The Society for Reproduction and Development

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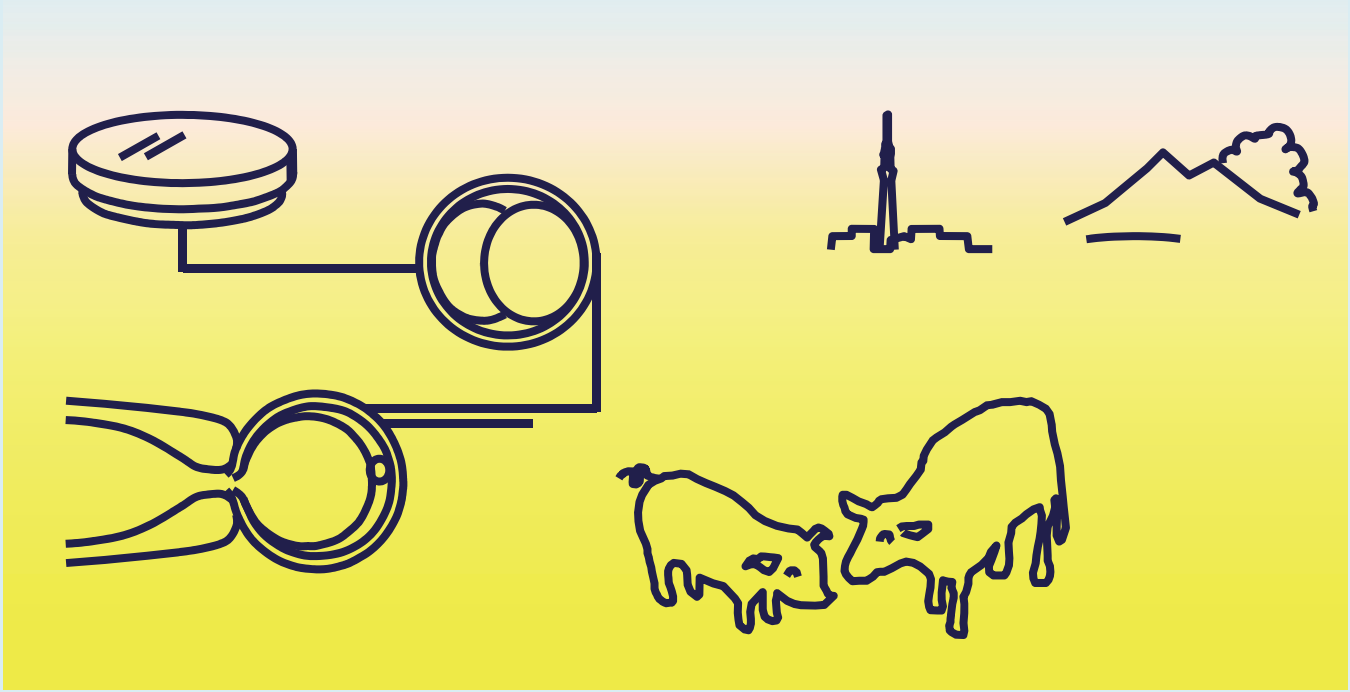
SRD-NILGS
The 2nd Japan– Korea–China
Joint Symposium

Current and Future Technologies for Animal Reproduction



September 5, 2012

University Hall
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SRD-NILGS 第 2 回 日・韓・中交流シンポジウム

SRD-NILGS the 2nd Japan-Korea-China Joint Symposium

Theme: “Current and Future Technologies for Animal Reproduction”
Location: University of Tsukuba, University Hall
Date and time: September 5 (Wed), 2012; 12:30-18:00

Society for Reproduction and Development (SRD)
http://reproduction.jp/index-j.php
NARO Institute of Livestock and Grassland Science (NILGS)
http://www.naro.affrc.go.jp/org/nilgs/eng/index-e.html

Program

Opening Remarks 12:30-13:00

Chair: **Noboru MANABE** and **Junyou LI**

Kei-ichiro MAEDA (President, The Society for Reproduction and Development).

Dong-Il JIN (President, The Korean Society of Animal Reproduction)

Shi-En ZHU (Chairperson, Reproduction Branch of Chinese Association of Animal Science and Veterinary Medicine)

Session 1. Efficient Offspring Production 13:00-14:00

Chair: **Hiroko TSUKAMURA** and **Deshun SHI**

Session 2. *In Vitro* Embryo Production, Transgenesis and the Related Studies 14:00-16:20

Chair: **Nam-Hyung KIM** and **Eimei SATO**

Coffee Break 15:00-15:20

Chair: **Li-guo YANG** and **Tae-Woan KIM**

JKC-5 **Productions of Transgenic Pigs for Xenotransplantation in Korea**

Seongsoo HWANG, Keon Bong OH, Dae-Jin KWON, Sun A OCK, Gi-Sun IM, Sung Soo LEE and Jin-Ki PARK (Animal Biotechnology Division, National Institute of Animal Science, Rural Development Administration, Korea) **P. 14**

JKC-6 **Roles of Neurotrophins in Mammalian Reproduction**

Xu ZHOU (College of Animal Science and Veterinary Medicine, Jilin University, Changchun, China) **P. 16**

Session 3. Future Possibility of Cloning and Stem Cells 16:20-17:50

Chair: **Yuji HIRAO**, **Gyu-Jin RHO** and **Xu ZHOU**

JKC-7 **Future Possibility and Current Status of Animal Stem Cell Technology**

Hiroshi KAGAMI (Faculty of Agriculture, Shinshu University, Nagano, Japan) **P.18**

JKC-8 **New Paradigm and Standard for Pluripotent Stem Cells in Domestic Animals**

Chang-Kyu LEE, Jin-Kyu PARK, Gwang-Hwan CHOI and Dong-Chan SON (Department of Agricultural Biotechnology, Animal Biotechnology Major, and Research Institute for Agriculture and Life Science, Seoul National University, Seoul, Korea) **P. 20**

JKC-9 **Generation of Buffalo Induced Pluripotent Stem cells**

Deshun SHI, Yanfei DENG, Qingyou LIU and Jianrong JIANG (Faculty of Animal Science and Technology, Guangxi University, Nanning, China) **P. 22**

Closing Remarks 17:50-18:00

Chair: **Kazuhiro KIKUCHI**

Takashi NAGAI (Chair, The 105th SRD Annual Meeting).

ABSTRACTS

JKC-1

Monitoring Metabolic Health of Transition Cows: The Keys to Improve Reproductive Performance in Dairy Cattle

Toshihiko NAKAO[1,2] and Martina HOEDEMAKER[1]

[1]University of Veterinary Medicine Hannover, Germany
[2]Alexander von Humboldt Foundation Alumni Research Fellow from Japan

Decrease in fertility of dairy cows has been reported worldwide for the last few decades. The underlying cause of the declining fertility is metabolic challenge facing cows during transition periods and concomitant occurrence of peri-parturient diseases. Cows with sever degree of metabolic challenge have reduced immune and reproductive function, leading to retention of fetal membranes, metritis, poor quality of the oocyte, and abnormal resumption of ovarian cycles postpartum. If the oocyte is damaged, any conventional hormone treatment or intra-uterine treatment could not be useful to improve fertility. The most prominent metabolic change in transition periods is the elevation of serum NEFA as a consequence of negative energy balance. High serum NEFA 1 to 2 w pre-partum is associated with increased incidence of peri-parturient diseases which are major causes of the decreased fertility. Cows with high serum NEFA pre- and/or postpartum showed a higher incidence of subclinical ketosis, delayed first ovulation, a lower pregnancy rate and a longer interval from calving to conception than those with the normal NEFA concentrations. Subclinical ketosis with high BHBA in early lactation was also associated with increased risk of metritis, displacement of abomasums, poor fertility, and decreased milk production. The changes in metabolism and immune function precede common reproductive diseases by a few or several weeks. It is, therefore, imperative to monitor metabolic changes of cows in transition period and to implement nutritional and management programs to minimize the negative energy balance in order to improve reproductive performance. Based on the energy status of cows monitored by serum NEFA, the following management program can be utilized; (1) high pre-partum NEFA- searching the causes of low dry matter intake (DMI) and increasing DMI, (2) high NEFA pre- and postpartum, or low NEFA pre-partum and high NEFA postpartum- increasing DMI, delaying rebreeding, or using embryo transfer instead of AI, (3) low NEFA pre- and postpartum- starting to rebreed shortly after a voluntary waiting period.

Keywords: dairy cow, transition, negative energy balance, NEFA, fertility

JKC-2

The Research Progress of Hormone Genetic Immunization Techniques Improving Growth and Reproduction in Animals

Liguo YANG, Aixin LIANG, Li HAN, Guohua HUA and Shujun ZHANG

Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan, China

We have successfully obtained two types of hormone DNA vaccines against somatostatin and inhibin for improvement of growth and fertility respectively in animals since the starting of the research in 1998. The research process and progress of hormone genetic immunization in 15 years are reviewed in this paper. Our researches focused on three fields to improve the safety, growth or fertility promoting effect, and simplicity of the vaccine applications. In order to enhance the immune effect of the hormone DNA vaccines, some adjuvant such as GM-CSF, CpG and VP22 was reconstructed into the hormone plasmids. In order to improve the safety of the vaccines, we set up an asd based host-plasmid balanced lethal system instead of antibiotic resistance genes. Moreover, attenuated *Salmonella entericaserovar Typhimurium* C500 with crp^- asd^- double deleted was used as a delivery system to improve the safety and simplicity. So far, we have constructed several decades of DNA vaccines and compared their results in safety, growth or fertility promoting effect. Our researches have proved that the better DNA vaccine against somatostatin is C500 (pVGS/2SS-asd) which caused 31.49% and 25.38% increase in growth of piglets after double immunizations 4 and 8 weeks after weaning compared with the controls. And the effective DNA vaccine against inhibin is C500 (pVAX-IS-asd) which could improve the average number of litter size (2.5) and mature follicles (4.9) of immunization group in mice, in comparison to control group. Furthermore, the genomic DNA was assayed for integrated plasmid using a sensitive PCR method, and the risk of hormone DNA vaccines due to integration in mice was negligible. Nowadays, assessment of the environmental release test of Ministry of Agriculture of genetically modified organisms safety for these hormone vaccines have been accomplished and the further production test is ongoing.

This study was financially supported by the National Natural Science Foundation of China (No. 30972099).

Session 2. *In vitro* embryo production, transgenesis and the related studies

JKC-3

Culture of Bovine Oocytes in an Individually Identifiable Manner and Its Applications

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Although cattle embryos usually develop alone *in vivo*, *in vitro* culture of single oocytes or embryos results in poor embryo development. To compensate for this limitation, *in vitro* culture of oocytes and zygotes generally takes place in groups to allow the autocrine and/or paracrine factors to support embryo development replacing the appropriate environment *in vivo* provided by their mother's reproductive tract. However, traditional embryo culture in groups makes tracking of individual embryo impossible. Furthermore, although, good quality immature oocytes are selected based on their morphology for *in vitro* maturation (IVM), fertilisation (IVF) and culture (IVC), this morphological criterion is inadequate; only 30 % of cultured oocytes develop to the blastocyst stage (Day 7), the reminder undergoing developmental arrest prior stage.

Recently, much effort had been made to develop embryo culture systems which facilitate both the identification/tracking of individual embryos and the supporting effect of autocrine and/or paracrine factors. Establishment of individual culture systems for oocytes and embryos would greatly facilitate the study of oocyte developmental competence including the assessment of the relationship between the follicle and oocyte. Previously, most reports of individual culture procedures have mainly focused on the post-insemination development of fertilised oocytes. A few studies have attempted the culture of single immature oocytes; however, these attempts were characterised by low blastocyst formation. Thus, it is necessary to improve individual culture from the immature oocyte to the blastocyst stage. In particular, it is important to overcome the compromised developmental ability of individually cultured oocytes.

We examined three systems that have been reported for the individual culture of mammalian embryos; 1) the well of the well, or WOW system, 2) culture on an adhesive matrix, such as Cell-Tak (a formulation of polyphenolic proteins extracted from marine mussel *Mytilus edulis*) and 3) culture within the opening of a monofilament polyester mesh. In each of these culture systems, 20 oocytes are cultured in the same droplet through IVM, IVF and IVC to enhance development. Fixing the

position of oocytes/embryos and the distance between them in the common culture droplet can provide the double benefit of stimulation by putative autocrine/paracrine growth factors as in group culture and the individual tracking of oocytes/embryos culture for the entire period of *in vitro* development.

We investigated the value of intrafollicular parameters for the non-invasive prediction of developmental potential of bovine oocytes (i.e. to development to the blastocyst stage) *in vitro* using culture methods which permit the individual tracking of oocytes and embryos. To indentify follicle-related makers of oocyte developmental competence, we investigated the relationship between the levels of several candidate factors including intrafollicular steroids, metabolomic profiles (amino acid, urea, glucose, citric acid, carbohydrate and fatty acid) in follicle fluid and mRNA abundance of candidate genes in theca, granulosa and cumulus cells and the *in vitro* development of oocytes/embryos. The concentration of testosterone, progesterone and estradiol in follicular fluid were not different between oocytes achieving or failing to develop to the blastocyst stage. L-alanine, glycine, L-glutamate and citric acid concentration were positively correlated, whereas urea and carbohydrate concentration were negatively correlated with blastocyst formation ($P<0.05$). Palmitic acid and total saturated fatty acid (SFA) levels were lower, whereas Linolenic acid level was higher in follicle fluid of competent oocytes compared with those of incompetent oocytes ($P<0.05$). Relative mRNA abundance of *ESR1* and *VCAN* genes in theca, *LHCGR* in granulosa cells and *TNFAIP6* in cumulus cells related to oocytes developing to blastocysts was significantly higher than those related to degenerated oocytes ($P<0.05$).

In summary, established individual oocytes/embryos culture systems can facilitate studies examining factors affecting oocyte developmental competence. Several follicular parameters including metabolite and gene expression in theca, granulosa and cumulus cells can be used as predictors for oocyte developmental competence.

This research was supported by Science Foundation Ireland (grant number SFI/07/SRC/B1156) and long term overseas dispatched staff under the NLBC program.

JKC-4

Production of Transgenic Pigs for the Translational Research

Yubyeol JEON, Seong-Sung KWAK, Seung-A JEONG, Eui Bae JEUNG and Sang-Hwan HYUN*

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Transgenic pigs have a potential to be a suitable source for xenotransplantation, disease models, disease resistance and bioreactors. Successful production of the cloned animals using by somatic cell nulcear trasnfer (SCNT) was reported in several species including pigs. Among the cloned animals, pigs are also essential large-animal models in various types of translational research including diabetes, cardiovascular disease, cystic fibrosis model, influenza resistant model, dermatology, toxicology and xenotransplantation. In anatomically and physiologically analogous, pigs are more similar to humans than mouse, rat, dog and cow. For this reason, a lot of research on the production of transgenic pigs has been reported for the field of biomedical studies. Transgenic pigs for the translational models provide our understanding of the causes and potential treatments of many human diseases. However, the use of transgenic pigs for the translational research has been very limited. This may be due to the low efficiency of the relatively process for producing transgenic pigs. Production technique of transgenic pigs consists of the following process; selection the specific gene for the disease model, design of the vectors and transfection's skill, SCNT including cytoplasm quality, donor cell conditions and reprogramming efficiency, and the selection of surrogate for surgical embryo transfer. Here, we report production of the SCNT-transgenic pigs through improvement of SCNT process.

This work was supported by a grant from Next-Generation BioGreen 21 program (# PJ008121012011), Rural Development Administration, Republic of Korea.

Keywords: somatic cell nuclear transfer, transgenic pigs, translational research

JKC-5

Productions of Transgenic Pigs for Xenotransplantation in Korea

Seongsoo HWANG, Keon Bong OH, Dae-Jin KWON, Sun A OCK, Gi-Sun IM, Sung Soo LEE and Jin-Ki PARK

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A shortage of organs from deceased human donors is a major problem limiting the number of organ transplants performed each year. In Korea, in February 2012, the Korean Network for Organ Sharing (KONOS) waiting list for solid organ transplantation approached 29,267 patients.

To overcome this serious problem, pigs are currently the preferred species for organ xenotransplantation. Pig seems to be the optimal xenograft donor animal because of a similar size as human organs, many physiological similarities with humans, short reproduction cycles and large litters and the maintenance is possible at specific (SPF) or designed pathogen free condition (DPF).

More than 10 different types (DAF, CD59, Fas ligand, HA-hHO1, and shTNFR1Fc, etc) of transgenic cloned pigs for xenotransplantation (xeno-pig) have developed in Korea. Especially 4 types of xeno-pigs such as α1,3-galactosyltransferase knock-out (+/-) (GalT KO +/-), GalT KO additionally knock-in for the human CD46 (GalT KO/hCD46 KI), human CD73 transgenic, and GalT KO -/- have generated in National Institute of Animal Science.

Now GalT KO -/- xeno-pigs have become herd by natural mating and SCNT using GalT KO -/- cells and their organs such as heart and kidney have applied pig-to-primate xenotransplantation.

JKC-6

Roles of Neurotrophins in Mammalian Reproduction

Xu ZHOU

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Neurotrophins (NTS) are required for the proliferation, differentiation, survival, and death of neuronal cells. The family of neurotrophins are composed of (nerve growth factor) NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), neurotrophin 4(NT4), three tropomyosin-related kinase (Trk) receptors (TrkA, TrkB and TrkC) and a low affinity receptor p75NTR. There is evidence to suggest that NTs not only have effects on the nervous system, but also play important roles in regulating the development of non-neuronal cells such as in reproductive systems. During the pass years, a series of work has been done in our laboratory to study the expression and function of NTs in female bovine and porcine reproductive systems, including oocytes and embryos, and in ejaculated spermatozoa from bull and men. We have shown that GV oocytes, MII oocytes, 4-cell and 8-cell embryos, morulae, and blastocysts were all shown to express BDNF. The highest expression was detected in MII oocytes and the lowest expression in 8-cell embryos. The mRNA for BDNF was highly expressed in the PA and IVF blastocysts compared to the NT blastocysts. BDNF caused a significant increase in the rates of *in vitro*-fertilized blastocyst formation and parthenogenetic blastocyst formation. BDNF, NGF, NT4, TrkA and TrkB mRNAs and proteins were detected granulosa cells, cumulus oocyte complexes, theca cells of follicles, as well as in the oviduct epithelial cells, endometrium in uterus of cows and sows. BDNF and NGF promoted the growth of granulosa cells. NGF/TrkA and NT-4/TrkB pathway can simulate the expression of FSHR and LHR mRNA expression in bovine granulosa cells. LH and FSH can stimulate the mRNA expression of NGF and its receptor TrkA. BDNF, NGF, NT-4, TrkA and TrkB exist in mature spermatozoa from bull and men. NTs can modulate the function of bovine and human spermatozoa and may be a biomarker for infertility of men.

JKC-7

Future Possibility and Current Status of Animal Stem Cell Technology

Hiroshi KAGAMI

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Recent progress on stem cell technology could lead breakthrough in animal reproduction. Reproductive technologies; artificial insemination, cloning, stem cell regeneration, gene transfers, *in vitro* fertilization and sex alteration, have been developed in cattle, swine, chicken etc. These innovations must be refined and performed in the next decades. Especially, stem cell technology could be one of the most promising subjects in animal reproduction (Sartori et al., 2012). Embryonic stem cells (ES cells) from ICM of mouse blastocysts was isolated (Martin and Evans, 1975). When the cells were injected into recipient embryos, somatic and germline chimeras were generated. Offspring from donor ES cells derived gamete could be generated. It was found that the stem cells contained self-renewal ability and maintained pluripotentcy both *in vivo* and *in vitro*. Successful application of these stem cell technologies could improve genetic performance of livestock effectively. Induced Pluripotent Stem cells (iPS cells) was developed by retrovirus-mediated transfection with four transcription factors; Oct-3/4, Sox 2, KLF 4 and c-Myc, into mouse fibroblasts (Takahashi and Yamanaka, 2006). The stem cell technology involved ES or iPS cells could expenditure to human health. A large number of drugs, diagnostic probes and vaccines are frequently applied to livestock prior to becoming available for use by humans. Many of the pharmaceutical industry have had much interests for livestock production since the research findings could be directly applicable to human medicine. One of the most promising applications of the stem cell technology should be regenerative medicine. The success could bring novel strategies for the medical treatment.

Thus, the stem cells should be one of the most useful tools (Kagami et al., 2006) for future production of transgenic animals, generation of pharmaceutical substrates, sexual manipulation of offspring, conservation of endangered animals and generation of resistant animal for infectious disease. Moreover, genetic modification into the stem cell will enhance improvement of livestock breeding. Accumulation of the scientific knowledge and technical innovations should open up new frontier for animal reproduction and frontier medicine (Robinton and Daley, 2012).

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JKC-8

New Paradigm and Standard for Pluripotent Stem Cells in Domestic Animals

Chang-Kyu LEE, Jin-Kyu PARK, Gwang-Hwan CHOI and Dong-Chan SON

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Since pluripotent cells were first derived from inner cell mass (ICM) of blastocyst in mouse, many effort have been made to establish pluripotent stem cells from domestic mammals. However for many domestic species including the pig it remains that authentic pluripotent stem cells from embryos of domestic animals have not yet been derived. It has proven difficult to maintain embryonic stem cells (ESCs)-like state in domestic animals due to the frequent occurrence of spontaneous differentiation into epiblast stem cells (EpiSCs)-like state during culture based on genomic background. A recent study has reported that pluripotent stem cells exist in one of two forms and can be categorized according to their pluripotent state. The first is a “naïve” state which is characterized by small, round or dome-shaped colony morphologies and LIF signaling, mouse ESCs (mESCs) represent this ES cells of this type. A second “primed” state has also been described within which EpiSCs or human ESCs (hESCs) can be placed. These primed state pluripotent stem cells display flattened monolayer colony morphologies and FGF/ Nodal/Activin signaling pathways. We suggest that pluripotent stem cells derived from embryos and iPSCs derived from embryonic fibroblasts in the domestic model, especially in pig, possess a primed pluripotent state similar to that of mEpiSCs or hESCs, rather than to that of mESCs. Meanwhile, A few studies have reported that non-permissive species could be derived to a naïve pluripotent state using various exogenous factors including GSK3β and MEK inhibitors (2i), LIF, hypoxic conditions and up-regulation of Oct3 or klf4. In present, we are trying to establish naive pluripotent stem cells with 2i and other factors and we have been able to successfully induce PEFs into a LIF- dependent naïve pluripotent-like cell line showing a mESC-like morphology. Further characterization of these cells is in progress.

This work was supported by the BioGreen 21 Program (PJ0081382011), Rural Development Administration, Republic of Korea.

JKC-9

Generation of Buffalo Induced Pluripotent Stem cells

Deshun SHI, Yanfei DENG, Qingyou LIU and Jianrong JIANG

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Ectopically expression of defined factors could reprogram mammalian somatic cells into induced pluripotent stem cells (iPSCs), which initiates a new strategy to obtain pluripotent stem cell lines. Attempts have been made to generate buffalo pluripotent stem cells by culturing primary germ cells or inner cell mass, but the efficiency is extremely low. Here, we report a successful method to reprogram buffalo fetal fibroblasts (BFFs) into pluripotent stem cells (biPSCs) by transduction of buffalo defined factors (Oct4, Sox2, Klf4 and c-Myc) using retroviral vectors. The established biPSCs displayed typical morphological characteristics of pluripotent stem cells, normal karyotype, positive staining of alkaline phosphatase, and expressed pluripotent markers including Oct4, Sox2, Nanog, Lin28, E-Cadherin, SSEA-1, SSEA-4, TRA-1-81, STAT3 and FOXD3. They could form embryoid bodies *in vitro* and teratomas after injecting into the nude BALB/C mice, and three germ layers were identified in the embryoid bodies and teratomas. Methylation assay revealed that, the promoters of Oct4 and Nanog were hypomethylated in biPSCs compared with BFFs and pre-biPSCs, while the promoters of Sox2 and E-Cadherin were hypomethylated in both BFFs and biPSCs. Furthermore, inhibiting p53 expression by co-expression of SV40 large T antigen and buffalo defined factors in BFFs or treating BFFs with p53 inhibitor pifithrin-a (PFT) could increase the efficiency of biPSCs generation up to 3-fold, and nuclear transfer embryos reconstructed with biPSCs could develop to blastocysts. These results indicate that BFFs can be reprogrammed into biPSCs by buffalo defined factors, and the generation efficiency of biPSCs can be increased by inhibition of p53 expression. These efforts will provide a feasible approach for investigating buffalo stem cell signal pathways, establishing buffalo stem cell lines and producing genetic modification buffaloes in the future

本シンポジウムは、社団法人つくば観光コンベンション協会からの協力を得て開催されます。

SRD-NILGS 第２回 日・韓・中交流シンポジウム　資料

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