocyst stage *in vitro*, nevertheless with reduced numbers of inner cell mass cells (Han et al., 1999 Biol Reprod 60:1110-3). Previous studies havesuggested that, under *in vivo* conditions, such embryos are able to recover their ploidy to a normal diploid status and to develop to term (Han et al., 1999 Biol Reprod 61:1340-6).

We have investigated if polyspermic porcine oocytes can develop to the early embryonic stages, and if they can recover their ploidy to the normal status during *in vitro* culture (IVC). Embryos were produced by IVF of *in vitro* matured oocytes. Monospermic and polyspermic oocytes were selected after visualization of pronuclei by centrifugation of putative zygotes at 10 h after IVF and *in vitro* cultured according to Kikuchi et al., (2002 Biol Reprod 66: 1033-41). Cleavage and blastocyst formation rates of polyspermic embryos were significantly lower compared to monospermic ones;however, the ability of cleaved embryos to develop to the blastocyst stage did not differ between them. Those zygotes that failed to undergo first cell division contained high numbers (an average of 5.6±0.2) of pronuclei. There were no differences in total cell numbers and rates of dead cells between polyspermic and monospermic blastocysts. The majority of monospermic blastocysts were diploid, whereas ploidy status of polyspermic blastocysts was very heterogeneous including polyploid, mosaic and a remarkable proportion (31.3%) of diploid embryos. At 36 h after IVF there was no difference in the number of nuclei/embryo bet-

ween the two groups, however, polyspermic embryos had higher blastomere numbers than that of monospermic embryos, due to an increased frequency of blastomeres without nuclei. These results confirmthat polyspermic embryos can develop to the blastocyst stage and suggest that similarly to *in vivo* development correction of embryo ploidy in polyspermic embryos may occur during IVC. Nevertheless increased frequency of partial fragmentation suggests altered cytoplasmic characteristics in polyspermic embryos.

Although the exact mechanism of ploidy correction is not clarified yet, specific cleavage patterns related to polyspermic fertilization during first cleavage have been suggested to be involved (Kola et al., 1987 Biol Reprod 37:395-401; Han et al., 1999 Biol Reprod 61: 1340-6 Funahashi 2003 Reprod Fertil Dev 15:167-77). There seems to be a debate if direct cleavage from one cell to 3 or more cells or the appearance of an extrusion (a mass that is extruded from the embryo simultaneously with the first cleavage) during is more likely related to ploidy correction. Our preliminaryinvestigations on *in vitro* produced cattle embryos using time lapse cinematography revealed that embryos showing direct cleavage to 3 or more cells have similar ability to develop to the blastocyst stage *in vitro* to those showing normal cleavage. On the other hand embryos showing extrusions during cleavage seem to have a reduced ability to develop to the blastocyst stage. Furtherexaminations for embryo quality are needed to clarify the mechanisms underlying ploidy correction in polyspermic embryos.

In conclusion, our results indicate that correction of ploidy in some polyspermic embryos occurs during *in vitro* culture in large animals. Therefore, we suggest that, in case of high rates of polyspermy (such as in pig IVP systems), embryos with a low grade of polyspermy (dispermic embryos) should be considered for further use for embryo transfer or cryopreservation.

This work was supported by a grant-in-aid for Japanese Society for the Promotion of Science Postdoctoral Fellowship for Foreign Researchers (P05648) and "Research and Development Program for New Bio-industry Initiatives".

IS 007

Metastin/kisspeptin Neurons: A Missing Link for Mechanism Generating Two Modes of GnRH/LH Release

Yoshihisa UENOYAMA, Hiroko TSUKAMURA and Kei-ichiro MAEDA
Lab. of Reproductive Science, Graduate School of Bioagricultural Sciences,
Nagoya University, Nagoya, JAPAN

Gonadal functions are controlled by two modes of GnRH/LH release in the female. Pulsatile GnRH/LH release has been shown to stimulate follicular development and steroidogenesis in the ovary. The negative feedback action of ovarian steroids, in turn, modulates GnRH/LH pulses to keep their levels stable. GnRH/LH surge, another mode of release is found only in female, and is induced by positive feedback action of estrogen derived from preovulatory follicles. The mechanism underlying the generation of the two modes of GnRH/LH release has not fully been understood. Metastin (also known as kisspeptin) was originally found in human placenta and shown to be encoded by KiSS-1 metastasis suppressor gene in 2001. This 54-amino acid peptide is now considered as a key neuropeptide involved in mechanism governing reproductive functions, because mutations in gene encoding GPR54, a metastin/kisspeptin receptor, result in the hypogonadotropic hypogonadism in human and mice. Increasing evidences suggest that metastin/kisspeptin or kisspeptin-10 (Kp-10), a C-terminal decapeptide, profoundly stimulates GnRH/LH release in several mammalian species including rodents, farm animals and primates. The present paper discusses the possible role of brain metastin/kisspeptin neurons as a key player controlling two modes of GnRH/LH release in the female rodent model.

Metastin/kisspeptin neurons were limitedly localized in the anteroventral periventricular nucleus (AVPV) and hypothalamic arcuate nucleus (ARC) of female rats, which are prospected regions controlling GnRH/LH surges and pulses, respectively. In male rats, metastin/kisspeptin neurons are not found in the AVPV. The sexual dimorphism of the AVPV metastin/kisspeptin population is well consistent with the absenceof LH surges in male rats. In addition, estrogen enhances KiSS-1 mRNA expressions in the AVPV of female. Thus, AVPV metastin/kisspeptin neurons would be responsible for female-specific surge mode of GnRH/LH release and be a target of positive feedback action of estrogen to evoke preovulatory GnRH/LH surge. On the other hand, role of ARC metastin/kisspeptin neurons remains largely unknown. The ARC KiSS-1 mRNA expression is increased by ovariectomy and decreased by estrogen replacement in female rats. This suggests that ARC metastin/kisspeptin neuronal population is associated with estrogen negative feedback and thus pulse mode of GnRH/LH release. Taken these together with the presence of estrogen receptors in metastin/kisspeptin neurons, two populations of metastin/kisspeptin neurons may regulate two modes of GnRH/LH release, thereby controlling gonadal activity.

IS 008

Functional Analysis of Maternally Expressed Transcripts in Oocyte Maturation and Embryogenesis

Kyung-Ah Lee, Ph.D.

Graduate School of Life Science and Biotechnology, Pochon CHA University College of Medicine, Fertility Center, CHA General Hospital, Seoul, Korea

The maturation promoting factor (MPF) and MAP kinase (MAPK) have been well-known as key signaling systems in regulating of oocyte maturation. Recently, by applying the ACP-PCR method, we have listed differentially expressed genes (DEGs) during oocyte maturation [1], and have evaluatedfunctions of several target genes, such as malate dehydrogenase in the mouse [2] and pig [3], Diva [4], Sebox [5], Obox4 [6], and many others yet published. The list per seand piling up functions of each target gene one-by-one would provide an insight for finding new regulators of the oocyte maturation process as well as in embryogenesis.

By using RNA interference (RNAi), we are finding the loss-of-function of target genes at interest. RNAi is very simple but powerful tool for learning functions of genes in specific cells at specific time point during development. Especially, RNAi by microinjection of dsRNA (300~400nt) compared to siRNA (19~21nt) in oocyte and embryo is an excellent tool for studying functions of genes, since dsRNA will produce many siRNAs inside of the oocytes cytoplasm due to oocytes Dicer activity [7]. In addition, it is well known that the oocytes and embryos do not reveal dsRNA-dependent protein kinase reaction that induce apoptotic cell death due to introduced long dsRNA [8, 9] and off-targeting, non-specific effects [9].

When we assemble data on functions of maternally expressed genes (MEGs) by microinjecting dsRNA of target gene into the cytoplasm of the germinal vesicle (GV) oocytes followedby *in vitro* maturation, RNAi effects were resulted into 3 groups; GV-arrested, MI-arrested, and MII oocytes developed normal despite of RNAi of certain genes. Oocyte quality is a key limiting factor in fertilization and further embryogenesis. In mammals, the first 2-3 cleavages are regulated by factors of maternal origin after that embryonic gene activation occurs. Therefore, knowledge about MEGs would extend our understanding on the mechanisms of the embryo development and the oocyte maturation. Results of RNAi for several genes, especially resulted in MII development despite of RNAi of those targets will be discussed.

Acknowledgement

This work was supported by the Korea Science and Engineering Foundation (KOSEF)

grantfunded by the Korean government (No. R01-2007-000-20451-0) and by a grant (SC-4021) from Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea.

References

1. Yoon SJ, Chung HM, Cha KY, Kim NH, Lee KA. Identification of differential gene expression in GV vs. MII mouse oocytes by using annealing control primers. Fertil Steril 2005; 83(S4):1293-1296.
2. Yoon SJ, Koo DB, Park JS, Cho KH, Han YM, Lee KA. Role of cytosolic malate dehydrogenase in oocyte maturation and embryo development. Fertil Steril 2006; 86(S4):1129-36.
3. Kim EY, Kim KH, Kim YS, Lee HS, Kim YN, Lee KA. Comparative functional analysis of the malate dehydrogenase (Mor2) during *in vitro* maturation of the mouse and porcine oocytes. Develop Reprod 2007; 11:263-372.
4. Lee KA, Kim EY, Lee EH, Kim YS, Bae J. Finding of a new regulator of maturation promoting factor (MPF) in meiotic progression of the mouse oocytes. 63rdAnnual Meeting of the American Society for Reproductive Medicine, Washington D.C., USA, October 13-17, 2007; O-62.
5. Kim KH, Kim EY, Lee HS, Lee KA. Functional analysis of SEBOX during oocyte maturation and embryogenesis by RNAi. Annual Meeting of the IETS, Denver, Colorado, USA, January 5-9, 2008; P-246.
6. Lee HS, Lee KA. Characterization and Functional Analysis of Obox4 during Oocyte Maturation by RNA Interference. Korean J Fertil Steril, 2007; 34:293-303.
7. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev, 2001; 15:188-200.
8. Gil J, Esteban M. Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. Apoptosis 2000; 5:107-114.
9. Stein P, Zeng F, Pan H, Schultz RM. Absence of non-specific effects ofRNA interference triggered by long double-stranded RNA in mouse oocytes. Dev Biol, 2005; 286:464-71.

IS 009

Protective Effects of Glucocorticoids on the Reproductive Function under Stress Conditions

Takashi Matsuwaki, Keitaro Yamanouchi and Masugi Nishihara
Department of Veterinary Physiology, Veterinary Medical Science, The University of Tokyo, Tokyo 113-8657, Japan

It is well establishedthat reproductive function is suppressed under stress conditions, and it has been widely accepted that this is partially due to stress-induced secretion of glucocorticoids. We have previously reported, however, that glucocorticoids counteract the suppressive effects of tumor necrosis factor-a on the pulsatile secretion of luteinizing hormone (LH), suggesting that glucocorticoids may have a protective effect on reproductive function under stress conditions. We therefore examined the possible protective effects of glucocorticoids on LH pulses under various acute stress conditions and the possible involvement of prostaglandins (PGs) in glucocorticoid actions. Three different types of stressors, a low level of infectious (lipopolysaccharide) and hypoglycemic (2-deoxy-D-glucose)stress and 1 h of restraint stress, were applied to ovariectomized rats. In ovariectomized rats, LH pulses were partially suppressed by restraint, but not by lipopolysaccharide or 2-deoxy-D-glucose. On the other hand, adrenalectomy (ADX) significantly enhanced the suppressive effects of all the stressors applied on LH pulses. Treatment with both corticosterone and indomethacin in ADX rats significantly attenuated the suppressive effects of these stressors on LH pulses. In addition, the immunoreactivity of cyclooxygenase-2, a PG-synthesizing enzyme, in the brain under stress conditions was much enhanced by ADX, and this was counteracted by corticosterone treatment. These results suggest that suppression of LH pulsatility by stress is mediated by PGs in the brain, and that increased release of endogenous glucocorticoids in response to stress counteracts this suppression by inhibiting PG synthesis, and thereby maintains reproductive function regardless of the nature of the stressor.

IS 010

Production of RFP Transgenic Cat and Its Application

Il-Keun Kong

Division of Applied Life Science (BK21) and Institute of Agriculture and Life Sciences, Gyeongsang National University

A method for engineering and producing genetically modified cats is important for generating biomedical models of human diseases. Here we describe the use of somatic cell nuclear transfer to produce cloned transgenic (TG) cats that systemically express red fluorescent protein (RFP). Immature oocytes were collected from superovulating cat ovaries. Donor fibroblasts were obtained from an ear-skin biopsy of a white male Turkish Angora cat, cultured for one to two passages, and subjected to transduction with a retrovirus vector designed to transfer and express the RFP gene. A total of 176 RFP cloned embryos were trasnferred into 11 surrogate mothers (mean = 16±7.5/recipient). Three surrogate mothers were successfully impregnated (27.3%), and delivered two liveborn and one stillborn kitten at 65 to 66 days of gestation. Analysis of nine specific microsatellite loci confirmed that the cloned cats were genetically identical to the donor cat. Presence of the RFP gene in the transgenic cat genome was confirmed by PCR and Southern blot analysis. Whole body ref fluorescence was detected 60 days after birth in the liveborn TG cat, but not in the surrogate mother cat. Red fluorescence was detected in tissue samples including hair, muscle, brain, heart, liver, kidney, spleen, bronchus, lung, stomach, intestine, tongue and even excrement of the stillborn TG cat.

To apply RFP TG cat, we tried production of re-cloned RFP TG cat using RFP TG cat as donor cells. Donor fibroblasts were obtained from a skin biopsy of a cloned transgenic cat and cultured for one to two passages, which was checked an expressing of the red fluorescent protein (RFP) gene. A total of 281 RFP cloned embryos were transferred into 13 surrogate mothers (mean = 21±7.7/recipient). One surrogate mother was successfully impregnated (7.7%), and delivered one liveborn at 65 to 66 days of gestation. However, RFP TG re-cloned cat was died 5 days after born, because the surrogate mother was not able to nurse the colostrum by aftereffect of Caesarean operation. Presence of the RFP gene in the re-cloned transgenic cat RNA and DNA were confirmed by PCR analyses. Red fluorescence was detected in whole body and tissue samples of the re-cloned transgenic cat. Although the cloning efficiency was low, the cloned transgenic cats can be used as donor cats to produce second generation cloned transgenic cats.

Cat RFP transgenic MSCs have differentiated into neuron, astrocyte and oligodendrocyte. However, survival of the grafted cells was decreased significantly four and five

weeks post implantation and even disappeared at post-grafting 6 weeks. In addition, migration of the grafted cells could be seen three and four weeks after transplantation. The fluorescence test of the grafts revealed that the survival cells increased from two to three weeks post-grafting and then dropped at beginning four weeks after transplantation. We also isolated cat embryonic stem (ES)-like cells from cat blastocysts generated *in vivo*. In an effort to identify genetic markers for the characterization of cat ES-like cells, we have determined the coding sequences (CDSs) of cat *POU5F1* (*cPOU5F1*) and *NANOG* (*cNANOG*). We also examined ES-like cells by immunocytochemistry, and demonstrated that cPOU5F1 and cNANOG are present at a high level in cat ES-like cells, and undetectable in cat fibroblast feeder cells.

These results suggested that this nuclear transfer procedure using genetically modified somatic cells could be useful for the efficient production of transgenic cats, and cat ES-like cells can be successfully isolated from *in vivo*-produced blastocysts, and that the expression of *cPOU5F1* and *cNANOG* can be used as a biomarker for the characterization of cat ES-like cells.

*Supported by KOSEF (#M10525010001-05N2501-00110).

Key words) C***at, cloned, SCNT, transgenic, RFP, MSCs, ES biomarker***

IS 011

Somatic Cell Nuclear Transfer with HDACi

Satoshi Kishigami[1,2], Kazuhiro Saeki[1], Kazuya Matsumoto[1], Akira Iritani[1], Teruhiko Wakayama[2] and Yoshihiko Hosoi[1]

[1]Department of Genetic Engineering, Kinki University, Wakayama, Japan

[2]Laboratory for Genomic Reprogramming, RIKEN Kobe, Kobe, Japan

Somatic cell nuclear transfer (SCNT) has been diffused into a variety of research fields including research for reprogramming and animal reproduction after Dolly the sheep, the first mammal to be cloned from an adult cell. However, the success rates have been inefficient in mammals as a result of incomplete reprogramming of the somatic genome. The incomplete reprogramming has been represented by abnormal epigenetic modifications, especially DNA methylation. Recently, we developed a new SCNT method using trichostatin A, a histone deacetylase inhibitor (HDACi). This new SCNT with HDACi not only allows us to increase their success rates 2-5 fold in F1 mice but also to clone a previously "unclonable" outbred strain, ICR. This success suggests that the acetylation level of histones after SCNT is the key to determine the reprogramming efficiency. Further, our and others' most recent studies indicate that SCNT with HDACi can be applied to other animals such as cow and pig.. In this talk, I will introduce this new SCNT method and their applications.

Key words) ***Somatic cell nuclear transfer, reprogramming, mouse***

IS 012

Analysis of Hypoplastic Umbilical Cords from Somatic Cell Nuclear Transfer-Derived Piglets: Implications for Early Postnatal Death

Jin-Hoi Kim

Department of Animal Biotechnology, Konkuk University

Hypoplastic umbilical arteries (HUAs) are associated with fetal malformations, karyotype anomalies, preterm birth, and low birth weight. In this study, we observed HUAs in nine of 65 somatic cell nuclear transfer-derived (scNT) term piglets. Two of these HUA-bearing piglets were stillborn, six died during the first week of life or puberty and one grown up to adulthood. We here analyzed the umbilical cords and organs of these piglets to determine how HUAs promote stillbirth and postnatal death. Microscopic analyses of the umbilical cords of the nine scNT-HUA piglets revealed complete occlusive thrombi and the absence of columnar epithelial layers. The scNT-HUA umbilical cords expressed significantly lower levels of PECAM-1 protein and angiogenesis-related genes than the umbilical cords of normal scNT (scNT-N) piglets that survived into adulthood. The scNT-HUA umbilical cord-derived endothelial cells migrated and formed tubules more slowly than control and scNT-N cells. This may reflect the underlying defect responsible for the HUA. Proteome analysis of the scNT-HUA umbilical cords revealed significant down-regulation of proteins preventing oxidative stress and controlling glycolysis and cell motility, while molecules involved in apoptosis were significantly up-regulated. Histomorphometricanalyses revealed severe calcification in the kidneys and placenta, peliosis in the liver sinusoidal space, abnormal stromal cell proliferation in the lungs, and tubular degeneration in the kidneys of scNT-HUA piglets. Elevated apoptosis was detected in all scNT-HUA piglet organs by TUNEL assays. This study shows for the first time how HUAs affect fetal growth and organ development. It also provides clues about the molecular mechanisms behind the angiogenic events in umbilical cord development.

IS 013

Epigenetics of Mouse Trophoblast Cells

Satoshi Tanaka

Animal Resource Science and Veterinary Medical Science,

The University of Tokyo, Japan

The relatively hypomethylated status of the genome of murine extraembryonic lineages, including trophoblast cells, has been rendering ageneral impression that DNA methylation may play less relevant a role in gene regulation in these cell lineages. However, we have previously reported that DNA methylation plays an important role in suppression of mouse Oct4 and Nanog genes in trophoblast cells. Restriction landmark genomic scanning (RLGS) analysis revealed that an equal number of NotI sites (15 each out of ~1,500 analyzed) in the genome of mouse trophoblast stem (TS) cells were methylated or demethylated as the cells were induced to differentiate, implying that DNA methylation may regulate dynamic changes in gene expression even during trophoblast differentiation. In fact, we have found that DNA methylation plays a causal role in the regulation of Ddah2 gene that is repressed in undifferentiated TS cells but is upregulated upon induction of differentiation. Further detailed analysis of genome-wide DNA methylation profile of TS cells, placenta and other somatic tissues by restriction tag-mediated amplification (D-REAM) revealed differentiation status-dependent or placenta-specific DNA methylation profiles. Thesesuggest that DNA methylation underlie transcriptional regulation of genes in the trophoblast cell lineage.