Oral Presentation

Opening Session

OPENING ADDRESS
HISTORICAL BACKGROUND OF REPRODUCTIVE TECHNOLOGIES IN DOMESTIC ANIMALS – FROM ARTIFICIAL INSEMINATION TO SOMATIC CELL CLONING –

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At the opening of the present workshop, we, the Japanese audience, express a heart felt welcome to the outstanding invited speakers from other countries. In looking at the rapid development of animal biotechnology over the past 50 years to the discovery of somatic cell cloning, I believe that the organization of this workshop is timely for promoting further progress of these technologies, and would like to sincerely thank the organizers, president, Dr. Yokouchi and program chairman, Dr. Nagai.

Since successful deep freezing of spermatozoa was reported by Polge et al. around 1950 the technique has been effectively used for artificial insemination (AI) in cattle. Following the progress of cryobiology, embryo freezing was also successful in 1972 (Whittingham, Leibo and Mazur). Freezing gametes also contributed to the wide spread use of embryo transfer (ET). Early work on embryo transfer in cattle (Sugie and Hafez) and establishment of the International Embryo Transfer Society (IETS) greatly accelerated the world wide application of ET and related techniques. Since the discovery of the phenomena of sperm capacitation (Austin and Chang), in vitro fertilization (IVF) has been successful in various mammals. IVF in large domestic animals such as cattle and pig was first reported in 1977~1978 (Iritani et al.), and the first IVF calf was born in 1982 (Brackett et al.). IVF techniques in large domestic species have contributed to the production of conventional and transgenic offspring in these species.

Approximately 20 years ago identical animals were produced by the separation of blastomeres (Willadsen et al.). Cloning has since been achieved by microblade bisection of morula or blastocyst stage embryos (Utsumi and Iritani), embryonic cell nuclear transfer (Smith and Wilmut) and somatic cell nuclear transfer (Wilmut et al.). Once the efficiency to clone animals by somatic cell nuclear transfer has further improved and ethical and legal aspects have been considered, this technique promises to be a useful tool for animal breeding, conserving endangered species and developing pharmaceutical and clinical applications.

References

1. Whittingham, D.G., Leibo, S.P., and Mazur, P. (1972) *Science* 178, 411-414.
2. Austin, C.R. (1951) *Aust.J.Res* 34, 581-589.
3. Chang, M.C. (1951) *Nature* 168, 697-698.
4. Iritani, A., Niwa, K., and Imai, H. (1978) *J Reprod Fertil* 1978, 54, 379-383.
5. Iritani, A., and Niwa, K. (1977) *J Reprod Fertil* 50, 119-121.
6. Brackett, B.G., Bousquet, D., Boice, M.L., Donawick, W.J., Evans, J.F., Dressel, M.A. (1982) *Biol Reprod* 27, 147-158.
7. Willadsen, S.M. (1979) *Nature* 277, 298-300.
8. Smith, L.C., and Wilmut, I. (1989) *Biol Reprod* 40, 1027-1035.
9. Utsumi, K., and Irutani, A. (1990) Theriogenology 33, 341.
10. Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., and Campbell, K.H. (1997) *Nature* 385, 810-813.

OPENING ADDRESS
CLONING: BIOTECHNOLOGY FOR THE NEW MILLENNIUM

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To my respected colleagues and fellow participants: Welcome to this symposium on the "Current Status of and Perspectives in Cloning and Related Studies." I have been involved in these fields for the past 3 decades. In the early 1970's, I began 10 years of basic and applied research in embryo transfer in North America. These experiences led to my involvement in the creation of the " International Embryo Transfer Society "in 1975. At the time, the ability to clone a living organism seemed like a bit of a dream.

Cloning research developed quickly after the somatic cell clone was first reported in 1997 in UK. In Japan, about 600 embryo clone cattle have been produced to date, many of which have already been shipped to market. An additional 200 somatic cell clones cattle have been produced for experimental purposes.

While cloning research progressed, great contributions were also being made by artificial insemination (AI), embryo transfer (ET), in vitro fertilization (IVF) and related technologies. I will be discussing the history and development of these and related technologies in Japan, as well as the commercial application of embryo transfer in North America.

Cloning will undoubtedly be at the forefront of biotechnological research in the 21st Century, and we can expect researchers in this field to make great contributions and breakthroughs. However, as scientists, we must not lose sight of the great responsibility we have to society at large to conduct our research ethically. This responsibility exists even if one takes the view that animals are a necessary component of our work.

Although both basic and applied research is crucial to any further progress in the life sciences, this research can only continue with a free and open exchange of information and the support and understanding of the general public.

Reference

1. Carmichael, R. A. (1980) *Theriogenology* 13, 3-11.

Opening Lecture

FACTORS INFLUENCING THE DEVELOPMENT OF CLONED EMBRYOS

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Offspring have been produced from somatic cells in five species, sheep, cow, mouse, pig and goat. However, no offspring have been produced from at least five others species, rat, rabbit, rhesus monkey, cat and dog despite considerable effort, by experienced laboratories. Although there are some small differences between species and donor cell type, in general cloning in mammals remains a very inefficient process with 0-4% of reconstructed embryos becoming live

offspring. The low overall efficiency is the cumulative result of death at all stages of development. As the proportion of embryos that reach the blastocyst stage is typically only 10-50%, depending upon cell type and species, this is the stage at which the greatest loss occurs. However, a greater than normal pre-and perinatal loss occurs at all stages, with the notable possible exception of the pig. In this species all piglets born alive in the first studies survived. Several factors influence development of cloned embryos including method of cell cycle co-ordination, method and time of oocyte activation, cell type in particular the stage of development from which the cell was derived.

The low efficiency and pattern of loss are the judged to be the result of inappropriate expression of a number of genes whose lethal effect is exerted at different times. The epigenetic errors in the regulation of gene expression that cause inappropriate expression are not well understood. However, two recent observations suggest that errors in methylation are involved in some cases. A change in methylation of regulatory elements of IGF2r was found to be associated with increased fetal weight after culture of sheep embryos (Young et al, 2001). Cloned bovine blastocysts were found to have unusually variable degrees of methylation and in some cases the pattern of methylation resembled that of the donor cell tissue, rather than a normal blastocyst (Yong-Kook Kang et al, 2001).

Improvements to the efficiency of cloning will depend upon a better understanding of the molecular mechanisms that regulate early development and the development of new strategies using that knowledge to create more appropriate environments during nuclear transfer.

References

1. Young, L.E., Fernandes, K., McEvoy, T.G., Butterwith, S.C., Gutierrez, C.G., Carolan, C., Broadbent, P.J., Robinson, J.J., Wilmut, I., and Sinclair, K.D. (2001) Nat Genet 27, 153-154.
2. Kang, Y.K., Park, J.S., Choi, Y.H., Chung, A.S., Lee, K.K., and Han, Y.M. (2001) Nat Genet 28, 173-177.

DONOR CELL TYPE AND CLONING EFICIENCY IN MAMMALS

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Cloned animals have been produced after nuclear transfer of somatic cells from various organs. It is not clear, however, which cell types or cell origins are most sufficient for mamalian cloning. The in vitro developmental ability of bovine, mouse, rabbit and pig oocytes receiving donor cells from various tissues was not largely different among donor cells. In bovine system, there was also no differences in the percentages of blastocysts that developed from oocytes containing adult, newborn or fetal calf nuclei, or between female and male nuclei. In the mouse system, the developmental ability of oocytes receiving ES cells from different cell lines was higher than those in somatic cells. So far, 33 cloned calves were obtained after embryo transfer of enucleated oocytes receiving somatic cells from various tissues but 17 of them died around or after parturition. Although the precise comparison of the relationship between donor cell origin and the production efficiency of normal cloned calves is difficult due to the limited data, we suppose there are some relationship. In this symposium, I will show recent data on mammalian cloning in my laboratory.

References

1. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, and Tsunoda Y. (1998) *Science* 282, 2095-2098.

2. Tsunoda, Y., and Kato, Y. (1998) *J Reprod Fert* 113, 181-184.
3. Kato Y, Yabuuchi A, Motosugi N, Kato J, and Tsunoda Y. (1999) *Bio. Reprod* 61, 1110-1114.
4. Kato Y, Rideout WM 3rd, Hilton K, Barton SC, Tsunoda Y, and Surani MA. (1999) *Development* 126, 1823-1832.
5. Kato Y, Tani T, and Tsunoda Y. (2000) *J Reprod Fert* 120, 231-237.
6. Yin, X.J., Tani T, Kato Y, and Tsunoda Y. (2000) *Theriogenology* 54, 1469-1476.
7. Tani, T., Kato Y, and Tsunoda Y. (2001) *Biol Reprod* 64, 324-330.
8. Yabuuchi, A. et al. (2001) *J Exp Zool.* 289, 208-212.
9. Amano T, Kato Y, and Tsunoda Y (2001) *Reproduction* 12, 729-733.

TRANSGENISIS AND NUCLEAR TRANSFER, PROGRESS IN GENE ADDITIONS AND KNOCKOUTS

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The development of techniques for somatic cell nuclear transfer in large animal species has provided a route for the precise genetic modification of livestock. Early studies demonstrated the feasibility of random gene addition by transfection and selection of cultured cell populations in cattle and sheep[1,2]. More recently targeted gene deletions and targeted gene additions have been reported[3,4]. The ability to perform genetic manipulation on cultured somatic cells is dependent upon a range of factors including the level of transcription of the gene of interest, the targeting construct, the frequency of homologous recombination in the cell population, the ability to select and culture the cells, their lifespan and their efficiency at promoting development of nuclear transfer embryos. This paper will review progress and problems in achieving transgenesis via somatic cell nuclear transfer and more specifically in achieving targeted gene knockouts and additions. In addition other routes to achieving tissue specific regulation of transcription and potential applications of the technology will be discussed.

References

1. Schnieke, A.E., Kind, A.J., Ritchie, W.A., Mycock, K., Scott, A.R., Ritchie, M., Wilmut, I., Colman, A., and Campbell, K.H. (1997). *Science* 278, 2130-2133.
2. Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J, Jerry, J., Blackwell, C., Ponce de Leon, F.A., and Robl, J.M. (1998) *Science* 280, 156-158
3. McCreath, K.J., Howcroft, J., Campbell, K.H.S., Colman, A., Schnieke, A.E., and Kind, A.J. (2000). *Nature* 405, 1066-1069.
4. Denning, C., Burl, S., Ainslie, A., Bracken, J., Dinnyes, A., Fletcher, J., King, T., Ritchie, M., Ritchie, W.A., Rollo, M., de Sousa, P., Travers, A., Wilmut, I., and Clark, A.J. (2001). *Nat Biotechnol* 19, 559-562.

Oral Presentation 1

CURRENT STATUS OF CLONING IN LIVESTOCK IN JAPAN

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The study of somatic cell nuclear transfer (SCNT) in livestock becomes active even in Japan as well as in foreign countries. Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF) and Agriculture, Forest and Fisheries research council (AFFRC) are promoting several collaborative research projects on the somatic cloning in domestic animals. And over 200 cloned calves have been born experimentally in Japan by March 2001 since the first adult somatic cell-cloned calves were produced in 1998 (1). The research on bovine somatic cloning was initiated to produce a number of cloned cattle for the meat and dairy production. However, any products have not been forwarded on a market, because their safeties have not guaranteed yet. The Ministry of Health, Labor and Welfare has been in charge of the evaluation of safety of products from cloned animals as a food. Since the production of the somatic cloned calves, the investigations on their body growth and physiological/reproductive functions have been carried out. No remarkable abnormality has been found in the growth rate, biochemical parameters of the blood, semen property and the conception rate following artificial insemination in the cloned cattle. In the first part this paper addresses the current status and problems of the production of cloned cattle in Japan.

Also in our laboratory, the study for the cloning and transgenesis in the goat and the rabbit has been carried out for several years, and a somatic cell-cloned goat was born in 2000. However, the goat died suddenly at day 16 after birth. Although the ectopic hematopoiesis was observed in some organs in the goat by the pathological examination, the cause of this abnormality could not be clarified. The somatic cell-cloned rabbit has not succeeded yet. By the aggregation of blastomares of a rabbit SCNT embryos with a normal embryo, it was succeeded to obtain the chimeric fetus and placenta, but not childbirth.

Reference

1. Kato Y., Tani T., Sotomaru Y., Kurokawa K., Kato J., Doguchi, H., Yasue H., Tsunoda Y. (1998) *Science* 282, 2095-2098

COMPARISON OF MICROINJECTION (PIEZO-ELECTRIC) AND CELL FUSION FOR NUCLEAR TRANSFER SUCCESS WITH DIFFERENT CELL TYPES IN CATTLE

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Cloning adult cattle by nuclear transfer is still an unpredictable and low efficiency procedure particularly if the end point is a live and viable offspring. Amongst the many variables that can determine success the source of nuclei, the procedure used for nuclear transfer and the activation of the reconstructed embryo are the most critical.

In this study we have looked at the results obtained using the two most common procedures for transferring nuclei to enucleated oocytes: cell fusion (CF) and piezo-electric

microinjection (PEM).
CF protocol (1) requires intact and viable cell of medium to large size to achieve membrane fusion and indirectly nucleus transfer. Microinjcetion of nuclei was developed for nuclear transfer in amphybia and more recently perfected (2; 3) with the aid of a piezo-electric manipulator. PEM requires a cell, possibly of small size, with a broken membrane or an isolated nucleus, not necessarily from a viable cell. For these reasons both techniques could find useful application in mammalian nuclear transfer.

The source of nuclei: granulosa cells and fibroblasts were grown to confluency to induce a quiescent state, lymphocytes were thawed immediately prior to use. Oocytes collected by dissection from ovaries of slaughtered donors (3) were matured in vitro for 16 h, decumulated and returned to maturation. As soon as polar bodies were extruded oocytes were enucleated. Embryos were reconstructed either with CF or PME by 21-23 h post maturation. For cell fusion one pulse of 1kVolt/cm for 30µsec was used, for PEM cell membrane was broken by repeated pipetting and transferred in a 12% PVP solution to facilitate injection. Manipulated oocytes were activated 1-4 h after reconstruction with ionomycin (5 µM for 6 min) and 6DMAP (1.9 mM for 5h) or cicloheximide (CHX, 10µg/ml for 5h). After activation embryos were cultured in microdrops of SOF with 16mg/ml FAF BSA, glutamine, EAA and NEAA. On day 7 (day 0: nuclear transfer) embryo development was evaluated and embryos were either transferred fresh or were frozen

Table. Development of oocytes reconstructed with different cell types by CF and PEM

Cell type	method (activation)	N. of constructs	N. fused or success. injected (%)	N. cleaved (%)	N. blastocysts D+7 (%)
lymphocytes	PEM (DMAP)	353	338 (95.6)[a]	289(85.5)[c]	54 (16.0)[f]
"	PEM (CHX)	234	224 (95.7)[a]	134 (59.8)[d]	24 (10.7)[g]
granulosa cells	CF (DMAP)	253	177 (70.0)[b]	164 (92.7)[c]	70 (39.5)[h]
"	PEM (DMAP)	273	250 (91.6)[a]	189 (75.6)[d]	46 (18.4)[f]
adult fibroblasts	CF (DMAP)	227	139 (61.2)[b]	123 (88.5)[c]	89 (64.0)[i]
"	CF (CHX)	192	117 (61.0)[b]	79 (67.5)[dc]	44 (37.6)[h]
"	PEM (CHX)	722	696 (96.4)[a]	459 (65.9)[c]	78 (11.2)[g]

Chi Square $p < 0.05$

Data are not presented for CF with lymphocytes because it was not technically possible. In general more embryos were successfully reconstructed with PEM than CF but a much higher number of oocytes reconstructed by CF developed to blastocyst at D+ 7. In addition in both systems more embryos were obtained after activation with 6DMAP than with CHX.

Forty-seven embryos obtained from the same ear fibroblast cell line with CF or PEM were transferred to recipients (1 embryo per recipient). Live offspring was obtained both for CF and PME. For CF (6DMAP) 10 pregnancies were established (50% pregnancy rate) with none to term; for CF (CHX), 3 pregnancies (21.4%) with 1 gone to term; with PEM (CHX) 7 pregnancies (54,8%) with 4 gone to term.
In conclusion, more embryos can be produced by CF than with PEM indicating a low tolerance of the bovine oocytes to microinjection (also demonstrated by the low efficiency of ICSI in this species), but the embryos obtained by PEM are viable in supporting the development of a normal offspring.

References

1. Campbell, K.H.S., McWhir, J., Ritchie, WA., and Wilmut, I. (1996) *Nature* 380, 64-66.
2. Wakayama, T., Perry, A.C.F., Zuccotti, M., Johnson, K.R. and Yanagimachi, R. (1998) *Nature* 394,

369-374.
3. Galli, C., Duchi, R., Moor, R.M., and Lazzari, G. (1999) *Cloning* 1, 161-170.

Oral Presentation 2

PATHOLOGY OF DISEASES IN CALVES CLONED BY NUCLEAR TRANSFER

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From April, 1999, 101 materials from calves were examined histopathologically. Among them, 22 calves were destroyed as scheduled, 42 calves were aborted or stillborn, 9 calves died just after birth, 5 died within 5 days, and 10 calves survived more than 6 days. The absence or decrease of thyroidal colloid in the thyroid were seen in 17 cases. Large fetuses of more than 50 kg of body weight were seen 12 cases. In 11 cases, the major lesions were seen in the placenta. Immunodeficiency was seen in 11 cases in which lymphatic tissues were hypoplastic. Abnormalities were seen in the muscles in 6 cases, in the umbilical cord in 5 cases and in the hip joint in 3 cases. Alveolar proteinosis was seen in 2 cases.

Among 9 calves which died just after birth, 5 calves were more than 50 kg of body weight, one was 40-50 kg, another one was 30-40 and other two was unknown.

Among 12 calves of more than 50 kg of body weight, 3 cases were aborted or stillborn. 5 calves died just after birth, as mentioned above, 2 cases died within 1 day, 1 case died at 3 days and other died at 22 days. Among the major lesions seen in these calves, hyperplasia of connective tissues in the liver were observed in 9 cases. Absence of thyroidal colloid was seen in 2 cases, irregular size of thyroidal follicles was seen in 2 cases. Immunodeficiency was seen in 2 cases.

Among 16 calves with thyroidal abnormalities, absence of thyroidal colloid was seen in 12 cases, irregular size of thyroidal follicles was seen in 4 cases, and goiter was seen in 1 case. 12 calves were aborted or stillborn and their age was from 146 to 265 of fetal days. 3 calves of more than 50 kg of body weight died just after birth and another calf died at 0 day and showed inhalation of amniotic fluid and muscular abnormality.

Among 11 calves with immunodeficiency, one calf was aborted, 6 calves died within 10 days, and 2 calves died 10 to 30 days and 2 calves survived more than one month. The transferred nucleus of 6 cases was that of fibroblast.

Among 11 cases with placental abnormalities, 4 twins (8 calves) were included. The transferred nucleus was that of cumulus cell in 7 calves and that of fibroblast in 4 cases. Calcium deposition was seen in 5 cases and fibrosis was seen in one case.

IMPLANTATION AND PLACENTAL DEVELOPMENT IN SOMATIC CELL CLONE RECIPIENT COWS

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The success of somatic cloned animal production has been reported in various domesticated species including cattle, goat, sheep, and pig, albeit, with a high rate of pregnancy failure. Significant early embryonic loss is reported, although, subsequent fetal death also occurs throughout the gestation period. There could be a number of plausible causes, as the entire interaction during normal pregnancy still remains vague. The low cloning yield could possibly arise from either an abnormal and/or poorly developed placenta. In the bovine, fetal trophoblast attaches to a specific area of the endometrium called caruncle, and certain specialised cells fuse with the epithelial cells in this region. The complex of both maternal and fetal tissue is called a placentome. A less number of placentomes were found in somatic cell nuclear transferred (SNT) recipient cows at days 30, 60 and 90 of gestation, particularly, at day 30, suggesting a retardation of fetal growth in SNT recipients. It is well known that endometrial remodeling is essential for implantation and placental formation; in addition, maternal-fetal dialogue may be one of crucial factors for the successful outcome of gestation. SNT recipients not only had less number of chorionic villi but also had poorly developed caruncles. However, over day 90 of gestation the latter progressed to typical in SNT recipients, but fetal death and/or stillbirth was the likely outcome. Macroscopical examination revealed atypical development of placentome in terms of shape and size. Many factors such as steroid hormones, placental specific proteins, cytokines may participate in the regulation of implantation and placental formation depending on humoral dispersion and genetic background. Histological disarrangement of chorionic villi and caruncle septum was found in SNT recipients. Of particular interest was that the gene and protein expression in placentomes showed a slight difference between SNT recipients and artificially inseminated cows, especially placental lactogen (PL) and pregnancy-associated glycoprotein (PAG). Trophoblastic cells, especially binucleate cells, produce both PL and PAG. The binucleate cells play a pivotal role for fusion and angiogenesis in bovine placentome. These data strongly supports a delay in trophoblastic development at day 30 of gestation. In summary, these findings suggest that placental specific proteins, including PLs, PAGs, are amongst the potential indicators of placental function in SNT recipient cows. This study was supported by grants for Organized Research Combination System, Science Technology Agency, Bio-oriented Research Advancement Institute and MAFF, Japan.

ENDOCRINE CHARACTERISTICS OF CLONED CALVES

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Birth of oversized calves or perinatal abnormalites such as lack of spontaneous parturition, prolonged gestation and increased perinatal mortality are frequently observed in association with the pregnancies of cloned calves. These problems limit application of cloning technology in the field of livestock production. In the course of normal pregnancy, there is an increase in the circulating glucocorticoid concentration in the fetus towards term (Fowden et al., 1998). This prepartum cortisol surge promotes maturational changes in several fetal organs during late gestation in preparation for extra-uterine life and decreases fetal growth rate during this period,

and it is considered to trigger the initiation of parturition. The insulin-like growth factor (IGF) system is developmentally regulated and is essential for normal growth. Expression of IGF genes is directly regulated by cortisol in late pregnancy, in which prenatal cortisol surge initiates the transition from fetal (paracrine/autocrine) to adult mode of IGF action (endocrine). I suspected that any disruption of normal maturational changes in endocrine function such as IGF system and hypothalamo-pituitary-adrenal axis is responsible, at least in part, for overgrowth or perinatal abnormalities associated with the pregnancies of cloned calves. To address this issue, endocrine status were compared between cloned and normal calves at birth.

Blood sample was obtained by venipuncture immediately after delivery from 13 cloned Japanese Black calves and 7 contemporary control calves. Plasma hormone concentrations were measured by radioimmunoassays and IGF binding proteins (IGFBPs) were semiquantified by Western ligand blotting. Five cloned calves were delivered by caesarian section and 8 cloned calves by vaginal delivery, while all of the control calves were delivered by spontaneous vaginal delivery. One of cloned calves died at birth and 4 cloned calves died within 1 week of age. Birth weights of cloned calves ranged from 25 to 56 kg and averaged 42 kg. Average birth weight of control calves was 31kg, which was lighter ($P < 0.01$) than that of cloned animals. Plasma IGF-I concentrations were lower ($P < 0.01$) in cloned calves than in controls. Cloned calves had extremely lower ($P < 0.001$) plasma cortisol concentrations compared to control animals (34 ± 11 *vs* 111 ± 6 ng/ml). Plasma levels of adrenocorticotropic hormone were not different between cloned and control calves. Striking difference was observed in plasma IGFBP profile. Cloned calves showed greater relative abundance of IGFBP-1 ($P < 0.05$) and IGFBP–2 ($P < 0.001$) compared with controls, although there was no difference in the relative abundance of IGFBP-3 and IGFBP-4 between the groups.

Observed differences in endocrine characteristics between neonatal cloned and control calves suggest that the lack of sufficient prenatal cortisol surge in cloned calves fail to switch to adult mode of IGF action during late gestation. Inappropriate developmental changes in endocrine system may be partly responsible for the birth of oversized cloned calves or perinatal abnormalities associated with the production of cloned animals.

Reference

1. Fowden, A. L., Li, J., and Forhead, A. J. (1998) *Proceedings of the Nutrition Society* 57, 113-122.

Oral Presentation 3

EPIGENETIC CHANGES INDUCED BY EMBRYO TECHNOLOGIES; PHENOTYPIC IMPLICATIONS FOR PRENATAL LOSS AND LARGE OFFSPRING SYNDROME.

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Cattle and sheep embryo development is perturbed during embryo technologies such as in vitro embryo culture and nuclear transfer. Resultant offspring can be oversized and have a wide range of organ, physiological, placental and skeletal defects, often resulting in prenatal or perinatal loss. We are investigating the possibility that these defects are caused by epigenetic changes induced during the preimplantation stages, focusing on the process of DNA methylation. In

order to examine whether gross, genome-wide aberrations occur we have developed techniques for whole-mount immunostaining of sheep embryos with anti methylcytosine antibodies, as well as single embryo, methylation sensitive PCR methods for analysis of specific components of the genome such as repetitive elements and single gene loci. We will discuss our findings as a possible cause of early embryonic loss.

In terms of specific fetal defects we have already linked the oversized phenotype with an epigenetic change in one imprinted gene, Igf2r (1). However we are investigating other imprinted genes that may contribute to both the growth anomalies and also to other observed phenotypes. For expression analysis we are optimizing techniques that allow reproducible measurements of transcript levels of several imprinted genes in single embryos and will present our latest results. In addition we are using studies of parthenogenetic and diploid, in vivo-derived sheep embryos and fetuses to determine which genes are imprinted during early development of the sheep. This will assist in the choice of candidate genes for further study.

Reference

1. Young, L.E., Fernandes, K., McEvoy, T.G., Butterwith, S.C., Gutierrez, C.G., Carolan, C., Broadbent, P.J., Robinson, J.J., Wilmut, I., and Sinclair, K.D., (2001). *Nature Genetics* 27;153-154.

OBSTACLES TO CLONING ANIMALS: INCOMPLETE REPROGRAMMING

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Although cloning systems using somatic cells as donor nuclei have been successfully achieved in a variety of animals such as sheep, cattle, mice, goats and pigs, till now the developmental competence of NT embryos is very low in that far less than 1% of the reconstructed embryos with somatic nuclei give rise to live-born animals. Moreover, the nuclear transfer has showed high developmental failures including lower in vitro development, high abortion rate and increased perinatal death. To clarify these developmental failures, we first examined developmental potential of nuclear transfer (NT) embryos and also compared cell numbers of NT blastocysts with in vitro fertilized (IVF) embryos. Blastocyst formation is the first differentiation process during early embryonic development in mammals, yielding two different cell lineages such as the inner cell mass (ICM) and trophectoderm (TE). Generally, the ICM cells contribute to develop all embryonic tissues as well as a part of the extra-embryonic membranes, and the TE cells mainly do to form the fetal placenta. Both cell lineages are vital and essential for embryonic and fetal survival. We found that bovine NT blastocysts had a smaller mean number of TE cells than IVF embryos, although in vitro developmental rate of NT embryos to blatocyst stage was similar to that of IVF embryos. Secondly, we have hypothesized that the anomalies observed in cloning system probably is due to incomplete epigenetic reprogramming of donor DNA. During early embryonic development a genome-wide demethylation may be a prerequisite for the formation of pluripotent stem cells that are important for the later development. In this study aberrant methylation patterns were detected on various genomic regions of bovine NT embryos. The NT blastocysts closely resembled donor cells in the overall genomic methylation status, which was quite different from normal embryos produced in vitro or in vivo. Consequently, our findings suggest that the developmental failures of NT embryos may be due to the insufficient formation of blastocyst and/or the incomplete epigenetic reprogramming of donor genomic DNA.

Oral Presentation 4

RE-PROGRAMMING OF THE rRNA GENES DURING NUCLEAR TRANSFER IN CATTLE AND SWINE

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Fibrillo-granular ribosome synthesizing nucleoli are established in conjunction with the major genomic activation during the 3rd or 4th post fertilization cell cycle in swine (1) and cattle (2), respectively. It was our aim to assess the re-programming of the rRNA genes as visualized by nucleolar ultrastructure and protein allocation in bovine and porcine embryos re-constructed by nuclear transfer.

Re-constructed bovine embryos were produced by nuclear transfer from serum starved granulosa cells to enucleated oocytes matured in vitro and either incubated for 20 min in ^{3}H-uridine and processed for autoradiography and transmission electron microscopy (N=18) or processed for immunocytochemistry and confocal laser scanning microscopy (N=427). The embryos were labeled with antibodies against nucleolar proteins important for rRNA gene transcription (topoisomerase I, RNA polymerase I, upstream binding factor (UBF)), early rRNA processing (fibrillarin) or late rRNA processing (nucleolin and nucleophosmin). During the 1st cell cycle (1-cell embryos) no autoradiographic labeling was detected and nucleoli in the process of inactivation were observed. Labeling of RNA polymerase I, fibrillarin, UBF and nucleolin was localized to nuclear entities. During the 2nd cycle (2-cell embryos) autoradiographic labeling was also lacking and the embryos displayed varying forms of nucleolar inactivation. Only labeling of RNA polymerase I and fibrillarin persisted. During both the 3rd (4-cell embryos) and 4th (tentative 8-cell embryos) cycles some embryos displayed autoradiographic labeling and associated re-formation of fibrillo-granular nucleoli. Labeling of RNA polymerase I and fibrillarin persisted in some embryos but lacked in others, and labeling of nucleophosmin and nucleolin appeared. During the 5th cycle (tentative 16-cell embryos) all embryos displayed autoradiographic labeling and fibrillo-granular nucleoli. Almost all embryos showed labeling of all proteins except for UBF, which lacked in more than half of the embryos. In conclusion, in bovine nuclear transfer embryos the re-formation of fibrillo-granular nucleoli may be initiated prematurely during the 3rd cell cycle and the nucleolar allocation of RNA polymerase I and, in particular, UBF may be delayed or even lacking.

Porcine embryos were produced either by parthenogenetic activation or by nuclear transfer from serum starved fetal fibroblasts to enucleated oocytes matured in vivo or in vitro. Parthenogenetic (N=265) and nuclear transfer embryos (N=277) were labeled with antibodies against UBF or RNA polymerase I and processed for confocal laser scanning microscopy. Among both types of embryos, a considerable proportion of the blastomeres were anucleated or, in fewer cases, polynucleated. About half of the parthenogenetic embryos showed localization of UBF and RNA polymerase I to nuclear entities during the 1st and 2nd cell cycle. During the 3rd cycle, none of the proteins were localized, whereas during the 4th cycle some blastomeres showed localization of both proteins to nuclear entities. None of the proteins were localized to nuclear entities in nuclear transfer embryos. In conclusion, the nucleolar allocation of proteins is disturbed in porcine parthenogenetic and, in particular, nuclear transfer embryos.

References

1. Hyttel, P., Laurincik, J., Rosenkranz, C., Rath, D., Niemann, H., Ochs, R.L. & Schellander, K. (2000): Nucleolar proteins and ultrastructure in pre-implantation porcine embryos developed in vivo. Biol. Reprod., 63, 1848-1856.
2. Laurincik, J., Thomsen, P.D., Hay-Schmidt, A., Avery, B., Greve, T., Ochs, R.L. & Hyttel, P. (2000): Nucleolar proteins and nuclear ultrastructure in pre-implantation bovine embryos produced in vitro. Biol. R eprod., 62, 1024-1032.

CELLULAR AND MOLECULAR CHARACTERIZATION OF DONOR CELLS AND NUCLEAR TRANSFER DERIVED EMBRYOS

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We have examined whether the cell death induced by serum deprivation occurs through an apoptotic pathway in porcine primary fetal fibroblasts and found that the degree of high molecular weight DNA fragmentation or single strand breaks was dependent on serum concentration and time of exposure affecting approximately 40% of the cells after 5 days when no serum was in the medium. Bax and Bak, members of the apoptosis related Bcl-2 gene family were upregulated upon serum deprivation. However, no signs for the internucleosomal DNA-cleavage characteristic for apoptosis could be detected. The unconventional form of apoptosis which was observed, suggests that changes in chromatin constitution could be reversible by nuclear transfer and that serum deprivation could improve the success rate of somatic nuclear transfer.

In the bovine we have measured to what extent modifications of the nuclear transfer protocol affect mRNA expression patterns in reconstituted blastocysts. Fusion either before, or simultaneously with, activation generated nuclear transfer derived blastocysts, in which heat shock protein (Hsp) mRNA could not be detected. However, Hsp mRNA was present in in vitro produced (IVP) control embryos. The use of either G_0 or G_1 donor cells or donor cells from either passage 5/6 or 8 significantly reduced the relative amounts of transcripts for DNA-methyltransferase (DNMT-1) and increased the relative abundance for Mash-2 compared to the control embryos. In addition, transcripts for interferon tau were significantly elevated in NT derived blastocysts from G_1 donor cells over the IVP controls and those constructed with G_0 cells. These findings indicate profound alterations of expression patterns in NT-embryos with regard to genes involved in stress adaptation, trophoblastic function and DNA methylation.

In current experiments, fetal and adult bovine fibroblasts as donor cells resulted in similar cleavage and blastocyst rates (70% and 76.3%; 23.5% and 26.2%, respectively). From 13 transfers of reconstituted embryos using fetal donor cells, four pregnancies (31%) were established all of which resulted in live calves upon caesarean section. The calves had an average birthweight of 68.4 kg. Using adult fibroblasts, 16 reconstituted embryos were transferred to 16 recipients resulting in seven initial pregnancies (43.7%).Up to now, one live calf was born with a normal birthweight of 43 kg. Gene expression analyses revealed a significant difference between reconstituted embryos derived from fetal or adult donor cells at the blastocyst stage with an increased expression of XIST in morulae and blastocysts derived from adult donor cells. This elevated XIST expression was also apparent when reconstituted embryos from adult cells were compared with in vitro produced, in vivo generated and parthenogenetic embryos.

Collectively, these data show that the origin and treatment of donor cells can have significant effects on gene expression patterns in NT-reconstituted embryos. The hypothesis of a "relaxed chromatin constitution" induced by a short period of serum deprivation warrants further study.

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References

1. W.A. Kues, M. Anger, J.W. Carnwath, D. Paul, J. Motlik, and H. Niemann. (2000) *Biol Reprod* 62, 412-419.
2. C. Wrenzycki, D. Wells, D. Herrmann, A. Miller, J. Oliver, R. Tervit, and H. Niemann. (2001) *Biol Reprod* 65, 323-331.

REMODELING OF NUCLEI UPON TRANSFER INTO ENUCLEATED MATURED MAMMALIAN OOCYTES

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Viable offspring have already been obtained in different mamalian species by using nuclear transfer into enucleated matured oocytes. The efficiency of this technique is however still limited because of the occurrence of developmental arrest. Our results in cattle showed that late fetal and neonatal mortality increases when shifting from an embryonic, to a fetal and to an adult source of nuclei. This increase could be attributed to the long lasting effect of nuclear transfer on fetal development. In mouse, we evidenced the same occurrence of long lasting detrimental effects with different types of cells, including a source of pluripotent embryonic cells. In both species, a high rate of blastocysts could be obtained from the reconstituted embryos cultured in vitro, and independentely of the source of nuclei used, but these blastocysts often exhibited a retarded development and a lower ratio of the inner cell mass cells to the trophoblastic cells. This indicates that a deregulation of the development was already present at the time of the first differentiation in the embryos. Early cellular and molecular events that take place immediately upon or just after the procedure of nuclear transfer appear to be apparentely very different between these two species. The successful development of mouse cloned embryo needs a prolonged exposure of the chromatin in a condensed state to the recipient cytoplasm, whereas an efficient procedure used in bovine does not imply the premature chromatin condensation of the donor nucleus after introduction into the enucleated oocyte. Thus, these macromolecular events are not the only one which modulates the potential of recontructed embryos for later development. New hypothesis can be drawn from recent data which will be presented.

Oral Presentation 5

INITIATION OF OVARIAN TERATOCARCINOGENESIS IN STRAIN LT MICE: An UNANSWERED QUESTION ABOUT THE ESSENTIAL MOUSE MODEL FOR CELL TOTIPOTENCY

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Females of LT/Sv and related strains exhibit a high incidence of ovarian teratomas arising from parthenogenetically activated oocytes. This unique mouse model of teratocarcinogenesis has been utilized extensively in both developmental and genetic studies, yet it is still not well understood why LT oocytes spontaneously activate. These oocytes exhibit a delayed entry into anaphase-I and, therefore, remain in a prolonged metaphase-I (MI) stage during meiotic maturation. A late entry into anaphase-I and progression to metaphase-II (MII) eventually occurs, but is often followed by spontaneous activation and entry into interphase. As a result of activation at this specific stage of meiosis, the parthenogenetic embryos are diploid. In recent studies, evidence has begun to emerge suggesting that protein kinase C (PKC) participates in the regulatory mechanisms that delay entry into anaphase-I in LT oocytes. Loss of regulatory control over PKC activity during oocyte maturation disrupts the critical MI to MII transition leading to a precocious exit from meiosis. We review our current understanding of the events involved in the initial meiotic defects that promote teratoma formation in LT mice.

CLONING MICE AND EMBRYONIC STEM (ES) CELLS BY NUCLEAR TRANSFER

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The absence of a model organism has impeded progress in understanding mammalian cloning by nuclear transfer (nt). Although the mouse is the pre-eminent candidate model due to our grasp of its biology, the cloning of mice from adult somatic cells has proved elusive. We recently developed a new method to generate cloned mice based on piezo-actuated nuclear transfer. Mice have been cloned from cumulus cells, tail-derived cells (most cells were fibroblast), adult/fetus males and females, and extensively passaged embryonic stem (ES) cell lines. ES cells have now been derived from cloned embryos produced by nt from somatic cells (ntES cells); ntES cells show full capacity for differentiation, including gametogenesis, after chimera production by blastocyst injection. The rate of full term development in cloning from adult somatic cell of hybrid strains, over all is invariably low, with only approximately 2% of reconstructed oocytes developing to term. When inbred strains were used as nuclear donor, the success rate of cloning is extremely low (0-0.3%) except 129 strains. All cloned mice are associated with abnormal placentae, often dying perinatally of unknown causes with a strain-dependent frequency. However, clones surviving to adulthood exhibit normal fertility, and re-cloned mouse which repeated 6 times also had a healthy body. In attributing causes to cloning phenomena, distinctions should be made between technical limitations, nuclear “reprogramming”, somatic mutation, genomic imprinting and incompatible cell cycle effects as contributing factors.

References

1. Wakayama, T., Perry, A.C.F., Zuccotti.,M., Johnson, K.R., and Yanagimachi, R. (1998) *Nature* 394, 369-374.
2. Wakayama, T., and Yanagimachi, R. (1999) *Nat Genet* 22, 127-128
3. Wakayama, T., Rodriguez, I., Perry, A.C.F., Yanagimachi, R., and Mombearts P. (1999) *Proc Natl Acad Sci USA* 96, 14984-14989
4. Wakayama, T., Shinkai, Y., Tamashiro, K.L., Niida, H., Blanchard, D.C., Blanchard, R.J., Ogura, A.,

Tanemura, K., Tachibana, M., Perry, A.C., Colgan, D.F., Mombaerts, P., and Yanagimachi, R. (2000) *Nature* 407, 318-319.
5. Wakayama, T., Tateno, H., Mombaerts, P., and Yanagimachi R. (2000) *Nat Genet* 24, 108-109
6. Wakayama, T., and Yanagimachi R. (2001) *Mol Reprod Dev* 58, 376-383
7. Wakayama, T., Tabar, V., Rodriguez, I., Perry, A.C., Studer, L., and Mombaerts, P. (2001) *Science* 292, 740-743
8. Wakayama, T., and Yanagimachi R. (2001) *J Reprod Fertil.* 122, 49-60.

CLONED MICE FROM GENETARGETED EMBRYONIC STEM CELLS

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Cloned individuals from differentiated somatic cells are now available in mammals. Studies so far revealed the capacity of unfertilized oocytes for reprogramming the DNA of differentiated cells, although the mechanism by which this work is not yet well understood. We have preciously developed a technique for cloning mice using embryonic blastomeres arrested at metaphase by serial nuclear transfer system. In this system a nucleus is first transferred to unfertilized oocyte, and then the resultant pronucleus is again transferred into enucleated fertilized eggs. Here we examined whether fertilized cytoplasm is effective to produce clone mice using fetal fibroblasts and embryonic stem cells as donor cells. In the case of using fetal fibroblast as donor cells, development of constructed oocytes to the blastocyst stage was similar in serial and single nuclear transfer (31% vs 37%). Five live pups (2%) were produced by serial nuclear transfer, and two of them grew up normally and matured. While the production of clones from the single transfer technique failed to yield any healthy offspring. When ES cells were used as donor cells, the proportions of blastocysts and implantation sites were significantly higher in the oocytes produced by serial nuclear transfer compared with those produced by single nuclear transfer. The proportion of pups was similar in both groups (3.2% vs 3.7%), however, postnatal death was observed only in the pups produced by single nuclear transfer. All 10 pups have been growing normally in serial nuclear transfer, except one case which the foster mother refused nursing. These results suggest that cytoplasm from fertilized eggs contains activities for supporting development of cloned embryos to term, although further precise experiments required to clarify nature of the activity.

References

1. Wilmut, I., Schnieke, AE., McWhir, J., Kind, AJ., and Campbell, KHS. (1997) *Nature* 385, 810-813.
2. Wakayama, T., Perry, AC., Zuccotti, M., Johnson, KR., and Yanagimachi R. (1998) *Nature* 394, 369-74.
3. Kwon, OY., and Kono, T. (1996) *Proc Natl Acad Sci USA* 93, 13010-13013.
4. Ono, Y., Shimozawa, N., Ito, M., and Kono, T. (2001) *Biol Reprod* 64, 44-50.

Oral Presentation 6

PLURIPOTENCY OF BOVINE EMBRYONIC CELL LINE DERIVED FROM PRECOMPACTING EMBRYOS

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We report herein the establishment of three bovine pluripotent embryonic cell lines derived from 8-16 cell pre-compacting embryos. Two cell lines were cultured ten passages and underwent spontaneous differentiation. One cell line (Z2) has been cultured continuously for over three years and has remained undifferentiated. These cells express cell surface markers that have been used routinely to characterize embryonic stem (ES) and embryonic germ (EG) cells in other species such as stage-specific embryonic antigens SSEA-1, -3, and –4 and c-Kit receptor. In the absence of a feeder layer these cells differentiated into a variety of cell types and formed embryoid bodies (EBs). When cultured for an extended period of time, EBs differentiated into derivatives of three embryonic germ layers, mesoderm, ectoderm and endoderm, that were characterized by detection of specific cell surface markers. Our results indicate that the Z2 cell line is pluripotent and resembles an embryonic stem cell line. To our knowledge this is the first bovine embryonic cell line that has remained pluripotent in culture for more than 150 passages.

DEVELOPMENTAL STUDY AND MANIPULATION OF MAMMALIAN GERM CELLS AND EMBRYONIC STEM CELLS

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Establishment of human pluripotent stem cell lines, which include embryonic stem (ES) cell lines derived from the inner cell mass of blastocysts and embryonic germ (EG) cell lines derived from primordial germ cells (PGCs), has pointed out their great potential for utilization in the regenerative medicine. For many years, mouse pluripotent stem cell lines have been important tools to investigate cell differentiation and to carry out gene targeting for analysis of gene function in mice. Gene targeting procedures depend on availability of very stable ES cell lines, which are so far limited to 129 and few other strains of the laboratory mouse. We have established many ES cell lines from several inbred mouse strains including C57BL/6, MSM and SWN. The latter two strains had been derived from wild mice in Japan and Korea, respectively, and they have wild-type behavioral phenotypes that are different from ordinary laboratory mouse strains, which frequently exhibit abnormal behavior. Thus, such ES cell lines may be useful for the investigation of brain function in gene-targeted mouse models. These ES cell lines produced chimeric mice that exhibited the germ line transmission.

Also, we have been studying development of mouse germ cells in culture. PGCs isolated from fetuses showed limited proliferation and autonomous growth arrest in culture. A combination of proliferation signals caused appearance of the EG cell colonies. Usage of antibodies against meiosis-specific proteins showed that PGCs before and after arriving at female gonads autonomously enter into meiosis when cultured as dispersed single cells on a feeder cell layer (1). Even the male PGCs showed transition into meiosis in the same conditions if they had been isolated before 12 days post coitum from the fetal testis. Using such culture system, we found that signals from LIF and its receptor gp130 caused strong inhibition of the meiotic entry by PGCs in culture. Thus, the LIF/gp130 signal has multiple and probably related effects of the maintenance of undifferentiated stem cells, growth/survival of PGCs and inhibition of entry into meiosis.

Recently, we have established several ES cell lines from blastocysts of the Cynomolgus

monkey (2). They can be maintained in culture as stem cell colonies expressing several stem cell markers. Also, they produced teratomas containing many types of tissues when transplanted into immuno-deficient SCID mice, thus indicating their pluripotency. Such primate ES cell lines are valuable tools in preclinical research for production of various functional cells and cell transplantation into disease-model monkeys.

References

1. Chuma, S., and Nakatsuji, N. (2001) *Dev Biol* 229, 468-479.
2. Suemori, H. et al. (2001) Submitted for publication.

IN VITRO DIFFERENTIATION OF EMBRYONIC STEM CELLS INTO GERM CELLS

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Embryonic stem cells can differentiate into all cell lineages when they are introduced into host blastocysts, and can generate various somatic cells lineage in vitro. Since ES cells can contribute to the germline of a host embryo, theoretically they should also be able to make germ cells in vitro. We previously showed that the mouse vasa homolog (Mvh) gene is specifically expressed in germ cells after their colonization of the gonad. Mvh provides a specific marker to distinguish cells of the germ cell lineage. To visualize generation of germ cells in vitro, we established ES cell lines, in which GFP or lacZ were expressed from the endogenous Mvh locus. During ES cell differentiation into embryoid bodies Mvh-positive germ cells appeared. Appearance of germ cells can be stimulated by direct induction by Bone Morphogenetic Protein-4 (BMP4). Transplantation experiments showed that ES cell-derived germ cells contribute to the germ cell compartment of reconstituted testicular tubules, demonstrating their developmental potency as germ cells in vivo. This system provides the first in vitro differentiation method to study germ cell formation and provides a new experimental paradigm for defining factors influencing fertility in mammals.

Oral Presentation 7

THE IMPROVEMENT OF FOLLICULAR DEVELOPMENT AND OVULATION BY PROMOTING FOLLICULAR ANGIOGENESIS IN *rdw* AND NORMAL RATS

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The present study was performed to investigate (i) the follicular development and ovulation by promoting follicular angiogenesis in immature stage and (ii) total egg production in an individual rat induced to ovulate up to three consecutive times at intervals of about 30 days in infertile hypothyroid *rdw* rats and normal counterparts. Animals were treated as described previously with or without gonadotrophins (10 IU eCG and hCG) and/or thyroxine (T4, 10 μg/100 g body weight) (1) in immature stage, and with T4 subsequently in drinking water at a

final concentration of 20 µg per 100 g body weight as described previously (2). Then, 30 iu eCG and 30 iu hCG were given on the day of metestrus and proestrus, respectively, at all subsequent treatments. The follicular development was examined by haematoxylin and eosin staining (3). Follicular angiogenesis was examined by light, scanning and transmission electron microscopy. mRNA expression of angiogenic factors was also analyzed by RT-PCR. Our results showed that, in immature *rdw* rats, T4 treatment promoted the development of follicular microvasculature and mRNA expression of angiogenic factor such as vascular endothelial growth factor, and markedly increased the number of healthy antral follicles 101-400 or larger than 500 µm in the absence or presence of eCG, respectively. This led to significantly more eggs (85±5) ovulated in the first gonadotrophin treatmentin in those treated with T4 and gonadotrophins compared with those treated with gonadotrophins alone (1-5±1-2). The total number of eggs collected in the first three treatments in *rdw* rats treated with T4 and gonadotrophins was significantly more than that in normal counterparts treated with gonadotrophis alone ($P<0.05$). T4 treatment also increased the total number of eggs ovulated in first three treatments in normal rats compared to those in normal treated with gonadotrophins alone. These results suggested that the improvement of follicular development and ovulation by improving follicular microvasculature might be important in assisted reproductive technologies such as cloning. This work was supported by grants from the Program for Promotion of Basic Research Activities for Innovative Biosciences.

References

1. Jiang, J.Y., Miyoshi, K., Umezu, M., and Sato, E. (1999) *J Reprod Fertil* 116,19-24.
2. Jiang, J.Y., Umezu, M., and Sato, E. (2000) *Biol Reprod* 63,1637-1641.
3. Jiang, J.Y., Umezu, M., and Sato, E. (2000) *J Reprod Fertil* 119,193-199.

IN VITRO GROWTH AND DEVELOPMENT OF BOVINE PREANTRAL FOLLICLES

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Cow contains thousands of immature oocytes in the preantral follicles. A large number of the immature oocytes are potentially useful for an application of embryo transfer, transgenesis, conservation of endangered species, and oocyte bank of genetic materials, In the first part, we investigated to determine an efficient isolation method of bovine early preantral follicles (diameter in 30 µm to 70 µm) and develop a culture system for sustaining a long-term survival and growth of the follicles (1). The mean number (157) of intact follicles per ovary isolated by a mechanical method with a grating device was significantly greater than that (26) of follicles isolated by collagenase treatment. After 30 days in culture, rates of viable follicles in coculture with bovine ovary mesenchymal cells (BOM) and fetal bovine skin fibroblasts (FBF) were significantly greater than those of follicles in the non-coculture or in coculture with bovine granulosa cells (BGC). In the second part, we studied the effects of insulin growth factor families (insulin, IGF-I, IGF-II) and FSH on the growth and development of late preantral follicles (mean diameter 218 ± 12.4 µm). The follicles were microsurgically dissected from bovine ovaries and the follicles embedded in 0.15% type I collagen gels were cultured in a serum-free medium. When the follicles were cultured in the control medium, follicle diameters were gradually decreased with no antrum formation and oocyte diameters were unaltered after 9 days of culture. Insulin, IGF-I or FSH individually increased the follicle and oocyte growth, and antrum formation. While insulin enhanced estradiol (E_2) production, FSH did not. E_2 production by insulin was further stimulated in the presence of FSH. Gene

expression of insulin and IGF-I receptors were dominant in both isolated and culture follicles than IGF-II receptor. The successful development and growth of bovine preantral follicles in vitro would take advantage not only for understanding the complex mechanisms of folliculogenesis, but also for producing a large number of animal stocks with high genetic values.

Reference

1. Itoh, T., and Hoshi H. (2000) *In Vitro Cell Dev Biol* 36, 235-240.

CRYOPRESERVATION OF MAMMALIAN OOCYTES

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For long-term storage of cells alive, they must be preserved at temperatures below the glass transition temperature, which normally requires storage in liquid nitrogen. During the processing and cooling of cells, and during recovery of stored cells in a physiological solution, the cells are at risk of injury by various factors, including chilling injury, the toxicity of cryoprotectants, injury from concentrated salts, physical injury by extracellular ice, fracture damage, formation and growth of intracellular ice, osmotic swelling, and osmotic shrinkage (1). To find the optimal procedure for cryopreservation of each type of cell, it is important to know the mechanism by which the cells are liable to be injured. It is known that oocytes are more sensitive to cryopreservation than embryos (2). So, oocytes are more sensitive to injuries by some of the factors. Using mouse oocytes at the metaphase II stage, cryobiological characteristics of oocytes were examined. The permeability of the oocytes to permeating cryoprotectants, such as, ethylene glycol and glycerol, was lower than that of 8 cell-morula stage embryos. However, it was not different from the permeability of 1-cell embryos. On the other hand, oocytes were much less resistant to osmotic swelling than 1-cell embryos (3). Therefore, for successful cryopreservation of oocytes, it is important to load cryoprotectant in steps before cooling for preventing cryoprotectant toxicity, and to use a high concentration of sugar in the initial process of removing the cryoprotectant after warming for preventing osmotic over-swelling. When mouse oocytes were vitrified in EFS40, an ethylene glycol-based vitrification solution, survival was greatly improved by pretreatment of oocytes with solutions containing lower concentrations of ethylene glycol, and diluting the oocytes with 1.5 M sucrose/PB1 medium after warming. However, since oocytes were also less resistant to osmotic shrinkage than 1-cell embryos (4), it would be preferable to reduce the concentration of the sugar once the initial swelling of oocytes was prevented. Another potential strategy for oocyte cryopreservation would be to increase the permeability of the cell membrane artificially. This may be possible by injecting cRNA of a certain type of aquaporins (5), a water channel that can transport not only water but also cryoprotectants, into oocytes. Actually, in mouse oocytes injected with cRNA of aquaporin 3, both water permeability and glycerol permeability increased significantly; these oocytes survived after vitrification in a solution based on glycerol, which scarcely permeates into intact oocytes, whereas none of oocytes injected with water survived after vitrification in the solution.

References

1. Kasai, M. (1996) *Anim. Reprod Sci* 42, 67-75.
2. Shaw, J.M., Oranratnachai, A., and Trounson, A.O. (2000) *Theriogenology* 53, 59-72.
3. Pedro, P.B., Zhu, S.E., Makino, N., Sakurai, T., Edashige, K., and Kasai, M. (1997) *Cryobiology* 35,

150-158.
4. Pedro, P.B., Sakurai, T., Edashige, K., and Kasai, M. (1997) *J Mamm Ova Res* 14, 66-71.
5. Edashige, K., Sakamoto, M., and Kasai, M. (2000) *Cryobiology* 40, 171-175.

Oral Presentation 8

MPF REGULATES AGING IN PORCINE OOCYTES MATURED *IN VITRO*

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Control of oocyte aging during manipulation of matured oocytes should have advantages for the recent reproductive technologies such as cloning after nuclear transfer. We have shown that the enhanced activation ability in porcine aged oocytes had a close relationship with the decreasing activity of maturation/M-phase promoting factor (MPF) (1), and that porcine aged oocytes contained plenty of MPF but inactivated by phosphorylation of catalytic subunit $p34^{cdc2}$, so-cold pre-MPF and, therefore, had a low MPF activity (2). In the recent study (3), in order to know the relationship between MPF activity and the aging phenomena, we incubated porcine oocytes with vanadate and caffeine, which affected the phosphorylation status and the activity of MPF. Subsequently oocyte activation ability and fragmentation were examined. Incubation of 48 h-matured oocytes with 500 μM vanadate for 1 h increased the phosphorylation of $p34^{cdc2}$ and decreased histone H1 kinase (H1k) activity is a similar way to aged oocytes. Their parthenogenetic activation and fragmentation rates after calcium ionophore A23187 treatment were significantly increased comparing to those of the control oocytes. On the other hand, treatment of aged oocytes (60 h-maturation) with 5 mM caffeine for 10 h decreased the level of the phosphorylation of $p34^{cdc2}$ and elevated H1k activity. These oocytes revealed significantly lower parthenogenetic activation rate and lower percentage of fragmentation than those of aged mature oocytes without treatment (70 h-maturation). The results suggest that MPF activity is a key mechanism of oocyte aging and control of MPF activity through phosphorylation of $p34^{cdc2}$ with these chemicals may enable manipulation of oocytes aging *in vitro*.

References

1. Kikuchi, K., Izaike, Y., Noguchi, J., Furukawa, T., Daen, F.P., Naito, K., and Toyoda, Y. (1995) *J Reprod Fertil* 105, 325-330.
2. Kikuchi, K., Naito, K., Noguchi, J., Shimada, A., Kaneko, H., Yamashita, M., Tojo, H., and Toyoda, Y. (1999) *Zygote* 7:173-179.
3. Kikuchi, K., Naito, K., Noguchi, J., Shimada, A., Kaneko, H., Yamashita, M., Aoki, F., Tojo, H., and Toyoda, Y. (2000) *Biol Reprod* 63, 715-722.

THE EFFECT OF ACTIVATION OF MAMMALIAN OOCYTES AND CELL CYCLE SYNCHRONIZATION OF DONOR NUCLEI ON THE DEVELOPMENTAL POTENTIAL AFTER NUCLEAR TRANSFER

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Activation of bovine oocytes by experimental procedures that closely mimic normal fertilization and allow to obtain haploid oocytes is essential both for intracytoplasmic sperm injection and for nuclear transfer. Therefore, with the goal of producing haploid activated oocytes, we evaluated whether butyrolactone I and bohemine, either alone or in combination with ionomycin, are able to activate young matured mammalian oocytes. Furthermore, the effect on the patterns of DNA synthesis after pronuclear formation as well as changes in histone H1 kinase and MAP kinase activities during the process of activation were studied. Our results with bohemine show that the specific inhibition of CDKs in metaphase II bovine oocytes induces parthenogenetic activation in a dose dependent manner (25, 50 and 100 µM, respectively), either alone (3%, 30% and 50%) or in combination with ionomycin (30%, 70% and 87.5%). A haploid pronucleus was observed (97%) when Ca^{2+} influx was stimulated in the presence of bohemine, although pronuclear formation without meiotic progression was observed when bohemine was used alone. Bohemine-activated oocytes start to synthesize DNA in 6-7 hours pos-activation (hpa) and a high synchrony in the S-phase was registered with more than 85% of parthenotes actively synthesizing DNA 8 hpa. A drop in histone H1 kinase activity was observed in bohemine- and butyrolactone I-activated oocytes. The activity of MBP kinase decreased later than histone H1 kinase and even 4 h after inomycin-bohemine treatment at least half of this activity was still detectable. Then, the MBP kinase activity decreased and the lowest level could be seen 6-8 hpa. The present data indicate that inhibitors of cdk kinases (butyrolactone I, bohemine) are able either alone or in combination Ca^{2+} ionophore activate the mouse, pig and cattle metaphase II oocytes. Since the activation allows the second polar body expulsion and formation of the haploid pronucleus in which the DNA sythesisi is synchronously initiated, inhibitors of cdk kinases can be successfully included in nuclear transfer techniques.

M-phase synchronized bovine blastomeres were used to study the effect of nuclear-cytoplasmic co-ordination on the developmental potential after nuclear transfer (NT). The capacity of nocodazole and benomyl to reversibly synchronize blastomeres of different developmental stage embryos in the M-phase of the cell cycle was evaluated. Nocodazole reversibly arrested bovine embryos at the studied stages and induced high rates of M-phases in morulae and compact morulae. In contrast, benomyl was less efficient than nocodazole to synchronize in M-phase. Premature chromatin condensation prevaled in the first hour post-fusion (hpf). The reformation of a metaphase plate (1-3 hpf) which acquired an organized structure over the time (3-7 hpf) was subsequently observed. Anaphase-telophase structures were predominant at 4-9 hpf. About 50% of embryos, activated at 3-4 and 6-7 hpf, extruded a polar body-like structure 5 h after activation. This was not observed in embryos activated immediately after fusion. Significantly lower activation rate was observed in oocytes activated 3-4 hpf compared to those activated 6-7 hpf. However, the ability to undergo first cleavage was significantly lower in the later. Reconstructed embryos activated immediately after fusion showed no differences in the rate of activation compared to those activated 6-7 hpf, while cleavage rate was higher. DNA synthesis was observed in a significantly higher rate in embryos activated immediately after fusion than those activated 3-4 hpf with a polar body-like structure. These data show that M-phase donor cells cannot be properly remodeled after NT in cattle to trigger normal embryo development. Our observations of chromatin structures together with DNA synthesis suggest that the failure in the development may be due to an improper chromatin remodeling of mitotic nuclei after NT, which may result in chromosomal abnormalities incompatible with normal embryo development.

References

1. Alberio, R., Kubelka, M., Zakhartchenko, V., Hajdúch, M., Wolf, E., and Motlík, J. (2000) *Mol Reprod Dev* 55, 422-432.
2. Alberio, R., Motlík, J., Stojkovic, M., Wolf, E., and Zakhartchenko, (2000) *Mol Reprod Dev* 57, 37-47.
3. Kubelka, M., Motlík, J., Schultz, R.M., and Pavlok, A. (2000) *Biol Reprod*, 62, 292-302.

REGULATION OF SPERM-INDUCED CALCIUM OSCILLATIONS IN MAMMALIAN EGGS AT FERTILIZATION

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Initiation of embryonic development of the egg, including nuclear-transplanted eggs, requires transient increases in the intracellular free calcium concentration (1-2). At fertilization in all mammalian species studied, it is a universal phenomenon that the sperm activates the eggs by inducing a series of calcium oscillations, which can last for several hours before pronuclear formation. There is evidence showing that these oscillations are triggered by as perm-derived protein factor which diffuses into egg cytoplasm after gamete membrane fusion. So far the identity of the protein factor and its precise mechanism of action is still a mystery. In this report, we describe our studies on the identification of the protein factor and its possible mechanism of initiating calcium release from the intracellular store in the mammalian eggs. We show that the sperm protein factor appears to be sperm specific and its calcium oscillation-inducing activity in mammalian eggs is not species specific in vertebrates. This factor induces calcium release from the intracellular store though InsP3 receptor-mediated mechanisms, and is functional only when microinjected into egg cytosol. The sperm factor can induce calcium oscillations in metaphase eggs, maturing oocytes, parthenogenetically activated eggs but not in the zygotes. We present evidence that the sperm factor induced calcium oscillations are mediated by maternal machinery that functions only once in mammalian eggs. This machinery is inactivated by a sperm-derived protein factor(s) but not parthenogenesis activation. In conclusion, our studies demonstrate that the orderly sequence of calcium oscillations in mammalian eggs at fertilization depends upon two essential players: a perm-derived protein factor which serves as the physiological trigger for inducing calcium release, and a maternal machinery that determines whether the oscillations can persist.

References

1. Sun, F.Z., and Moor, R.M. (1995) *Current Topics in Dev Biol* 30, 147-176.
2. Tang, T.S., Dong, J.B., Huang, X.Y., and Sun, F.Z. (2000) *Development* 127, 1141-1150.

SPINDLE OBSERVATION IN LIVING MAMMALIAN OOCYTES WITH THE POLARIZATION MICROSCOPE AND ITS PRACTICAL USE

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In unfertilized metaphase II oocytes, the meiotic spindle is crucial for normal chromosome alignment and separation of maternal chromosomes during meiosis. Stabilization of spindles is important for normal meiosis during in vitro manipulation of oocytes, such as in vitro fertilization, intracytoplasmic sperm injection (ICSI) and nuclear transfer. Conventional methods to image the meiotic spindles rely on fixation and transmission electron microscope or immunofluorescence staining and fluorescence microscope, so they provide limited value to studies of spindle dynamics. We used a new orientation-independent polarized light microscope, the LC polscope (1), to examine the birefringent spindles in living mammalian oocytes and found that spindles could be imaged with the polscope in living oocytes in all mammals so far examined, including hamster (2, 3), mouse (3, 4), cattle (3), human (5) and rat. The first polar body did not accurately predict spindle location in most metaphase II oocytes (2, 5). ICSI could be performed after oocytes were rotated to place spindles at 90 degree relative to injection needle. This technology overcomes traditional methods by rotating the first polar body at 90 degree relative to injection needle, which may disrupt spindles during injection in some oocytes and hence induce abnormal fertilization. The studies in the human indicated that after ICSI, higher fertilization and embryonic developmental rates could be achieved in oocytes with than without spindles, indicating that ICSI by monitoring spindle position is safer and/or the presence of spindles in oocytes can predict oocyte's quality. Spindles in human oocytes are extremely sensitive to slight changes, not only in low temperature, but also in high temperature. Maintenance of temperature at 37C is crucial for normal spindle function. It seems that sensitivity of spindles to fluctuation in the temperature is species-specific. Spindles in mouse oocytes are stable even at room temperature although meiosis stops at room temperature. When a rigorous thermal control produced by a novel heating system was used, it stabilized spindles and increased the fertilization and pregnancy rates achieved after ICSI in human oocytes. As chromosomes are usually associated with spindle fibers, the position of chromosomes could be indirectly located by imaging spindles. Removing spindles under polscope can achieve an enucleation efficiency rate of 100% in mouse oocytes. Also enucleation with polscope overcomes the disadvantages by traditional methods, such as blind enucleation or fluorescence staining of chromosomes and then exposure of oocytes to fluorescence microscope. The polscope imaging of spindles also is useful for the examination of spindle dynamics or for study of the effect of environmental changes on spindles as spindles can be observed continuously over time in their living state.

References

1. Oldenbourg, R. (1999) *Methods Cell Biol* 61, 175-208.
2. Wang W.H., Gao, S.R., Meng, L., and Keefe, D.L. (1999) *Biol Reprod Suppl* 60, 180.
3. Silva, C.P., Kommineni, K., Oldenbourg, R., and Keefe, D.L. (1999) *Fertil Steril* 71, 719-721.
4. Lui, L., Oldenbourg, R., Trimarchi, J.R., and Keefe, D.L. (2000) *Nat Biotech* 18, 223-225.
5. Wang, W.H., Meng, L., Hackett, R.J., and Keefe, D.L. (2000) *Biol Reprod Suppl* 62, 191.
6. Wang, W.H. , Meng, L., Hackett, R.J., Oldenbourg, R., and Keefe, D.L. (2001) *Fertil Steril* 75, 348-353.

Oral Presentation 9

CLONING IN PIGS

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Many live births of cloned animals have been achieved in sheep, cattle and mice by somatic cell transfer. In pigs, nuclear transfer had been less successful for a long time; only a single piglet was reported after electric fusion of the blastomere of four-cell embryo with an enucleated oocyte. The application of somatic cell nuclear transfer to animal breeding is potentially important. In addition, pig cloning combined with genetic modification is expected to apply to xenotransplantation because the size of pig organs are considered most likely compatible with humans. PPL Therapeutics announced the first press release about successful cloning of pigs by somatic cell transfer. After their announcement, three scientific papers (1,2,3) including our successful work were published in succession.

These three papers demonstrate the different procedures for pig cloning. The method of nuclear transfer can be classified into two types; typical electrofusion and piezo-actuated microinjection method. Microinjection can separate into two steps the introduction of cell nuclei into cytoplasm and activation of oocyte development. On the contrary, electrofusion tends to cause both fusion of nucleus to the oocyte and activation at the same time. If exposure of the transferred nucleus to maturation promoting factor in the cytoplasm of enucleated oocyte is critical for genome reprogramming, microinjection is suitable for nuclear transfer. Actually, we showed pigs could be cloned by microinjection of somatic cell nuclei into enucleated oocytes.

Cloned animals frequently die before and soon after birth. Increased placental and birth weight are also observed in many cases. However, there seems to be no report about abnormalities in cloned pigs at present. Further study is necessary whether cloned pigs display similar abnormalities or not.

References

1. Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T., Hanada, H, and Perry, A.C.F. (2000) *Science* 289, 1189-1190.
2. Polejaeva, I.A., Chen, S.H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D.L., Colman, A., and Campbell, K.H.S. (2000) *Nature* 407, 86-90.
3. Betthauser, J., Forsberg, E., Augenstein, M., Childs, L., Eilertsen, K., Enos, J., Forsythe, T., Golueke, P., Jurgella, G., Koppang, R., Lesmeister, T., Mallon. K., Mell, G., Misica, P., Pace, M., Pfister-Genskow, M., Strelchenko, N., Voelker, G., Watt, S., Thompson, S., and Bishop, M. (2000) *Nat. Biotechnol*, 18, 1055-1059.

SOMATIC CELL NUCLEAR TRANSFER IN SHEEP AND PIG: CHALLENGES AND RECENT ADVANCES IN THE ROSLIN INSTITUTE

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Somatic-cell nuclear transfer offers new opportunities in the area of genetic modification of experimental and farm animals for agricultural production of biopharmaceutics and research applications. To realise the potential of this technology we are working to improve the efficiency of animal cloning. In the Roslin Institute recent progress in sheep and pig cloning included new observations on the effect of the breed of oocyte donors; the effect of the culture system; and the application of gene deletion technology. Details of methods and materials were published previously (1-4). We attribute our progress in pig cloning mainly to an accurate control of the ovulation age of the oocytes via ultrasound scanning; and an improved pregnancy

maintenance method by the co-transfer of parthenogenetically activated embryos with the cloned ones. Nuclear transfer with fetal fibroblast cells resulted in the birth of a healthy cloned piglet. In sheep our results indicated that the genetic origin of the recipient oocytes influences the in vitro development of fetal fibroblast nuclear transfer sheep embryos to the blastocyst stage. Both in vitro and in vivo culture in temporary recipients to the blastocyst stage resulted in lambs following embryo transfer into a final recipient. Deletion of the PRP-gene in cultured fetal fibroblast cells and subsequent nuclear transfer resulted in the birth of a live lamb, however, it was euthanized 12 days after birth due to a non-resolving respiratory problem. The low efficiency of the large animal cloning technology hinders progress towards full-scale commercialisation of this technology. Reduction of the embryo and fetal mortality will likely come from our efforts to develop technical improvements and a better understanding of the somatic-cell reprogramming process. In vitro produced oocytes and in vitro culture systems are beneficial for reducing the cost and number of animals needed for the experiments. Selection of developmentally competent embryos prior to transfer would be highly beneficial both from an animal welfare and an economical point of view.
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References

1. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., and Campbell, K. H. S. (1997) *Nature* 385, 810-813.
2. Dinnyes, A., King, T., Wilmut, I., and De Sousa, P. A. (2001) *Theriogenology* 55, 264.
3. Denning, C., Burl, S., Ainslie, A., Bracken, J., Dinnyes, A., Fletcher, J., King, T., Ritchie, M., Ritchie, W. A., Rollo, M., de Sousa, P., Travers, A., Wilmut, I., and Clark, A. J. (2001) *Nat. Biotechnol* 19, 559-562.
4. De Sousa, P., King, T., Zhu, J., Archibald, A., Dinnyes, A., Dobrinsky, J.R., and Wilmut I. (in press) *Reproduction Supplement*

CULTURE OF PIG ZYGOTES IN A CHEMICALLY DEFINED MEDIUM

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In vitro culture systems for preimplantation embryos in domestic species are important to study the physiology of embryos in early pregnancy and to control animal reproduction, including embryo transfer, transgenesis and cloning. Although pig embryos can develop from the zygote to the blastocyst stage in vitro, the pregnancy rate and average litter size after transfer of cultured pig embryos is apparently low. Thus, culture conditions for pig zygotes have yet to be substantially improved. A chemically defined medium is useful for analyzing the physical action of substances, such as inorganic compounds, energy substrates, hormones, cytokines and vitamins, on the development of preimplantation embryos, since it eliminates unknown factor(s) in which serum and commercial serum albumin are contaminated. Such medium could also be used as a powerful tool for optimizing embryonic growth and maximizing the number of embryos that survive after transfer. However, to date, no piglet has been obtained from embryos cultured in a chemically defined medium after transfer into recipients.

We developed a novel medium (Porcine Zygote Medium; PZM) for in vitro culture of porcine zygotes, based on the composition of pig oviduct fluid, which was supplemented with amino acids. The suitability of this medium for in vitro development of porcine zygotes was investigated in comparison with in vivo or in vitro (NCSU-23 medium) controls. Moreover, in

vivo viability of embryos that were cultured in a chemically defined PZM was also determined after transfer to recipient gilts.

Porcine single-cell zygotes were collected on Day 2 (Day 0 = the day of hCG injection) from gilts stimulated with eCG and hCG. The percentages of zygotes that developed to the blastocyst stage on Day 6 and the hatching blastocyst stage on Day 8 in PZM containing 3 mg/ml BSA (PZM-3) were higher ($P<0.05$) than in NCSU-23. The mean numbers of inner cell mass (ICM) cells and total cells in Day-8 embryos cultured in PZM-3 were significantly greater ($P<0.05$) than in NCSU-23. In culture with PZM-3, embryo development was optimized in an atmosphere of 5%CO_2:5% O_2:90%N_2 compared to 5%CO_2 in air. The ICM and total cell numbers in Day-6 morulae/blastocysts cultured in PZM-3 or PZM-3 in which BSA was replaced with 3 mg/ml polyvinyl alcohol (PZM-4), were also greater ($P<0.05$) than those of NCSU-23, while they were less ($P<0.05$) than those developed in vivo. However, there was no difference in the ratio of ICM to total cells among embryos developed in PZM-3, PZM-4 or in vivo. When the Day-6 embryos that developed in PZM-4 (99 embryos) or in vivo (100 embryos) were each transferred into 6 recipients, 5 gilts were pregnant and farrowed in both treatments. A total of 33 and 42 piglets were born that were derived from in vitro- and in vivo-developed embryos, respectively. No significant difference in the numbers (total, alive and litter size) and body weight of piglets born were observed between the treatments.

Our results demonstrated that our novel developed PZM is efficient for in vitro development of porcine zygotes. We also showed that porcine zygotes could successfully develop to the blastocyst stage in a chemically defined medium (PZM-4) and the cultured Day-6 embryos have developmental competence to full term. Our culture medium should be useful as a basal medium for in vitro production systems in the pig and for embryo manipulation techniques in transgenesis and cloning.

Oral Presentation 10

NEW STRATEGIES FOR EFFICIENT PRODUCTION OF TRANSGENIC PIGS

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Transgenic pigs have been so far practically produced only by means of pronuclear DNA microinjection into in vivo produced zygotes, in spite of rapidly increasing demands from agricultural and biomedical researches. We therefore have been developing alternative strategies for more efficient and economical production of transgenic animals using reproductive technologies such as in vitro maturation of oocytes, intracytoplasmic sperm injection (ICSI) and nuclear transfer.

1. Developmental competence of IVM porcine oocytes: Aiming at employing IVM oocytes for the production of transgenic pigs, we examined the developmental ability of the parthenogenetic IVM oocytes in vitro and in vivo. Oocytes matured in modified NCSU23

(1) were electrically activated by a single DC pulse (150V/mm, 100 μ sec), followed by 7.5 μ g/ml cytochalasin B treatment for 3-4 hr. Activated oocytes were then cultured for 7 days or transferred to the oviducts of recipient gilts. A total of 45 (26.9%) somite stage fetuses were obtained from 167 parthenogenetic oocytes in 4 of 9 recipients. When the activated oocytes were cultured for 7 days 40 - 60% of them developed to the blastocysts. This result clearly shows that IVM oocytes can be a source of competent materials for the production of transgenic pigs.

2. Gene transfer by intracytoplasmic sperm injection into IVM oocytes: Porcine sperm cryopreserved in BTS (2) were diluted to the concentration of 2 x 105 and co-incubated with EGFP DNA (2.5ng/ μ l) for 5 min. An isolated sperm head was microinjected into an IVM oocyte using Piezo micromanipulator, followed by electric activation. Sperm injected oocytes were cultured for 6 days in NCSU23. Of 101 sperm-injected oocytes, 21 (20.8%) developed to the blastocysts of which 6 (28.6%) expressed GFP. When parthenogenetic oocytes (n = 120) at pronuclear stage were injected with DNA, expression of GFP was observed in 12 (37.5%) of 32 blastocysts obtained. These data demonstrate that transgenic porcine embryos can be produced by ICSI of the cryopreserved DNA-bound sperm into IVM oocytes.

3. Nuclear transfer of somatic cells: IVM oocytes were enucleated as described previously (3). As nuclear donor cumulus cells collected from IVM oocytes and fetal fibroblast cells were used. Nuclei of the donor cells were injected into the enucleated IVM oocytes using Piezo manipulator (4). Some of the reconstructed embryos were fixed and stained to examine the nuclear remodeling. To examine developmental ability of the reconstructed embryos, they were electrically activated 2 - 2.5 or 3.5 - 4 hr post nuclear transfer and cultured in NSCU23 for 7 days. Premature chromosome condensation was observed (3 hr post nuclear transfer) in 84 and 93% of the embryos reconstructed with cumulus cells and fetal fibroblast cells, respectively. Blastocyst formation rates of the reconstructed embryos were between 5 - 11 %, regardless of the donor cell type and the intervals between nuclear transfer and activation. These results demonstrated that nuclear remodeling could be efficiently induced following intracytoplasmic injection of somatic cell nuclei into IVM porcine oocytes. Nuclear transfer embryos reconstructed with IVM oocytes and cultured somatic cells were shown to have developmental ability to the blastocyst stage in vitro.

References

1. Funahashi, H., and Day, B.N. (1993) *J Reprod Fertil* 98, 179-185.
2. Pursel, V.G., and Johnson, L.A. (1975) *J Anim Sci* 40, 99-102.
3. Nagashima, H., Ashman, R.J., and Nottle, M.B. (1997) *Mol Reprod Dev* 48, 339-343.
4. Wakayama, T., Perry, A.F.C., Zuccotti, M., Johnson, K.R., and Yanagimachi, R. (1998) *Nature* 394, 369-374.

PRODUCTION OF TRANSGENIC MINIATURE PIGS FOR BIOMEDICAL AND AGRICULTURAL RESEARCH

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Miniature pig has many advantages over domestic pigs for biomedical and agricultural research,

regarding its small size, the possibility of breeding it under restrict environmental controls, and a physiology that has potential similarity to that of human. We describe the production of transgenic miniature pigs by introducing Huntington's disease candidate gene. The huntingtin gene isolated miniature pig genomic library was fused with the rat neuron-specific enolase promoter region and the constructs were injected into fertilized eggs. A total of 402 eggs were transferred to 23 miniature pig recipients. Sixteen of the recipients maintained pregnancy and 65 young were delivered. Of these 65 young and 3 aborted fetuses, five were determined to be transgenic by PCR and Southern analysis. The overall rate of transgenic production was 1.24 %. The phenotype of the transgenic individuals is under an investigation.

Oral Presentation 11

GENETICALLY MODIFIED NON-HUMAN PRIMATES: MODEL FOR HUMAN DISEASES

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Animal models that represent human diseases are important tools in understanding the pathogenesis of the diseases and the development of effective medications. However, how could an animal model represent a patient without having similar symptoms? A model that can not mimic human disease is only good for safety evaluation rather than for accurately evaluating the efficacy of medication and therapy for patients. Through animal models like mice, scientists have learned a lot about human physiology and disease that lead to the development of treatment for patients. However, the fact that mice may not be the best model for human disease is also recognized. Differences between humans and mice include life span, brain complexity, as well as reproductive function. Due to the high physiological and genomic similarity between human and non-human primates (NHP), NHP are considered one of the best models for human diseases. Transgenic technology has opened a new era for animal modeling in biomedicine, which accelerates the development of appropriate animal models and results in better understanding of diseases and in development of better medication for patients. An efficient gene delivery method is the crucial factor in transgenic NHP production because of the limited number of animals and ethical concerns. Four major gene transfer methods have been used to produce transgenic animals: 1. Pronuclear microinjection, 2. Retrovirus infection, 3. Nuclear transplantation, and 4. Sperm mediated gene transfer. Although transgenic NHP have been produced by infection of mature oocytes with VSV-G pseudotyped retroviral vector, other gene transfer methods are also being considered in future experimentation to overcome the disadvantages of a retroviral vector system and to develop the best gene delivery system.

References

1. Chan, A.W.S. (1999) *Cloning* 1, 25-46.
2. Chan, A.W.S, Luetjens, C.M., Dominko, T., Ramalho-Santos, J., Simerly, C.R., Hewitson, L., and Schatten, G. (2000) *Mol Hum Reprod* 6, 26-33.
3. Chan, A.W.S., Chong, K.Y., Martinovich, C., Simerly, C., and Schatten, G. (2001). *Science* 291, 309-312.

NUCLEUS REPLACEMENT IN MAMMALIAN OOCYTES

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The replacement of nuclei in human oocytes seems to be the procedure which is ethically acceptable and may serve, for example, as a tool for the prevention of certain diseases and correction of cytoplasmic abnormalities. Currently, the transfer of germinal vesicles (GV) between oocytes has been extensively studied. The results in the mouse showed that germinal vesicles can be effectively exchanged between oocytes with a subsequent very high rate of maturation up to the second metaphase. Interestingly, the isolated GVs surrounded with a very thin rim of the cytoplasm remain intact even after the prolonged culture and can be also effectively frozen. Under some conditions the transfer of GVs into an asynchronous cytoplasm is possible and has a minimal effect upon further maturation. When embryonic nuclei (G2) from 2 cell staged blastomeres replace GVs maturation proceeds up to the second metaphase like stage. On the other hand, transfer of somatic cell nuclei into enucleated immature oocytes rarely resulted in matured oocytes. Our contribution discussed the above results and some other possible applications.
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THE CHALLENGES OF DEVELOPMENT OF NUCLEAR TRANSFER IN BIRDS

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The goal of developing a method for nuclear transfer in birds is very challenging. The process can be considered as involving three separate processes that must be developed: 1). enucleation of the recipient egg, 2). preparation of suitable donor cells and 3). nuclear transfer, allowing development to take place. Experiments with the chick will be informed by the extensive literature available on nuclear transfer in mammals. The classic experiments in amphibians will also be informative as in many ways the early development of the chick embryo is more similar to the development of an amphibian embryo than a mammalian embryo. Avian embryos develop from large, yolky eggs and the initial rounds of cell division are very fast. The chick embryo develops from the first cleavage division to the blastoderm stage, present in a new laid egg, in less than 20 hours, indicating a cell cycle time of approximately 30 minutes. The avian oocye or zygote is large and therefore not easy to manipulate, and can only be obtained by sacrificing a laying hen shortly after ovulation. The choice of cells for nuclear donors will focus on the use of cells which can be genetically modified before nuclear transfer. A successful method for nuclear transfer in the chick will have many applications as the basis of an efficient route to genetic modification.

Closing Session

CLOSING REMARK

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