Cloning/Nuclear Transfer

24 TRANSGENESIS AND NUCLEAR TRANSFER USING STEM CELLS FROM CULTURED PORCINE PRIMORDIAL GERM CELLS

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Embryonic germ (EG) cells are undifferentiated stem cells isolated from cultured primordial germ cells (PGC). These cells share many characteristics with embryonic stem cells including their morphology and pluripotency. Undifferentiated porcine EG cell lines demonstrating capacities of both *in vitro* and *in vivo* differentiation have been established (Shim H *et al*. 1997 Biol. Reprod. 57, 1089–1095). Since EG cells can be cultured indefinitely in an undifferentiated state, whereas somatic cells in primary culture are often unstable and have limited lifespan, EG cells may provide an inexhaustible source of karyoplasts in nuclear transfer (NT). This would be particularly advantageous in maintaining nuclear donor cells carrying a transgene. In addition, genome-wide demethylation of DNA occurs in pre-implantation embryos as well as PGC. Nuclear transfer embryos using EG cells rather than somatic cells may be close to embryos from normal fertilization in their DNA methylation status. If combined with NT technique, EG cells may potentially be useful for genetic manipulation in pigs. In this study the efficiencies of transgenesis and NT using porcine fetal fibroblast and EG cells were compared. Two different techniques were used to perform NT. When conventional NT procedure (Roslin method) involving fusion of donor cells with enucleated oocytes was used, the rates of development to the blastocyst stage were 16.8% (59/351) and 14.1% (50/354) in EG and somatic cell NT, respectively. In piezo-driven micromanipulation (Honolulu method) involving direct injection of donor nuclei into enucleated oocytes, the rates of blastocyst formation in EG and somatic cell NT were 11.9% (15/126) and 7.5% (12/160), respectively. Although the differences between EG and somatic cell NT were statistically insignificant, the rates of blastocyst development in EG cell NT were comparable to the somatic cell counterpart regardless of NT methods used in the present study. To investigate if EG cells can be used for transgenesis in pigs, GFP gene was introduced into porcine EG cells. Nuclear transfer embryos using transfected EG cells gave rise to blastocysts (29/137, 21.2%), and all embryos that developed to the blastocyst stage expressed GFP, based on observation under fluorescence microscope. In this study, the possibility of using EG cells as karyoplast donors in NT procedure was tested. The results suggest that EG cell NT may be used as an alternative to somatic cell NT, and transgenic pig embryos may be produced using EG cells.

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25 *IN VITRO* DEVELOPMENT OF AGGREGATED NUCLEAR TRANSFERRED EMBRYOS DERIVED FROM BOVINE CUMULUS CELLS

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It has been reported that aggregation of two nuclear transfer (NT) mouse embryos shows an improvement in full-term development (Boiani M *et al*. 2003 EMBO J. 22, 5304–5312). In this study, we examined the effect of aggregation on *in vitro* development of bovine NT embryos. As donor cells for NT, cumulus cells of passage 3–5 were used following culture in serum-starved medium for 5–7 days. NT was performed as previously described (Akagi S *et al*. 2003 Mol. Reprod. Dev. 66, 264–272). NT embryos were cultured in a serum-free medium (IVD-101, Research Institute of Functional Peptide Co., Ltd., Shimojo, Yamagat, Japan). Eight-cell-stage embryos on Day 2 or 16- to 32-cell-stage embryos on day 4 were used for embryo aggregation after removal of the zona pellucida. A small depression was made in a 25-μL drop of TCM-199 with 50 μg/mL phytohemagglutinin (TCM199/PHA) or IVD-101 using a darning needle. Two or three NT embryos were placed into the depression in the drop of TCM199/PHA for 20 min. NT aggregates were then moved into the depression in the drop of IVD-101 and cultured until Day 7. *In vitro* development of NT aggregates was summarized in Table 1. There were no differences in the cell number and ICM ratio of blastocysts between non-aggregated zona-intact and zona-free embryos. All aggregates of three NT embryos developed to the blastocyst stage and the cell number of these blastocysts was significantly higher than that of non-aggregated NT blastocysts. These results indicate that removal of the zona pellucida does not affect the cell number and ICM ratio of blastocysts and that aggregates of three NT embryos can develop to blastocysts with high cell numbers which are equivalent to *in vivo*-derived embryos (166 ± 11, Knijn HM *et al*. 2003 Biol. Reprod. 69, 1371–1378).

Table 1. Development, cell number, and ICM ratio of NT aggregates

Embryo	No. aggregates	No. blastocysts (%)	Total cells	%, ICM: Total cells
Non-aggregated (Zona-intact)	–	10	89 ± 6^{a}	32 ± 2^{a}
Non-aggregated (Zona-free[1])	–	12	84 ± 6^{a}	34 ± 5^{a}
Day 2 NT × 2	9	8 (89)	123 ± 7^{ab}	40 ± 3^{ab}
Day 4 NT × 2	11	9 (92)	135 ± 12^{ab}	40 ± 3^{ab}
Day 2 NT × 3	9	9 (100)	218 ± 34^{c}	41 ± 2^{ab}
Day 4 NT × 3	10	10 (100)	163 ± 18^{bc}	48 ± 2^{b}

[1] Zona pellucida was removed on Day 2 or 4.

[abc] Values without common characters in same column differ significantly ($P < 0.05$).

26 ASSESSMENT OF CHROMOSOME ABNORMALITIES IN SHEEP PARTHENOGENETIC AND NUCLEAR TRANSFER EMBRYOS: EFFECT OF 6-DMAP AND CYCLOHEXIMIDE ON PLOIDY

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In current somatic cell nuclear transfer (NT) protocols, the reconstructed embryos are activated by incorporation of secondary oocyte activation compounds such as 6-DMAP or cycloheximide (CHX). The effects of these compounds on the chromosome complement of sheep NT embryos have not been studied in detail. Therefore, the aim of this study was to assess the chromosome abnormalities using sex chromosome specific probes of Day 6 blastocyst-stage sheep embryos produced from parthenogenetic activation and NT. Following 20–22 h of IVM, the oocytes were activated by electric pulsing followed by 30-min culture in cytochelasin B. They were reactivated using ionomycin (5 min) followed by 2-h culture in 6-DMAP or CHX. In contrast, NT embryos were produced using standard NT procedures using male sheep fetal fibroblasts. Reconstructed embryos were activated using the same methods described earlier. The embryos (compact morulae and blastocysts) were fixed and subjected to FISH analysis using cattle X and Y chromosome painting probes. The data were analyzed using Fisher's exact test. Of the parthenogenetic embryos (6-DMAP, $n = 28$; CHX, $n = 32$) analyzed, none of the embryos was totally haploid (X) or totally polyploid. When all of the nuclei per embryo were considered, normal (XX) genotype embryos were 6.2% and 0.0% in CHX and 6-DMAP groups, respectively. The rest of the embryos were abnormal due to mixoploidy (100% vs. 93.8%, $P < 0.05$) in 6-DMAP and CHX treatment groups, respectively. The abnormal nuclei per embryo ranged from 7.3% to 72.2%. The mean total cell number of parthenogenetic blastocysts was 91.2 ± 4.3 and 81.8 ± 6.2 (mean $\pm$ SE) in 6-DMAP and CHX, respectively. Among NT embryos analyzed, (6-DMAP, $n = 30$; CHX, $n = 32$) only 40.0% and 43.8% of embryos were completely normal for XY chromosomes in 6-DMAP- and CHX-treated groups, respectively. The rest of the embryos were abnormal due to mixoploidy (60.0% vs. 56.2%, $P > 0.05$) in 6-DMAP and CHX groups, respectively. Monosomy (XO or OY), trisomy (XXY), and tetrasomy (XXYY) were the common abnormalities detected in mixoploid embryos. The abnormal cells per embryo ranged from 3.8% to 41.8% in both treatment groups. The mean total cell number of NT blastocysts was 71.2 ± 9.8 and 63.8 ± 8.4, in 6-DMAP and CHX treatment groups, respectively. In conclusion, the 6-DMAP-treated embryos derived from parthenogenetic activation had significantly higher chromosomal abnormalities than CHX-treated embryo groups ($P < 0.05$). In contrast, the NT embryos derived from either 6-DMAP or CHX treatment did not show any significant difference in producing chromosomally abnormal embryos at the blastocyst stage. This study also highlights the feasibility of using bovine chromosome painting probes on ovine embryo spreads.

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27 MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL CHARACTERIZATION OF DAY 21 IVP AND NT BOVINE EMBRYOS

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A major limitation of somatic cell nuclear transfer (NT) for the production of cloned calves is that only 1–5% of cloned embryos produce viable calves. The high rate of mortality is attributed to both pre- and post-natal losses and is primarily due to incomplete reprogramming of donor cells. Almost 50% of the pregnancy losses occur in the first trimester of pregnancy, indicating a major disruption in normal embryo development at NT. The aim of this study was to analyze germ layer formation by stereomicroscopy and immunohistochemical techniques for both NT embryos and their *in vivo* counterparts on Day 21 and to compare deviations from normal embryonic development as a measure of developmental capacity. Blastocyts derived by IVF ($n = 20$), conventional NT ($n = 20$), or hand made cloning (HMC; $n = 20$) were non surgically transferred to each synchronized recipient cow ($n = 3$). Each group of twenty embryos was transferred to one recipient. Cows were slaughtered on Day 21 and uterine tracts recovered and flushed with phosphate-buffer solution with 10% serum. Recovered Day 21 embryos were fixed in 4% paraformaldehyde, and embedded in paraffin; serial sections were stained with hematoxylin and eosin and evaluated by light microscopy. Immunohistochemical localization of cytokeratin 8 was used as a marker for potential ectoderm, alphafetoprotein for potential endoderm, and vimentin for potential mesoderm. Four IVF (20%; 4/20) embryos, six NT (30%; 6/20) embryos, and ten HMC (50%; 10/20) embryos were recovered following flushing. No obvious morphological differences were seen in the formation of a neural tube, differentiation of mesoderm, and number of somites among IVF (25%; 1/4), NT (33.3%; 2/6), and HMC (20%; 2/10) embryos. Delayed development with respect to the formation of neural groove and mesoderm differentiation was observed in 25% (1/4) of IVF, 16.7% (1/6) of NT, and 30% (3/10) of HMC embryos. In addition, 25% (1/4) of IVF, 16.7% (1/6) of NT, and 33.3% (2/6) of HMC embryos had not initiated gastrulation (i.e. displayed hypoblast and epiblast), suggesting a more substantial developmental delay. The remaining embryos showed gross abnormalities compared to their *in vivo* counterparts, including degenetration of epiblast and hypoblast cells. Cytokeratin was detected in the trophoblast, ectoderm, hypoblast, and endoderm. Alpha-fetoprotein was detected in the hypoblast while vimentin was seen in the mesoderm. In conclusion, although localization of staining in IVF and cloned embryos was consistent with that of *in vivo* embryos, the intensity was weaker, suggesting compromised or delayed development. It is also possible that the differences observed were due to the recipient and not to the treatment group.

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28 CLONED EMBRYOS CAN BE PRODUCED USING DONOR CELLS OBTAINED FROM A 72-HOUR COOLED CARCASS

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There are few reports on the use of cells from a dead mammal for nuclear transfer (NT). So far, most calves have been cloned from live adult cows or fresh fetal samples. The ability to produce cloned animals using postmortem tissue can provide an additional application to the field of NT. This study was conducted to investigate whether viable cells could be obtained from tissues chilled for 72 h and whether these cells could be used for NT. Bovine oocytes isolated from slaughterhouse ovaries were matured in TCM199 supplemented with 10% fetal calf serum (FBS), 50 μg/mL sodium pyruvate, 1% v:v penicillin-streptomycin (10,000 U/mL penicillin G, 10,000 μg/mL streptomycin), 10 ng/mL EGF, 0.5 μg/mL FSH, and 5 μg/mL LH. A cell line (MC) was established from leg muscle of a cow carcass stored at 0°C for 72 h. Tissues from muscle were cut into small pieces. Tissue explants were cultured in DMEM-F12 supplemented with 10% FBS at 37°C in 5% CO_2 in air. Bovine granulosa cells (GC) were isolated from ovarian follicles and used for NT as control cells. Prior to NT, all somatic cells were allowed to grow to confluency (G1/G0) in DMEM-F12 medium supplemented with 10% FBS. Cumulus cells were removed by vortexing with hyaluronidase at 18 h after the start of maturation. Matured oocytes labeled with DNA fluorochrome Hoechst 33342 were enucleated under UV to ensure full removal of the chromatin. A single cell was inserted into the perivitelline space of the enucleated oocyte. Oocyte-cell couples were fused by a DC pulse of 133V/500 μm for 25 μs. After fusion, NT units were activated using a combination of calcium ionophore (5 μM), cytochalasin D (2.5 μg/mL) and cycloheximide (10 μg/mL) and cultured for 7 days in BARC or G1.3-G2.3 medium. Differences (developmental potential and cell numbers) among groups were analyzed by one-way ANOVA after arcsin square transformation. The results are summarized in Table 1. The results suggest that viable cells can be obtained from muscle of a cow carcass stored at cold temperature for 72 h and that these cells have ability to generate NT blastocysts at rates similar to those obtained with fresh GCs. In addition, G1.3 and G2.3 culture medium supported embryo development better than BARC medium.

Table 1. *In vitro* development of NT embryos

Donor cell	Medium	NT units	Cleaved (%)	Blastocyst (%)	No. of cells
MC	G1.3-G2.3	78	46 (58.9)[a]	18 (23.0)[a]	96.0 ± 11.5
MC	BARC	66	24 (36.3)[b]	6 (9.0)[b]	100.6 ± 15.0
GC	BARC	43	22 (51.2)[a]	5 (11.6)[b]	93.7 ± 14.6

[ab] Values within each column with different superscripts are significantly different ($P < 0.05$).

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29 COMPARISON OF TERM PLACENTAS IN CLONED AND CONTROL PREGNANCIES IN CATTLE

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Somatic cell nuclear transfer is associated with high incidence of fetal loss, late-term pregnancy complications, perinatal mortality, and abnormal placental development. Several groups have described abnormalities of early and mid-gestation cloned placentas (Hill *et al*. 2000 Biol. Reprod. 63, 1787–1794; Lee *et al*. 2004 Biol. Reprod. 70, 1–11). The objective of our study was to characterize differences in the placentas of clones and control calves at term delivery. Clones were produced from ovarian cell lines from two donors (Holstein, $n = 5$; Hereford, $n = 2$). Breed-matched controls included AI (Holstein, $n = 3$) and embryo transfer (Holstein, $n = 3$; Hereford $n = 3$) calves. All calves were delivered alive with no visible birth defects between Days 273 and 280 of gestation, and placentas were recovered for measurement and morphological analysis. When possible, pregnancies were delivered via caesarian section, and the entire uterus was recovered for classification of anatomical shape of placentomes. Each placentome was measured, weighed, and classified by type as (A) engulfing mushroom-like; (B) sub-engulfing mushroom-like; (C) flattened, non-engulfing; and (D) convex (adapted from Penninga and Longo 1998 Placenta 19, 187–193, for sheep). Mean number of placentomes per placenta was significantly greater in controls than clones, while total mass of placentomes in the pregnant horn was significantly greater in clones than in controls (Table 1). Total surface area of placentomes in the pregnant horn tended to be larger and more variable in clones (range: 2710–7450 cm^2) than in controls (range: 3120–5030 cm^2; $P < 0.10$). A two-fold increase was observed in cloned placentas, as compared with control placentas, in mean surface area per placentome and mass per placentome. Anatomically, cloned placentas differed from controls in the percentage of placentomes classified Type A (controls > clones) and Type C (clones > controls). Other abnormalities noted in cloned placentas included moderate to severe edema, teratomas, enlarged vessels, and large areas devoid of placentation. All clones and 2/9 controls displayed enlarged umbilical vessels. Significant placental abnormalities were observed in all cloned pregnancies.

Table 1. Placental characteristics of term cloned and control pregnancies

Mean/placenta[1]	Clones (*n*)	Controls (*n*)
Total placentomes	67.4 (7)[a]	98.3 (9)[b]
Placentome mass, preg horn (kg)	6.05 (7)[a]	3.84 (5)[b]
Mean/placentome[1]		
Surface area (cm^2)	117[c]	67[d]
Mass (g)	120[c]	67[d]
Placentome morphology[2]	(%)	(%)
A, engulfing	20[c]	26[d]
B, mushroom-like	37	38
C, flattened	40[c]	16[d]
D, convex	3	0

[1] Student's *t*-test. [a–d] Values between columns with different superscripts differed significantly; $P < .05$.
[2] Contrasts made with 99% confidence intervals on sample proportions.

30 WITHDRAWN

31 CONSTRUCTION OF A TARGETING VECTOR SPECIFIC FOR THE BOVINE BETA-CASEIN GENE

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Although using livestock as animal bioreactors is a powerful tool to produce valuable therapeutic proteins in the milk, there are still some limitations to the technology such as a low frequency of transgenesis and a low expression of the transgenes by random integration. In this study, we constructed gene-targeting vectors for a mammary gland-specific gene and then obtained two homologous recombinant cell clones after transfection of the vectors into bovine somatic cells. Two targeting vector cassettes, BCKI I and BCKI II, which have homology regions for a bovine beta-casein gene, were constructed. The beta-casein gene is expressed only in the mammary gland and is the most abundant milk protein in the cow. The targeting sequence lengths of the BCKI I and BCKI II vector cassettes were 13.1 kb and 9.1 kb, respectively, and contained different long arm lengths. A neo gene was inserted into the vectors as a selection marker, and a few restriction enzyme sites were made in front of the neo gene. The human thrombopoietin (TPO) gene was inserted into the restriction enzyme sites of the vector cassettes, named BCTPOKI I and BCTPOKI II vectors. The BCTPOKI I

and BCTPOKI II vectors were transfected into bovine embryonic fibroblasts (bEF) and ear skin fibroblasts (bESF) using Lipofectamine™ 2000 reagent (Invitrogen, Seoul, South Korea). In order to determine the highest transfection efficiency, a variety of factors such as DNA concentration, lipid volume, and exposure time to DNA-liposome complexes based on the manufacturers' guideline, was optimized. The 2:1 and 1:2 ratios of DNA (μg) to transfection reagent (μL) were efficient for bEF and bESF, respectively, under overnight exposure to DNA-Lipofectamine™ 2000 reagent. Seventeen percent (51/304) of bESF clones and 6% (9/149) of bEF clones were normally expanded into passage 8. PCR and Southern blotting indicated that 6.3% (2/32) of the clones carrying with BCTPOKI II vectors was homologously targeted at the beta-casein gene. However, none (0/60) of the clones carrying BCTPOKI I was targeted. Additionally, both of the targeted clones were from bESF. When the targeted cells were transferred into enucleated oocytes and cultured, 83% (43/52) of the cloned embryos were transgenic. Thus, we found that homologous recombinant events using gene-targeting vectors might be dependent on cell types, vector sizes, and transfection procedures. In conclusion, mammary gland-specific gene-targeting vectors coupled with somatic cell nuclear transfer technology will be very useful for developing animal bioreactors that produce therapeutic proteins in milk.

32 EFFICIENCY OF FEMALE-DERIVED DONOR CELLS ON HIGH POSTNATAL SURVIVAL IN PIG CLONING

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The present study was conducted to investigate the developmental competence of male and female somatic cell derived nuclear transfer (NT) porcine embryos and also the production and survival efficiency of cloned male and female piglets. Maturation of porcine COCs was accomplished by incubation in NCSU-23 medium supplemented with 0.6 mM cysteine, 10% porcine follicular fluid, 1 mM dibutyryl cyclic adenosine monophosphate, and 0.1 IU/mL human menopausal gonadotrophin for 20 h and then culture without dbcAMP and hMG for another 18 to 24 h. Fetal cells were isolated from a male fetus and two female fetuses, and cultured in ES-DMEM medium containing 10% FCS. Enucleated oocytes were fused with fetal fibroblasts (passage 4 to 15). Reconstructed embryos were cultured in NCSU-23 with 4 mg/mL BSA under mineral oil at 39°C in 5% CO_2 in air for up to 6 days. NT eggs that had been activated with electric pulses and cultured for 1 or 2 days were transported to the experimental station in modified NCSU-23 with antibiotics. NT embryos were surgically transferred into the oviducts of recipients between Day 27 and Day 30; pregnancy was determined by ultrasound. The potential of NT embryos to develop into blastocysts was not different among donor cells of different origins. However, the mean cell number of *in vivo* female and male blastocysts (83.8 ± 46.2 to 99.2 ± 55.7) was higher than in *in vitro* culture of NT groups (31.4 ± 8.29 to 33.2 ± 10.15). A total of 11,535 NT embryos (1- to 8-cell stage) were surgically transferred into 66 surrogate gilts. Among fourteen pregnant gilts, four recipients aborted during the period of conception. Five pregnant gilts delivered fifteen female piglets, 1.28 ± 0.33 kg (0.48∼1.83 kg) in female piglets and 0.84 ± 0.25 kg (0.45∼1.25 kg) in male piglets. Nine live cloned female (60.0%) and four male piglets (18.2%) were produced. According to these results, survival rates and birth weights of female cloned piglets were higher than those of cloned male piglets ($P < 0.05$). This study suggests that use of female, compared with male, fetal fibroblast cells as nuclear donors may increase cloning outcomes.

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33 PRODUCTION OF TRANSGENIC-CLONED PIGS CARRYING HDAF, GnT-III AND HETEROZYGOUSLY DISRUPTED α-1,3-GALACTOSYLTRANSFERASE GENES

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In pig-to-human xenotransplantation, transplants are rapidly rejected by binding of human natural antibodies to porcine xenoantigen, mostly Gal α-1-3Gal oligosaccharides, and subsequent complement attack. To overcome this rejection, we so far have produced transgenic pigs expressing both human CD55/DAF (decay-accelerating factor, a complement-regulatory protein) and GnT-III (N-acetylglucosaminyltransferase III, a sugar chain modifying enzyme). In the present study, we heterozygously disrupted the α-1,3-galactosyltransferase (GT) gene, which catalyses the biosynthesis of Gal α-1-3Gal epitopes, in the fetal fibroblast cells from the DAF/GnT-III transgenic pigs by homologous recombination, and successfully produced GT-knockout pigs by nuclear transfer. Fibroblast cells isolated from Day 30 fetuses of DAF/GnT-III transgenic pigs were transfected with a GT-targeting vector. The targeting event in drug-resistant colonies was confirmed by PCR analysis, and targeted cells were used as nuclear donors. The reconstructed embryos were electrically activated and transferred to estrus-synchronized recipient pigs. At pregnancy Day 27 of gestation, fetuses were collected and their fibroblast cells were isolated for secondary nuclear transfer. The genomic DNA of live-born piglets produced by the secondary nuclear transfer were analyzed for the presence of DAF and GnT-III genes as well as the heterozygous disruption of the GT gene. From a total of 5.5×10^7 cells transfected with the GT-targeting vector, 2,749 drug-resistant colonies were obtained. Eighteen colonies were judged positive for targeting events by PCR analysis. After transfer of 321 cloned embryos reconstructed with the knockout cells to three recipients, four knockout fetuses were obtained from one recipient. Transfer of 633 cloned embryos reconstructed with the knockout fibroblast cells from one knockout fetus to six recipients gave rise to two live knockout piglets. PCR analysis of genomic DNA confirmed that the cloned piglets carried both DAF and GnT-III transgenes as well as the heterozygously disrupted GT gene.

34 LIFESPAN AND CHROMOSOMAL STABILITY OF BOVINE AND PORCINE FETAL FIBROBLAST CELLS CULTURED *IN VITRO*

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The low efficiency of nuclear transfer (NT) has been related to factors such as mitochondria heteroplasmy, failure of genomic activation, and asynchrony between the donor karyoplast and recipient cytoplast. Few studies have characterized donor cell lines in terms of proliferative capacity and chromosomal stability. It is known that suboptimal culture conditions can induce chromosomal abnormalities, and the use of aneuploid donor cells during NT can lead to a high incidence of abnormal cloned embryos (Giraldo *et al*. 2004 Reprod. Fertil. Dev. 16, 124 abst). The purpose of this study was to determine the lifespan and chromosomal stability of bovine and porcine fetal cells. Four bovine and four porcine fibroblast cells lines were established from 50-day and 40-day fetuses, respectively. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin at 37°C in 5% CO_2. Each cell line was passaged to senescence. Total population doublings (PDs) and cell cycle duration were calculated. To determine the chromosome numbers at different PDs, cells were synchronized in metaphase, fixed, and stained. ANOVA and chi-square tests were used to analyze differences in PDs and proportion of aneuploid cells between cell lines, respectively ($P < 0.05$). The results show that proliferative capacity was not different between cell lines derived from the same species. Cell lines derived from bovine and porcine fetuses had different *in vitro* lifespans (33 PDs vs. 42 PDs, respectively; $P < 0.05$). The mean length of the cell cycles for both bovine and porcine fetal fibroblasts was ~28 h. The percentage of aneupliod cells in both bovine and porcine fetal cell lines increased progressively with duration of culture (see Table) and was high throughout the study. The proliferative capacity of cultured cells was similar within individuals of the same species, but growth characteristics differed between fetal bovine and porcine cell lines. The progressive increase of aneuploid cells could be due to suboptimal culture conditions or unusual chromosome instability in the particular fetuses used. These data demonstrate the importance of determining chromosome content and the use of cells at early passages to decrease the percentage of aneuploid reconstructed embryos and increase the efficiency of NT.

Cell type	Percentage of aneuploid cells (± SEM)		
	<10 PDs	10–20 PDs	>20 PDs
Bovine fetal fibroblasts	47.9 ± 3.08 *n* = 240	65.5 ± 2.53 *n* = 360	85.0 ± 2.00 *n* = 240
Porcine fetal fibroblasts	77.5 ± 3.48 *n* = 240	92.5 ± 1.04 *n* = 240	95.8 ± 2.09 *n* = 120

n = number of metaphases analyzed.

35 PRODUCTION OF CLONED MINIATURE CALVES USING CYTOPLASTS FROM COWS OF STANDARD SIZE

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Numerous genetically similar cattle for use in research or teaching can be produced with chromatin transfer technology (Sullivan *et al.* 2004 Biol. Reprod. 70, 146–153). Miniature cattle can provide advantageous biotechnological models for teaching and study of human and animal diseases. Miniature cattle are approximately one fourth the size of standard cattle and, therefore, represent a potentially less expensive, safer, and more easily managed animal model. Our limited attempts to reproduce miniature cattle via *in vivo* and *in vitro* production of embryos resulted in poor response to superovulation and logistical challenges in recovering embryos or oocytes due to the small size of the animals. Thus, the purpose of this study was to evaluate the potential of embryos derived by chromatin transfer from fibroblasts of a miniature cow and cytoplasts from cows of standard size to produce viable offspring after transfer into recipient cows of standard size. The donor of somatic cells was a heifer that weighed 7.7 kg at birth. Chromatin transfer resulted in 19% (82/428) blastocyst formation. A total of 66 cloned blastocysts (65 excellent/good quality, 1 fair quality) were transferred into 26 synchronized recipients. While ultrasound revealed 13 pregnancies prior to 67 days of gestation, only 4 pregnancies of 5 fetuses were maintained beyond 100 days. Parturition was induced with dexamethasone and prostaglandin on Day 286 of gestation. One singleton (12.3 kg) and a set of twins (10.2 and 11.1 kg) were healthy at birth and normal at 1 week of age. Two fully developed singletons, weighing 21.4 and 13.6 kg, died *in utero*. The latter fetus exhibited a fixed dorsolateral deviation of the neck that complicated delivery despite a caudal obstetrical presentation. No abnormalities were noted in the size or structure of any placenta. Our results indicate that healthy miniature calves can be gestated by recipient cows of standard size after transfer of embryos derived by chromatin transfer. Unfortunately, the fetal wastage, fetal anomalies, and stillbirths observed with standard sized cattle also may occur.

36 IMPROVING THE APPLICATION OF NUCLEAR TRANSFER FOR PRODUCING NON-DOMESTIC FELIDS

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One of the most remarkable aspects of somatic cell nuclear transfer (NT) is the possibility of avoiding extinction when there are few remaining animals of a specific felid population. Previously, we produced live male African Wildcat (AWC; *Felis lybica*) cloned kittens using inter-species nuclear transfer (Gomez *et al*. 2004 Cloning and Stem Cells 6, 217–228). The production of females is a primary objective of most breeding programs. Therefore, the purpose of the present study was to determine (1) if we could produce live female AWC cloned kittens at a proportion similar to that previously demonstrated with males, and (2) if our inter-species NT technique used to produce AWC is applicable to *in vitro* production of another non-domestic felid species. Specifically, we evaluated the *in vivo* developmental competence of NT embryos derived by fusion of Black footed cat (BFC, *Felis nigripes*) and AWC fibroblasts with domestic cat (DSH, *Felis catus*) cytoplasts, after transfer into domestic cat recipients. Fibroblast cell lines were established from skin biopsies of BFC (6-year-old), and AWC (12-year-old) adult females. After at least three passages, cells were serum-starved for 5 days and injected into the perivitelline space of enucleated domestic cat oocytes. Fusion of cell-cytoplast couplets was induced by applying a 3-s AC pre-pulse of 20 V, 1 MHz, followed by two 30-μs DC pulses of 240 V/mm. Fused couplets were activated 2 to 3 h after fusion by exposure to two 60 μsec DC pulses of 120 V/mm, followed by 4 h incubation with 10 μg/mL cycloheximide and 5 μg/mL cytochalasin B. Reconstructed BFC ($n = 16$) and AWC ($n = 536$) NT Day 1 embryos were transferred by laparoscopy into the oviducts of 1 and 12 gonadotrophin-treated DSH recipients, respectively, on Day 1 after induced ovulation. Pregnancy was assessed by ultrasonography on Day 22. One cat (100%) receiving BFC NT embryos and 5 (41.6%) cats receiving AWC NT embryos became pregnant. Twenty-three AWC cloned embryos implanted and 11 kittens were born. Three BFC NT embryos implanted and the pregnancy is currently ongoing. AWC cloned kittens were phenotypically and genetically identical to their somatic cell donor. Their clonal identity was assessed by multiplex PCR amplification of 20 microsatellite markers, including seven markers that are known to be on the X chromosome.

In summary, these results indicate that female AWC cloned kittens can be produced and BFC pregnancy can be established in domestic cat recipients. The embryo implantation rate and viability of AWC female cloned embryos was higher than that observed after the transfer of AWC male cloned embryos. The difference may be due to improvements in the NT procedure, rather than to differences in the sex of the cell lines.

Table 1. Implantation rate and fetal survival to term of AWC and BFC NT embryos in pregnant domestic cat recipients

Species	DSH pregnant recipients	No. Embryos transferred	No. Embryos implanted (%)	No. Kittens to term (%)
BFC	1	16	3 (18.7)	Ongoing
	1	34	8 (23.5)	7 (20.6)
	2	40	1 (2.5)	0 (0)
AWC	3	63	4 (6.3)	0 (0)
	4	39	2 (5.1)	2 (5.1)
	5	42	8 (19.0)	4 (4.7)

37 PREGNANCY-ASSOCIATED GLYCOPROTEIN (PAG) PROFILES DURING THE PERI-IMPLANTATION PERIOD IN RECIPIENTS CARRYING BOVINE SOMATIC CLONES: PRELIMINARY RESULTS

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Bovine pregnancy-associated glycoproteins (bPAGs) are secreted by binucleate cells of the placenta and can be assayed in maternal plasma as indicators of pregnancy and markers for the functional status of the trophoblast. The concentrations of PAGs vary differentially during the peri-implantation period. Large offspring syndrome (LOS) and abnormal placentation have been associated in cloned fetuses with abnormally increased pregnancy-specific protein 60 (PSP60). The aim of this study was to investigate the evolution of plasma levels of PAGs measured by the use of three different RIA systems in heifers after transfer of somatic cloned embryos and to compare that to plasma levels of control recipients during early pregnancy to distinguish which ones vary differentially. Cloned embryos were derived from nuclear transfer from fibroblasts of an adult cell line to *in vitro*-matured bovine oocytes according to Vignon *et al*. (1998 Theriogenology 49, 392). Control embryos were obtained *in vitro* after IVF and cultured in serum-free medium up to the blastocyst stage. By Day 7, cloned ($n = 29$) and control embryos ($n = 10$) were transferred to synchronous recipient heifers of the Normande breed (one embryo per recipient). Blood samples were taken every 2–3 days between Days 25 and 50 of pregnancy from recipients that had a positive progesterone test on Day 21, and the plasma was stored frozen until assay. Pregnancy status was monitored by repeated ultrasound scanning from Day 35 to Day 90. Concentrations of PAGs were determined by validated RIA assays for the different forms (PAGI67, PAG55+62, PAG55+59) using 3 antisera (AS 497, AS 706, AS 708, respectively) (Perenyi *et al*. 2002 Reprod. Domest.

Anim. 37, 100–104). profiles of only the recipients that were confirmed pregnant by scanning over Day 50 ($n = 18$ clones, $n = 4$ controls) were analyzed and compared. Concentrations of PAGs measured by AS 497 and AS 706 were significantly higher in clones than in control recipients during the second month of pregnancy, indicating that the placenta of clones secreted these forms in a different manner compared to controls. Moreover, we found that concentrations of PAGs as determined with the two same antisera were higher for recipients with a cloned fetus that developed to term compared to those which had fetal loss before 3 months. For instance, using the AS 706, respective values of PAGs at Day 45 were 17.64 ± 7.59 and 10.96 ± 6.38 ng/mL for pregnancies with cloned and control fetuses, respectively, ($P < 0.05$) and 21.29 ± 4.77 and 12.88 ± 8.90 ng/mL in pregnancies with cloned fetuses that developed normally to term or died before 3 months of age, respectively, ($P < 0.05$). We conclude that our assays using two antisera could be a predictive test for fetal loss in clone pregnancies. Until now, recipients carrying cloned embryos that develop LOS during late pregnancy could not be detected by conventional assays in maternal serum or by scanning during the period of 35–50 days. This study provides a new diagnostic tool to detect them. Investigations are in progress to check the localization of these different forms of PAG in placentomes removed from recipients carrying somatic clones.

38 HIGH OSMOLARITY AT EARLY CULTURE STAGE IMPROVES *IN VITRO* DEVELOPMENT OF PRE-IMPLANTATION PORCINE NUCLEAR TRANSFER EMBRYOS

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Fragmentation occurs during early developmental stage of electrically activated oocytes and nuclear transfer (NT) embryos, and it might be the cause of the low developmental rate of pre-implantation porcine nuclear transfer embryos. The present study was conducted to investigate whether addition of sugars such as sorbitol and sucrose suppresses fragmentation and supports the development of NT embryos. Oocytes derived from a local abattoir were matured for 42–44 h and enucleated. Fetal fibroblast cells were obtained from a Day 35 porcine fetus. Parthenogenetically activated oocytes or NT embryos were cultured in PZM-3 for 6 days, or in PZM-3 supplemented with sorbitol or sucrose for 2 days and then cultured in PZM-3 for the remaining 4 days. The osmolarity of PZM-3, PZM-3 supplemented with 0.05 M or 0.1 M sorbitol, and PZM-3 with 0.05 M sucrose was 269 ± 6.31, 316 ± 3.13, 362 ± 4.37 and 315 ± 5.03 mOsm, respectively. When the parthogenotes were cultured in PZM-3 supplemented with 0.05 M sorbitol or sucrose for the first 2 days and then cultured in PZM-3 without sugar, a significantly higher ($P < 0.05$) cleavage rate and blastocyst rate was observed. Interestingly, sugar addition into PZM-3 at early culture stage for 2 days reduced the fragmentation rate compared to the rate in PZM-3 without sugar. In NT embryos, sugar addition into PZM-3 significantly ($P < 0.05$) increased the cleavage rate (67.6 ± 5.80 vs. 77.3 ± 3.03) and developmental rate to the blastocyst stage (10.2 ± 0.79 vs. 19.4 ± 1.77). There was no significant difference between treatments for the number of nuclei in the blastocysts. In addition the fragmentation rate in sugar-supplemented PZM-3 was reduced compared to that in PZM-3 without sugar (26.1 ± 4.30 vs. 14.5 ± 1.74). In conclusion, the increased osmolarity of PZM-3 with sugar supplementation at an early developmental stage for 48 h could increase the cleavage and developmental rate to the blastocyst stage by reducing the fragmentation rate.

39 CHROMOSOME ABNORMALITIES IN BOVINE NUCLEAR TRANSFER EMBRYOS PRODUCED BY "HANDMADE CLONING"

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Embryos produced by nuclear transfer using somatic cells as donors exhibit a lower developmental competence compared to *in vivo* developed and IVM/IVF/IVC embryos. The so-called handmade somatic cell nuclear transfer (HSCNT or handmade cloning) that was recently presented (Vajta *et al*. 2003 Biol. Reprod. 68, 571–578) has, however, improved the reconstructed embryo and subsequent blastocyst rate. Further, the high total cell number ($n = 216$) and the high ratio of cells allocated to the inner cell mass (35%) as well as the initial pregnancy rate of 48% on Day 28 following nonsurgical transfer of HSCNT embryos indicated a high average quality. But still the pregnancy loss of HSCNT embryos was high and resulted in a birth rate of 8%. The aim of this study was to estimate the chromosomal variation in HSCNT embryos using fluorescent *in situ* hybridization (FISH) and to evaluate this as an additional parameter of embryo quality. Nuclei from 49 Day 7 HSCNT embryos from five independent trials were isolated by hypotonic treatment and fixed. Then the nuclei were hybridized with differentially labelled cJAB8 and p33E39 probes that hybridize specifically to the centromeric region of chromosome 6 and 7 (Viuff *et al*. 2000 Biol. Reprod. 63, 1143–1148). A total of 6715 nuclei were analyzed for chromosomal abnormalities, and the percentage of nuclei with false negative scores represented 1.8%. Only 4.1% of the embryos (2 of 49) had a completely normal diploid composition, while the remaining 47 embryos exhibited different types of mixoploidy. Of the 47 mixoploid embryos, 87% contained more than one type of chromosomal variation with diploid/triploid/tetraploid being the most frequent constitution (43% of the 47 mixoploid embryos). No pure polyploid embryos were observed. The percentages of HSCNT embryos in the groups of 0%, 1–25%, and 26–100% polyploid cells were 4.1%, 67.5%, and 28.6%, respectively. This is significantly higher than the corresponding figures produced and analyzed by comparable methodology of bovine IVM/IVF/IVC embryos (Viuff *et al*. 1999 Biol. Reprod. 60, 1273–1278). However, 71.4% of the HSCNT embryos contained less than 25% polyploid cells; this low level may not have compromised the developmental competence of these embryos.

40 CHARACTERIZATION OF EARLY G_1 CELLS AS NUCLEAR DONORS FOR SOMATIC CELL CLONING IN CATTLE

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In somatic cell cloning, the cell cycle phase of the donor cells has critical impact on nuclear reprogramming and chromosomal normality of the reconstructed embryos. Recently, enhanced development to full term was obtained with embryos reconstructed with bovine fibroblasts soon after cell division (early G_1 cells, Kasinathan P *et al.* 2001 Nat. Biotech. 19, 1176–1178; Urakawa M *et al.* 2004 Theriogenology 62, 714–728). In this study, to investigate the detailed cell cycle characteristics and gene expression of the early G_1 cells as nuclear donors, we examined the cell proliferating and nuclear activity by detecting PCNA and Ki-67 in the cells, and the gene expression in the cells transfected with the luciferase gene. Bovine fibroblasts were transfected with chicken β-actin/firefly luciferase fusion gene (*β-act/luc+*), and stably transfected; cloned cells were used for cell analysis. We compared cell cycle characteristics for quiescent cells (0.4% serum for 7 days), cell doublets (early G_1 cells) prepared by the "shake-off" method, and proliferating (30 to 40% confluency) cells. The presence of PCNA and Ki-67 and the incorporation of BrdU in the cells were determined by immunohistochemical analysis. The LUC+ signal (luminescence) in the cells was detected with an imaging photon counter for 10 consecutive min. Embryos reconstructed with these cells were cultured for 168 h for examination of blastocyst development. Experiments were repeated three times, and the data were analyzed with Fisher's PLSD test following ANOVA. Incorporation of BrdU was observed only in proliferating cells (24% of the cells). Neither PCNA nor Ki-67 signals were detected in the quiescent cells. PCNA was detected but Ki-67 was not detected in early G_1 cells. Both PCNA and Ki-67 were detected in the proliferating cells. A strong LUC+ signal (6354 ± 673 pixels/cell) was detected in the proliferating cells, and weak signals were detected in the early G_1 (2044 ± 303 pixels/cell, $P < 0.05$) and quiescent cells (617 ± 59 pixels/cell, $P < 0.05$). The rate of blastocyst development with early G_1 cells was higher (45/133, 32%) than that with starved and proliferating cells (47/233, 21%, and 41/258, 14%, respectively, $P < 0.05$). These results indicate that early G_1 cells were actively proliferating cells because of the positive PCNA signals, but their nuclei were silent because of the absence of Ki-67 signals and the weak LUC+ signals. These characteristics of the early G_1 cells might enhance the development of the reconstructed embryos.

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41 EFFECT OF CELL TYPES AND PASSAGES ON DEVELOPMENT AND APOPTOSIS OF PORCINE CLONED EMBRYOS

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The purpose of this study was to improve the efficiency of somatic cell nuclear transfer (SCNT) in pigs by assessing the development, cell numbers and apoptosis when using different cell types as nuclear donors and different numbers of passages. Primary cultures of the donor cells, porcine fetal fibroblasts (pFFF) from a female fetus at ~30 days of gestation and adult female ear skin cells (pAESC), were established in DMEM + 15% FCS. For nuclear donor, cells at different passages were cultured for 5 days until confluent. Cumulus-oocyte complexes were matured and fertilized *in vitro* as controls by the following methods (2000 Theriogenology 54, 787–797). Following enucleation, oocytes were reconstructed by transfer of donor cells and fusion with two DC pulses (1.4 kV/cm, 50 μs) in 0.28 M mannitol containing 0.01 mM $CaCl_2$ and $MgCl_2$. Eggs were then cultured in NCSU23 + 1.9 mM 6-dimethylaminopurine for 3 h. SCNT and IVF embryos were cultured in NCSU23 for 54 h and subsequently in the same medium with 5.55 mM glucose for 90 h at 38.5°C in 5% CO_2 in air. In Experiment 1, when the rates of development between IVF and SCNT embryos constituted with cells at 5–7 passages were compared, no significant ($P < 0.05$) differences were observed in the cleavage rates. The rates of blastocyst formation were significantly ($P < 0.05$) higher in IVF than in SCNT embryos with pFFF and pAESC (21% vs. 15% and 10%), but it did not differ between SCNT embryos. Total cell numbers in IVF blastocysts (35.4 ± 12) were significantly ($P < 0.05$) higher than in SCNT blastocysts with pFFF and pAESC (28.4 ± 8 and 26.2 ± 10, respectively). The apoptosis signal by TUNEL was initiated at Day 3 in IVF and SCNT embryos. Apoptosis rates in SCNT blastocysts with pFFF and pAESC (13.1 ± 2.5 and 16.6 ± 4.3, respectively) were significantly ($P < 0.05$) higher than in IVF embryos (3.6 ± 1.4). As the embryos developed, the rates of apoptosis were increased. On Day 6, the rates of apoptosis in IVF (4.8%) were significantly ($P < 0.05$) lower than those in SCNT embryos with pFFF (13.1%) and pAESC (16.6%). However, both total cell number and apoptosis in SCNT embryos with pFFF and pAESC revealed no significant differences. In Experiment 2, SCNT embryos with pFFF in different cell passages were compared for the development and apoptosis. No significant ($P < 0.05$) differences were observed in the cleavage rates of SCNT embryos among different cell passages. The rates of blastocyst formation were significantly ($P < 0.05$) higher in SCNT embryos with 5–7 passages than those with other numbers of passages (14% vs. 6–8%, respectively). Although total cell numbers of SCNT blastocysts did not differ among different cell passages, apoptosis rates were significantly ($P < 0.05$) higher when the number of cell passages was increased. These results suggest that fetal fibroblasts at 5–7 passages are ideal nuclear donor cells for obtaining high-quality porcine SCNT embryos.

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42 PRODUCTION OF CLONED PIGS FROM SOMATIC STEM CELLS DERIVED FROM SALIVARY GLAND

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The aim of the present study was to investigate whether a somatic stem cell derived from the salivary gland can be an efficient donor cell for pig cloning. Somatic stem cells (salivary gland progenitor cells, SGP) were isolated from the salivary gland of a 4-month old male pig (Matsumoto *et al*. 2004). Briefly, tissue sections of salivary gland were gently digested by collagenase/hyaluronidase and dispersed into single cells. Isolated cells (5×10^4) were cultured on a type I collagen-coated dish in William's medium E; then colonies having epithelial-like morphology were picked to establish the primary culture of SGP cells (CD49, intracellular laminin-positive) which have the potential to differentiate *in vitro* into hepatic and pancreatic endocrine cells after spherical body formation *in vitro*. SGP cells to be used as nuclear donors were cultured for 2 days under serum starvation. Fetal fibroblast (FF) cells were used as control nuclear donors. IVM oocytes were obtained from abattoir ovaries and matured in NCSU23. Donor cells were fused with the enucleated recipient oocytes by a single DC pulse of 190 V/mm for 10 μs in 0.28 M mannitol + 0.15 mM $MgSO_4$. Reconstructed embryos were electrically activated by DC pulse of 150 V/mm for 100 μs in 0.28 M mannitol + 0.05 mM $CaCl_2$ + 0.1 mM $MgSO_4$ at 1–1.5 h after the NT, followed by cytochalasin B treatment for 3 h. Development of the NT embryos was assessed *in vitro* by fixing and staining at either 2 h post NT or after culture for 7 days in NCSU23, or *in vivo* by transfer to the oviducts of estrus-synchronized recipient gilts. Incidence of premature chromosome condensation was similar regardless of donor cell type. Development of the NT embryos reconstructed with SGP to the blastocyst stage was significantly higher compared to that of the FF group (38/137, 27.7% vs. 19/168, 11.3%, respectively; $P < 0.05$). Transfer of 278 cloned embryos reconstructed with SGP to two recipients resulted in the production of three live piglets. Production efficiency of piglets from the cloned embryos reconstructed with FF was 2/263. Based on the *in vitro* development of the reconstructed embryos, SGP is a promising nuclear donor cell for pig cloning; further transfer experiments are to be carried out.

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43 DNA SYNTHESIS, PREIMPLANTATION DEVELOPMENT AND Oct-4 EXPRESSION OF BOVINE CLONES RECONSTRUCTED WITH OOCYTES PREACTIVATED OR ENUCLEATED AFTER SPINDLE DISASSEMBLY

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Enucleation procedures applied in mammalian cloning remove not only the oocyte's chromosomes but presumably also the spindle-associated factors. If these factors are beneficial for reprogramming, alternative protocols that limit enucleation of factors in addition to the chromosomes may improve cloning efficiency. In this study, we evaluated the enucleation in combination with various activation protocols on clone development and gene expression. Clones produced by nuclear transfer into pre-activated bovine oocytes rather than non-activated oocytes can develop *in vitro* (Kurosaka *et al.* 2002 Biol. Reprod. 67, 643–647; Tani *et al.* 2003 Biol. Reprod. 69, 1890–1894). We produced bovine clones using four different nuclear transfer protocols and, in clones of all groups, examined timing of DNA synthesis in the first cell cycle, pre-implantation development, and gene expression at the blastocyst stage. Protocols applied were: (A) donor cells were fused with non-activated oocytes; (B) donor cells were fused with oocytes at 2 hours after activation with ethanol (7%, 7 min); (C) oocytes were enucleated after spindle disassembly with nocodazole treatment (0.3 μg/mL, 30 min) and donor cells were fused with non-activated oocytes; and (D) oocytes were enucleated after spindle disassembly and donor cells were fused with oocytes at 2 h after activation. Fused couplets in all treatment groups were treated with 10 μg/mL cycloheximide for 6 h, and cultured *in vitro* in SOF supplemented with fetal bovine serum at 39°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The onset of DNA synthesis was determined by an immunofluorescence assay of 5-bromo-deoxyuridine incorporation at 6 and 9 h post-fusion (hpf). Oct-4 mRNA distribution in clone blastocysts was examined by whole mount *in situ* hybridization using a bovine Oct-4-specific antisense riboprobe. Data were statistically analyzed with Student's *t*-test. In the majority of clones DNA synthesis had not commenced 6 hpf but had initiated 9 hpf. Although the cell cycle of activated oocytes (protocols B and D) was 2 hours advanced compared to non-activated oocytes (protocols A and C), clones produced by all protocols had a similar onset of DNA synthesis at 6 to 9 h post-fusion. Developmental rates to the blastocyst stage of clones were not significantly different between the four protocols (48.5%–57.7%, $P < 0.05$). Oct-4 distribution in clones produced by all four protocols was not different from that of IVF embryos used as a control in that Oct-4 mRNA signal was typically restricted to the ICM (87.0%–100.0%, $P < 0.05$). We conclude that in bovine clones produced in this study, nocodazole-treated enucleation and activation status of recipient oocyte did not influence the pre-implantation development and spatial pattern of Oct-4 expression.

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44 IMPROVED DEVELOPMENT OF BOVINE NUCLEAR TRANSFER EMBRYOS BY THE TREATMENT OF NUCLEAR DONOR CELLS WITH APOPTOSIS INHIBITORS DURING SERUM STARVATION

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In somatic cell nuclear transfer, serum starvation is a widely used method to synchronize donor cells at the quiescent stage (Go) of the cell cycle. However, it has been shnown that serum starvation during culture of mammalian cells could induce cell death via apoptosis by removing growth

factors and increasing intracellular stress. Therefore, apoptosis caused by serum starvation in somatic cells could induce damages to nuclear DNA contributing to a lower efficiency of nuclear transfer. This study was performed to characterize apoptosis during serum starvation of bovine embryonic fibroblasts (BEFs) and to determine the effects of BEFs treated with apoptosis inhibitors on the development of bovine embryos after nuclear transfer. BEFs, collected from a fetus with a 3–4-cm crown-rump length, were cultured for 7 days in starvation medium consisting of Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum to induce quiescence. Cells were also placed in starvation medium containing the apoptosis inhibitors, β2-macroglobulin (broad-range protease inhibitor: MAC; 1.4 pM) and glutathione (GSH: reactive oxygen species scavenger; 2.0 mM). Apoptosis of serum starved BEFs with or without apoptosis inhibitors were analyzed morphologically with light and electron microscope, and biochemically using a TUNEL assay. Somatic cell nuclear transfer was performed by our standard procedure as follows. Bovine oocytes were matured *in vitro* and enucleated after 22 h. Nuclear donor cells were collected randomly before injection. The reconstructed embryos were placed into the fusion chamber in a solution containing 0.28 M mannitol and aligned manually. A double pulse of 1.8 kV/cm for 15 μs was used to fuse the cells and activate the embryos simultaneously. The fused embryos were cultured for 4 min in 5 μM ionomycin and 4 h in 2 mM 6-DMAP. Then, embryos were moved to culture media and cultured in 5% CO_2 and 39°C in 100% humidity. Development of NT embryos was recorded at 120 h post NT (morulae) and 168 h (blastocysts) with experiments being repeated three times. Serum starved BEFs showed typical morphology of apoptotic cells such as chromatin condensation and membrane blebbing. Also, when stained for DNA fragmentation by TUNEL assay, 22.6% of BEFs showed apoptosis, in contrast to 0.1% in actively growing cells. However, when BEFs were cultured with MAC and GSH, the proportions of apoptotic BEFs were greatly reduced, 6.0% and 2.1%, respectively. After nuclear transfer with BEFs, embryos reconstructed with BEF treated with apoptosis inhibitors showed significant improvement in *in vitro* development compared to the controls (Table 1). In conclusion, while there are a number of factors affecting the nuclear transfer procedure, damage to the donor nuclei by serum starvation is likely to reduce the efficiency of the procedure; the addition of apoptosis inhibitors could reduce this unnecessary damage to donor nuclei and result in improvement in the development of nuclear transferred embryos. Further experiments are needed to assess the effect of apoptosis inhibitors on improvement of overall nuclear transfer efficiency.

Table 1. Development of bovine embryos nuclear transferred with embryonic fibroblasts treated with or without apoptosis inhibitors

Treatment	No. oocytes	No. fused (%)	No. cleaved (%)	No. developed (%) Morula	Blastocyst
SS	131	90 (68.7)	69 (76.6)	6 (6.7)	14 (15.6)[a]
SS + MAC	150	98 (65.3)	73 (74.5)	6 (6.1)	23 (23.5)[b]
SS + GSH	135	88 (65.2)	69 (78.4)	4 (4.5)	18 (20.5)[b]

SS = serum starved; MAC = 2-macroglobulin; GSH = glutathione.
[a,b] Values with different superscripts within same column differed significantly ($P < 0.05$).

45 ANALYSIS OF DIFFERENTIALLY EXPRESSED PROTEINS IN THE PLACENTA OF SOMATIC CELL CLONED AND ARTIFICIAL INSEMINATION PIG PLACENTA USING PROTEOMICS

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Somatic cell cloning has been hampered by biological and technical problems. A major limitation of cloning procedures is the extreme inefficiency of producing healthy offspring for reasons such as sudden intrauterine unexplained death and infant death syndrome. In this study, we analyzed differentially expressed protein profiles in the placenta of somatic cell cloned (SCNT) and artificial insemination (AI; control) pigs by proteomics. Protein expression pattern of placentas was established in the pH range 4–7, IEF cell system, and 7.5–17.5% gradient 2-dimensional polyacrylamide gel electrophoresis. Image analysis following silver staining used the spot and PDQuest analysis system. Peptide mass fingerprinting with matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and SWISS-PROT database search were utilized to identify proteins. Around 1000 protein spots were recognized by image analysis on each placenta. In SCNT pig placentas, 37 spots were changed compared with those of AI pig placentas. Among them 11 proteins such as serum albumin precursor and annexin A5 showed an increased protein expression level whereas the expression level of 26 other proteins such as aldose reductase and tropomyosin decreased. Annexin A5 and aldose reductase are implicated in the apoptosis process: the former is an apoptotic marker of oxidative stress; on the other hand, the latter is a critical regulator of TNF-α-induced apoptotic signaling in endothelial cells. Also, tropomyosin is implicated in stabilizing cytoskeleton actin filaments. The expression of these proteins was confirmed by western blot and real-time PCR analysis. These results suggest expression of abnormal placental protein and apoptotic-related protein in SCNT pig placentas affects the regulation of placental growth and development.

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46 EFFICIENT TRANSFECTION OF PLASMID DNA INTO CELLS FOR USE AS NUCLEAR DONORS

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Somatic cell nuclear transfer (SCNT) has the potential to significantly improve the production of valuable livestock that produce recombinant proteins, such as pharmaceutical proteins for human disease or biomaterials for medical use. The success of this potential depends on efficient

and optimized protocols for introducing exogenous DNA into cells. In this study, we compared two methods of transfection, Effectene (Qiagen, Inc., Valencia, CA, USA) and electroporation. Plasmid DNA (pEGFF-N1, Clontech, Seoul, Korea) was transfected into fetal fibroblasts (FFB), cumulus cells (CUC), and adult ear skin cells (ESC). Transfection efficiency, chromosome normality, gene expression, and apoptosis were assessed. Cells cultured in α-modified Eagle's medium (α-MEM; BioWhittaker, Walkersville, MD, USA) + 10% FBS were transfected with pEGFP-N1. For electroporation, cells (5×10^6 cells/mL) were mixed in 300 μL perrim buffer (75% Cytosalts with 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 5 mM $MgCl_2$, and 25% α-MEM) + 15 μg pEGFP-N1, and subjected to two pulses of 0.38 kV and 400 μF delivered by Gene Pulser (Bio-Rad; BMS, Ltd., Seoul, South Korea). For Effectene transfection, the procedure suggested by the manufacture was followed. Transfected cells were selected with 600 μg/mL G418 (Gibco; KDR Biotech Co., Ltd., Seoul, South Korea) and cultured at 39°C, 5% CO_2 in air. Assessments of EGFP transfected cells by green fluorescence was carried out under an inverted epifluorescence microscope (Nicon, Kanagawa, Japan) equipped with a filter for FITC (excitation maximum = 488 nm; emission maximum = 507 nm). Differences among the groups were analyzed using one-way ANOVA after arc-sine transformation of proportional data. Most cells (>80%) in confluence were at G0/G1 phase, and transfection of the gene into all three cell types did not affect the incidence of chromosomal abnormality or change morphology. In addition, the rates of apoptosis assessed by TUNEL did not differ in all three cell types by either method of transfection at different cell passages. However, the efficiency of gene transfection into FFB by Effectene reagent ($14.2 \pm 1.7\%$) was significantly ($P < 0.05$) higher than that by electroporation ($5.1 \pm 1.0\%$). Among the three type cells, the efficiency of gene transfection by Effectene and electroporation of FFB (14.2 ± 1.7 and $5.1 \pm 1.0\%$, respectively) was significantly ($P < 0.05$) higher than those of CUC and ESC (9.4 ± 1.5 and 3.3 ± 0.8; 8.8 ± 0.7 and $2.1 \pm 0.4\%$, respectively). In conclusion, although there were no differences in the alteration of chromosomes, cell morphology, and apoptosis among three cell types transfected with or without plasmid DNA, FFB is the most effective cell type to be transfected. Effectene is superior to other currently available methods for introducing plasmid DNA into a variety of cells. The high level of transfection achieved by Effectene will encourage its use as a tool for producing transgenic embryos and animals by SCNT.

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47 CHROMATIN AND CYTOSKELETAL REORGANIZATION OF RABBIT OOCYTES AFTER CUMULUS CELL NUCLEAR TRANSFER

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It has been reported that a preincubation of the reconstructed oocytes prior to activation treatment might enhance the efficiency of nuclear reprogramming after somatic cell nuclear transfer in many species. The objectives of this study were to evaluate chromatin and cytoskeletal reorganization of nuclear-transferred rabbit oocytes before activation treatment. Sexually mature New Zealand White rabbits (6-month-old) were superovulated by six consecutive injections of FSH (0.4 mg) at 12-h intervals, followed by a single hCG (200-IU) injection. Mature oocytes were collected by flushing the oviducts 14–15 h post-hCG treatment. Cumulus cells were introduced into enucleated oocytes by piezo-driven microinjection. The reconstructed oocytes were randomly allocated to two treatment groups, with or without electric pulses (EP, 2 kV cm^{-1}), and the progressive changes of the chromatin and cytoskeleton were examined by immunocytochemical staining at 0, 2, and 4 h after EP. The oocytes ($n = 59$) without successful enucleation served as the control, in which 30 out of 59 oocytes were electrically pulsed. After immunocytochemical staining, the proportions of reconstructed oocytes possessing dense chromatin decreased from 0 to 4 h (40-0% vs. 82-9%, respectively) in both groups, but only the oocytes without EP showed a significant reduction ($P < 0.05$). In contrast, the proportions of reconstructed oocytes with spread chromatin increased significantly from 0 to 4 h in both groups (20–95% vs. 5–82%, respectively; $P < 0.05$). The oocytes at 0 h after EP had the greatest percentage (35%) of normal chromosome alignment on the spindle compared to the other EP groups. The EP treatment also caused significant increase of the cytasters in both the reconstructed (5 to 45%, $P < 0.05$) and the control oocytes (20 to 78%) at 0 h. These changes were recovered at 2 h after EP, but the proportions of cytasters in the reconstructed oocytes increased to 59% in the non-EP group and 81% in EP group at 4 h after EP. The percentages of reconstructed oocytes with multiple spindles increased from 0 to 27% and 43% in EP and non-EP groups, respectively. These results demonstrated that EP changed the proportions of chromatin configurations from the condensed form to normal alignment on the metaphase plate. The extended preincubation period for the reconstructed oocytes prior to activation treatment might increase the incidences of chromatin and spindle abnormalities of the reconstructed rabbit embryos.

48 PREGNANCY AND FETAL CHARACTERISTICS AFTER TRANSFER OF VITRIFIED *IN VIVO* AND CLONED BOVINE EMBRYOS

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This study was conducted to examine pregnancy progression and fetal characteristics following transfer of vitrified bovine nuclear transfer (NT) vs. *in vivo*-derived (Vivo) embryos. Nuclear transfer was conducted using skin fibroblast cells derived from cultured ear explants taken from an elite Holstein-Friesian dairy cow. Expanding and hatching blastocysts on Day 7 were vitrified using liquid nitrogen surface vitrification (Shen *et al*. 2004 Reprod. Fertil. Dev. 16, 158). Day 7 *in vivo* embryos, produced using standard superovulation procedures applied to Holstein-Friesian heifers ($n = 6$), were vitrified in the same way. Following warming, embryos were transferred to synchronized recipients (2 per recipient; NT: $n = 81$ recipients; Vivo: $n = 20$ recipients). Pregnancies were monitored by ultrasound scanning on Days 25, 45, and 75, and a sample of animals was slaughtered at each time point to recover the fetus/placenta for further analyses. On Day 25, the entire conceptus was weighed together; on Day 45, the fetus and

placenta were weighed separately; on Day 75, the fetus was dissected and the major organs were weighed. Significantly more animals remained pregnant after transfer of *in vivo*-derived embryos than NT embryos at all time points: Day 25 (95.0 vs. 61.7%, $P < 0.01$), Day 45 (93.3 vs. 39.7%, $P < 0.001$), and Day 75 (90.9 vs. 19.8%, $P < 0.001$). There was no significant difference ($P = 0.10$) in the weight of the conceptus on Day 25 from NT transfers (1.14 ± 0.23 g, $n = 8$) vs. Vivo transfers (0.75 ± 0.19 g, $n = 8$). On Day 45, there was no significant difference in the weight of either fetus ($P = 0.393$) or membranes ($P = 0.167$) between NT embryos (fetus: 2.76 ± 0.40 g, $n = 12$; membranes 59.0 ± 10.0 g, $n = 11$) or *in vivo*-derived embryos (fetus: 2.60 ± 0.15 g, $n = 6$; membranes 41.8 ± 5.2 g, $n = 4$). However, on Day 75, the weights of the fetus and several of the major organs were heavier from NT embryos (Table 1). These data suggest that the large calf syndrome is manifested after Day 45.

Table 1. Fetal characteristics on Day 75 of gestation following transfer of vitrified *in vivo*-derived vs. NT blastocysts

Organ weight (g)	*in vivo* ($n = 8$)	NT ($n = 7$)	*P* value
Fetus (g)	69.9 ± 4.2	83.2 ± 5.6	0.036
Placenta (g)	207.1 ± 39.8	242.7 ± 30.1	0.244
No. of cotyledons	48.3 ± 4.3	58.3 ± 3.8	0.054
Lungs (g)	2.06 ± 0.24	2.98 ± 0.21	0.006
Liver (g)	2.88 ± 0.19	3.64 ± 0.41	0.051
Kidneys (pair) (g)	0.75 ± 0.05	0.81 ± 0.08	0.281
Spleen (g)	0.03 ± 0.01	0.12 ± 0.06	0.065
Heart (g)	0.60 ± 0.04	0.84 ± 0.11	0.028
Brain (g)	2.52 ± 0.17	2.55 ± 0.08	0.437

Analyzed with Student's *t*-test.

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49 EFFECT OF REPEATED CELL FREEZINGS ON PREGNANCY RATE OF BOVINE NUCLEAR TRANSFER DERIVED EMBRYOS

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Cell line cryopreservation is nowadays one of the most useful tools in somatic cell nuclear transfer. Although this technique guarantees the genetic storage for an unlimited period of time, many studies have shown that it produces different kinds of cellular damage such as DNA fragmentation (Men *et al*. 2003 Mol. Reprod. Dev. 64, 245–250) and ultrastructural cell anomalies (Taddei *et al*. 2001 Cryobiology 42, 244–555; Nardid *et al*. 1997 Cryobiology 34, 107–113). The aim of the present study was to evaluate how repeated cell freezing/thawing processes could affect the pregnancy rate of bovine nuclear transfer-derived embryos. Two adult fibroblast cell lines from different animals were separated into two groups according to the number of freezing/thawing processes they went through (1 vs. 3). For both groups, the first freezing process was performed with cells from passage 1. Cells from passages 3 and 4 were used for the second and third freezings, respectively. The time interval between thawing and next freezing was 20 days. Cells were harvested at 80% confluence using trypsin, and cryopreservation was performed in D-MEM with 35% FCS and 10% DMSO. Enucleation and nuclear transfer (NT) were performed as described by Cibelli *et al*. (1998 Science 280, 1256–1258) with modifications. For both groups, cells from the same number of passages were used for the NT assays (between passages 2 and 7). Cytoplasts were activated using 5 μM ionomycin for 4 min and the couplets were subsequently fused. The fused units were cultured in 10 μg/mL cycloheximide and 5 μg/mL cytochalasin B for 6 h. Embryo culture was performed at 38.5°C in a 5% O_2, 5% CO_2, 90% N_2 atmosphere, in 50-μL drops of KSOM. On Day 3 of culture, the KSOM was supplemented with 2% FCS and 0.2 mM glucose. After 6–7 days, the embryos were non surgically transferred to synchronized recipients. Pregnancy at 30 and 60 days was recorded by ultrasonography using an Aloka 500® scanner (Aloka Co., Tokyo, Japan). Data were analyzed by ANOVA (InfoStat, Austin, TX, USA) (Table 1). The results show an association between the number of cell freezing/thawing processes and a higher pregnancy loss at 60 days. This could be related to the cellular damages caused by multiple cryopreservation procedures, which could lead to chromosomal abnormalities in the donor cells and thus in the nuclear transfer (NT) embryos and pregnancies derived from them. Further studies should be done in order to evaluate the chromosomal status of the cell lines used in this work.

Table 1. Numbers of cell freezings/thawings and their effect on pregnancy rate of bovine NT-derived embryos

No. of cell freezings/ thawings	NT assays (*g*)	NT units (*g*)	Fused/ NT units (%)	Blastocysts/ fused (%)	Pregnancy 30 days/embryos transferred (%)	Pregnancy 60 days/embryos transferred (%)
1	15	770	492/770 (64[a])	137/492 (28[a])	36/99 (36[a])	25/99 (25[a])
3	6	279	204/279 (73[a])	78/204 (38[a])	30/61 (49[a])	2/61 (3[b])

[a,b] Values with different superscripts within a column are significantly different (ANOVA, $P < 0.05$).

50 INVESTIGATION OF THE EFFECT OF BUTYROLACTONE I AND CYCLOHEXIMIDE TREATMENT DURING *IN VITRO* MATURATION OF SWINE OOCYTES

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Butyrolactone I and cycloheximide specifically inhibit MPF activation and prevent the resumption of meiosis. The aim of this study was to investigate the kinetics of *in vitro* maturation of butyrolactone I- and cycloheximide-treated swine oocytes in an attempt to produce cytoplasts for nuclear transfer. Oocytes from slaughterhouse ovaries were randomly allotted to one of 3 treatments; group 1 ($n = 102$ – control – 22 hours of *in vitro* maturation in TCM199 supplemented with 3.05 mM glucose, 0.91 mM sodium pyruvate, 10% follicular fluid, 0.57 mM cysteine, 10 ng/mL EGF, 10 IU/mL eCG and 10 IU/mL of hCG and 22 hours of culture); Group 2 ($n = 191$ – blocking for 10 hours in 12.5 M butyrolactone I and *in vitro* maturation for 44 hours); and Group 3 ($n = 175$ – blocking for 10 hours in 5 M cyclohexemide and *in vitro* maturation for 44 hours). After *in vitro* maturation, oocytes were fixed and stained for evaluation of meiotic division. The percentage of oocytes at metaphase II (MII) in Groups 1 and 3 (75.49% and 70.29%, respectively) were higher ($P < 0.05$) than Group 2 (63.35%). Based on these results and in order to increase enucleation rates, we also investigated the proximity of the first polar body (PB) with the metaphase plate (MP) in Groups 1 and 3. After *in vitro* maturation (36, 40, and 44 hours), oocytes were gently decumulated and incubated in microdroplets (50-μL) of bisbenzimide solution (5 g/mL) to analyze the MP and PB positions. Group 1 (control) at 44 hours of maturation (47.05% – 48/102) and Group 3 at 40, and 44 hours (60.20% – 59/98 and 55.46% – 61/110, respectively) showed similar rates, that were higher ($P < 0.05$) than Group 1 at 36 hours and 40 hours (4% – 4/100 and 36% – 36/100, respectively) and Group 3 at 36 hours (34.58% – 37/107). In conclusion, Group 1 at 44 hours and Group 3 at 40 or 44 hours provide the best oocytes for enucleation because they showed a high number of matured oocytes with the first polar body and the metaphase plate located proximally.

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51 PHENOTYPIC VARIATION IN CLONED SWINE IS CORRECTED IN THE F1 GENERATION

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Systematic studies of cloned animals generated from adult somatic cell nuclei are critical in assessing the utility of somatic cell cloning in various applications, including the safety of food products from cloned animals and their offspring. Studies in mice show that abnormalities seen in the cloned parents are not transmitted to the siblings. To our knowledge, however, there are no studies on the F1 progeny of clones from food animals. Previously, we compared somatic cell derived cloned pigs with naturally bred control pigs on a series of physiological and genetic parameters. Phenotypic and genetic analyses indicated that there are two classes of traits, one in which the cloned pigs have less variation than controls and another characterized by variation that is equally high in cloned and control pigs. We have extended our studies to the F1 progeny of these clones to see whether these phenotypic differences are transmitted to the next generation. Age-, sex-, and breed-matched cloned and control pigs, housed together since weaning, were used in this study. Starting with their second estrus cycle, standing gilts were mated two consecutive days. All gilts were mated to the same boar. Pregnant cloned ($n = 9$) and naturally bred ($n = 5$) gilts (F0) were allowed to farrow naturally, and number and sex of live offspring at birth (F1) recorded. There was no difference in the average litter size between litters from cloned gilts and naturally bred controls (7.78 ± 2.6 and 7.40 ± 3.0, respectively; mean ± SD) or in the degree of litter size variation (coefficients of variation of 33.4% and 40.5% for litters of clones and controls, respectively). Similarly there were no statistical differences between sex ratios from cloned litters (51%:49%; M:F) and control litters (59%:41%; M:F). Blood profiles among cloned pigs, control pigs, and their progeny were compared at two time points, i.e. 15 and 27 weeks, to quantify the effect of cloning on various blood parameters and their transmission to next generation. Although the range of values for all traits overlapped between different classes, the variation differed between F0 clones and F0 controls. In the clones there were two groups of traits: one in which cloned pigs had less variability than controls, and the other in which clones had the same variability as control pigs. In contrast, the variability between all of the traits in F1 progeny of both the clones and the control pigs was similar at 15 and 27 weeks, with one exception. Combined, our data and previous results in mice strongly support the hypothesis that offspring of clones are to all intent and purposes indistinguishable from offspring of naturally bred animals, and as such there should not be any increased risks associated with consumption of products from these animals.

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52 NUCLEAR REMODELING AFTER SOMATIC CELL NUCLEAR TRANSFER (SCNT) IN THE RHESUS MONKEY

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Successful reprogramming of somatic cell nuclei after nuclear transfer requires active remodeling by factors present in the nonactivated cytoplast. High levels of maturation promoting factor (MPF) activity are associated with this remodeling process which includes nuclear envelope breakdown (NEBD), premature chromosome condensation (PCC), and spindle formation. MPF degradation, caused by fertilization or artificial activation, is in turn required for pronuclear formation and subsequent embryonic cleavage, and involves cyclin B catalyzed proteolysis by the proteasome system.

In the rhesus monkey, SCNT results in the production of cleaving embryos, but development arrests at the morula stage presumably because of abnormal or incomplete reprogramming. We undertook this pilot study to examine the timing and extent of nuclear remodeling events (NEBD, PCC) in monkey SCNT embryos. The proteasome inhibitor MG-132 was employed to avoid or delay premature MPF degradation (Zhou *et al*. 2003 Science 302, 1179). Monkey fetal fibroblasts employed as nuclear donor cells were fused with nonactivated cytoplasts and incubated in the presence ($n = 20$) or absence (control; $n = 35$) of MG-132. Embryos were fixed and co-labeled with DAPI (DNA) and monoclonal antibody against lamin A/C (nuclear envelope). In monkey germinal vesicle-stage oocytes ($n = 5$) and zygotes ($n = 6$), a lamin A/C signal was detected at the nuclear periphery while matured MI ($n = 6$) and MII ($n = 12$) oocytes were negative for lamin A/C staining, consistent with the absence of a nuclear membrane. Donor fetal fibroblasts arrested at the G1 stage of the cell cycle exhibited a lamin A/C signal. Minimal or no changes were observed in donor nuclei within 1 h after fusion. The majority of control SCNT embryos sampled 4 h after fusion exhibited only slight chromatin condensation; however, they failed to form metaphase chromosomes. Positive lamin A/C staining indicated the presence of intact nuclear membranes. Following activation these SCNT embryos cleaved, but arrested at the 8–16 cell stage. In initial experimentation we determined the minimal efficient concentration of MG-132 to be 5 μM, that is capable of inhibiting first polar body extrusion during the MI–MII transition. Subsequent *in vitro* development to the blastocyst stage (53%) of fertilized oocytes treated with 5 μM MG-132 for up to 4 h was similar to that of nontreated controls. Incubation of SCNT embryos ($n = 20$) for 4 h with MG-132 resulted in robust chromosome condensation, spindle formation, and weak or partial lamin A/C signal. Our observations suggest that incomplete nuclear remodeling events in monkey SCNT embryos may be due to premature MPF inactivation perhaps caused by the fusion pulse. Future studies will address the developmental potential of monkey SCNT embryos exposed to MG-132.

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53 CELL CYCLE SYNCHRONIZATION OF DONOR CELLS AT G1 PHASE AND DEVELOPMENTAL ABILITY OF NUCLEAR TRANSFER EMBRYOS IN MINIATURE PIGS

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The cell cycle of donor cells is one of the essential factors for the success of somatic cell nuclear transfer (SCNT), and G0/G1-phase cells have been widely used as donor cells. However, cells synchronized at the G0/G1-phase also have a population of cells with cell cycles other than G0/G1-phase, and we cannot precisely know the cell cycle of donor cells being used for SCNT. In this experiment, we reconstructed SCNT embryos from donor cells that were synchronized at the G1-phase or at the G0/G1-phase and compared their developmental ability in two different culture media. Immature oocytes were isolated from ovaries collected from domestic gilts at a local slaughterhouse and were co-cultured with follicle shells for *in vitro* maturation (Hoshino *et al*. 2003 Theriogenology 59, 260). Donor cells were collected from fibroblast cells of miniature Potbelly pigs. Cells synchronized at the G1-phase were prepared shortly after dividing M-phase cells that had been synchronized using 2-methoxyestradiol, as described by Urakawa *et al*. (2004 Theriogenology 62, 714–728). The G0/G1 cells were also prepared from a fully confluent culture of cells. Donor cells were fused with enucleated oocytes and simultaneously activated by two electric pulses. Reconstructed embryos were cultured in two different media [Whitten and Biggers medium supplemented with 0.5 mg/mL hyaluronic acid sodium salt (WM) and porcine zygote medium-3 (PZM-3, Yoshioka *et al*. 2002 Biol. Reprod. 66, 112–119)] under 5% CO_2 in air. Cleavage rate and development rate to the blastocyst stage were assessed after 48 and 168 hr of culture, respectively. The results are summarized in Table 1. Developmental rate to the blastocyst stage of SCNT embryos reconstructed from G1 cells and cultured in PZM-3 (40%) was significantly higher than that of embryos cultured in WM (25%). The SCNT embryos of the G1 cells showed significantly lower cleavage rate (51%) than that of the G0/G1 cells (69%). However, the developmental rates to the blastocyst stage per cleaved embryo in WM were significantly higher in G1 cells (50%) compared with G0/G1 cells (32%). In addition, the total cell number of the SCNT blastocysts was comparable between the cultures in WM (58 ± 4) and PZM-3 (46 ± 5), although the ratio of inner cell mass cells to the total cell number was significantly higher in PZM-3 (32%) compared with WM (14%). These results suggest that PZM-3 may fit with the culture of SCNT embryos, and that the G1 synchronized cells could be stably reprogrammed for early embryonic development in SCNT embryos and be useful as donor cells for analyzing the processes of nuclear reprogramming.

Table 1. Development of nuclear transfer embryos reconstructed from G1 or G0/G1 cells in different culture media

Cell cycle of donor cells	Medium	No. trials	No. embryos examined	No. embryos cleaved (%)	No. embryos to blastocysts (%)	% of blastocysts/ cleaved embryos
G1	WM	6	85	45 (51)	24 (25)	50[b]
	PZM-3	3	45	26 (67)*	22 (40)*	60
G0/G1	WM	6	88	61 (69)[a]	19 (22)	32
	PZM-3	3	37	26 (70)	11 (30)	44

[a,b] Values significantly differ within the same medium and
* Values significantly differ within the same donor cells ($P < 0.05$).

54 COMPOSITION OF ALLANTOIC FLUID IN CATTLE PREGNANT WITH AI-, IVP-, OR NUCLEAR TRANSFER-GENERATED EMBRYOS

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Abnormal placentation, pregnancy failure, and hydroallantois are associated with somatic cell nuclear transfer (SCNT) in cattle. Identification of diagnostic markers for abnormal placentation in early gestation would permit therapeutic intervention. Ultrasonography and transvaginal sampling of amniotic and/or allantoic (fetal) fluid enables regular monitoring of fetal health. We report on the composition of serial samples of fetal fluid from individual cows between Days 70–130 of gestation and the potential of steroid and electrolyte composition as an early diagnostic marker for the subsequent occurrence of hydroallantois in SCNT pregnancies in cattle. On Day 70, pregnancy rates were 50% and 60% for cows or heifers implanted with single *in vitro*-fertilized (IVP, 20/40) or SCNT (25/42) embryos, respectively, and 67% for pregnancies generated by artificial insemination (AI, 12/18). Resulting fetuses were either clones (SCNT) or offspring (IVP/AI) of a donor Holstein bull. Fetal fluids, sampled using ultrasound-guided transvaginal puncture, were collected on Days 70, 100, and 130 of gestation ($n = 12$ and 139 for amniotic and allantoic samples, respectively). Placental and fetal morphological data were collected following slaughter between Days 135–163 of gestation ($n = 14$, 20, and 10 for SCNT, IVP, and AI groups, respectively). Fetal fluids were analyzed for progesterone, estrone sulphate, sodium, chloride, potassium, creatinine, urea, calcium, magnesium and phosphate. Pregnancy outcomes for the SCNT group were retrospectively classified as: Fail 100 (pregnancies failing between Days 70–99; $n = 6$); Fail 130 (failing between Days 100–129; $n = 5$); Hydrops (greater than 10 L combined amniotic and allantoic fluid at postmortem between Days 135–163; $n = 8$) and SCNT Pregnant 150 (pregnant between Days 135–163; $n = 6$). IVP and AI pregnancies were classified as IVP or AI Pregnant 150. Fluid composition was analyzed by ANOVA on log-transformed data. On Day 70, allantoic progesterone and estrone sulphate concentrations were significantly higher ($P < 0.05$) for the SCNT cows compared to the IVP/AI Pregnant 150 cows. On Day 70, allantoic potassium, chloride, creatinine, and urea concentrations were significantly higher ($P < 0.05$) for the SCNT Hydrops cows compared to the IVP/AI Pregnant 150 cows. In addition, Day 70 allantoic creatinine and urea concentrations were significantly higher ($P < 0.05$) for the SCNT Hydrops cows compared to other SCNT groups. By Day 100, allantoic chloride, creatinine, and urea concentrations in SCNT Hydrops cows were significantly lower ($P < 0.05$) than in IVP/AI Pregnant 150 groups. We conclude that elevated Day 70 allantoic urea and creatinine concentrations are potential early diagnostic markers predicting hydroallantois in recipient cattle carrying SCNT fetuses. Further investigation of these markers in other somatic donor cell lines used for nuclear transfer is warranted to determine their general utility.

55 NUCLEUS CHANGES AND DEVELOPMENT OF PORCINE RECONSTRUCTED (NT) AND PARTHOGENETICALLY ACTIVATED (PA) EMBRYOS

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Nuclear reprogramming is characterized by functional modification(s) of the transferred nucleus that allows it to direct normal embryo development with the potential to grow to term. The aim of our study was to investigate the process of nuclear changes in reconstructed and activated embryos as well as their developmental competence. All chemicals used were from Sigma Chemicals (St. Louis, MO, USA). Cumulus-oocyte complexes were aspirated from slaughterhouse ovaries of prepurbetal gilts and matured for 42 h *in vitro*. The cumulus cells were removed by adding in 1 mg mL $^{-1}$ hyaluronidase in TLP-HEPES. For the NT experiment, oocytes with first polar body were cultured in 0.4 μg mL^{-1} demecolcine for 1 h. A protruding membrane was removed by micromanipulator and a single donor nucleus from fetal fibroblast was injected subzonally. Fusion was conducted immediately after transfer in 0.3 M mannitol, 0.5 mM HEPES, 0.1% PVA, and 0.1 mM $MgCl_2$ in a fusion chamber with parallel electrodes set 1 mm apart using a singe DC pulse of 125 V mm^{-1} for 80 s. Activation was done 2–4 h after fusion in the same medium as fusion but with 0.1 mM $CaCl_2$ added; embryos were cultured in 5 μg mL^{-1} cytochalasin B and 10 μg mL^{-1} cyclohexamide for 6 h. The embryos were cultured in glucose-free NCSU-37 containing 4 mg mL^{-1} BSA as basic medium supplemented with 0.17 mM sodium pyruvate and 2.73 mM sodium lactate from Days 0 to 2, and then in basic medium with 5.55 mM D-glucose from Days 2–6 (Kikuchi K *et al*. 2002 Biol. Reprod. 66, 1033–1041). Non-manipulated oocytes (PA) were electrically activated as stated above. For observing the changes of donor cells, some reconstructed oocytes were fixed 2 h after fusion, prior to activation, and some 12 h after activation in acetic acid:ethanol (1:3) and stained in 1% orcein. The activated oocytes were fixed at 12 h and stained as stated above. There were 47.5% (38/80) of reconstructed oocytes with premature chromosome condensation (PCC), and 23.7% (19/80) with nuclear swelling two hours after fusion. Pronuclear like formation 12 h after activation was 45% (27/60) and 83.3% (50/60) in NT and PA, respectively. The blastocyst rate was 8.3% (5/60) and 46% (69/150) for NT and PA, respectively. The results suggest that porcine oocyte cytoplasm can successfully reprogram somatic cell nuclei and support the development of NT embryos to the blastocyst stage.

56 SOMATIC CELL NUCLEAR TRANSFER IN NON-HUMAN PRIMATES: THE POSSIBILITY OF USING OOCYTES MATURED *IN VITRO* FOR UP TO 3 DAYS AS HOST OOPLASTS

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Production of cloned nonhuman primate embryos has been reported using mature oocytes obtained from donors treated *in vivo* with a high dose of recombinant human FSH (r-hFSH, 35 IU per day for 10 days). The disadvantages of this approach are the high cost of hormones and the need to use

the oocytes shortly after collection. Our study aimed to investigate the possibility of using initial *in vivo* treatment with a reduced FSH dose followed by *in vitro* culture for long periods of up to 3 days to produce mature monkey oocytes as host ooplasts for somatic cell nuclear transfer (SCNT). Adult female long-tailed Macaque (*Macaca fascicularis*) monkeys were treated with r-hFSH (Serono, Aubonne, Switzerland, 35 IU per day, i.m.) either for 10 days with an injection of hCG (1000 IU, i/m) 34 h before oocyte collection (G.I) or with only r-hFSH for 7 days (G.II). Cumulus oocyte complexes (COCs) were collected by follicular aspiration and then cultured in TCM-199 medium (GIBCO) supplemented with estradiol-17β, FSH, LH, and 10% FCS at 39°C in an incubator with 5% CO_2 in air. The maturation rate based on the level of cumulus expansion and the presence of the first polar body was recorded at the moment of collection and during 24 h, 48 h, and 72 h of *in vitro* maturation (IVM). For SCNT, the mature Metaphase II oocytes were separated from cumulus cells and selected for enucleation in the presence of cytochalasin B (Sigma, St. Louis, MO, USA). Skin fibroblasts obtained from adult monkeys were cultured in DMEM+ 10% FCS and induced to quiescence in DMEM 0% FCS 2 days before use. A single cell was transferred under the zona of each enucleated oocyte. Couplets were fused with two direct current (DC) pulses of 220 V/mm for 25 μs in Zimmerman medium. Fused oocytes were cultured in medium containing cyclohexamide for 6 h before placing them into monkey culture medium (Cook, Brisbane, Australia). The average number of oocytes collected per animal were 21.2 ($n = 18$) and 18.6 ($n = 12$) for the G.I and G.II treatments, respectively. For G.I, the rate of COCs with fully expanded cumulus was 42% at collection and was maximal (80%) at Day 1 of IVM. For G.II, fully expanded cumulus was not observed at the time of collection and during the first 2 days of IVM, but 75% of COCs had full cumulus expansion by Day 3 of IVM. The rates of intact and fused oocytes were 50.3% for G.I and 55.4% for G.II. From the fused oocytes, 67.8% and 64.4% developed to the 4- to 8-cell stages at Days 2–3 after nuclear transfer for G.I and G.II, respectively. From these data, it can be concluded that this approach can be applied to optimize production of mature oocytes for non-human primate SCNT and ART (assisted reproductive technologies) programs.

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57 INVESTIGATION OF CYNOMOLGUS MONKEY (*MACACA FASCICULARIS*) FETUS FIBROBLAST CELL NUCLEAR TRANSFER

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Use of nuclear transfer (NT) in the cynomolgus monkey to establish tailor-made ES cells with the final goal of cloned embryo production was investigated. Activation stimulus conditions previously confirmed in parthenote production were used. Injection method NT was conducted using cynomolgus monkey fetus fibroblast cells in order to investigated the time it takes, from injection to activation, to reprogram the donor nuclei. Oocytes were collected under laparoscopic observation from mature cynomolgus monkeys 40 h after hCG (400 IU/kg) administration 9 days after follicle stimulation by i.m. injection of FSH (25 IU/kg). Donor cells, 40-day-old fetus fibroblast cells, were cultivated and synchronized at G0/G1 phase. After mature (MII) oocytes were enucleated using a Piezo-drive unit, donor cells were injected. At 2 h (Experiment 1, E1) and 4 h (Experiment 2, E2) after donor cell injection, activation was carried out by 2-min treatment with ionomycin and cultivation by 6-dimethylaminopurine for 4 h. As a control, parthenote production was carried out under the same activation conditions as NT. After activation, *in vitro* culture was carried out for about 9 days under conditions of 38°C, 5% CO_2, and 5% O_2. Whole-mount specimens of NT embryos were made immediately post-injection, 2 and 4 h post-injection, and 2 h after activation. Pronuclear formation (PN) and cleavage rates of NT embryos were 82.1% and 95.7% for E1, and 53.8% and 92.8% for E2, respectively. Control PN and cleavage rates were each 100%. Subsequent embryo development arrested at the 6-cell stage (8.7%) in E1 and 5-cell stage (7.1%) in E2 but proceeded to blastocyst stage (27.3%) in the control. For whole mount specimens, donor nuclei caused premature chromosome condensation in enucleated oocyte cytoplasm, and decondensation due to activation was seen, so injected donor nuclei reconstruction had occurred. No difference was seen between E2 and E1 embryo development and whole mount specimens, but E1 PN rate was clearly higher than that of E2. So 2 h of reprogramming time is more appropriate than 4 h. In this study, most NT embryos arrested at the 4-cell stage. These results suggest that development did not proceed beyond MET (maternal-embryonic transition) which is believed to occur between the 4- and 8-cell stage in cynomolgus monkey. Further study will be necessary to find the condition that completely reprograms injected donor nuclei for cloned embryo production.

Table 1. Development of cynomolgus monkey fibroblast nuclear transfer embryos

Experiment	No. oocytes	Reprogramming time (hours)	Pronuclear formation (%)	Cleaved (%)	6-cell (%)	Blastocyst (%)
E1	28	2	23/28 (82.1)	22/23 (95.7)	2/23 (8.7)	0/23 (0)
E2	26	4	14/26 (53.8)	13/14 (92.8)	0/14 (0)	0/14 (0)
Parthenogenesis	11	–	11/11 (100)	11/11 (100)	11/11 (100)	3/11 (27.3)

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58 HANDMADE CLONING IN TRANS-SPECIES NT: CULTURE MEDIUM HAS AN EFFECT ON THE ABILITY OF RECONSTRUCTED BOVINE-MURINE EMBRYOS TO DEVELOP BEYOND THE 8-CELL STAGE

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The objectives of studies of trans-species nuclear transfer (NT) include epigenetic reprogramming and stem cell technology. The present study evaluated the effect of culture media on the development of reconstructed bovine-murine embryos. Bovine NT served as a technical control. The NT embryos were produced with the hand made cloning (HMC) technique (Vajta G *et al*. 2003 Biol. Reprod. 68, 571–578), and to our knowledge, this is the first report on the application of HMC in trans-species NT. Abattoir-derived bovine oocytes were matured for 21 h and enucleated by hand as described (Vajta G *et al*. 2003). Bovine cytoplasts were fused with either bovine granulosa cells or murine fetal fibroblasts. The bovine NT embryos were cultured for 7 days in modified SOFaaci (Holm P *et al*. 1999 Theriogenology 52, 683–700) containing either 5% FBS (8 trials) or 4 mg mL^{-1} fatty acid free albumin (FAFBSA, 6 trials). Bovine-murine NT embryos were cultured for 4.5 days in SOFaaci + FAFBSA (5 trials), or for the first 12 h in SOFaaci + FAFBSA and until 4.5 days in KSOMaa (Biggers JD 1991 J. Reprod. Fertil. 91, 543) containing 1 mg mL^{-1} embryo-tested BSA (4 trials). The results are shown in Table 1. In bovine NT embryos, both cleavage (day 2) and day 7 blastocyst rates were significantly improved was SOFaaci + FAFBSA was used as culture medium. Culture medium did not affect the cleavage rate of bovine-murine NT embryos at 12–16 h after start of culture. The development of reconstructed bovine-murine embryos beyond the 8-cell stage was significantly improved when SOFaaci + FAFBSA was replaced with KSOMaa + BSA after 12 h culture. Fourteen of a total of 464 (3.0%) Day 4.5 bovine-murine reconstructed embryos reached early morula stage with signs of compaction. The study showed that the development of the reconstructed NT embryos was significantly affected by the culture medium. Contrary to earlier findings (Park SH *et al*. 2004 Mol. Reprod. Dev. 68, 25–34), the bovine-murine reconstructed embryos developed beyond 8-cell stage, even until early compaction. The gene expression of species-specific and development-related genes of the reconstructed embryos is under characterization.

Table 1. Effect of culture medium on cleavage and development of reconstructed NTt embryos

Culture medium (cultured)	Donor cell	Cleaved (%)	Developed embryos (%) 2–8 cells	>8 cells	Blastocysts
SOFaaci + 5% FBS ($n = 201$)	bovine	154 (77.2)[a]	107 (54.1)[a]	27 (13.5)[a]	20 (10.1)[a]
SOFaaci + FAFBSA ($n = 138$)	bovine	129 (94.0)[b]	63 (55.4)[a]	16 (15.3)[a]	26 (23.6)[b]
SOFaaci + FAFBSA ($n = 215$)	mouse	178 (84.2)[a]	148 (69.8)[a]	30 (14.4)[a]	0
SOFaaci/KSOMaa ($n = 249$)	mouse	232 (93.2)[a]	146 (59.0)[a]	86 (34.2)[b]	0

[a,b] $P < 0.05$, Student's *t*-test.

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59 A PRELIMINARY STUDY OF THE *IN VITRO* DEVELOPMENT OF ASIAN ELEPHANT, CLONED EMBRYOS, RECONSTRUCTED USING A RABBIT RECIPIENT OOCYTE

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Interspecies nuclear transfer is an important tool for studying the interaction between the cytoplasm of one cell and the donor nucleus of another (Chen *et al*. 2002 Biol. Reprod. 67, 637–642). The aim of this experiment was to investigate the possibility of developing *in vitro* an asian elephant cloned embryo using a rabbit recipient oocyte. The elephant donor cells were obtained from the ear skin of a stillborn Asian elephant (*Elephus maximus*) and the *in vivo*-matured recipient oocytes were obtained from FSH-stimulated New Zealand White doe rabbits. Enucleation was accomplished by aspiration of the first polar body and the metaphase II plate together with a small amount of cytoplasm. Successful enucleation was confirmed by UV examination after staining with 5 μg mL^{-1} Hoechst 33342. The donor cells were introduced into the perivitelline space of the enucleated oocytes immediately after enucleation. The elephant-rabbit reconstructed embryos were fused in 0.3 M manitol with 0.1 mM Ca^{2+} and Mg^{2+} using two types of electrical pulses: E1 ($n = 61$): 3.2 kV/cm, 3 pulses, 20 μs (Chesne *et al*. 2002 Nat. Biotechnol. 20, 366–369); E2 ($n = 69$): 2.0 kV/cm, 2 pulses, 20 μs (Chen *et al*. 2002 Biol. Reprod. 67, 637–642). The fused embryos were activated 1 h after fusion by electrical pulses to those used for fusion and then incubated in 5 μg mL^{-1} cyclohexamide and 2 mM 6-DMAP for 1 h. Subsequently, the activated embryos were cultured in B2 medium containing 2.5% fetal calf serum. The developmental rate was observed every 24 h for 7 days and the differences in the percentages of embryos developing to a particular stage were determined by chi-square analysis. The results showed that the fusion and cleavage rates of elephant-rabbit cloned embryos fused and activated by E1 were significantly higher than for E2 ($P < 0.05$; see Table 1). Compared with rabbit-rabbit cloned embryos using adult skin fibroblast as a donor cell and E1 for both fusion and electrical activation, we found that the cleavage and blastocyst rates of elephant-rabbit cloned embryos was higher than for the rabbit-rabbit ones (65% (28/43) versus 58% (28/48) and 7% (3/43) versus 4% (2/48) respectively). Results from this study showed that either of the electrical pulses, 3.2 kV/cm, 3 pulses, 20 μs or 2.0 kV/cm, 2 pulses, 20 μs, can be used to fuse elephant somatic cells to rabbit ooplasm and the rabbit oocytes can be served as recipient oocytes to support the development of elephant cloned embryos up to the blastocyst stage.

Table 1. Developmental rate of elephant–rabbit cloned embryos after being fused by different electrical pulses

Electrical pulses	Reconstructed *n*	Fused/Culture *n* (%)	Cleavage *n* (%)	Blastocyst *n* (%)
E1	61	43 (70.5[a])	28 (65[a])	3 (7[a])
E2	69	36 (52.2[b])	17 (47[b])	1 (3[a])

[a,b] Values with different superscripts within the same column differ significantly ($P < 0.05$).

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60 HISTOLOGICAL COMPARISONS BETWEEN NUCLEAR TRANSFER AND *IN VIVO* PORCINE EMBRYOS

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Due to the high rate of embryonic loss during a nuclear transfer pregnancy, cloning is considered a relatively inefficient process. However, as the only method of producing knockout domestic animals it is considered an invaluable tool for the biotechnical industry. By histologically comparing embryos at significant stages in the porcine pregnancy (Days 10, 12, and 14), factors contributing to embryonic loss may be revealed. Many consider the period between days 10 and 14 to be critical for determining survivability as the embryos must undergo rapid changes to signal for maternal recognition of pregnancy as well as adapt to a changing environment. This study included three gilts per stage of pregnancy and four different experimental groups for each stage studied: nonpregnant animals, *in vivo*-pregnant animals, nuclear transfer (NT) recipients, and *in vitro*-manipulated recipients (IVM). IVM embryos were *in vitro*-produced embryos upon which a mock nuclear transfer has been performed in an effort to account for the variability introduced by the actual technique. Animals either were bred or underwent a surgical embryo transfer on Day 1 of the estrous cycle according to their assigned experimental group. Fifty embryos were transferred per embryo transfer. Embryos were flushed from the uterine horns at time of collection (Day 10, 12, or 14) and preserved in 10% neutral buffered formalin. All embryonic disc diameters and gross morphology were evaluated as parameters for normal development. Embryos were then dehydrated in ethanol, paraffin-imbedded, sectioned, stained with hemotoxylin and eosin, and Day 14 embryos were evaluated for abnormalities such as higher-than-normal nucleoli numbers, increased cytoplasmic vacuoles, and higher than normal numbers of mitotic figures. All results were analyzed using ANOVA. There were significant differences ($P < .0001$) between diameters of the embryonic disks, with the diameters of the NT embryonic disks being smaller than those of the *in vivo* controls at all stages studied. Morphologically, the *in vivo* controls were more developmentally competent than their NT counterparts by the time they reached Day 14 ($P = 0.0002$) in that most had achieved the more advanced elongated form of growth as opposed to remaining spherical in shape. Significant histological differences in the number of nucleoli per nuclei were also found between *in vivo* and NT embryos ($P = 0.05$) as well as between MNT and NT embryos ($P = 0.05$). Therefore, nuclear transfer embryos develop at a much slower rate than their *in vivo* counterparts and often exhibit histological abnormalities that could contribute to this slow growth. Due to the apparent increase in nucleoli, it is possible that NT embryos are being arrested at a specific stage in the cell cycle.

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61 CLONED MOUSE PRODUCED USING A ZONA FREE METHOD OF NUCLEAR TRANSFER

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Mice have been cloned from somatic and embryonic cells; however, only 0–3% of the reconstructed embryos develop into viable offspring. In addition, the piezo microinjection method widely used for mouse nuclear transfer (NT) is difficult to master. Our objective was to compare cumulus and ES cells as nuclear donors using a simplified method of zona-free NT. In cattle, zona-free NT is simpler, faster, easier to learn and more reproducible than zona-intact NT (Oback *et al*. 2003 Cloning Stem Cells 5, 3–12). Oocytes were recovered at metaphase II stage (13 h after hCG injection) from the oviducts of C57BL/6J × DBA/2 F1 females (8–10 weeks of age). Cumulus cells were removed with hyaluronidase (300 units/mL) and the zona pellucida digested with pronase (0.5%) at 37°C for 3 min. Oocytes were then enucleated under UV light in cytochalasin B (5 μg/mL) after a 5-min staining with Hoechst (5 μL/mL). The metaphase DNA was removed in an enucleation pipette (16–20 μm, perpendicular break) by separating karyoplast and cytoplast with a simple separation pipette (60–80 μm, perpendicular break, closed round tip). Embryonic stem (ES) cells were cultured for 3 days and serum-starved for 16 h before use. Cells from this line had yielded offspring by the piezo procedure. Cumulus cells were used freshly. Donor cells were attached to the cytoplasts with phytohemagglutinin (10 μg/mL) and couplets were electrically fused in 0.2 mM mannitol buffer. Reconstructed embryos were activated 1–2 h after fusion for 5–6 h in CZB medium containing 10 mM strontium chloride and 5 μg/mL of cytochalasin B. Embryos were cultured individually in 5-μL droplets in CZB. Morulae and blastocysts were transferred into the uteri

(Day 2.5) of pseudopregnant surrogate mothers (C57BL/6J × CBA/2J). Recipient mothers were sacrificed at 19.5 days postcoitum and pups removed. Airways were cleaned to remove fluid and the pups were held in a warm box before being fostered by a lactating mother. During development of the technique, we assessed the frequency of fusion, cleavage of reconstructed embryos, and development to morula/blastocyst stage. Fusion (58.1 ± 6.7% vs. 24.2 ± 1.7%, $P < 0.001$) and cleavage (66.4 ± 4.2% vs. 50.5 ± 5.4%, $P < 0.05$), all respectively, were higher when cumulus cells were used as donors, as compared with ES cells. However, the percentage of embryos developing to morula/blastocyst stage was greater when ES cells were used (22.2 ± 4.2% vs. 5.3 ± 2.7%, $P < 0.01$). Using ES cells as donors, 19/94 (20.2%) reconstructed embryos reached compacted morula/blastocyst stage. After transfer to five recipients, one pup was born (5.2%). It was larger and heavier than uncloned pups of the same age. The pup is healthy and now 12 weeks old. Genotype was confirmed by microsatellite analysis. The birth of a healthy cloned mouse pup from zona-free NT provides "proof of principle" of a technology that promises to increase throughput, ease of operation, and reproducibility of mouse cloning.

62 RELATION OF INTENSITY OF GENE EXPRESSION IN BOVINE RECONSTRUCTED EMBRYOS TO SUBSEQUENT DEVELOPMENT

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During embryo development, embryonic gene activation (EGA) is the first critical event. We previously showed that EGA is also critical for further development in somatic cell-cloned embryos (Saeki K *et al*. 2004 Reprod. Fertil. Dev. 16, 157–158 abst). To show this, we reconstructed bovine embryos with bovine somatic cells transfected with chicken β-actin/firefly luciferase fusion gene (*β-act/luc+*) and showed that only luminescent embryos at 60 hours post-fusion (hpf) developed to the blastocyst stage. In this study, we examined the relation between the intensity of expression of the same reporter gene in embryos reconstructed with bovine *β-act/luc+* fibroblasts and their subsequent development to the blastocyst stage. Bovine fibroblasts were transfected with *β-act/luc+* as described earlier (Saeki K *et al*. 2004 Reprod. Fertil. Dev. 16, 157–158 abst). The stably transfected and cloned cells were cultured for several passages. The cells were cultured under serum starvation (0.4% FCS) for 7 days and then used as donor cells. *In vitro*-matured bovine oocytes derived from slaughterhouse ovaries were enucleated at 20 h post maturation. Enucleated oocytes were electrofused with the cells, and activated with a calcium ionophore and cycloheximide. The LUC+ signal (luminescence) in the embryos was detected in medium containing 500 μg mL^{-1} luciferin with an imaging photon counter (ARGUS 50, Hamamatsu, Japan) for 30 consecutive min at 60 hpf. The intensity of luminescence in embryos (4- to 8-cell stage) was graded as being strong ($>10 \times 10^4$ pixels/embryo), intermediate (5 to 10×10^4 pixels/embryo), weak ($<5 \times 10^4$ pixels/embryo), or absent. The embryos were cultured separately until 168 hpf, and examined for blastocyst development. Experiments were repeated four times, and the data were analyzed with Fisher's PLSD test following ANOVA by Stat View software (Ver. 5.0; abacus Concepts, Berkeley, CA, USA). Of 125 embryos that were reconstructed, 74 (59%) developed to the 4- to 8-cell stage at 60 hpf. The luminescence was strong in 29 (39%) of the embryos, intermediate in 12 (16%), weak in 19 (26%), and absent in 14 (19%). Blastocysts were obtained from a group of embryos that exhibited strong luminescence (10/29, 34%), but none of the embryos from the other groups developed to blastocysts. These results suggest that active gene expression in embryos reconstructed with somatic cells is important for their subsequent development.

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63 IMPROVED *IN VITRO* DEVELOPMENT OF PORCINE EMBRYOS PRODUCED BY NUCLEAR TRANSFER, IVF AND PARTHENOGENESIS

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Porcine nuclear transfer (NT) is an inefficient process and it is necessary to use as many as 120 NT embryos for each foster mother to obtain small litters of live piglets. In these experiments, we evaluated the effects of culture atmosphere and medium on the development of NT embryos by monitoring blastocyst rate and cell number of Day 6 blastocysts. Age matched IVF and parthenogenetic embryos were also evaluated for comparison. For all experiments a pool of oocytes was aspirated from ovaries collected in a local abattoir. Following aspiration, oocytes were allowed to mature for 40 h in North Carolina State University (NCSU)-37 medium (supplemented with cAMP and hCG/eCG for the first 22 h). After removal of the cumulus cells, denuded oocytes with polar bodies were selected for NT, enucleated, fused with fetal fibroblasts, and sequentially activated electrically and chemically by 3 h of treatment with 6-dimethylaminopurine (6-DMAP). A second group of oocytes from the same denuded pool were maintained in TL-HEPES medium and activated in parallel with the NT group to produce parthenogenetic embryos. A third group was fertilized with frozen-thawed epididymal semen and co-cultured for ~12 h to give IVF embryos. All three treatment groups were subdivided into a control subgroup and an experimental subgroup. In the first experiment, we compared the effects of atmosphere (20% vs. 5% oxygen) on *in vitro* embryonic development in NCSU-23 medium. In the second experiment, we used only the 5% oxygen concentration and compared different culture media. One subgroup was maintained in standard NCSU-23 medium and the second subgroup was cultured in a two-step system for the first 58 h in modified NCSU-23 (without glucose but supplemented with 2.0 mM lactate and 0.2 mM pyruvate), followed by addition of glucose to give a final concentration of 5.55 mM. Data were statistically analyzed by analysis of variance and chi square test. Blastocyst rate and mean cell number in all three embryo groups were improved under 5% oxygen. The most dramatic effect was observed in the NT group, in which the blastocyst rate increased significantly ($P < 0.001$) from 6.7% ± 5.9 ($n = 279$) to 19.6% ± 8.9 ($n = 250$) and mean cell number increased from 17.7 ± 12.1 to 25.8 ± 10.3 cells

per blastocyst. With 5% oxygen there was also an increase of blastocyst rates and mean cell numbers in both IVF and parthenogenetic groups. In the second experiment, blastocyst rate for NT embryos increased significantly ($P < 0.05$) from 21.8% ± 7.6 ($n = 242$) in conventional NCSU-23 to 31.5% ± 11.0 ($n = 271$) in the modified system whereas there was almost no difference in the mean cell number of both groups (29.2 ± 4.3 vs. 31.5 ± 5.3). In the groups of IVF and parthenogenetic embryos no difference was found. These results indicate that both the reduced oxygen and the modified culture medium are important for pre-implantation development of porcine nuclear transfer embryos.

64 OOPLASMIC TRANSFER AFTER INTERSPECIES NUCLEAR TRANSFER: PRESENCE OF FOREIGN MITOCHONDRIA, PATTERN OF MIGRATION, AND EFFECT ON EMBRYO DEVELOPMENT

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Interspecies somatic cell nuclear transfer (iSCNT) using the bovine cytoplast as universal recipient has potential applications in the conservation of exotic species. However, an *in vitro* developmental block has been observed using this approach. It has been suggested that mitochondrial mismatch between donor cell and recipient oocyte could be a cause for the embryonic developmental arrest. A series of experiments were conducted to investigate the effect of mixed mitochondrial populations (heteroplasmy) on early development of cloned embryos. In Experiment 1, we examined the effect of combining the technique of ooplasmic transfer (OT) with somatic cell nuclear transfer (SCNT) in the bovine model. In addition, presence and pattern of migration of foreign mitochondria after OT were examined by MitoTracker® (Molecular Probes, Inc., Eugene, OR, USA) staining. In Experiment 2, we examined the effect of transferring caprine ooplasm into bovine enucleated oocytes (iOT) used as recipients for goat iSCNT. Ooplasm from donor oocytes was aspirated until the oolema was ruptured and filled about 200 μm of the micropipette. Aspirated ooplasm was injected into recipient oocyte; the oolema of the recipient oocyte was also ruptured by partial aspiration into the micropipette to ensure mixing. Mean cleavage rates and embryo development were compared by chi-square analysis. Percentages (except for parthenogenic controls) were calculated from number of fused couplets. In Experiment 1, there was no significant effect of the sequence of events (OT-SCNT or SCNT-OT) on the number of fused, cleaved, blastocyst (BLST), or hatched blastocyst (HCHD) embryos (Table 1). MitoTracker Green FM staining of donor oocytes used for OT revealed foreign mitochondria were introduced by the procedure. Their pattern of distribution remained in a distinct cluster after 12, 74 and 144 h of *in vitro* culture. However, when goat ooplasm was injected into bovine enucleated oocytes used for iSCNT, there was a significant reduction in fusion (52 vs. 82%) and cleavage rates (55 vs. 78%) ($P < 0.05$). In addition, the procedure of iOT prior to iSCNT was not effective in overcoming the 8- to 16-cell *in vitro* developmental block and only parthenogenic cow and goat controls reached blastocyst (36 and 32%) and hatched blastocyst (25 and 12%) stages, respectively (Table 1). This study demonstrates that foreign mitochondria are introduced at the time of OT and these mitochondria remain in a cluster without relocation after a few mitotic divisions. Although the bovine cytoplast appears capable of supporting mitotic divisions after iOT-iSCNT, heteroplasmy or mitochondrial incompatibilities may affect nuclear-ooplasmic events occurring at genomic activation. To our knowledge, this is the first scientific report of iOT used in combination with iSCNT in an attempt to overcome the *in vitro* developmental block. Further research is needed to determine characteristics of foreign mitochondrial dynamics as well as replication of foreign mitochondria introduced into NT embryos.

Table 1. Intraspecies (cow) and interspecies (goat-cow) ooplasmic transfer and nuclear transfer

Treatment group	No. M-II oocytes	No. (%) fused	No. (%) cleaved	No. (%) BLST	No. (%) HCHD	No. (%) BLST with nuclei
OT-SCNT	48	31 (65)[a]	27 (87)[a]	15 (48)	10 (32)	15 (100)
SCNT-OT	45	33 (73)[a]	28 (85)[a]	13 (39)	9 (27)	13 (100)
iSCNT-iOT	60	31 (52)[b]	17 (55)[b]	0	0	–
iSCNT only	55	45 (82)[a]	35 (78)[a]	0	0	–
Parthenogenic (cow)	55	–	44 (80)[a]	20 (36)	14 (25)	20 (100)
Parthenogenic (goat)	25	–	22 (88)[a]	8 (32)	3 (12)	8 (100)

[a,b] Values within columns with different superscripts differ significantly; chi-square: $P < 0.05$.

65 PRODUCTION OF PORCINE NUCLEAR TRANSFER EMBRYOS USING FETAL FIBROBLAST CELLS ANALYZED ON APOPTOSIS

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One of the most important factors that determine the developmental potential of mammalian cloned embryos is the structuro-functional quality of nuclear donor cells. Biochemical changes that are some of the earliest symptoms of apoptosis signal transduction are not reflected in the morphological features of somatic cells. Therefore, an appropriate system of cell selection would enable the sorting of donor nuclei with high morphological and biochemical susceptibility to somatic cloning. The aim of our study was to examine the *in vitro* developmental competencies of porcine nuclear transfer (NT) embryos reconstructed with fetal fibroblast cells that had been analyzed for apoptosis by live-fluorescent labelling. Frozen/thawed fetal

fibroblast cells, which had been *in vitro*-cultured to a confluent state, were used for analysis. To detect the early apoptotic changes in the fibroblast cells, a single cell suspension of nuclear donor cells was subjected to dyeing with live-DNA green fluorochrome YO-PRO-1. The recipient cells were *in vitro*-matured oocytes. Maternal chromosomes were removed by a chemically assisted microsurgical technique. Then, single nuclear donor cells were inserted into the perivitelline space of enucleated oocytes. Fibroblast cell-ooplast couplets were simultaneously fused and activated with two consecutive DC pulses of 1.2 kV/cm for 60 μs. Reconstructed embryos were *in vitro* cultured in 50-μL drops of NCSU-23 medium supplemented with 0.4% BSA-V for 6 to 7 days at 38.5°C in a humidified atmosphere of 5% CO_2 and 95% air. The rates of cleavage and development to morula/blastocyst stages were examined on Days 2 and 6/7, respectively. After fluorescent analysis of approximately 50 different random samples collected from the population of fetal fibroblast cells, that had been labelled with YO-PRO-1 dye, it was found that a relatively high proportion of donor cells revealed ultrastructural apoptotic changes. The percentage of late apoptotic cells with advanced morphological transformations was about 40% of the total pool of the fibroblast cells. A total of 262/270 (97.0%) enucleated oocytes were subjected to reconstruction and 141/262 (53.8%) were successfully fused with non-apoptotic nuclear donor cells. Following the simultaneous fusion/activation protocol, reconstituted oocytes were selected for *in vitro* culture. Out of 262, 133 (50.8%) cultured NT embryos cleaved. The frequencies of cloned embryos that reached the morula and blastocyst stages were 48/133 (36.0%) and 10/133 (7.5%), respectively. In conclusion, morphology is a sufficient selection factor for detection of apoptosis in the cultured (confluent) fetal fibroblast cells to be used for cloning. Moreover, it was found that YO-PRO-1 fluorochrome may be not able to detect the early phases of apoptosis, because only the morphologically abnormal cells emitted the YO-PRO-1-derived fluorescence.

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66 *IN VITRO* DEVELOPMENT OF YAK (*POEPHAGUS MUTUS*) CLONED EMBRYOS BY INTERSPECIES SOMATIC NUCLEAR TRANSFER

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Interspecies nuclear transfer (NT) is an important tool for preservation of endangered animal species. This study was carried out to clone Yak (*Poephagus mutus*) embryos by using Yak skin fibroblasts and bovine (*Bos taurus*) recipient cytoplasts, and to compare the efficiency of YAK interspecies NT (bovine cytoplast-Yak donor cell) and bovine somatic NT (bovine cytoplast-bovine donor cell). Recipient oocytes were extracted from antral follicles of bovine ovaries, and subsequently cultured in maturation medium for 18–20 h in 5% CO_2 and 95% humidified air at 39°C. Cumulus cells were removed from the oocytes by vortexing also facilitated further enucleation. Yak skin fibroblast cells were prepared from cultured ear explants of an adult 5-year-old female. Fibroblasts were cultured at passage 6–9 in 10% FBS DMEM at 37°C in 5% CO_2 humidified air. The donor cell at a diameter of 19–20 μm was inserted into the perivitelline space of an enucleated oocyte. A bovine female cell line at similar passage number was used for bovine somatic NT as control. Somatic cell-cytoplast pairs were then fused by applying two direct current pulses at 2.0 kV/cm for a duration of 6–10 μs/pulse. Fused embryos were activated in 10 μg/mL cycloheximide and 2.5 μg/mL cytochalasin D in M199 plus 7.5% FBS for 5 h. Reconstructed Yak embryos were cultured in CR1aa plus 6 mg/mL BSA for 2 days (initiation of activation = Day 0) at 39°C, 5% CO_2, 5% O_2, and 90% N_2, and then in 7.5% FBS CR1aa medium for 5 successive days on bovine cumulus monolayers. Expanding and hatching blastocysts on Day 7 were recorded and cryopreserved for further embryo transfer trials. The percentage of cleavage and the development to morulae and blastocysts were statistically analyzed using a General Linear Model (GLM, Univariate, SPSS 9.0, SPSS Inc, Chicago, IL, USA). As indicated in Table 1, the results demonstrated that the efficiencies of fusion rate as well as developmental potential *in vitro* were significantly higher in the bovine somatic NT group compared to those of the Yak interspecies NT group. However, the morphology and cell number per embryo of interspecies Yak cloned embryos were indistinguishable from those of bovine NT embryos. Our data suggest that bovine oocytes possess the capability of reprogramming/reactivation of the genome from differentiated somatic Yak nuclei.

Table 1. Comparison of yak interspecies and bovine somatic nuclear transfer

Donor cells				No. (%) of embryos developed to			
	Cytoplast	No.	No. fused (%)	2–8 cell	Morula	Blastocyst	Total NT efficiency (%)
YAK skin fibroblast	Bovine	394	178 (45)[a]	110 (62)[a]	54 (30)[a]	33 (18)[a]	8)[a]
Bovine skin fibroblast	Bovine	339	205 (60)[b]	180 (88)[b]	91 (44)[b]	63 (31)[b]	19)[b]

[a,b] Values within columns with different superscripts are significantly different ($P < 0.05$).

67 PREMATURE CHROMOSOME CONDENSATION IS NOT ESSENTIAL FOR BOVINE SOMATIC NUCLEAR REPROGRAMMING

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The objective of this study was to determine the effect of donor nuclear exposure in MII oocyte cytoplasm on nuclear reprogramming events and subsequent development of cloned embryos in cattle. Somatic nuclear transfer (NT) was performed by electrofusion of the enucleated MII oocytes

with cultured cumulus cells by ovum pickup. Donor cell-cytoplast pairs were fused by applying two direct current pulses at 2 kV/cm for 10 μs. Fused NT embryos were randomly divided in to Treatment A (immediate activation) and Treatment B (delayed activation, 4-h exposure in MII cytoplast before activation). In both treatment groups, the activation protocol was identical and consisted of incubation in cycloheximide (10 μg/mL) plus cytochalasin D (2.5 μg/mL) in M199 + 7.5% FBS for 1 h, followed by culture in cycloheximide (10 μg/mL) for an additional 4 h. Reconstructed embryos in both groups were subsequently cultured in CR1aa in 5% CO_2, 5% O_2, and 90% N_2 at 39°C. Samples from both treatments were fixed at 0, 1, 2, 4, 6, 12, 18, 24, 30, 36, and 44 h after fusion. All fixed samples were double stained for tubulin and DNA, and observed with a laser-scanning confocal microscope for changes in nuclei and microtubules. The experiment was replicated three times. Cleavage rate and blastocyst rate were recorded and analyzed by Studen's *t*-test (SPSS 11.0, Chicago, IL). The staining revealed an absence of premature chromosome condensation (PCC) in all embryos in Treatment A. However, delayed activation (Treatment B) resulted in a high incidence of PCC, probably due to high levels of MPF in the MII cytoplasts. Chromosome condensation was observed in Treatment B at 4 h (82%, $n = 17$), 6 h (80%, $n = 10$), 12 h (36%, $n = 25$), 18 h (71%, $n = 24$), 24 h (50%, $n = 16$) and 30 h (6%, $n = 18$) after fusion. Subsequent culture results of these cloned embryos (Table 1) indicated that there were significantly higher cleavage rates and blastocyst development in Treatment A than in Treatment B. This study clearly demonstrated that PCC is not essential to support bovine cloned blastocyst development. Direct exposure of donor nuclei in a MII cytoplast enviroment for a very short time was sufficient for nuclear reprogramming.

Table 1. Effect of donor nuclear exposure duration in MII cytoplasm on the development potential of cloned bovine embryos

Treatment	No. reconstructed embryos (Replicates)	No. (%) Cleaved	No. (%) Blastocyst
Immediate activation (A)	112 (3)	106 (95)[a]	73 (65)[a]
Delayed activation (B)	182 (3)	62 (34)[b]	27 (15)[b]

[a,b] Values in the same column with different superscripts differ significantly ($P < 0.05$).

68 REPRODUCTIVE PERFORMANCE OF CLONED BULLS

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The generation of cloned animals by somatic cell nuclear transfer has been reported in a number of countries worldwide. However, progress has been impeded by the extremely low efficiency of cloning and health of some of the cloned animals. Surprisingly, little is known of the reproductive performance of viable clones when compared to the original cell donor, given that a major motivation of cloning is dissemination of superior genotypes. The aim of this study was to compare semen collected from three cloned bulls (Clones A1, B1, and B2) to that of the original donor bulls (Donors A and B). Parameters examined included ejaculate volume, sperm concentration and the motility characteristics of frozen/thawed semen using computed-assisted semen analysis (Hamilton Thorne Biosciences, Inc., Beverly, MA, USA). The fertilization ability of each semen sample was examined using *in vitro* matured oocytes derived from abattoir-source ovaries. Frozen/thawed semen samples from donor and cloned bulls were prepared on a Percoll gradient and diluted with fertilization medium to a concentration of 1 million sperm/mL prior to fertilization (IVF). The number of blastocysts and total cell counts were analyzed on Day 7 of culture. Finally, *in vitro*-fertilized blastocysts (Day 7) were transfer to synchronized recipients ($n = 49$) to examine *in vivo* development. Proportional data for the *in vitro* development of embryos and subsequent pregnancy rates were analyzed by chi-square test, and embryo cell numbers were analyzed using Student's *t*-test. Progressive motility percentage between donor and cloned bull did not differ: Donor A (62.25 ± 3.89, $n = 12$); Donor B (66.69 ± 4.47, $n = 13$); Clone A1 (71.37 ± 8.57, $n = 8$); Clone B1 (73.75 ± 2.42, $n = 12$); Clone B2 (72.41 ± 3.26, $n = 12$). No obvious differences in kinetic motility parameters were evident between cloned and non-cloned donor animals. However, blastocyst rates were significantly higher in cloned bulls (Clone A1: 30.9%, 81/262; Clone B1: 34.4%, 98/285; and Clone B2: 42.9%, 120/280) compared to donor bulls (Donor A: 20.7%, 54/261; Donor B: 20.9%, 76/364). Total embryo cell numbers did not differ significantly between donor bulls (Donor A: 138.3 ± 5.3, $n = 39$; Donor B: 133.2 ± 5.2, $n = 47$) and cloned bulls (Clone A1; 126.3 ± 4.4, $n = 45$; Clone B1: 134.4 ± 7.1, $n = 26$; and Cloned B2: 140.1 ± 3.9, $n = 46$). Initial pregnancy rates on Day 30 were also not different between Donor A (42.3%, 11/26) and Clone A1 (47.8%, 11/23). Preliminary observations from the small data set on postpubertal cloned bulls indicate that semen production, semen morphology, and reproductive performance (*in vitro* and *in vivo*) were similar in terms of semen characteristics and reproductive performances when compared to their original donor bulls.

69 PRODUCTION OF CLONED PIGS BY NUCLEAR TRANSFER OF PREADIPOCYTES

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Since the first success in producing cloned pigs, donor cells have been limited to fetal fibroblasts and a few other cell types. The aim of the present study was to determine if porcine preadipocytes can be efficient donor cells for somatic cell nuclear transfer (NT) in pigs. Preadipocytes established

from subcutaneous adipose tissue of a male adult pig were used as nuclear donor cells. Cell cycle synchronization was carried out by serum starvation (5 days), confluency (5 days), roscovitine treatment (15 μM, 2 days), or differentiation induction by 0.5 mM 3-Isobutyl-1-methylxanthine, 0.25 μM dexamethasone, and 5 μg/mL insulin (5 days). Cell cycle synchronization and apoptosis of the donor cells were examined by flow cytometry and Annexin V staining and TUNEL. IVM oocytes were obtained from abattoir ovaries and matured in NCSU23. Donor cells were fused with the enucleated recipient oocytes by a single DC pulse of 200 V/mm for 10 μs in 0.28 M mannitol + 0.15 mM $MgSO_4$. Reconstructed embryos were electrically activated at 1–1.5 h after the NT, followed by cytochalasin B treatment for 3 h. Development of the NT embryos was assessed by fixation/staining at 3 h after NT, culture for 7 days in NCSU23, and transfer to the oviducts of estrus-synchronized recipient gilts. The cells immediately entered the G0 phase by differentiation induction (92.5 ± 0.4%), with higher efficiency of synchronization than for the other methods (roscovitine: 80.3 ± 0.2%; confluency: 79.9 ± 0.3%, $P < 0.05$) except for serum starvation (89.8 ± 0.6%). The proportion of apoptotic cells in the differentiation group was significantly lower than the other groups (Annexin V: 7.7% vs. 15.7 to 19.3%, TUNEL: 8.3% vs. 12.8 to 14.0%, $P < 0.05$). Incidence of premature chromosome condensation following NT (88.0%) was as high as that observed after NT with fetal fibroblasts previously (data not shown). *In vitro* developmental rates of the NT embryos did not differ significantly among the cell cycle synchronization methods of the donor cells (7.2 to 10.8%). Cell number of the blastocysts was highest in the differentiation group (49.0 vs. 30.2 to 41.9, $P < 0.05$). Transfer of 1004 cloned embryos of the serum starvation group to 5 recipients resulted in the production of 4 live and 1 stillborn piglets from 1 recipient. Transfer of cloned embryos reconstructed of donor cells treated by differentiation induction is currently underway. These data demonstrate that preadipocytes collected from an adult pig are promising nuclear donor cells for pig cloning.

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70 RECONSTRUCTED BOVINE BLASTOCYSTS COMPRISING NUCLEAR TRANSFER-DERIVED INNER CELL MASS AND TROPHECTODERM FROM IVF EMBRYOS DO NOT IMPROVE *IN VIVO* DEVELOPMENT OF CLONES

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The cloning of cattle by somatic cell nuclear transfer (NT) is associated with a high incidence of abnormal placentation, excessive fluid accumulation in the fetal sacs (hydrops syndrome) and fetal overgrowth (Lee RSF *et al*. 2004 Biol. Reprod. 70, 1–11). Early embryonic loss in bovine NT pregnancies may also be due to immunological rejection (Hill JR *et al.* 2002 Biol. Reprod. 67, 55–63). As a means of overcoming placental abnormalities and improving pregnancy outcome in bovine NT, reconstructed blastocysts were produced by combining immunosurgically isolated inner cell masses (ICM) from Day 7 NT embryos with the trophectoderm (TE) of Day 7 IVF embryos. Oocytes for the production of NT and IVF embryos were obtained from abattoir-collected ovaries of dairy cows. The semen used for IVF was from the bull from which the cell line for NT was derived. The NT blastocysts were produced as described previously (Oback B *et al.* 2003 Cloning Stem Cells 5, 3–12) except that two one-cell embryos were aggregated together after NT (2NT). Blastocyst reconstruction was achieved using a modified procedure (Rorie RW *et al.* 1994 Vet. Record 135, 186–187). Embryos from four experimental groups were transferred individually to synchronized recipient heifers on Day 8 of culture: (1) ICM from 2NT embryos reconstructed with IVF TE (R-2NT, $n = 15$); (2) ICM from IVF embryos reconstructed with IVF TE (R-IVF, $n = 15$); (3) control 2NT ($n = 10$); and (4) control IVF ($n = 10$). Pregnancy rates were recorded and treatments compared using Fisher's exact test. After slaughter between Days 149 and 161 of gestation, morphometric measurements were determined for the fetuses, fetal organ weights, fluid volumes, and placentomes. Data were rank transformed; treatments were compared using Student's *t*-test with standard errors calculated from the pooled variation. Pregnancy rates on Day 35 were R-2NT (60%), R-IVF (47%), 2NT (90%), and IVF (10%). Pregnancy rates on Day 150 were R-2NT (40%), R-IVF (40%), 2NT (70%), and IVF (10%). The reason for the low IVF pregnancy rate was unknown. Previously, pregnancy rates using the same sire and cell line (but using Day 7 embryo transfer) on Day 35 were 63% ($n = 40$) and 69% ($n = 42$) for IVF and single, non-aggregated NT, respectively, and 50% and 33% for IVF and NT on Day 150. The single NT pregnancy rate was not significantly different from that for the 2NT embryos. There was no significant difference in pregnancy rates on Day 35 and Day 150 between R-2NT v. 2NT, R-2NT v. R-IVF, or 2NT v. R-IVF. The blastocyst reconstruction procedure did not have any impact on fetal development or influence pregnancy rates. All fetuses recovered were male. No significant differences were found between R-2NT and 2NT fetuses in terms of fetal weight, fluid volume, total placentome weight, and placentome numbers or in the relative and absolute weights of the brain, heart, liver, and kidneys. Thus, replacement of the TE in NT embryos with TE from IVF embryos did not overcome placental abnormalities or decrease fetal overgrowth prevalence.

71 DEVELOPMENTAL DELAY OF PRE-IMPLANTATION OVINE *IN VITRO* CULTURED AND SOMATIC CELL NUCLEAR TRANSFER EMBRYOS

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Despite advances in the production of somatic cell nuclear transfer (SCNT) embryos, significant embryo losses are persistent, particularly around implantation. Malformations of the placenta and in a variety of organs are seen, and have been linked to deviant epigenetic reprogramming. The aim of the present study was to examine the formation of germ layers, which are prerequisites for formation of the embryo proper and placenta, in *in vivo*-derived (*in vivo*), partly *in vitro*-cultured (IVC), and SCNT ovine embryos. Embryos were derived as follows: *In vivo* embryos ($n = 27$) were

flushed from the uterus on Days 7, 9, 11, and 13. For IVC embryos ($n = 22$) *in vivo* zygotes were flushed, followed by culture in the presence of 20% human serum, transfer to the uterus on Day 6, and flushing as *in vivo* embryos. SCNT embryos ($n = 41$) were produced by fusion of serum starved granulosa cells with enucleated oocytes, followed by activation, culture in SOF, transfer to the uterus on Day 6, and flushing as described for *in vivo* embryos. Recovered embryos were processed for light microscopy (LM) and transmission electron microscopy (TEM), and paraffin sections were immunohistochemically labelled for the germ layers: alpha-1-fetoprotein for potential endoderm, cytokeratin-8 for potential ectoderm, and vimentin for potential mesoderm. A consistent delay of the IVC and particularly the SCNT embryos was noted throughout all time points: On Days 7 and 9, differentiation of the inner cell mass into hypoblast and epiblast was evident in 7 out of 12 *in vivo* embryos, whereas this phenomenon was less prominent or absent in 9 out of 13 IVC and 13 out of 15 SCNT embryos. Furthermore, 6 of the IVC and 12 of the SCNT embryos lacked an identifiable embryonic disc. On Day 11, half of the *in vivo* embryos had initiated gastrulation, evidenced by localization of endoderm and mesoderm precursor cells between the hypoblast and the epiblast. This feature was noted in only a single IVC and in none of the SCNT embryos. On Day 13, all *in vivo* embryos had completed gastrulation including the formation of somatic and visceral mesoderm. This feature was noted in only 1 out of 3 IVC and in none of the SCNT embryos. Likewise, amniotic folds were seen in one third of the *in vivo* embryos at this stage, but not observed in any IVC or SCNT embryos. The immunohistochemical markers displayed the same cell lineage localization in all three groups of embryos, but a developmental delay in the IVC and in particular the SCNT embryos was evident. In conclusion, ovine IVC and SCNT embryos develop at a slower rate than *in vivo* embryos at least up until Day 13 of gestation.

72 EFFECT OF CELL CYCLE PHASE OF GENE-MANIPULATED FETAL FIBROBLASTS ON THE DEVELOPMENT OF CLONED BOVINE EMBRYOS

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Transgenic bovine fetuses and offspring can be produced by using gene-modified somatic cells and clones of these cells. In this study, we examined the effects of specific cell cycle (early G1 phase) of donor cell (gene-manipulated fibroblasts) on the development of the nuclear transfer (NT) embryos into blastocysts and on the fetus production after embryo transfer. The gene-manipulated (tg; targeting of one or both alleles of gene encoding α-1,3-galactosyltransferase) or non-manipulated (control) bovine fetal fibroblasts were used for NT. The fibroblasts transfected with the targeting vector were selected with 0.4 mg mL^{-1} G418. The G418-resistant cells were monitored by PCR and Southern blot analysis. The cells (tg cells) in which homologous recombination occurred were used for NT. For NT, both tg cells and control cells were cultured in DMEM with 10% FCS. Early G1 cells were prepared by choosing pairs of bridged cells derived from mitotic phase cells (Urakawa M *et al.* 2004 Theriogenology 62, 714–728), and non-synchronized cells were obtained from a culture plate that had reached 60–80% confluence. Each donor cell was inserted into an enucleated, *in vitro*-matured (19 h) oocyte. Oocyte-cell couples were electrofused and activated with calcium ionophore and cycloheximide. The NT embryos were then co-cultured with bovine oviduct epithelial cells in CR1aa with 5% CS. The blastocyst rates were determined at 6 days after NT. The blastocysts were nonsurgically transferred to recipient heifers, and the developmental rate to the normal fetus was examined by the recovery of fetus or by using ultrasonography at Days 35–42. Data were analyzed by ANOVA. The developmental rate to the blastocyst stage did not differ significantly between tg (28.4%, 128/425) and control (25.4%, 181/739) cell groups. In the control group, the blastocyst rate of embryos constructed from early G1 phase fibroblasts (25.7%, 80/311) was not significantly different from that of embryos constructed with non-synchronized fibroblasts (23.6%, 101/428). In contrast, the blastocyst rate of tg cell derived-embryos was lower ($P < 0.05$) in early G1 phase (23.5%, 71/302) than in non-synchronized cell phase (46.3%, 57/123). The rate of development to a normal fetus in the tg group (15.4%, 4/26) was significant lower than that in the control group (62.5%, 25/40). For both the tg group and the control group, the rate of development to fetus tended to be higher ($P > 0.05$) for blastocysts derived from cells at the early G1 phase than for blastocysts derived from non-synchronized cells (tg group, 25.0%, 3/12 v. 7.1%, 1/14; control group, 90.0%, 9/10 v. 53.3%, 16/30). These results demonstrate that gene modification of fetal fibroblasts affects the development of NT embryos to fetuses. In addition, the synchronization of genetically modified donor cells to the early G1 phase may increase the potential to develop to a normal fetus.

73 ABNORMAL REPROGRAMMING OF HISTONE ACETYLATION IN CLONED BOVINE EMBRYOS

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Histone acetylation plays an important role in the chromatin structure prior to zygotic gene expression during early embryonic development. Successful animal clones indicate that differentiated somatic nuclei must be reprogrammed to some extent during pre-implantation development. However, the molecular mechanisms regarding epigenetic reprogramming of somatic nuclei in the early-stage embryos are poorly understood. To test this, the patterns of hyperacetylated histone H4 lysine 5 (AcH4K5) in the nuclear-transferred (NT) embryos were monitored, comparing *in vitro* fertilized (IVF) embryos and Trichostatin A (TSA)-NT embryos with TSA-treated cells. The intensity signals of AcH4K5 were observed in early-stage embryos and somatic cells (bovine ear skin fibroblasts composed of about 80% at G0/G1 stage) by immunofluorescence analysis with anti-AcH4K5 using image the analyzer system, SigmaScan-pro V5.01 (SPSS, Inc., Chicago, IL, USA). Our data were analyzed by analysis of variance (ANOVA) using an SAS package (SAS Institute, Inc., Cary, NC, USA). Somatic cells were exposed to TSA (1 μM for 60 h), a specific inhibitor of histone deacetylase (HDAC), to induce hyperacetylation prior to somatic cell nuclear transfer. Signal intensity for AcH4K5 in TSA-treated cells ($n = 80$)

was significantly increased ($P < 0.05$), which was approximately double compared to that of normal cells ($n = 80$). In normal cells, histone H4 acetylation was profoundly reduced from the pro-metaphase to the early telophase and then reappeared at the late telophase. Acetylation signals of TSA-treated cells gradually increased to the early anaphase, abruptly decreased at the late anaphase and the early telophase, and recovered during late telophase. During early embryonic development (1 cell to 8 cell stage), NT embryos ($n = 8$) were hypoacetylated at the metaphase, whereas IVF ($n = 10$) and TSA-NT embryos ($n = 8$) were hyperacetylated. Our findings demonstrate that aberrant epigenetic reprogramming of histone modification occurs as early as the pronuclear stage in cloned embryos.

74 ANATOMICAL ABNORMALITIES IN CALVES PRODUCED BY NUCLEAR TRANSFER

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Two limitations currently restrict the acceptability and adoption of bovine cloning as a commercial reality. The first of these is its low rate of efficiency and the second is that some of the cloned calves are not healthy. Abnormalities in the placenta are thought to contribute to many of the losses in early gestation; however, less is known of the pathology of clone deaths in the perinatal period. To date, the majority of perinatal deaths have been attributed to the "large offspring syndrome" characterized by increased birth weight and a range of morphological abnormalities thought to be associated with *in vitro* culture and manipulation. This report describes multi-systemic abnormalities in aborted, stillborn, and neonatal genetically modified and unmodified cloned calves weighing less than 60 kg at birth and aged between 6 months gestation and 3 weeks postnatal, generated in various experiments. Three of 14 genetically modified cloned calves had cystic renal dysplasia and osteopetrosis. All three and a fourth had irregular nodular, fibrotic livers with biliary abnormalities. Another two had marked flexion of the fetlock joints. Eleven calves derived from an unmodified cloned cell line by nuclear transfer had nodular, fibrotic livers with biliary anomalies, 9 of 11 had cystic renal dysplasia and cardiomegaly, two had osteopetrosis, and two had contracted tendons. In addition, three calves had polymicrocerebral gyri, two had retinal dysplasia, and one had an aortic aneurysm. Only one calf from a second unmodified cloned cell line produced by nuclear transfer had no significant congenital abnormalities. All calves were negative for bovine virus diarrhea virus (BVDV) by competitive-antigen ELISA, and by virus isolation and no BVDV antibodies were detected by AGID assay. Furthermore, all cell lines and media used were negative for BVDV by virus isolation. Two calves were tested and found to be negative for Akabane virus and Aino virus. There are very few reports of the pathological abnormalities of cloned animals. Similar multi-systemic abnormalities have not been found in non-cloned calves, but several analogous conditions occur in humans, including Simpson-Golabi-Behmel and Zellwegers syndromes. Further ultrastructure studies and genetic analysis are needed to investigate the mechanisms of these multi-systemic disorders, which may ultimately elucidate mechanisms for improved reprogramming and increase the efficiency of generating cloned animals with somatic cells by nuclear transfer.

75 RABBIT NUCLEAR TRANSFER WITH CULTURED SOMATIC CELLS

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Cloned rabbits have been obtained by somatic cell nuclear transfer (SCNT) only with fresh, non-cultured cumulus cells (Chesne *et al*. 2002 Nat. Biotechnol. 20, 366–369). For the purpose of generating transgenic animals by SCNT, donor cells must be cultured and modified prior to use as nuclear donors. The objective of this study was to optimize the SCNT procedure using cultured cumulus or fibroblast cells. MII oocytes were harvested from superovulated Zika rabbits, and maternal chromosomes were removed by demecolcine-assisted enucleation (Yin *et al*. 2002 Biol. Reprod. 67, 442–446). Two types of somatic cells originating from Ali/Bass rabbits were used as nuclear donors: cumulus cells collected from *in vivo*-matured oocytes and cultured for 1–5 passages, and primary fetal fibroblasts obtained from Day 16 fetuses and grown to confluence or starved for 4–5 days. Somatic donor cells and recipient cytoplasts were fused with 2 electric pulses (1.95 kV/cm, 25 μs each, 1 s interval). Twenty to 40 min after fusion, cloned embryos were activated first with the same electropulses as for fusion, and then immediately followed by 1 h incubation in 2 mM 6-dimethylaminopurine and 5 μg/mL cytochalasin B in culture medium (B2 medium supplemented with 10% FCS). Cloned embryos were either transferred at the 2- and 4-cell stage to asynchronized recipients or cultured in vitro for 6 days. Data were compared using chi-square test, and differences were considered significant when $P < 0.05$. Our results demonstrate that cloned rabbits can be produced by SCNT with cultured cells but the efficiency of this technique is still very low irrespective of the type of donor cells.

Table 1. Development of cloned embryos derived from somatic cells

Donor nucleus	Fused	Cleaved	Blastocysts	Transferred embryos	Recipients	Pregnancies	Offspring
Cumulus cell	346/376[a]	208/240[a]	17/53[a]	172	5	2	1*
Confluent fibroblast	931/978[ab]	800/860[b]	41/124[a]	408	10	2	0
Starved fibroblast	413/425[b]	352/407[a]	37/114[a]	199	5	1	0
Control[#]		112/114[c]	110/114[b]				

[abc] Values in the same column with different superscripts differ significantly ($P < 0.05$).

* One live rabbit was delivered on Day 27 after embryo transfer and confirmed to be Ali/Bass origin.

Parthenotes.

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76 PRODUCTION OF CLONED BOVINE TRANSGENIC EMBRYOS WITH VARIOUS TYPES OF MONO-COLONY CELLS AND OVUM PICKUP

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The objective of this study was to determine the effects of genetic manipulation, cell type, and culture conditions on developmental potential of bovine nuclear transfer (NT) embryos. Ovum pickup (OPU) technology was developed to obtain the oocytes for NT. A total 4044 cumulus-oocyte complexes (COCs) were obtained during 492 OPU sessions, with an average of 8.2 COCs recovered each session. Cultured granulosa cells (CGC), bovine fetal (150 days) oviduct epidermic cells (FOEC), and adult ear skin fibroblasts (ASFC) were used as donor cells for NT and were transfected with the expression vector including human FIX coding sequence directed by goat β-casein promoter and neomycin gene. The cells were screened under 800 $\mu g\,mL^{-1}$ G418 for 10–14 days until the apperance of a "mono-colony" of cells which were then picked. Each cell population was expanded by consecutive passage culture under 300 $\mu g\,mL^{-1}$ G418 until used for NT, ensuring that the majority of cells were transgenic. Oocytes were enucleated at 20 h post-maturation and a single donor cell was transferred into the perivitelline space of a recipient oocyte. After fusion and activation, the reconstructed embryos were co-cultured with vero cells in B2 medium for 7 days. NT efficiency between primary granulosa cells (PGC) without in vitro culture and CGC, as well as among CGC, FOEC and ASFC that were transfected with exogenous DNA (named TCGC, TFOEC, TASFC, respectively), were compared (Table 1). Differences between groups were verified by chi-square test using SAS 6.12 (SAS Institute, Inc., Cary, NC, USA) program. CGCs presented a higher fusion rate ($P < 0.01$) for reconstructed embryos and higher development to the blastocyst stage for NT embryos than did PGC (67% vs. 54% and 41% vs. 21%, respectively). There were no significant differences ($P > 0.05$) in cleavage rate (65%, 71%, and 69%, respectively) and development to the blastocyst stage for NT embryos (36%, 30% and 40%, respectively) for TCGC, TFOEC, and TASFC. A total of 86 blastocysts were selected for transfer into uteri of 86 cows, resulting in 26 pregnancies (30%) at 60 days by ultrasound scanning. Among these, 12 cows remain pregnant and 14 have aborted. The results indicated that oocytes recovered from OPU can be successfully used for NT with development to the blasocyst stage. PGC, CGC, FOEC, and ASFC can all be used for generating transgenic cattle by NT, although this needs to be verified by the birth of live calves.

Table 1. Nuclear transfer efficiency with various cell types

Donor cell	Oocytes, *n*	Maturation (%)	Karyoplast–cytoplast complexes, *n*	Fused (%)	Culture	Cleaved Day 2 (%)	Blastocysts Day 7 (%)
CGC	950	773 (81)[a]	638	425 (67)[a,b]	335	232 (69)[a]	95 (41)[a]
PGC	787	635 (81)[a]	525	281 (54)[a]	178	106 (60)[a]	37 (21)[a]
TCGC	735	580 (79)[a]	486	301 (62)[c,d]	234	152 (65)[a]	55 (36)[a]
TFOEC	398	336 (84)[a]	298	121 (41)[c]	114	81 (71)[a]	24 (30)[a]
TASFC	289	230 (80)[a]	204	115 (56)[b,d]	91	63 (69)[a]	25 (40)[a]

[a,b,c,d] Values with the same superscript within a column are not significantly different.

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Cryopreservation/Cryobiology

77 FREEZING OF MOUSE SPERM BY THREE DIFFERENT CRYOPROTECTANTS

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The cryopreservation of sperm has contributed greatly to animal breeding and reproduction. This study was designed to examine the effect of raffinose, sucrose, and trehalose as cryoprotectants for freezing of mouse sperm. The cryoprotectant solution (CPA) consisting of 3% skim milk (Skim Milk dehydrated, Bacto, Difco, Seoul, Korea) as buffer or extender was prepared and supplemented with 0.3 M raffinose (D[+]raffinose pentahydrate, Sigma) or sucrose or trehalose as non-permeating cryoprotectants. Sperm samples for cryopreservation were collected from caudae epididymides and vas deferens of males of four mouse strains (ICR, FVB, C57BL/6, and CBA). Sperm samples from individual males were aliquoted in cryotubes, placed immediately in the vapor phase of a liquid nitrogen storage container for 10 min, and then plunged directly in liquid nitrogen. For thawing, frozen cryotubes were removed from liquid nitrogen and placed directly into a water bath kept at 37°C for approximately 2 min until the ice melted. Survival of mouse sperm was measured by vital staining. Survival rates of spermatozoa frozen and thawed in freezing solution supplemented with raffinose were higher (ICR: 51.0%, FVB: 27.6%, C57BL/6: 25.7%, and CBA: 23.3%) than those supplemented with sucrose (35.8, 19.6, 12.3, and 19.7%) or trehalose (16.0, 25.4, 25.3, and 24.7%), all respectively. Furthermore, *in vitro* fertilization of mouse oocytes with sperm frozen in raffinose gave cleavage rates of 61.1 (FVB: 217/355 eggs), 59.4 (C57BL/6: 165/278 eggs), and 57.1% (CBA: 144/252 eggs), respectively. These cleaved embryos (FVB: 163, C57BL/6: 137, and CBA: 134) were transferred to 7, 5, and 6 pseudopregnant females, respectively. A total of 4, 3, and 4 females in the respective strains became pregnant and delivered 17 (10.4%), 17 (12.4%), and 18 (13.4%) offspring, respectively. Our results suggest that raffinose is a good cryoprotectant for freezing of sperm for production of inbred mice.