CURRENT STATUS AND PERSPECTIVES IN ANIMAL REPRODUCTION AND SAFETY ASSESSMENT OF CLONING ANIMALS

The 3rd Korea-Japan Joint Symposium of Animal Reproductive Biology and Biotechnology

(09:50-17:30, 18th October, Thursday, 2007)

Yayoi Auditorium-Ichijo Hall
(http://www.a.u-tokyo.ac.jp/yayoi/)
College of Agricultural and Life Sciences, The University of Tokyo
Yayoi 1-1-1, Tokyo 113-8657, Japan

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PROGRAMM

18th October, Thursday, 2007

09:50 – 10:00 **Opening Remarks**
Dr. Masugi NISHIHARA
President of Japanese Society of Animal Reproduction (JSAR)
The University of Tokyo, Japan

10:00 – 10:30 **Recent Advances in Animal Biotechnology in Korea**
Dr. Hoon-Taek LEE
President of Korean Society of Animal Reproduction (KSAR)
Konkuk University, Korea

10:30 – 11:00 **Recent Research Trends in Animal Reproduction and Biotechnology in Japan**
Dr. Eimei SATO
Former President of Japanese Society of Animal Reproduction (JSAR)
Tohoku University, Japan

11:00 – 11:30 **Present status of somatic cell cloned cattle produced in Japan**
Dr. Shinya WATANABE
National Institute of Livestock and Grassland Science, Japan

11:30 – 12:00 **MicroRNA system in fertilized and cloned mouse embryos**
Dr. Nam-Hyung KIM
National Research Laboratory of Molecular Embryology
Chungbuk National University, Korea

12:00 – 13:30 Lunch

13:30 – 14:00 **Production of cloned cats and dogs by somatic cell nuclear transfer**
Dr. Il-Keun KONG
Sunchon National University, Korea

14:00 – 14:30 **From somatic cell to stem cell through nuclear transfer**
Dr. Teruhiko WAKAYAMA
Center for Developmental Biology, RIKEN-Kobe, Japan

14:30 – 15:00 **Characterization of somatic nuclear remodeling and reprogramming** in mouse cloned embryo
Dr. Nguyen Van THUAN
Konkuk University, Korea

15:00 – 15:30 **Transgenesis and assisted reproductive technologies in rat**
Dr. Naomi KASHIWAZAKI
Azabu University, Japan

15:30 –16:00 **Improvement of culture media for in vitro embryo production**
Dr. Hiroyoshi HOSHI
Functional Peptide Research Institute, Japan

16:00 – 16:20 Coffee break

16:20 – 17:20 **Cellular and molecular mechanisms regulating function of the corpus luteum**
Dr. Joy L. PATE
Former President of the Society for the Study of Reproduction (SSR)
Ohio State University, USA

17:20 – 17:30: **Closing Remarks**
Dr. Noboru MANABE
The University of Tokyo, Japan

Correspondence: Dr. Noboru MANABE
Animal Resource Science Center, The University of Tokyo
Ago 3145, Kasama 319-0206, Japan
Tel: +81-299-45-2606, Fax: +81-299-45-5950
E-mail: amanabe@mail.ecc.u-tokyo.ac.jp

ABSTRACTS

PREFACE: CURRENT STATUS AND PERSPECTIVES IN REPRODUCTIVE BIOTECHNOLOGY AND SAFETY ASSESSMENT OF CLONING ANIMALS

Noboru MANABE
Animal Resource Science Center, The University of Tokyo
Ago 3145, Kasama 319-0206, Japan
(amanabe@mail.ecc.u-tokyo.ac.jp)

Since “Dolly”, the first somatic cell cloned-sheep, was born in 1996, studies on the somatic cell cloning technique have been of interest around the world. The studies will bring fast and efficient improvement in farm animals, with precise sex determination and multiplication of elite animals including genetically modified ones. When combined with transgenic technologies, it will open new fields in medical applications such as the establishment of genetically defined models for xeno-transplantation and a source for human therapeutics. In Japan, the first somatic cell cloned calves was born in 1998. To date, more than 500 cloned calves have been born. The somatic cell cloning technique will be considered as a standard technique for beef cattle breeding. Recently, Japanese people have interesting in the food safety including the beef of somatic cell cloned cattle. To assess the safety of the cloning animals as food, it is essential to understand the current status and perspectives in reproductive biotechnology. The 3rd Korea-Japan joint symposium of animal reproductive biology and biotechnology will be held to understand the current status and perspectives in reproductive biotechnology in Korea and Japan. Professor Hoon-Taek LEE (President of Korean Society of Animal Reproduction: KSAR) will give a lecture on the recent advances in animal biotechnology in Korea. Professor Eimei SATO (Former President of Japanese Society of Animal Reproduction: JSAR, and President of Japanese Society of Animal Sciences) will give a lecture on the recent research trends in animal reproduction and biotechnology in Japan. In Korea and Japan, the importance of animal biotechnology is highly appreciated. Animal biotechnology researchers in government, university, institutes and industries have tried its vast application not only in production of superior quality animals but also in medicine, genetic engineering, transgenesis, cloning and stem cell. Professor LEE will focus on animal transgenesis, cloning and stem cell researches in Korea. Professor SATO will present the history and importance of research activities of animal reproduction in Japan. Dr. Shinya WATANABE will give a lecture on the present status of somatic cell cloned cattle produced in Japan. As mentioned above, from 1998 to September 2006, 511 somatic cell cloned cattle have been produced in Japan. He is a major member of government committee to assess the safety of somatic cell cloned cattle-beef as food in Japan, and will report the studies on genetic similarities and muzzle prints, hematology and clinical chemistry findings, pathology, growth performance, reproductive performance, meat productive performance and milk productive performance. Moreover, he will present that the somatic cell cloning technology does suffer from problems such as the large calf syndrome at birth, extremely low survival rates before weaning in cattle and abnormal gene expression of embryos. Professor Nam-Hyung KIM will present the study on micro RNA, small non-coding RNA molecules of 22 nucleotides in length that can post-transcriptionally regulate gene expression by base-pairing to mRNA, in fertilized and cloned embryos. Professor Il-Keun KONG will show us the somatic cell nuclear transfer (SCNT) for production of cloned-cats and dogs. Dr. Teruhiko WAKAYAMA, who obtained the first somatic cell cloned mouse, will give a lecture titled “From somatic cell to stem cell through nuclear transfer”. Mice can be cloned from cultured and non-cultured, adult-, fetus-, male- or female-derived cells. Recently, he was able to improve the success rate of cloning in mice up to six times using dimethyl sulfoxide, trichostatin A or by serial cloning using nuclear transfer- embryonic stem (NT-ES) cell technique. However, the overall efficiency of mouse cloning is less than 10% and the offspring that survive to term have shown many abnormalities, which are probably caused by incomplete genomic reprogramming after nuclear transfer into the oocyte cytoplasm. Moreover, he could get the mouse from nuclear transferred-embryonic stem (ntES) cell lines. He will talk about the similarity between ntES and embryonic stem (ES) cells, and the possibility to establish ES/ntES cell lines without ethical problem. Professor Nguyen Van THUAN, who recently moved from Wakayama’s laboratory to Lee’s laboratory in Konkuk University, will talk about the histone deacetylase, scriptaid, rescues full-term development in cloned inbred mice by enhancing zygotic nascent mRNA production. Professor Naomi KASHIWAZAKI will show us the transgenesis in rat, very important experimental animal. He will explain the detailed techniques on advancements in assisted reproductive technologies (ARTs) and the application of cryopreserved sperm, ovaris and intracytoplasmic sperm injection (ICSI). Dr. Hiroyoshi HOSHI, who is

a leader of the researches for improvement the follicle, oocyte and embryoculture system, will give a focused lecture on *in vitro* growth, maturation and development of oocytes derived from bovine early antral follicles. Finally, Professor Joy L. PATE (Former President of the Society for the Study of Reproduction: SSR) will give a special lecture on cellular and molecular mechanisms regulating function of the corpus luteum in cow. The corpus luteum (CL) dominates the estrous cycle, and so the regulation of CL function is very important for management the transplanted embryos with SCNT. Her lecture will be fruitful for all members of KSAR and JSAR.

RECENT ADVANCES IN ANIMAL BIOTECHNOLOGY IN KOREA

Sang-Jun UHM, Mukesh Kumar GUPTA, Ji-Hoon YANG, Young-Tae HEO, Hoon-Taek LEE

Departments of Biotechnology and Bioscience, and Animal Biotechnology
Bio-Organ Research Center, Konkuk University
1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Korea
(htl3675@konkuk.ac.kr)

The importance of animal biotechnology is highly appreciated in Korea. Recently, animal biotechnology researchers in government, university, institutes, and industries have tried its vast application in bio-medicine, genetic engineering, transgenesis, cloning, stem cell, and production of superior quality animals. This review focuses on animal transgenesis' cloning, and stem cell research in Korea. Through transgenesis, the mouse models for human diseases and animal bioreactors such as chicken (enhanced green fluorescent protein gene), goat (bovine growth hormone gene), pig (human erythropoietin and thrombopoietin genes), and cow (human lactoferrin gene) have been produced for production of proteins of pharmaceutical importance. These animals were produced by utilization of techniques such as pronucleus microinjection, gene recombination and transfer in somatic cells, and somatic cell nuclear transfer (SCNT) techniques. The SCNT technique has also been used for conservation as well as production of superior and rare animals. This technique has also been applied for the production of bio-organs for xenotransplantation in human. In the arena of stem cell research, embryonic and adult stem cells have been applied for therapy of human incurable disease using mice and pig as model systems. The Korean government promotes advancement of these researches for the next generation under network with industry, university and institute. In the present and future, the successful achievements of these animal biotechnology researches would contribute to bio-medicine, genetic engineering as well as agricultural industries.

RECENT RESEARCH TRENDS IN ANIMAL REPRODUCTION AND BIOTECHNOLOGY IN JAPAN

Eimei SATO

Laboratory of Animal Reproduction, Tohoku University
Sendai 981-8555, Japan
(eimei@mail.bios.tohoku.ac.jp)

Scientific activities regarding animal reproduction and technology in CJK regions are strong. Japanese scientists in this field have joined several domestic societies including the Japanese Society of Animal science, Japanese Society of Veterinary Science, Japanese Society of Animal Reproduction (JSAR), Japan Embryo Transfer Society, Japan Society of Fertilization and Implantation, Japanese Society of Mammalian Ova Research and others.

JSAR has a role as a branch of the International Congress of Animal Reproduction and has joined a working group to establish a new international society, the World Congress of Reproductive Biology (WCRB) with the Australian Society of Reproductive Biology, Society for the Study of Reproduction(SSR) in USA, Society for the Study of Fertility (SSF) in the UK and the first meeting is scheduled to be held in Hawaii in 2008.

History and activities of JSAR

JSAR was established in 1948 to encourage the study of reproductive biology in wild, domestic, laboratory, and companion animals. This was two years before the establishment of the British Society for Reproduction and Fertility, SSF and 19 years before the US Society for the Study of Reproduction, SSR. Members of JSAR have contributed to the cutting edge of research in reproductive biology in this country, which promotes the use and development of high level biotechnology. Previously, members of JSAR have played a key role in developing techniques for artificial insemination, fertilized egg transfer, and in vitro fertilization to increase the productivity of animals. Currently, members are actively involved in developing new technologies, such as transgenic techniques and cloned animals. They are also involved in research on endocrine disruptors.

The society has about 850 members from academic, governmental, and business institutions as well as veterinary clinicians. The interests of the members are necessarily diverse including sexual maturation, oogenesis, spermatogenesis, ovulation, gestation, and lactation. There is also research on practical themes such as the reproductive failure of dairy cows and in vitro fertilization. Several cloned domestic animals, including cows, pigs and goats have been produced by members of this Society.

The Society holds an annual fall meeting at locations throughout the country at which members and guests make presentations and hold symposia. Special lectures by foreign researchers are a highlight of the meetings. The Society has been publishing an official journal, the Journal of Reproduction and Development (JRD) since 1955. This bimonthly journal is currently up to volume number 53. Both the abstracts and full-text articles are accessible online at: http://jrd.jstage.jst.go.jp/en/. Impact factor of JRD was announced to be 1.300 and 1.149 on May, 2006 and 2007, respectively.

The JSAR Outstanding Research Award is given for outstanding basic and applied research in animal reproduction. The JSAR Innovative Technology Award is given for research which contributed to the development of innovative technology in animal reproduction. The JSAR Young Investigator Award is given for outstanding research in animal reproduction performed by an exceptionally promising young investigator who has not reached the age of 35 before April 1st of the year when the award is presented. JSAR made an academic agreement with the Korean Society of Animal Reproduction (KSAR) in 2005 to hold a joint symposium of JSAR and KSAR every year. In 2007, the third Korean-Japanese Seminar on Reproductive Biology is scheduled to be held at the University of Tokyo, on October 18 under the title of "Current Status and Perspectives in Reproductive Biotechnology and Safety Assessment of Cloning Animals".

Papers in the last 99th annual meeting of JSAR in 2006

The animal species in the papers presented at the 99th annual meeting1) are shown in Table 1. Some 51.9% of the papers refer to work with laboratory animals. Domestic animals accounted for 43.0%. The ratio of cattle and pigs is high in domestic animals. As for subjects, 29.1% of the papers were

classified as technology in animal reproduction, including freezing and drying of gametes and transgenic, animal cloning (Table2). A total of 70.9% of the papers were classified as reproductive biology. The fields of endocrinology, gametogenesis and oocyte maturation, follicular development and luteinization were numerous in the paper on reproductive biology. Our papers published in the international journal were presented originally at the annual meeting of JSAR [2-5].

Recent progress in biotechnology research has been remarkable in Japan, as well as Korea. Since the report of a cloned sheep by nuclear transfer using adult mammary gland cells, the birth of cloned calves, pigs, goats and other domestic animals has been reported first or in succession in Japan. The ratio of papers related to animal cloning is also dramatically increasing for domestic and laboratory animals at the annual meeting of JSAR.

Cloned animals have the greatest impact in animal biotechnology

The cloned sheep was produced from the viewpoint of animal husbandry and more efficient breeding of good domestic animals. In the field of animal husbandry, animal biotechnology is often considered to consist of two categories. One is techniques such as animal reproduction, animal breeding, animal feeding, animal hygiene, and animal management, which have developed on the basis of "traditional technology" and have already yielded a number of practical techniques that contribute to the industry. The other is the technology that has emerged with the development of modern biology and consists primarily of cell manipulation such as gene recombination and cell fusion. Techniques represented by "cloned sheep" have been developed on the basis of both of these categories, and techniques such as artificial insemination, microscopic insemination, selective production of males or females, in vitro fertilization and embryonic transplantation, and storage of sperm and embryos by freezing have advanced until they now play important roles in medicine, as well as animal industry.

The birth of the cloned sheep is influencing the frontier of research in animal biotechnology in Japan, as well as worldwide. It has effects on research of animal biotechnology itself by requiring new research subjects, modifications to the strategy of ongoing research projects, and challenges to schemes formerly considered impossible [6-7].

I object to restrictions on cloning research

Discussion over restrictions on cloning research has become heated since the birth of the "cloned sheep". I agree that application of the cloning technique to humans must be made carefully, because it may shake the foundation of humans as higher animals: Individuals die and they produce the next generation by sexual reproduction. However, I object to the contention raised by some people that cloning research of even domestic animals should be restricted. Cloning research has a major impact on various fields of animal biotechnology, and restricting research would cause great loss to society. Also, if the restrictions are applied formally, it may lead to restrictions on animal biotechnology research itself, which would have massive negative effects on society. I personally think that on cloning research using domestic animals should go no further than associations formulating independent guidelines that investigators respect.

External report of the Science Council of Japan in 2000

Public interest in cloned animals is extremely high. However, lack of information and the difficulty of the available information result in anxiety and skepticism over cloned animals. In these circumstances, we thought that it is necessary to establish a “Guideline Concerning Research on Clone Individuals of Industrial Animals” prepared by scientists directly and indirectly involved in research into clones of industrial animals, thus, promoting research on biotechnology, including research on cloned animals. Thus, the Japan National Committee of Animal Science of the 17th Science Council of Japan organized the “Subcommittee For the Manual of Research on Cloning of Industrial Animals” and has intensively evaluated the issue. Then, in March 27, 2000, we announced our basic attitude to research on cloned individual industrial animals and the desirable state of research in the future. We made proposals concerning the importance of public information and educational activities concerning clone research on industrial animals and the need to establish a pioneer biotechnology research organization under the name of the Chairman of Japan National Committee of Animal Science, Science Council of Japan, Dr. Seiki Watanabe.

Guide for Studies on Industrial Animal Clones

The guide was released under the name of the Japan National Committee of Animal Science, Japan National Committee of Veterinary Medicine, and Japan National Committee of Breeding of the 17th

Science Council of Japan. In this guide, several points were emphasized. In studies related to industrial cloned animals, all investigators should promote studies beneficial for life science and strive to promote both animal and human welfare, aim to move research forward with the ultimate goal of improving human welfare. To achieve these objectives, investigators must adhere to the basic principles listed below:

(1) Investigators must adhere to national laws, regulations, policies and/or guidelines.
(2) In regard to foreign laws, regulations, policies and/or guidelines, basic common provisions must be adhered to with due consideration of Japanese national laws, regulations, policies and/or guidelines unless the content is based on specific religious beliefs and/or cultures.
(3) Studies that may conflict with (1) and (2) and those that may provoke social or ethical arguments must be deliberated upon by consultation with related academic associations and society in general. For this purpose, an ethical committee must be established in each research institution before the execution of a study, and the scientific necessity, significance, influence on society and/or the ethical aspects of the study must be fully investigated. Discussion at the ethical committee must be recorded and archived, and must be immediately disclosed upon request. In areas of controversy, the opinions of competent authorities must be collected via the head of the institute.

Future State of Research stated by the Science Council of Japan in 2000

The development of animal biotechnology research represented by animal cloning studying will have a very significant impact on the future of the Japanese animal industry in the 21st century. Applied life science research is rapidly progressing worldwide, and to keep up with this, Japan must develop long-term plans and redress the balance. A keen desire to learn in future generations, inspiring the spirit of inquiry, the training of researchers with a strong desire to advance science, and the execution of creative studies are very important not only for the Japanese animal industry, but also for the general progression of applied life science. In regard to these points, the promotion of the following items should be particularly focused on:

(1) Educational activity for industrial animal cloning research: To obtain sound public understanding of industrial animal cloning research and animal biotechnology, it is necessary to promote educational activities to correct social prejudice and excess expectations. To establish the position of industrial animal cloning research in the life science field, insemination of knowledge ranging from the basic research level to general life-supporting mechanisms must be promoted without excessive bias of application, and the educational efforts intended for the sound intelligent development of junior, high school and university students aspiring to careers in applied life science will be supported. For students in non-scientific fields, balanced and accurate knowledge will be made accessible to increase and maintain high standard of public knowledge of animal biotechnology.
(2) Establishment of research organizations for advanced animal biotechnology including clone research: For the development of advanced animal biotechnology in Japan, it is essential to establish a strong research system including basic life science such as embryology, reproductive biology, cell biology, molecular biology, genetics, animal behavior, and ecology, in addition to related fields such as traditional animal production, veterinary medicine, and breeding. Success in the production of cloned animals is based on the long history of basic research that started with the production of *Xenopus* laevis by somatic cell nuclear transfer achieved by Dr. J.B. Gordon in the 1960's in England. The production of cloned sheep would not have been possible without the tradition and tremendous accumulation of the results of basic studies in England on the molecular and cell biology related to the cell cycle and regulatory mechanisms underlying expression of genetic information stored in chromatin. When the reprogramming mechanisms in the nucleus of differentiated cells are elucidated in the near future following the rapid progress in genome research in domestic animals, epoch-making biotechnology may be developed. The same will be true life sciences fields other than animal biotechnology.

To develop new biotechnology for the animal industry keeping pace with Western countries, it is essential to establish a comprehensive research organization for advanced applied animal science with a strong research system. Thus, the Japan National Committee of Animal Science strongly and actively promote the establishment of the research organization described above.

References

1) Journal of Reproduction and Development, 52 (suppl.): J1-146, 2006 (in Japanese)
2) Yokoo,M., Sato,E. Cumulus-oocyte complex interactions during oocyte maturation. International Review of Cytology, 235: 251-291, 2004.

3) 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. Biology of Reproduction, 72:1416-1420, 2005.
4) Tanemura,K., Ogura,A., Cheong,C., Gotoh,H., Matsumoto,K., Sato,E., Hayashi,Y., Lee,H.W., Kondo,T. Dynamic rearrangement of telomeres during spermatogenesis in mice. Developmental Biology, 281:196-207, 2005.
5) Miyake,Y., Matsumoto,H., Yokoo,M., Miyazawa,K., Kimura,N., Tunjung,W.A.S., Shimizu,T., Sasada,H., Aso,H., Yamaguchi,T., Sato,H.: Expression and glycosylation with polylactosamine of CD44 antigen on macrophages during follicular atresia in pig ovaries. Biology of Reproduction, 74: 501-510, 2006.
6) Sato,E. A new frontier of animal biotechnology research with the birth of the cloned sheep. Journal of Mammalian Ova Research, 16: 83-85, 1999.
7) Sato,E., Yoshida,N., Kimura,N., Yokoo,M. New frontiers of animal biotechnology in the field of animal reproduction. Asian-Australasian Journal of Animal Sciences, 14 (suppl.): 19-27, 2001.
8) Japan National Committee of Animal Science: External report of Science Council of Japan-Establishment of the "Guideline concerning research on clone individuals of industrial animals". Journal of Mammalian Ova Research, 18: 130-132, 2001 (the original was published in 2000 in Japanese).

Table 1. Animal species in the papers at the annual meeting of JSAR and SSR

		JSAR	SSR
Domestic animals		77 (43.0%)	195 (36.8%)
	cattle	39	76
	horse	1	8
	pigs	24	44
	sheep	0	49
	goat	5	1
	mink	0	1
	dog	3	1
	cat	2	3
	chicken	3	11
	quail	0	1
Human		1 (0.6%)	40 (7.6%)
Laboratory animals		92 (51.9%)	235 (44.3%)
	mouse	50	151
	rat	32	59
	hamster	2	5
	guinea pig	0	2
	rabbit	1	2
	monkey	3	16
	Mastomys natalensis	1	0
	Suncus murinus	1	0
Wild animals		2 (1.1%)	15 (2.8%)
	fish	2 (1.1%)	15 (2.8%)
Other(cultured cells)		1 (0.6%)	30 (5.7%)

JSAR: The 99th annual meeting of JSAR at Nagoya, SSR: The 39th Annual meeting of SSR at Omaha.
Note: There are papers using two or more animal species.

Table 2. Subjects in the papers at the annual meeting of JSAR and SSR

		JSAR	SSR
Reproductive technology		51(29.1%)	54(10.4%)
	Freezing and freeze-dry of gametes	13	11
	X and Y sperm	1	1
	Transgenic	9	18
	IVF and ICSI	5	1
	ES cells and stem cells	7	5
	Clone and parthnogenesis	13	17
	Transplantation of gonads	3	1
Reproductive biology		124(70.9%)	464(89.6%)
	Sex differentiation	2	12
	endocrine	25	75
	gametogenesis and maturation	30	76
	gonadal function 8 18	8	18
	follicular development and luteinization	17	77
	fertilization	0	4
	embryogenesis	10	37
	implantation and placenta	6	37
	Fallopian tube and uterus	4	30
	pregnancy and delivery	5	17
	sexual maturation	0	4
	Reproductive immunology	0	4
	Comparative animals	1	4
	Stress, chemicals and irradiation	5	22
	DNA imprinting	5	8
	cancer	0	22
	sexual behavior	4	1
	lactation	2	2
	others	0	12
Total		175	516

JSAR: The 99th annual meeting of JSAR at Nogoya, SSR: The 39th Annual meeting of SSR at Omaha
Note: There ere papers working in 2 or 3 categories. ICSI: intracytoplasmic sperm injection. ES cells: embryonic stem cells

PRESENT STATUS OF SOMATIC CELL CLONED CATTLE PRODUCED IN JAPAN

Shinya WATANABE

National Institute of Livestock and Grassland Sciences
Tsukuba 305-0901, Japan
(shw@affrc.go.jp)

Since the first somatic cell cloned calves were born in Japan (Kato et al., 1998), 511 somatic cell cloned cattle have been produced in this country [as of September, 2006; press release from Ministry of Agriculture, Forestry and Fisheries, Japan (http://www.s.affrc.go.jp). According to the press release data, which is accumulated from 1998 to 2006 (September), 68% of newborns were surviving the perinatal period. However, improvement in efficiency of cloned cattle production is still on the way. Although voluntary moratorium on cloned cattle products was made by the Japanese government on November, 1999, and has remained in effect even now, studies on cloned cattle and their offspring have been conducted by many institutes in Japan. Namely, it have been reported at least 64 papers, which employed 171 clones and 32 offspring of clones, since 2000. These studies covered following seven research fields: (1) Genetic similarities and muzzle prints, (2) Hematology and clinical chemistry findings, (3) Pathology, (4) Growth performance, (5) Reproductive performance, (6) Meat productive performance and (7) Milk productive performance. No remarkable differences in health status and reproductive performance were found among conventionally bred cattle, somatic cell cloned cattle surviving to adult-hood and offspring of somatic cell cloned cattle. Similarities in growth performance and meat quality were observed between nuclear donor cattle and their clones. Also, growth curves of the offspring and their full siblings were resembled. As for the milk and meat produced from cloned cattle, studies on composition analysis and toxicology have been carried out in our country (Japan Livestock Technology Association, 2002). This study concluded that there were no remarkable differences between products from clones and comparators, and no harmful effects attributable to these products. Another data accumulation concerning milk and meat produced from offspring of clones is progressing since 2004 in a “Research Project for Utilizing Advanced Technologies in Agriculture, Forestry and Fisheries” from the Agriculture, Forestry and Fisheries Research Council, Ministry of Agriculture, Forestry and Fisheries, Japan. Conclusions of this study might appear in this autumn.

MICRORNA SYSTEM IN FERTILIZED AND CLONED MOUSE EMBRYOS

Nam-Hyung KIM

National Research Laboratory of Molecular Embryology, Department of Animal Science, Chungbuk National University
Cheongju, 12 Gaeshin-dong, Heungduk-gu, Vheongju, Chungbuk 361-763, Korea
(nhkim@chungbuk.ac.kr)

MicroRNAs (miRNAs) are small non-coding RNA molecules of 22 nucleotides in length that can post-transcriptionally regulate gene expression by base-pairing to mRNAs. To elucidate the role of microRNA pathway in early embryo development, we initially evaluated gene expression of dicer, GW182 and Ago2 in mouse oocytes and embryos *in vitro*. The transcript levels of these genes in GV stage oocytes steadily decreased up to the 2-cell embryo stage, and expression remained stable during morulae and blastocyst formation. Their protein syntheses were additionally observed in mouse oocytes and early embryos. Silencing of their mRNA expression by RNA interference (siRNA) did not inhibit development up to the blastocyst stage. However, significantly, enhanced apoptosis and lower cell numbers were observed in siRNA-treated blastocysts. Real time RT-PCR experiments confirmed the decreased expression of selected transcription factors, including POU domain, class 5, transcription factor 1 (*Pou5f1*), SRY-box containing gene 2 (*Sox2*), and Nanog homeobox (*Nanog*). Moreover, POU5F1 protein expression was suppressed by *Dicer1* or QW182 siRNA. Our data showed that microRNA pathway is implicated in gene transcription regulation at the blastocyst stage.

In addition, in order to get insights into the role of miRNAs in early embryogenesis, mouse fertilized and cloned embryos were chosen as model to investigate various miRNAs, such as miR-15a, -16, -21 and -302. TaqMan® MicroRNA Assays were employed to quantify the precise expressions of these miRNAs. The results revealed that expression of miR-15a, miR-16 and miR-21 expression were down-regulated from zygote to 2-cell and then gradually increasing from 4-cell to blastocyst *in vitro*. Interestingly, the significantly higher expressions in *in vivo*-and cloned derived blastocysts were found for three miRNAs compared with that in *in vitro*-derived blastocysts. In general miR-15a was expressed lowestly in preimplantation embryos among the three miRNAs, while miR-16 was the highest one. As the low levels of miR-15a, we carried out the microinjection of mature miR-15a and -302 into fertilized and cloned zygotes. Data showed that miR-15a and 302 expression were significantly increased in microinjected blastocysts. Although their microinjection did not affect the embryo development up to blastocyst stage, significantly higher apoptosis in microinjected blastocysts was observed ($p<0.05$). Taken together, the changes in miR-15a, 16, 21 and 302 expressions in fertilized and cloned embryos strongly indicated their possible role in early embryogenesis.

PRODUCTION OF CLONED CATS AND DOGS BY SOMATIC CELL NUCLEAR TRANSFER

Il-Keun KONG

Division of Applied Life Science, Gyeongsang National University
Jinju 660-701, Gyeong-Nam Province, Korea
(ikong@sunchon.ac.kr)

The somatic cell nuclear transfer (SCNT) technique has been used to produce cloned animals in various species, including cattle, sheep, goat, pig, cat and dog. Domestic cat and dog is a useful research model to develop assisted reproductive technologies for the conservation of endangered felids and canis. The current applications for cat and dog cloning include targeted genotype replication of pets and the production of models for the study of human and animal diseases. A second point of application is the production of transgenic cats and dogs, genetically engineered "designer pets" with favorable attributes, such as an allergen-free cat and a mini-cat. To date several groups have successfully cloned kittens and pups (Gomez et al., 2004; Shin et al., 2002; Yin et al., 2005; Lee et al., 2005). The cloned male cats have normal reproductive success and lie within the normal range of gonadal hormone production. All F1 kittens were produced by natural breeding and delivery, and are still alive and display normal healthy growth. Serial cloning is an excellent way to study the effect of progressively accumulating somatic mutations on development, health and reproductive performance. Moreover, recloning existing cloned fetuses or animals has the distinct advantage of reproducing unique genetic characteristics. We were also the first to successfully produce a cloned transgenic cat expressing an exogenous red fluorescence protein. Whole body red fluorescence was detected in the liveborn transgenic cats, but not in the non-TG cat at more than 2 months after birth. Red fluorescence was detected in tissue samples, including hair, muscle, brain, heart, liver, kidney, spleen, bronchus, lung, stomach, intestine, tongue and even excrement from the transgenic cat. Our nuclear transfer procedure using genetically modified somatic cells will be useful for the efficient production of transgenic cats. The cloned dog, Snuppy, was produced from in vivo ovulated and matured oocytes instead of in vitro matured oocytes, because nobody has been able to establish an in vitro maturation system yet. A cloned toy poodle was also cloned from somatic cells derived from an aging (14 year-old) female dog, and a wolf. The application of SCNT procedure to produce genetically modified cat and dog will be valuable for the biomedical modeling of human disease, as well as for the production of designer pets.

FROM SOMATIC CELL TO STEM CELL THROUGH NUCLEAR TRANSFER

Teruhiko WAKAYAMA

Laboratory for Genomic Reprogramming, Center for Developmental Biology, RIKEN
Kobe 650-0047, Japan
(teru@cdb.riken.go.jp)

Mice can now be cloned from cultured and non-cultured, adult-, fetus-, male-, or female-derived cells. Recently, we were able to improve the success rate of cloning in mice up to six times using dimethyl sulfoxide (DMSO), trichostatin A (TSA) or by serial cloning using NT-ES cell technique. However, the overall efficiency of mouse cloning is less than 10% and the offspring that survive to term have shown many abnormalities, which are probably caused by incomplete genomic reprogramming after nuclear transfer into the oocyte cytoplasm. Even the technique itself remains imperfect, embryonic stem (ES) cell lines can be generated from adult somatic cells *via* nuclear transfer. These cells, called ntES cells, exhibit full pluripotency in which they can be made to differentiate along prescribed pathways *in vitro*, and contribute to the germ line following injection into blastocysts. We have demonstrated that those ntES cells are indistinguishable from ES cell derived from fertilized embryos. For safety application of these techniques to regenerative medicine, there are several problems which have to be solved. For example, it has been widely assumed that fresh oocytes are required for nuclear transfer and the establishment of ntES lines, or derivation of ES or ntES cells arises from the need to destroy the embryos. Several approaches that circumvent this problem have recently been reported, such as ES cell derivation from single blastomere without destroy the embryo, or use aged fertilization-failure (AFF) oocytes as recipient, which usually discarded in infertility clinics. In this symposium, I will talk about the similarity between ntES and ES cells, and the possibility to establish ES/ntES cell lines without ethical problem.

THE HISTONE DEACETYLASE, SCRIPTAID, RESCUES FULL-TERM DEVELOPMENT IN CLONED INBRED MICE BY ENHANCING ZYGOTIC NASCENT MRNA PRODUCTION

Nguyen Van THUAN

Department of Animal Biotechnology, Konkuk University
1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Korea
(Laboratory for Genomic Reprogramming, Center for Developmental Biology, RIKEN, Kobe, Japan)
(vanthuan@konkuk.ac.kr)

Inbred mouse models serve as a biological test ground and genetic background is an important criterion when selecting a strain for a particular experiment. Moreover, inbred mouse strains such as C57BL/6 are used for transgenesis research to identify functional genes or as genetic models for disease susceptibility via somatic cell nuclear transfer (SCNT). Since Cumulina, the first B6D2F1 mouse clone produced by somatic cell SCNT (Wakayama, 1998), the success rate of cloning in mice has been extremely low compared with other species. Most of the important inbred mouse strains, such as C57BL6 and C3H, have never been cloned, except for the DBA2 strain (Wakayama, 2001) and mouse strain 129 (Inoue et al., 2003) but this was achieved with extremely low efficiency. A variety of approaches have been used with the aim of improving the efficiency of SCNT cloning: changing the timing and conditions of activation and fusion, treating donor cells or SCNT embryos with dimethyl sulfoxide, cyclin-dependent kinase inhibitor (roscovitin), or 5-aza-2′-deoxycytidine; altering the timing of removal of the oocyte chromosomes; serial nuclear transfer; removal of donor cell cytoplasm; and clone–clone aggregation. Recently, our laboratory found that treatment of SCNT mouse embryos with 50–100 nM Trichostatin A (TSA) for 10 h after nuclear transfer can enhance the generation of cloned B6D2F1 mice (Kishigami, 2006). However, application of these methods to inbred mouse strains such as B6 and C3H has never been successful.

Here, we show for the first time that treatment of SCNT embryos with scriptaid (SCR), a histone deacetylase inhibitor with low toxicity that enhances transcriptional activity and protein expression, all the important inbred mouse strains can be cloned, such as C57BL6, C3H, DBA2 and 129. Moreover, using SCR treatment, we obtained healthy GFP-expressing male ICR mice with donor cells collected from the tail of a 2.5 year-old GFP-expressing ICR mouse. Most importantly, the placentae of cloned mice were significantly smaller than those of untreated control clones, although they were still heavier than the placentas of ICSI-fertilized fetuses. In addition, our results clearly show that the full-term developmental ability of cloned mouse is donor cell strain-dependent. Moreover, the success of somatic reprogramming and cloning efficiency via NT technique is clearly linked to the competent synthesis of nascent mRNA at the 2-cell embryo stage. We also found that increases of histone acetylation in the pronuclei of cloned 1-cell embryos is essential for somatic cell chromosome reprogramming, but is not sufficient for full reprogramming of somatic cells to allow full-term development. In this symposium, I will discuss the characterization of somatic nuclear remodeling and reprogramming and the correlation between histone acetylation and transcriptional activities during preimplantation and full-term development of SCNT embryos in mice.

TRANSGENESIS IN RATS

Naomi KASHIWAZAKI

Laboratory of Animal Reproduction, School of Veterinary Medicine, Azabu University
Sagamihara 229-8501, Japan.
(*nkashi@azabu-u.ac.jp)*

The rat is one of the most important experimental animals for biomedical and physiological research. During the last two decades, there have been advancements in assisted reproductive technologies (ARTs), genetic engineering and molecular biology. The synergy has allowed genetic modifications including transgenesis. Transgenic rats are extremely valuable for studying, and can be produced by exogenous DNA microinjection into the pronucleus as well as intracytoplasmic sperm injection (ICSI) with exogenous DNA. Transgenesis through DNA microinjection accounts for most of the rat transgenic lines, which were over one hundred. Cryopreservation of rat gametes and embryos has also been developed to bank the genetic resources efficiently. However, transgenesis in rats is still hampered by the lack of embryonic stem cell lines in which generation of knockout (KO) mice by homologous recombination is routinely used. Although French research group reported generation of cloned rats to control oocyte activation through the targeting of proteasome and kinase inhibitors in 2003, this cloning approach that enables to produce KO rats, is not available yet. The first KO rat models, on the other hand, were generated in 2003 by the combination of N-ethyl-N –nitrosourea (ENU) mutagenesis and a screening assay. The application of cryopreserved sperm, ovaris and ICSI will likely increase the generation of KO rats from ENU-mutagenized males. In this symposium, I will talk about ARTs related to transgenesis in rats.

IN VITRO GROWTH, MATURATION AND DEVELOPMENT OF OOCYTES DERIVED FROM BOVINE EARLY ANTRAL FOLLICLES

Hiroyoshi HOSHI, Joon-Ho CHO, Takehiro ITOH and Yutaka SENDAI

Research Institute for the Functional Peptides
Yamagata 990-0823, Japan
(hoshih@func-p.co.jp)

Mammalian ovary contains a large number of resting follicles. Limited numbers of these follicles are activated to enter the growth phase characterized by proliferation of granulosa cells and by increase in size of the oocytes. However, most of these follicles gradually become atretic during in vivo growth. The large store of these small follicles creates a potential source of oocytes for animal reproduction, conservation of endangered animals and human infertility therapy. So far, it has been more difficult to establish a complete in vitro culture system for growing oocytes of early antral follicles from cattle. Essential factors that could achieve a full growth of oocytes from the small follicles have not been fully understood yet. We have recently established a novel culture system “cumulus-oocyte complexes with granulosa cells (COCGs) cultured on collagen-coated flat substratum” which was beneficial for a long-term survival of growing oocytes from bovine early antral follicles. This improved culture system was applied for identifying factors which could attain the complete growth of oocytes from bovine early antral follicles (follicle diameter; 400-700 μm, oocyte diameter; 90-99 μm).

In the present study, KGF, also known as FGF 7, was found to be important factor for in vitro growth of oocytes. After a subsequent in vitro maturation, the in vitro grown oocytes exposed to KGF increased the rate of meiotic competence (MII phase) compared to the control. Furthermore, the in vitro grown oocytes cultured in KGF-containing medium resulted in high rate of developmental competence after in vitro maturation, fertilization and embryo culture. Preliminary results indicated that KGF stimulated the oocyte growth directly via KIT ligand (KITLG), KITLG receptor (KIT) signaling pathway in bovine COCGs from the early antral follicles. These results may support the in vitro production of large numbers of fully grown oocytes for embryo transfer and nuclear cloning.

CELLULAR AND MOLECULAR MECHANISMS REGULATING FUNCTION OF THE CORPUS LUTEUM

Joy L. PATE

Department of Animal Sciences, Ohio Agricultural Research and Development Center,
Ohio State University,
2029 Fyffe Road, Columbus, OH, USA
(pate.1@osu.edu)

In cows, the corpus luteum (CL) dominates the estrous cycle, because it persists for the majority of the cycle, and as long as progesterone is elevated, ovulation of another follicle is prevented, due to negative feedback on GnRH/LH release. Near the end of the estrous cycle, the uterus releases prostaglandin $F_{2\alpha}$, which causes regression of the CL, allowing a new follicle to ovulate. If an embryo is present, it is critical that the CL be rescued from luteolysis and continue progesterone production, an event known as maternal recognition of pregnancy. Despite the central importance of the CL in regulation of the estrous cycle and maintenance of pregnancy, the exact mechanisms that regulate whether the CL regresses or is maintained are poorly understood. For some time, a focus of our research has been to understand the mechanism of action of $PGF_{2\alpha}$ on luteal cells that would initiate luteolysis. During the studies of functional effects of the prostaglandin on luteal cells, it was observed that, although $PGF_{2\alpha}$ effectively inhibited LH-stimulated progesterone, it did not kill luteal cells in culture. This was very surprising, because it is well accepted that $PGF_{2\alpha}$ is the luteolytic agent in the cow. The latter observation provided the first clue that the action of $PGF_{2\alpha}$ in the CL might require participation of cell types in addition to the steroidogenic cells represented in the culture system. The CL is a very heterogeneous tissue. Abundant endothelial cells, pericytes, extracellular matrix fibroblasts, and immune cells all contribute to support of the steroidogenic cells. Much of the work on the role of endothelial cells has been done here in Japan. In the past few years, considerable data have been collected in this laboratory and others to support the hypothesis that the immune system may be involved in regression of the corpus luteum. The proinflammatory cytokines, IFN-γ and TNF-α, are cytotoxic to luteal cells. Class II MHC molecules are expressed in the CL, suggesting that antigen presentation may serve to activate the resident lymphocytes to produce the proinflammatory cytokines. In fact, cultured luteal cells are potent stimulators of T cell proliferation in vitro. However, we have recently determined that the majority of MHC molecule expression within the CL is localized to the capillary endothelial cells at midcycle, and may serve a completely different role than activation of immune cells to a proinflammatory state. Further, analysis of luteal cell-stimulated T lymphocytes by flow cytometry revealed that the class of T cells that proliferated to the greatest extent was the γδ set of T cells. Since γδ cells can be either pro- or anti-inflammatory and typically respond to self antigens rather than being MHC restricted, entirely new possibilities for immune cell regulation of luteal function will be proposed.