ABSTRACTS FOR POSTER PRESENTATION

Cloning/Nuclear Transfer

22 PRODUCTION OF A CLONED CALF USING KIDNEY CELLS OBTAINED FROM A 48-HOUR COOLED CARCASS

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The ability to produce cloned livestock using postmortem tissue could incorporate an additional application into the field of nuclear transfer. This study examined the feasibility of producing cloned cattle using a primary cell line established from a postmortem beef carcass. A market beef heifer processed at a USDA-certified slaughterhouse was used to develop a primary somatic cell line. Tissue samples were taken from the kidney and forelimb regions either 1) immediately following slaughter (fresh) or 2) 48 h postslaughter (cooled) where the carcass was housed at 2 to 4°C. Tissue was removed and placed on ice in PBS + 5.0% (v:v) penicillin/streptomycin. A primary culture was established using standard techniques and cultured in supplemented DMEM F-12 medium. Once established, cells were trypsinized and either frozen or continually passaged. Cells used for nuclear transfer (NT) were passaged (48 h before use) and cultured with 15 μM roscovitine roughly 24 h prior to nuclear transfer. Cells were approximately 80% confluent and between passage numbers 1 and 11 at the time of NT. Selected slaughterhouse-derived oocytes were matured in supplemented TCM 199 medium for 18–20 h at 39°C in 5.0% CO_2 and air. Mature Metaphase II oocytes were vortexed and stained with Hoechst 33342 to help with chromatin removal. Following enucleation, roscovitine-treated carcass cells were placed in the perivitelline space of the oocyte. Reconstructed NT embryos were fused in Zimmermann's medium and pulsed using needle-like electrodes. This was followed by activation using a combination of calcium ionophore (5 μM), cytochalasin D (5 $\mu g\,mL^{-1}$), and cycloheximide (10 $\mu g\,mL^{-1}$) in TCM + 10% FBS. Fused NT embryos were cultured in 50-μL drops of BARC medium (USDA, Beltsville, MD) for 7 days at 39°C in a 5% CO_2, 5% O_2 and 90% N_2 environment. Embryo development for all four groups (Table 1) was assessed with blastocysts (grade 1 or 2) being transferred into recipient cows 7 days post-estrus. Cleavage rates were not significantly different between groups, and the use of either fresh or cooled cells did not impact blastocyst formation. However, there was a significant difference ($P = 0.05$) in % blastocyst based on the source of the donor cell. Overall, one live calf resulted from 34 transferred NTs produced using kidney cells taken from a 48 h cooled carcass. These results display the feasibility of producing cloned calves from cells collected post mortem, which ultimately could be used as a tool to select breeding bulls based on their own steer carcass characteristics.

Table 1. Embryo development and pregnancy data for the production of beef carcass clones

Cell source status of carcass	# Oocytes	# Mature (%)	# Cultured	# Cleaved (%)*	# Blasts (%)*	# ETs	Initial pregnancies	Live calves
Forelimb—Cooled	543	432 (80 ± 6)	203	138 (68 ± 18)	18 (9 ± 8)[a]	5	1	0
Kidney—Cooled	811	638 (79 ± 8)	409	301 (74 ± 13)	75 (18 ± 10)[b]	34	6	1
Forelimb—Fresh	438	336 (77 ± 7)	175	112 (64 ± 20)	27 (15 ± 10)[a]	0	0	0
Kidney—Fresh	520	414 (80 ± 5)	268	196 (73 ± 19)	36 (13 ± 7)	0	0	0

Values with different superscripts are significantly different based on cell source ([a,b] $P = 0.05$). *Percentages calculated based on # NTs cultured.

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23 REPROGRAMMING MAMMALIAN CELLS IN *XENOPUS* EGG EXTRACTS: ROLE OF EMBRYONIC LAMIN B3

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The generation of animals by nuclear transplantation has demonstrated that a fully differentiated cell can be reversed into totipotency when transferred into an oocyte. Identification of oocyte specific molecules responsible for the reprogramming of somatic cells may contribute to the understanding of cell differentiation and embryo development. We have developed a heterologous system to investigate the effect of lamin B3, a major component of *Xenopus laevis* egg cytoplasm, on DNA replication of mammalian somatic cells. Bovine fetal fibroblasts were arrested at G1/S by incubation in aphidicolin for 18 h. After permeabilization with digitonin, the cells were incubated in either (1) lamin B3 depleted, or (2) whole *Xenopus* egg extracts (1000 cells μL^{-1} extract) supplemented with an energy regenerating system for a period of 3 h at 21°C. *Xenopus* lamin B3-depleted egg extracts were prepared by three rounds of incubation with Dynabeads coated with a mouse monoclonal lamin B3 antibody (mAbLB3). Immunodepletion was confirmed by western blotting. Purified lamin B3 was obtained by dialysis of the beads after immunodepletion, and the purified lamin B3 was used for rescue experiments. DNA replication of cells incubated in the extracts was assessed by adding 25 μM Biotin-11-dUTP for 3 h. After treatment cells were fixed in 70% methanol at −20°C and incubated in mAbLB3 for 30 min at 37°C. This was followed by incubation in FITC-conjugated sheep anti-mouse antibody and in 5 mg mL^{-1} Texas Red-conjugated Streptavidin for 40 min at 37°C. After three hours' incubation in egg extracts, DNA replication was detected in 60% of cells and more than 95% of cells were lamin B3 positive. In contrast, DNA replication in immunodepleted extracts was significantly lower ($P \leq 0.01$, by one-way ANOVA) than in cells incubated in whole extracts and was coincident with the few lamin B3-positive cells observed. More than 95% of cells were lamin B3-negative and did not replicate DNA. When purified lamin B3 was re-added to depleted extracts, DNA replication was detected in 60% of cells. DNA synthesis resumed in 93% of control cells 3 h after release from aphidicolin into culture medium at 39°C. These experiments show that somatic nuclei, which possess a nuclear envelope with somatic variants of lamins, are able to synthesize DNA in egg extracts only when *Xenopus* lamin B3 is incorporated into the nuclear envelope. This heterologous system provides new information on the role of an embryonic molecule, namely *Xenopus* lamin B3, in the reprogramming of DNA replication of somatic cells incubated in egg environment. These results open new questions as to whether embryonic lamins also exist in mammals, and whether failure in development of cloned animals is in part due to abnormal or incomplete replacement of somatic variants of proteins with their embryonic counterparts.

24 ASSESSMENT OF TELOMERE LENGTH IN NUCLEAR TRANSFER DERIVED SHEEP CLONES, THEIR OFFSPRING, AND CONTROL ANIMALS

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The technique of somatic cell nuclear transfer (SCNT) involves the transplantation of a cultured somatic cell into an enucleated oocyte. Following activation, the nucleus of the somatic cell should be reprogrammed to a state of totipotency, which involves the resetting of epigenetic modifications of the somatic genome and also rebuilding of the chromosomal ends (telomeres). This study was carried out to investigate whether the nuclear transfer technique could rebuild the telomeres in sheep clones and their offspring when compared to age-matched control animals. Skin and blood samples were collected from three SCNT-derived sheep clones, and three of their offspring generated by natural mating. Control samples were collected from age-matched animals ($n = 17$), spanning an age range from 1 month to 36 months of age. Ovine genomic DNA was extracted from samples using a Qiagen DNA extraction kit according to the manufacturer's instructions. TeloTAGGG telomere length chemiluminescent assay kit (Roche Diagnostics) was used to determine the telomere length of all samples. Samples of ovine genomic DNA (2 mg) were digested at 37°C for 2 h using Hinf 1 and Rsa 1 endonuclease master-mix at the rate of 40 IU per sample. The resulted terminal restriction fragments (TRF) were separated on 1.2% agarose gel by pulsed field electrophoresis at 6V with 0.1 s switch time for 4 h in 0.5% TBE buffer at 14°C. The DNA fragments were southern-transferred to a nylon membrane and hybridized with telomere-specific $(TTAGGG)_n$, digoxigenin (DIG)-labeled hybridization probe at 42°C. Following post-hybridization washes the membrane was incubated with DIG-specific antibody covalently coupled to alkaline phosphatase. The immobilized telomere probe was visualized by adding a chemiluminecence substrate to the membrane and exposed to X-ray film. Mean TRF length was determined by comparing the telomere densitometry signals to a molecular weight standard. High resolution bands resulting from pulsed field gel electrophoresis revealed that ovine TRF size distribution was in the range of 10–19 kb. Linear regression analysis resulted in significant ($t = 25.84$, $P < 0.05$) decrease of mean TRF size with increasing age, at a mean rate of 0.08 kb per every 6 months. Mean TRF length of SCNT-derived sheep clones, Matilda (28 months old), Macather (14 months old) and Frida (13 months old) were 11.12 kb, 11.40 kb, and 13.41 kb, respectively. Their mean TRF values were significantly ($P < 0.05$) shorter than those of their age-matched control animals: 16.95 kb, 14.76 kb, and 14.76 kb, respectively ($n = 3$ per group). The mean TRF length of the 3 offspring (21 months old) derived from natural mating was not significantly ($P < 0.05$) different from age-matched controls (17.75 ± 0.69 kb v. 16.28 ± 0.72 kb). These results demonstrate that sheep clones derived from cultured somatic cells have shorter telomere lengths compared to age-matched controls, but this telomere loss is reset in the subsequent generation through natural breeding of the clones. (Funded by NSERC, OMAFRA and International Council for Canadian Studies.)

25 COLD STORAGE OF TISSUES AS SOURCE FOR DONOR CELLS DOES NOT REDUCE THE IN VITRO DEVELOPMENT OF BOVINE EMBRYOS FOLLOWING NUCLEAR TRANSFER

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So far, most calves have been cloned from live adult cows or fresh fetal samples. There are few reports on using cells from a dead mammal for nuclear transfer (NT). This study was conducted to investigate whether different kind of viable cells could be obtained from tissues stored in cold for different duration 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 (FCS), 50 $\mu g\,mL^{-1}$ sodium pyruvate, 1% v:v penicillin-streptomycin (10.000 $U\,mL^{-1}$ penicillin G, 10.000 $\mu g\,mL^{-1}$ streptomycin), 10 $ng\,mL^{-1}$ EGF, 0.5 $\mu g\,mL^{-1}$ FSH, and 5 $\mu g\,mL^{-1}$ LH. First cell line (CC) was established from articular cartilage of the leg of a slaughtered cow stored at 0°C in a cold storage room for 48 h. Second cell line (MC) was established from leg muscle of a cow carcass stored at 0°C for 24 h. Tissues from articular cartilage and 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 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 $\mu g\,mL^{-1}$), and cycloheximide (10 $\mu g\,mL^{-1}$), and cultured for 7 days. Differences 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 articular cartilage and muscle of a cow carcass stored at cold temperature for 24 and 48 h and these cells have ability to generate NT blastocysts at rates similar to that of the controls. This study was supported by a grant from TUBITAK, Turkey (VHAG-1908-102V048). F Ergin is a volunteer young researcher.

Table 1. In vitro development of NT embryos from different cell lines

Donor cell	NT units	Fused (%)	Cleaved (%)	NT blastocyst (%)	No.of cells
CC	113	60 (53)	40 (66.6)	6 (10)	107.0 ± 40.6
MC	90	44 (48.8)	24 (54.5)	4 (9.0)	89.0 ± 11.5
GC	72	43 (59.7)	22 (51.2)	5 (11.6)	93.7 ± 14.6

Values within each column are not significantly different ($P > 0.05$).

26 ABERRANT REPROGRAMMING OF IMPRINTED GENE EXPRESSION IN ENLARGED PLACENTAS OF MICE CLONED FROM ES CELLS TREATED WITH TSA OR 5AzaC

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The success rate of producing cloned animals is very low, and in many cases is associated with the formation of enlarged placentas. Increasing evidence has pointed towards epigenetic deregulation of imprinted genes due to incomplete or abnormal resetting of DNA methylation and/or histone acetylation patterns during development. It has previously been shown that drugs that alter DNA methylation (5AzaC) and histone acetylation (TSA) over-express imprinted genes in mouse ES cells (Baqir and Smith, 2001, Theriogenology 55, 410). Our objective in this study was to determine whether nuclear transfer is able to reprogram imprinted gene expression patterns in the placenta of mice cloned from ES donor nuclei exposed to 5AzaC and TSA. ES donor cells were treated with either TSA or 5AzaC prior to injection into enucleated oocytes. Total RNA was extracted from placentas of day 14–15 fetus clones, and reversed transcribed; the expression pattern of imprinted genes (Ipl, Mash2, Igf2, H19, Igf2r, p57, Peg1), non-imprinted placental-specific genes (Esx1, Dlx3, Tpbp) and a housekeeping gene (Gapdh) was examined by Real Time PCR. Samples were standardized with an exogenous control (Globin) and expressed as fold changes in relation to placentas of cloned fetus derived from non-treated donor cells. Data were analyzed by ANOVA and mean gene expression values were compared using the Tukey-Kramer test. Our results show that several imprinted genes (Mash2, H19, Ipl) and placenta-specific genes (Esx1 and Dlx1) were properly reprogrammed in non-enlarged (71 mg) placentas of fetus clones derived from the TSA and 5AzaC treated ES donor cells. Although Gapdh expression did not differ among normal and enlarged (210 mg) placenta groups, the expression level of Igf2 and Mash2 was higher in enlarged placentas from fetus clones produced from TSA-treated ES donor cells (4.6 and 3.5 fold) compared to non-enlarged placentas from non-treated ES cells (1 fold). Conversely, oversized placentas from cloned fetuses derived from TSA-treated donor ES cells under-expressed Peg1, H19 and Ipl (0.5, 0.2 and 0.2 fold, respectively) compared to control placentas (1 fold). In addition, enlarged placentas from the TSA- and 5AzaC-treated group displayed down-regulation of placenta specific genes Esx1 and Dlx3 and up-regulation of Tpbp, suggesting the presence of abnormal distribution of placental layers. These results indicate that while several imprinted and non-imprinted placenta specific genes were correctly expressed in normal size placentas of fetus clones derived from TSA and 5AzaC treated donor ES cells, enlarged placentas displayed aberrant gene expression patterns, suggesting that improper resetting of the epigenetic program after nuclear transfer is directly related to altered DNA methylation and histone acetylation patterns. Funded by NSERC & CIHR.

27 BIRTH OF AFRICA'S FIRST NUCLEAR-TRANSFERRED ANIMAL PRODUCED WITH HANDMADE CLONING

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Cloning technology has the potential to stimulate the development of the animal biotechnology industry in southern Africa, as well as provide conservationists with an additional tool to possibly assist with conserving critically endangered wildlife species sometime in the future. The aim of this study was to determine whether cloning could produce blastocysts and possibly live progeny in a field-type laboratory without micromanipulators and CO_2 incubator. Approx. 1 × 1-cm ear skin notches were surgically removed from a physically immobilized 9-year-old Holstein cow, a former South African milk production record holder. The tissues were placed into physiological saline and transported to the laboratory at 4°C within 2 h, cleaned with chlorohexidine gluconate and sliced finely in Minimal Essential Medium supplemented with 10% fetal calf serum. The resultant tissue explants were treated as previously described (Bartels *et al.*, 2003 Theriogenology 59, 387) and actively growing fibroblast cultures were made available for the nuclear transfer process. Bovine oocytes from slaughterhouse-derived ovaries were collected and matured for 21 h in modified TCM-199 medium supplemented with 15% cattle serum, 10 IU mL^{-1} eCG and 15 IU mL^{-1} hCG. Nuclear transfer was performed using the HMC technique (Vajta *et al.*, 2003 Biol. Reprod. 68, 571–578). At 21 h after the start of maturation, cumulus cells and zonae pellucidae were removed and oocytes were randomly bisected by hand. Cytoplasts were selected using Hoechst staining and a fluorescent microscope. After a two-step fusion, reconstructed embryos were activated with calcium ionophore and dimethylaminopurine. Culture was performed in SOFaaci medium supplemented with 5% cattle serum using WOWs (Vajta *et al.*, Mol. Reprod. Dev. 50, 185–191). All incubations including culture of donor cells were performed in the submarine incubator system (SIS; Vajta *et al.*, 1997 Theriogenology 48, 1379–1385). In two consecutive experiments, 6 blastocysts were produced from 52 reconstructed embryos. On Day 7, 5 blastocysts were selected for transfer into 3 previously synchronized recipients. All three recipients became pregnant, but two of the recipients aborted at six and seven months, respectively. Post-mortem examination on the first aborted fetus did not reveal any identifiable etiology, but coincided with 6 abortions from natural pregnancies during a heat wave, while the organism *Brucella abortis* was isolated from the second aborted fetus. The third pregnancy went to term, and a healthy calf, weighing 27 kg, was delivered by Caesarean section. The three-month-old calf is being raised by a surrogate Jersey cow under standard dairy conditions and is expected to join the dairy in eighteen months' time. The birth of 'Futhi', meaning 'replicate' in Zulu, is Africa's first cloned animal and signifies an important milestone in the development of animal biotechnology in Africa.

28 PREGNANCIES RESULTED FROM GOAT NT EMBRYOS PRODUCED BY FUSING COUPLETS IN THE PRESENCE OF LECTIN

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The procedure of nuclear transfer (NT) using somatic cells remains inefficient partly due to low fusion rates between donor cells and recipient ooplasm. Lectin is a glycoprotein which specifically binds to carbohydrates to induce a tight contact of membrane to membrane (Booth *et al.*, 2001 Cloning and Stem Cells, 3, 139–159). The purpose of this study was to examine the fusion rates and developmental competence of NT embryos following pre-incubation of couplets in medium containing lectin prior to electrical pulsing. Oocytes were collected by laparoscopic ovum pick-up from hormonally primed goats or by aspiration from culled goat ovaries, and cultured for maturation at 38.5°C, 5% CO_2. At approximately 24 h after the onset of IVM, the cumulus cells were stripped off by brief vortexing in medium containing 0.2% hyaluronidase. Oocytes with first polar bodies were selected for NT. Successful enucleation was confirmed by the absence of MII chromosomes in ooplasm by means of brief exposure of the Hoechst 33342-stained oocytes to UV light. Three cumulus-granulosa cell lines from transgenic goats were used as donor cells. They were cultured to confluency in DMEM + 20% FCS for 6 days prior to NT. Individual donor cells were transferred into the perivitelline space of the enucleated oocytes. Couplets were incubated for 15 minutes in TCM199 + 10% FCS containing 75 or 150 μg mL^{-1} lectin (L-9132 Sigma, St.Louis, MO, USA) prior to being subjected to electrical pulsing (lectin treatment) with one DC pulse at 2.4 kV/cm (1st pulsing). The fusion rate was determined 40–60 minutes after the 1st electric pulsing. Non-fused couplets were exposed to a 2nd pulsing. Approximately 30 minutes later, non-fused couplets were exposed to a 3rd pulsing. Couplets without the lectin treatment served as controls. Reconstructed embryos were activated with 5 μM ionomycin followed by 5 h of incubation in 10 μg mL^{-1} cycloheximide with 7.5 μg mL^{-1} cytochalasin B. A group of 10 to 13 embryos was transferred into a recipient after 12 to 14 h of culture in G1.3. Statistical analysis was performed using the χ^2 analysis. Results are shown in the following table. This study demonstrated that fusion rate could be improved by pre-incubating couplets in the medium containing 150 μg mL^{-1} lectin prior to electrical pulsing and the embryos derived from the lectin treatment could establish the early pregnancies.

Lectin (μg mL^{-1})	No. of couplets	No. of total fused couplets (%)			No. recip. transferred	No. (%) of recip. pregnant	
		1st pulse	1st + 2nd pulse	1st + 2nd + 3rd pulse		Day 30	Day 60
Control	339	136 (40)[a]	217 (64)[a]	265 (78)[a]	21	10 (48)	10 (48)
75	80	31 (39)[a]	48 (60)[a]	60 (75)[a]	4	1 (25)	0 (0)
150	94	46 (49)[a]	75 (80)[b]	88 (89)[b]	5	1 (20)	1 (20)

Data in one column with different superscripts differ significantly ([a,b] $P < 0.05$).

29 HEALTH AND REPRODUCTIVE PROFILES OF NUCLEAR TRANSFER GOATS PRODUCING THE MSP1-42 MALARIA ANTIGEN

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Somatic cell nuclear transfer (NT) using transfected primary cells is an efficient approach for the generation of transgenic large animals. However, abnormalities associated with the NT process could compromise the usefulness of cloned animals. In order to evaluate whether cloned animals could be used for the production of recombinant therapeutic proteins in milk, we examined the health, reproductive performance and milk production of 4 transgenic does derived from somatic cell NT. Two versions of the MSP1-42 transgene encoding either a glycosylated or a non-glycosylated MSP1-42 protein were constructed. Following somatic cell NT, 4 healthy transgenic does were born (Chen LH *et al*., 2002 Theriogenology 57, 777 abst). Does 1A and 2A were obtained from the same cell line carrying the glycosylated MSP1-42 transgene, whereas 3A and 4A were from a cell line carrying the non-glycosylated transgene. Health and growth of these NT animals was monitored with the supervision of a veterinarian and compared to age-matched control dairy does. All animals were bred naturally and, except for one control doe, delivered healthy kids. The 4 NT does produced a total of 9 kids of which 8 were live and one was stillborn. The control does delivered 5 kids, one fetus being lost during early pregnancy. Three of the eight kids from the NT animals were transgenic; all kids were normal and healthy. All does were milked after parturition, and recombinant protein expression in the milk of NT does was evaluated. There were no differences in birth and weaning weights or fertility between NT and control animals. The NT does expressing the glycosylated antigen (1A, 2A) produced only small amounts of milk at induction and postpartum. The reduced milk yield for that line is possibly caused by the expression of the transgene. However, normal lactations were achieved with the 2 does expressing the non-glycosylated antigen (3A, 4A), each producing in 100 days enough MSP1-42 protein to generate 5 million doses of the Malaria vaccine. These data demonstrate that transgenic goats produced by somatic cell NT can be used successfully for the recombinant production of therapeutic proteins.

	NT				Controls			
	1A	2A	3A	4A	1	2	3	4
Birth weight (kg)	2.9	3.3	4	3.5	3.5	3.1	3.2	2.9
Weaning weight (kg)	11.3	13.2	13.7	12.7	14	14.4	13	14.3
Breeding age (months)	18	17	15	15	18	14	12	18
Gestation length (days)	150	152	150	150	148	147	148	resorbed
# offspring	3	2	3	1	2	2	1	0
100-day milk volume (l)	0.057	0.017	270	250	350	320	340	0
MSP1-42 levels (gL^{-1})	>1	>1	>1	>1	NA	NA	NA	NA

NA, not applicable.

30 USE OF ADULT STEM/PROGENITOR CELLS AS NUCLEAR DONORS TO PRODUCE CLONED PORCINE EMBRYOS

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Incomplete or defective nuclear reprogramming may be responsible for low cloning efficiencies. Less differentiated stem cells are thought to be more easily reprogrammed, resulting in improved survival of cloned mice (Rideout WM III *et al.*, 2000 Nat. Genet. 24, 109–110). Our objective was to establish porcine mesenchymal stem cell (MSC) cultures and use these as donor cells in nuclear transfer (NT). A bone marrow (BM) aspirate was collected from an anesthetized gilt. BM mononuclear cells were isolated by centrifugation over a density gradient (Histopaque-1077; Sigma, St. Louis, MO, USA), resuspended in low glucose DMEM (Gibco) plus 10% FBS and plated on flasks; fibroblast-like MSCs were later passaged. Ear skin fibroblast (SF) cultures from the same BM donor gilt were established. Cultures of MSC and SF were exposed to lipogenic, osteogenic or chondrogenic differentiation media (Pittenger MF *et al.*, 1999 Science 284, 143–147) for 14 days. Cells cultured in DMEM with 10% FBS served as controls. Differentiation was assessed by histochemical methods. Calcium deposits and alkaline phosphatase (AP) activity (Vector Red AP Substrate Kit, Vector Labs) were indicative of osteogenic differentiation. MSCs cultured under osteogenic conditions were positive for AP activity and developed a black color after von Kossa staining, indicative of calcium deposition. Oil red O stain identified cellular lipid accumulation. When exposed to adipogenic differentiation media, 10–15% of MSCs developed an adipocyte phenotype with lipid droplet accumulation and oil red O staining. Lipogenic differentiation was not observed in SF and control cultures. Presence of acidic mucopolysaccharides associated with cartilage formation was determined by alcian blue stain. MSCs exposed to chondrogenic conditions were alcian blue-positive, and SF and control cultures were alcian blue negative. For NT, confluent (passage 2) MSC and SF cultures were exposed to roscovitine (15 μM; Sigma) for 24 h. In vitro-matured oocytes were enucleated and a single cell (MSC or SF) was transferred into the perivitelline space. Cell-oocyte couplets were fused in Zimmerman's medium with a single electric pulse (250 V/mm for 20 μs) delivered through a needle-type electrode. NT units were electrically activated (2 pulses of 100 V/mm for 60 μs separated by 5 s) in a chamber 1 h after fusion and transferred to NCSU-23 medium. Embryos were examined for cleavage and blastocyst formation at 2 and 7 days after NT, respectively. Cleavage rates were 53.3% (40/75) for MSC and 59.7% (46/77) for SF NT embryos.

Development to blastocyst stage was 6.6% (5/75) in the MSC group and 1.2% (1/77) in SF group. In conclusion, we established an adult MSC line from a live animal using a minimally invasive BM aspiration technique. Additionally, MSC donor-derived NTs developed to the blastocyst stage. Further experiments will determine nuclear reprogramming in MSC-derived NT embryos.

31 EFFECTS OF CYCLOHEXIMIDE ON CAPRINE SOMATIC CELL NUCLEAR TRANSFER EMBRYO AND FETAL DEVELOPMENT

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The use of protein synthesis inhibitors to down regulate the levels of Maturation Promoting Factor (MPF) following fusion and activation are widely used in the field of Nuclear Transfer (NT). Cycloheximide is a protein synthesis inhibitor that blocks the levels of cyclin B, a component of MPF which is required to maintain MII stage arrest in oocytes. However, it is unclear what, if any, effects these broadbased inhibitors may have on nonspecific protein expression in the oocytes or karyoplasts. The purpose of this study was to examine the effects of treatment with and without cycloheximide on embryo and fetal development to term. Ovulated in vivo MII oocytes from superovulated does during the traditional breeding season were surgically collected and then enucleated and reconstructed with either transfected fetal or adult skin cell karyoplasts or transgenic primary somatic skin cells. Couplets were simultaneously fused and activated with a single electrical pulse between 2.6 and 3.0 kV cm^{-1} for 20 μs. An additional electrical pulse was given to fused couplets and non-fused couplets were re-fused. Reconstructed embryos were either treated with 5 μg mL^{-1} cyclohexamide (Sigma, St. Louis, MO, USA) for a minimum of 3 h or cultured directly post-fusion and activation. The embryos were cultured in SOF + BSA at 38°C in a humidified modular incubation chamber (Billrups-Rothenberg, USA) containing 6% O_2, 5% CO_2, and 89% N_2 for 24–48 h. Cleavage was assessed, and nuclear transfer embryos, with age appropriate development (up to 2 cells at 24 h or 2 to 8 cells by 48 h), were surgically transferred to the oviduct of surrogate recipient does. There were 18 and 11 confirmed pregnancies by Day 50 post-fusion and activation from the cycloheximide and non cycloheximide groups, respectively. A total of 12 recipients that received cycloheximide treated embryos produced term pregnancies and yielded 15 offspring. Alternatively, 9 recipients that received untreated embryos produced term pregnancies which yielded 12 offspring. A total of 27 NT offspring were produced and one offspring from a set of quadruplets died several days post-natally. While significantly more cycloheximide treated NT embryos cleaved, there was no significant difference in pregnancy rate or offspring born between treatment groups. These results suggest that the use of cycloheximide for embryo development in somatic cell nuclear transfer may not be necessary for establishing and maintaining caprine NT pregnancies.

Treatment	Enucleated	Reconstructed	Fused (%)	Cleavage (24 + 48 hr) (%)	Pregnant preg/ recips. (%)	Development offspring/ embryo (%)
Cycloheximide	1474	1320	1122[a] (85)	440[a] (39)	18/104[a] (17)	15/741[a] (2)
Non Cycloheximide	1328	1164	955[a] (82)	261[b] (27)	11/84[a] (13)	12/591[a] (2)

Values total from 46 experiments. Data were analyzed by the Chi-square test. [a,b]within columns differ significantly, $P < 0.01$. Cleavage includes development at 24 h (1–2 cell) and 48 h (2–8 cell) post fusion activation.

32 ULTRASOUND MEASUREMENTS OF BOVINE SOMATIC CLONES AT DAY 50 AND DAY 64 OF PREGNANCY

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The overall efficiency of cloning has stayed low since this technique appeared. Most of the losses in the bovine species take place in early gestation between Day 35 and Day 70, when deficient placental development has been described. Late fetal losses are associated with excessive fetal growth and placental hydrops (Large Offspring Syndrome or LOS). One study measuring crown-rump length (CRL) by ultrasound (US) did not show any difference between clones and controls (Pace *et al*., 2002). The objective of this work was to compare fetal and placental measurements at Days 34, 50, and 64 for bovine clones and control pregnancies to determine whether these could be predictive of a further development of LOS. Twenty-five clone and 32 control (28 AI and 4 IVF) singleton Holstein pregnancies were used for US with a 5-MHz rectal probe. Clones originated from adult skin fibroblasts (three genotypes), used after serum starvation, as previously published (Vignon *et al*., 1998). CRL and head length (HL), orbital diameter (OD), vesicle width (VW), and length and width of the placentome closest to the fetus were measured at each stage. CRL were also recorded from six clone, six IVF and five AI, Day 34 and Day 60 fetuses recovered at slaughter. Maternal clinical parameters, US images with increased fetal fluids, excessive weight, and clinical features at surgical removal or birth were used to diagnose LOS. Data were analysed using multiple regression analysis with SAS software. There were no differences between IVF and AI fetal and placental measurements by ultrasound and the two groups were pooled as a single control group. LOS was diagnosed in 29% of the clones. CRL and HL were significantly smaller in clones at all stages (CRL: 10.2 ± 3.8 mm, $n = 5$ v. 13.6 ± 2.4 mm, $n = 7$, at retrieval at Day 34; 31.8 ± 5.6 mm v. 36.0 ± 4.6 in controls at Day 50, by US, and 48.6 ± 11.4 v. 63.3 ± 4.9 at Day 64, by US, $P < 0.05$, in clones and controls, respectively) but there was no difference between the clones developing LOS and those that did not. There was no difference for OD or VW at any stage. Placentome width but not length was statistically smaller in clones compared to controls (4.8 ± 1.5 v. 7.2 ± 1.7 mm at Day 50 and 5.7 ± 2.0 v. 9.4 ± 4.2 at Day 64, in clones and controls, respectively, $P < 0.05$). There were only two clones retrieved at Day 64, one degenerated and the other similar to controls. Placentome development was delayed. These data clearly show

that although a large proportion of clones develop LOS in late gestation, they are actually smaller than controls in early pregnancy, whether or not they will develop LOS. This is probably due to delayed or abnormal early placental development, which can be detected by US. US cannot be used, however, to predict further development of LOS, and other means such as maternal plasma PSP60 concentrations (Heyman *et al*., 2002) must be used.

33 DEVELOPMENT OF BOVINE EMBRYOS CLONED WITH FETAL FIBROBLASTS ARRESTED AT G0/G1 PHASE BY DIFFERENT TREATMENTS

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Numerous factors have an effect on the development of cloned embryos, and one of the most important might be the synchronization between donor nuclei and recipient ooplasts. The objective of this study was to examine the effect of donor cell treatments for G0/G1 synchronization and the donor cell type on development and incidence of apoptosis in cloned cattle embryos. Primary cultures were established from a female fetus on Day 50 of gestation and adult ear skin biopsies. The cells were used for assessements of cell cycle and apoptosis, and for nuclear transfer. Cells were randomly allocated into 3 experimental treatment groups after 6–8 passages: Group 1 (confluent), cells were cultured in DMEM supplemented with 10% FBS until 90% confluent; Group 2 (serum-starvation), cells were cultured in DMEM supplemented with 0.5% FBS for 5 days; Group 3 (Roscovitine), cells were cultured in DMEM supplemented with 10% FBS and 30 μM Roscovitine for 12 h. Cell cycle and apoptosis were analyzed using flow cytometry after labelling with DAPI and YO-PRO-1, respectively. At 19 h post-maturation (hpm), enucleated oocytes were reconstructed with donor cells and fused by a single DC pulse (1.6 kV/cm, 60 μs) delivered by a BTX 200. After activation with the combination of ionomycin (5 μM, 5 min) and cycloheximide (10 $\mu g\,mL^{-1}$, 5 h), the eggs were cultured in CR1aa medium for 3 days and additionally cultured in CR1aa medium supplemented with 30 $mg\,mL^{-1}$ BSA for 5 days at 39°C in a humidified atmosphere of 5% CO_2 in air. Differences between groups were analyzed using one-way ANOVA after arc-sine transformation of the proportional data. There were no significantly differences in the incidence of cells arrested at G0/G1 for fetal fibroblasts cultured in the three treatment groups (87%, 83% and 80%; confluent, serum starvation and Roscovitine, respectively). More cells were apoptotic in Group 2 compared to the cells in Groups 1 and 3 (12% v. 6 and 6%, respectively) ($P < 0.05$). Blastocyst development of cloned embryos was significantly ($P < 0.05$) higher when fetal fibroblasts from Group 1 were used, compared to Groups 2 and 3 (35.1%, v. 31 and 29.7%, respectively). Similar results were observed in the use of ear skin fibroblasts as nuclear transfer donor cells (32.7%, v. 24 and 24%, respectively). These results suggest that fetal fibroblasts can be effectively synchronized at G0/G1 by three different treatments, including growth to confluence, serum-starvation and Roscovitine treatment. However, based on blastocyst development and levels of apoptosis, the use of confluent fetal fibroblasts as donor cells is more effective than using cells synchronized by serum-starvation or Roscovitine treatment in the production of cloned bovine embryos. [Supported by High Technology Development Project for Agriculture and Forestry Korea, MAF-SGRP, 30012-05-3-SB010 and Cho-A Pharm. Co. LTD.]

34 EFFECT OF GENOTYPE AND CELL LINE ON THE EFFICIENCY OF LIVE CALF PRODUCTION BY SOMATIC CELL NUCLEAR TRANSFER

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The efficiency of production of live calves using somatic cell nuclear transfer was compared among 52 different cell lines representing 43 different genotypes. Cell lines were not genetically modified. Nuclear transfer was performed according to methods described by Cibelli *et al.*, 1998 Science 280, 1256–1258, with modifications. All cells were derived from either explant cultures or enzyme digests of skin biopsies and were cyropreserved and thawed at least 48 hours prior to nuclear transfer. Cells were harvested using either pronase or trypsin at 70 to 90% confluence. Oocytes were either activated prior to fusion or immediately after fusion using ionomycin. The couplets were then cultured in cycloheximide and cytochalsin B for 6 hours. In 36 cases (84%), at least one healthy calf was produced from the initial trial which included transfer to 10 to 20 recipients for each cell line. For 4 of the 7 cases where the initial cell line failed to produce a live calf, a new cell line was derived and the process repeated. In one case where the data are available from the second cell line, 5 live calves were produced from 20 recipients receiving embryos (25%). Results from the other repeated cell lines are pending. For 5 of the different genotypes, nuclear transfer was done at about the same time using two different cell lines, and 4 of these have produced healthy calves from both cell lines. In one case, one cell line produced live calves, and no calves were produced from the other cell line. In total, 167 calves were born, of which 107 are alive and healthy as of this writing (64%), and range in age from 1 to 25 months. There are 86 calves older than 6 months of age and no losses have occurred as calves have aged into early adulthood. Forty-four (26%) of the calves were stillborn, failed to convert to neonatal circulation or were euthanized within 48 hours of birth. The most frequent reason for euthanasia was severe contracture of the limbs (arthrogryposis). This defect occurred even within cell lines that also gave rise to healthy calves, although it was more prevalent with certain cell lines. Other complications among the normal calves born were those of an abnormally large umbilicus or umbilical vessels. In addition, 16 calves were lost after the first 48 hours (13%). Two of these losses were due to accidents and 9 of them were due to complications from umbilical infections. The other 5 calf loses resulted from complications common to young calves such as clostridial infection and ruptured abomasum. Recent improvements in cell line derivation and embryo culture techniques, as well as a higher incidence of natural birth and improved neonatal management, have resulted in healthy calf production efficiencies (from embryos transferred) greater than 30% for 5 independent genotypes. The number of healthy calves produced per embryo transferred was 11 of 20 (55%), 5 of 10 (50%), 5 of 10 (50%), 4 of 11 (36%), and 3 of 10 (30%), for each of these genotypes, respectively. There was no correlation between the efficiency of blastocyst production and pregnancy outcome for the cell lines evaluated in this study. In conclusion, the efficiency of live healthy calf production using somatic cell nuclear transfer

remains variable, depending on both the cell line and the genotype. However, efficiencies approaching those obtained using conventional embryo transfer is possible.

35 ANALYSIS OF EPIGENETIC MODIFICATIONS AND GENOMIC IMPRINTING IN NUCLEAR TRANSFER DERIVED *BOS GAURUS* × *B. TAURUS* CONCEPTI

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Somatic cell nuclear transfer in cattle is an inefficient process hindered by low pregnancy rates and fetal placental abnormalities. Improper or incomplete epigenetic reprogramming of the donor genome has been implicated as a cause for these aberrations and has been investigated extensively in mice. Here we report the use of a bovine interspecies model (*Bos gaurus* × *B. taurus*) for the assessment and characterization of epigenetic modifications and genomic imprinting in 40-day-old female nuclear transfer (NT)-derived fetuses and placentas. Previously, we identified genomic imprinting at the IGF2, GTL2 and XIST loci in the *Bos gaurus* × *B. taurus* fetuses. These results indicated maternal and paternal imprinting of the IGF2 and GTL2 loci, respectively, in the chorion, allantois, liver, lung and brain, whereas the XIST locus was maternally imprinted solely in the chorion of females. We extended this analysis to 40-day-old NT fetuses derived from a hybrid lung fibroblast cell line (female). Analysis of the donor cell line indicated conservation of imprinting of the IGF2 and GTL2 loci and bialleic expression of the XIST locus, presumably from the random patterns of X-chromosome inactivation. Analysis of three NT and three control pregnancies indicated disruption of genomic imprinting at the XIST locus in the chorions of all three clones compared to controls. In contrast, proper allelic expression of the IGF2 and GTL2 loci was observed in all fetuses and placentas. Quantification of maternal and paternal XIST transcripts in the chorion of clones and controls demonstrated a significant skewing from preferential paternal expression in controls (95.0 ± 0.882, mean ± S.E.) to mixed paternal and maternal expression in clones (73.6 ± 5.2), (*t*-test; $P < 0.05$). In an attempt to determine the cause for the abnormal allelic expression of the XIST locus in the chorion of the clones, methylation analysis of the XIST Differentially Methylated Region (DMR) was performed. Methylation-sensitive restriction digests and subsequent PCR of the XIST DMR indicated patterns were not different between controls and clones. However, when genome-wide and promoter-specific methylation analysis (bisulfite sequencing) was extended to the satellite I repeat element and epidermal cytokeratin promoter, hypermethylation was observed in the chorion of clones. These results demonstrate disruption of genomic imprinting in XIST locus in the placenta of 40-day-old clones independent of DMR methylation. They also indicate that cloning is associated with increased levels of methylation in selected genomic regions in the chorion.

36 COMPARISON OF THE DEVELOPMENTAL POTENTIAL OF CAPRINE NUCLEAR TRANSFER EMBRYOS DERIVED FROM IN VITRO AND IN VIVO MATURED OOCYTES

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The objective of this study was to compare the development to the blastocyst stage of reconstructed caprine nuclear transfer (NT) embryos derived from two sources of ova. In vivo oocytes were flushed from the oviduct of superovulated donors by exposing the reproductive tract via a small ventral laparotomy. In vitro oocytes were collected from ovaries supplied by an abattoir located in Purdue, IN. Oocytes were aspirated, cultured in maturation medium (M199 +10% goat serum, 3 $\mu g\,mL^{-1}$ LH, 3 $\mu g\,mL^{-1}$ FSH and 0.22 mM sodium pyruvate), and shipped overnight (38°C, air). Donor cell preparation and NT procedures were as previously reported (Behboodi *et al*., 2001 Theriogenology 55, 254 abst). Donor cells were transfected female fetal fibroblasts that were synchronized by 4 days of serum starvation, followed by a 10-hour exposure to medium containing 10% FCS. Oocytes were enucleated, karyoplast-cytoplast couplets were reconstructed, fused and then activated simultaneously by a single electrical pulse. Couplets containing in vitro oocytes were incubated in the presence of 5 $\mu g\,mL^{-1}$ ionomycin after fusion. Fused couplets were co-cultured in TCM199 with 10% FCS and oviductal epithelial cells for 8–10 days (38°C, 5% CO_2). Embryos that developed in vitro to the blastocyst stage were surgically transferred to recipients. Pregnancies were confirmed by ultrasonography. One live kid was delivered on Day 150 of gestation via elective C-section. Southern blotting analysis confirmed that it was derived from the transgenic donor cell line. These experiments show that in vivo matured oocytes not only better support caprine NT embryo development to the blastocyst stage, but also can result in live birth (table). Although fusion and cleavage rates were similar in the two groups, development to the blastocyst stage was significantly higher (Student's *t*-test) in the group utilizing in vivo-matured oocytes. In conclusion, this is the first live goat produced from goat NT blastocysts developed in vitro. This suggests that in vivo matured oocytes may be superior to oocytes developed in vitro for generating live animals from NT blastocysts.

Oocytes	Cell lines	No. couplets	No. fused	No. cleaved	No. blastocysts	No. recipients	No. births
In vivo	T66	30	27	17	4	2	1
	T93	30	14	10	2	1	0
	T321	50	34	24	8	4	0
Total		110	75 (68%)[a]	51 (68%)[a]	14 (26%)[a]	7	1
In vitro	T65	30	25	20	1	1	0
	T66	32	25	18	2	1	1 Stillbirth
	T321	60	27	22	2	1	0
Total		122	77 (63%)[a]	60 (78%)[a]	5 (8%)[b]	3	1 Stillbirth

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

37 DEVELOPMENTAL POTENTIAL OF CLONE CELLS IN MURINE CLONE-FERTILIZED AGGREGATION CHIMERAS

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In both murine and porcine preimplantation stage clones, mosaicism in gene expression has been observed, indicating variation in transcription of some genes between cells of the individual clone (Boiani M *et al.*, 2002 Genes Dev. 16, 1209–1219; Park KW *et al.*, 2002 Biol. Reprod. 66, 1001–1005). This observation raises the question as to whether all blastomeres within one early-stage clone are equivalent, or whether there are differences in developmental potential. To address this, we aggregated preimplantation-stage clone embryos with fertilized embryos and assessed contribution of Oct4-GFP expressing cells of clone origin in blastocysts and in vitro outgrowths. In normal embryos, the Oct4-GFP transgene is expressed during preimplantation stages and reflects expression of Oct4 protein. Mouse cumulus cell clones were produced from cells transgenic for Oct4-GFP (Szabó PE *et al.*, 2002 Mech. Dev. 115, 157–160) as described (Boiani M *et al.*, 2002 Genes Dev. 16, 1209–1219). Four-cell-stage clones and synchronous fertilized non-transgenic embryos were aggregated in micro-wells after removal of the zona pellucida using acid Tyrode's solution. Aggregates were cultured to the blastocyst stage in -MEM supplemented with bovine serum albumin (0.4% w/v). All control chimeras produced from four-cell-stage fertilized non-transgenic and Oct4-GFP transgenic embryos formed blastocysts, and 15 of 20 had GFP-expressing cells. The majority of clone-wild-type aggregates developed to the blastocyst stage (35/40); however, contribution of GFP-expressing cells was observed in fewer blastocysts compared to controls (12/35; $P < 0.05$). Contribution of GFP expressing clone cells to the ICM varied between 30% and 100% of cells as determined by subjective evaluation of GFP fluorescence overlaying bright-field images. During in vitro outgrowth formation of synchronous aggregation chimeras of clone and wild-type embryos, maintenance of clone contribution to the ICM mound was observed, but at a lower frequency (12% v. 34% at the blastocyst stage). The results suggest that aggregation with fertilized cells does not provide benefit to clone blastomeres during preimplantation stages. Possibly, clone blastomeres may not be competitive with wild-type blastomeres, or are developmentally asynchronous, which will be tested using asynchronous chimeras.

38 BIRTH OF AFRICAN WILD CAT CLONED KITTENS

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The African wild cat (AWC, *Felis silvestris lybica*; $2n = 38$) is one of the smallest wildcats, and it's future is threatened by hybridization with domestic cats (*Felis silvestris catus*; $2n = 38$). Nuclear transfer (NT) is a potentially valuable tool for retaining genetic variability, and could assist in the continuation of species with few remaining individuals. Inter-species nuclear transfer into domestic cat (DSH) supports development of somatic cell nuclei from AWC (Gomez *et al*., 2003, Biol Reprod 69, 1032–1041). Therefore, the purpose of the present study was to evaluate the in vivo developmental competence of nuclear transfer embryos derived by fusion of African wildcat fibroblasts with domestic cat cytoplasts, after transfer into domestic cat recipients. In vivo- and in vitro-matured domestic cat oocytes were mechanically enucleated in modified Tyrodes salt solution supplemented with 20 μg mL^{-1} of cytochalasin B (CCB) and 2 mg mL^{-1} of sucrose, and reconstructed with AWC fibroblast cells derived from an adult male; cultured and passaged 1 to 3 times before serum-starved with DMEM + 0.5% FBS and cultured for 5 additional days before use. Fusion took place in fusion medium (0.3 M mannitol and 0.1 mM Mg + 2), and membrane fusion was induced by applying a 3 s AC pre-pulse of 20 V, 1 MHz; followed by two 30 μs DC pulses of 240V/mm at intervals of 0.5 s. Fused couplets were activated 2–3 h after fusion by placing the couplets between two electrodes in a fusion chamber containing 3 mL of fusion medium and exposing them to two 60 μs DC pulses of 120 V/mm. Then, couplets were incubated in 30 μL drops of Tyrodes solution containing 1% MEM nonessential amino acids, 3 mg mL^{-1} BSA (IVC-1 medium), and supplemented with 10 μg mL^{-1} cycloheximide and 5 μg mL^{-1} CCB at 38°C in 5% CO_2 for 4 h. After activation, cloned embryos were cultured in 500 μL of IVC-1 medium until the day of the transfer. Derived AWC NT embryos were transferred into the oviducts (Day 1) or uteri (Days 5, 6, 7) of 36 gonadotrophin-treated DSH recipients on Day 1 after ovulation or on Days 5, 6, or 7 after oocyte aspiration, respectively. Pregnancy was assessed by ultrasonography on Days 21 to 23. One domestic cat was still pregnant and ongoing on Day 60. Kittens were delivered by Cesarean section in each of the seven pregnant recipients on days 61 to 67 of gestation. The kittens weighed an average of 86.2 g (50.0 to 103 g) and died within 36 h after delivery. The post-mortem pathology reports revealed that most of them had an immature respiratory system. The clonal status of the kittens was assessed by multiplex PCR amplification of 20 microsatellite markers, including seven markers that are known to be on the X chromosome. Results from these assays confirmed that the AWC kittens had originated from the AWC donor somatic cell line and were not related to the DSH recipient cats. In summary, these results indicate that AWC cloned kittens can be produced by ET of embryos derived from AWC cells into DSH cytoplasts. Research was funded partially by the John & Shirley Davies Foundation.

Embryos			Recipients		Pregnant no. (%)	Fetuses no.	Kittens no.	Kittens/fetuses (%)
Day	Total	Avg./cat	Day	No.				
7/8	142	15.7	7	9	0	0	0	0
6	150	18.7	6	8	0	0	0	0
6	148	37.0	6	4	2 (50.0)	3	3	100
5	49	24.5	5	2	1 (50.0)	1	1	100
5	295	49.1	5	6	2 (33.3)	2	1	50.0
1	260	37.1	1	7	3 (42.8)	8	3	37.5

39 MATERNAL ORIGIN OF OOCYTES AFFECTS IN UTERO DEVELOPMENTAL CAPACITY AND PHENOTYPE OF BOVINE NUCLEAR TRANSFER FETUSES

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Epigenetic phenomena have been recognized as an important issue in somatic cell nuclear transfer (SCNT) and have been linked to SCNT success rate. We have previously reported a significant maternal lineage effect of oocyte donors on the number of transferable embryos obtained after SCNT (Brüggerhoff *et al.*, 2002 Biol. Reprod. 66, 367–373), pointing to ooplasmic maternal genetic/epigenetic factors important for early embryonic development. We have now extended this study and generated a set of cloned embryos using recipient oocyte pools from defined or random maternal sources that were identified by pedigree data and mitochondrial DNA (mtDNA) haplotype, to study effects of nuclear-cytoplasmic interactions on in utero developmental capacity and fetal phenotype. Embryos were reconstructed from granulosa cells of a *Bos taurus* (Brown Swiss) cow using oocytes with *B. taurus* A (Simmental), *B. taurus* B (Simmental), *B. indicus* (Dwarf Zebu), or random *B. taurus* (Brown Swiss) cytoplasm. Two to three embryos per recipient heifer (Simmental) were transferred on Day 7 after SCNT, and fetuses were recovered after slaughter of recipients on Day 80. The number of pregnancies obtained per transferred embryo on Day 28 was similar for the *B. taurus* combinations (0.11–0.15) but much higher for a limited number of embryos reconstructed with *B. indicus* cytoplasm (0.50). Developmental capacity of embryos derived from the four oocyte pools varied considerably and resulted in 8 (11.1%), 3 (4.6%), 2 (33%) and 10 (15.2%) viable fetuses with *B. taurus* A, *B. taurus* B, *B. indicus* and random *B. taurus* cytoplasm, respectively. When fetuses in resorption were included, however, the percentages for recovered fetuses on Day 80 were similar (12.5, 13.8 and 16.7%) in all *B. taurus* combinations. Body weight, crown rump length, thorax circumference ($P < 0.05$), and femur length ($P < 0.01$) of viable fetuses with *B. taurus* A cytoplasm differed from fetuses with random *B. taurus* cytoplasm and showed less variation. Furthermore, body weights of fetuses with *B. taurus* A or B, or *B. indicus* cytoplasm, were not significantly different from those of *B. taurus* (Brown Swiss) control fetuses produced by artificial insemination, but fetuses reconstructed with cytoplasts from randomly collected *B. taurus* oocytes indicated fetal overgrowth (80.6 ± 6.5 g v. 107.9 ± 5.8 g, $P < 0.01$). Our data point to complex ooplasm-dependent epigenetic modifications and/or nuclear DNA mtDNA interactions that affect developmental capacity and fetal weight and dimensions in SCNT. This might also be relevant for crossbreeding in farm animals and other reproductive technologies such as ooplasmic transfer in human-assisted reproduction

40 PREDICTORS OF CLONED CALF VIABILITY

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The high rate of gestational loss in nuclear transfer (NT) pregnancies is a major economic and animal cost, preventing the widespread use of the technique. It is currently not possible to predict which early gestation fetuses will remain viable to term. As placental abnormalties are commonly associated with nonviable fetuses, placental proteins such as pregnancy specific protein (PSP 60, Heyman *et al.*, 2002 Biol. Reprod. 63, 1787–1794; or PSPb Hill *et al.*, 2000 Biol. Reprod. 66, 6–13) have been investigated as possible markers of viable pregnancy. To build upon these studies, we explored the predictive value of Day-35 maternal serum PSPb value to final cloned-calf viability; PSPb profiles throughout pregnancy; and the relationship of maternal plasma estradiol levels to calf viability. Maternal serum was sampled for PSPb at the time of the first ultrasound pregnancy examination (Day 35) to determine fetal number and viability (heartbeat). PSPb values were determined using an RIA by Dr. Garth Sasser (BioTracking, Moscow, ID). Median PSPb values were compared using the Kruskal-Wallis one-way ANOVA test on ranks. PSPb levels from failed single and twin pregnancies were significantly different from those of AI bred controls ($P < 0.05$). PSPb levels from failed singles were not significantly different from those of viable single pregnancies although only nonviable cloned pregnancies had PSPb values >7.5. As PSPb levels at Day 35 were not highly predictive of viable outcome, we detailed the gestational PSPb profile for a different group of cows ($n = 40$) that carried cloned pregnancies beyond Day 90. PSPb was determined at Days 35, 50, 65, and 90, then monthly to Day 240. Although there was no clear statistical significance, PSPb levels from nonviable cloned pregnancies were consistently higher at each time point than for viable pregnancies (14 cows gave birth to live calves) and significantly higher than those of controls. At term, plasma estradiol concentrations were assessed in a group of 5 NT pregnancies. Estradiol was investigated as cloned pregnancies often show a lack of readiness for parturition (e.g. lack of udder development, prolonged gestation, dysmature calves). These 5 pregnancies produced 3 highly viable NT calves: 1 with lowered viability that survived only with a high level of intensive care, and 2 nonviable despite a high level of assistance. In this small group, estradiol levels on the day of birth rose in parallel with viability (viable, 662 pg; lowered viability, 170 pg; nonviable, 76 pg). This is a clinical observation rather than a statistical trend that suggests further investigation may be warranted. In summary, our results suggest maternal PSPb levels have value in identifying the outliers in a population of cloned pregnancies and that term-cloned pregnancies may show abnormally low maternal estradiol concentrations at term.

Outcome	No. tested at Day 35	Mean PSPb at ng mL^{-1}	SD
Died at term	5	5.4	1.5
Single abort	38	5.0	2.1
Single viable	12	4.2	1.1
Single control	11	2.8	0.8
Twin abort	18	4.9	1.4
Twin viable	5	4.4	0.7

41 EFFECTS OF MATURATION PERIOD OF PORCINE OOCYTES ON DEVELOPMENT FOLLOWING SOMATIC CELL NUCLEAR TRANSFER

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Despite intensive research, porcine nuclear transfer is still characterized by low success rates. To determine the effect of maturation period of porcine oocytes on subsequent development following nuclear transfer, we investigated fusion rate, induction of activation and development to blastocyst stage of somatic cells. For this we used MII-oocytes after 38, 40, and 42 h of maturation culture as recipients. Oocytes surrounded by a compact cumulus mass were selected and placed into North Carolina State University (NCSU) 37 oocyte maturation medium supplemented with 0.1 mg mL^{-1} cysteine, 10 ng mL^{-1} epidermal growth factor, 10% porcine follicular fluid, 50 μm 2-mercaptoethanol, 0.5 mg mL^{-1} cAMP, 10 IU each of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) for 22 h in humidified air with 5% CO_2 at 38.5°C. Subsequently the oocytes were moved to fresh NCSU37 maturation medium without cAMP, eCG and hCG and incubated for an additional 16, 18, and 20 h. In the first experiment, a total of 878 MII-arrested oocytes were enucleated, fused with pig fetal fibroblasts in calcium-free medium and activated approximately 3 h later with an electrical stimulus. This was followed by incubation in 6-dimethylaminopurine for 3 h and subsequent analysis of development in vitro. Maturation period had no effect on the frequencies of fusion (87% v. 75% v. 84%, respectively), and cleavage (82% v. 81% v. 87%, respectively), but when MII-oocytes recovered at 40 h of maturation were used as recipients, 41/279 (14,8%) the numbers of cloned embryos developing to the blastocyst stage on Day 7 of culture was significantly (ANOVA followed by multiple pairwise comparisons using Tukey test, 6 replicates, $P < 0{,}05$) higher than those of embryos reconstituted with oocytes collected at 38 h (27/285, 9.6%) and 42 h (16/314, 4.9%). In the second experiment, reconstructed embryos derived from oocytes matured for 40 h were surgically transferred to the oviducts of synchronized German Landrace gilts. Transfers were made on the first day of standing oestrus within 3 h of activation to assess their development in vivo. Synchronization was achieved by injections of 1500 IU eCG followed by 500 IU hCG 3 days later. Of 4 recipients receiving an average of 150 zygotes (range, 136 to 163), 2 became pregnant as determined by ultrasound between Days 25 and 35 of gestation. Of the two pregnant recipients, one subsequently farrowed 4 piglets on Day 115 of pregnancy. These results indicate that the maturation period is critical and affects development of porcine nuclear transfer embryos. This study was funded by the Deutsche Forschungsgemeinschaft (DFG; SFB265).

42 ANGIOGENESIS IN CLONED AND IVF-DERIVED BOVINE PREGNANCIES AT DAY 30 OF GESTATION

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New and expanded microvascular networks in maternal and embryonic tissue are vital to development of a functional placenta. Typically a large proportion of bovine pregnancies derived from somatic cell nuclear transfer (NT) are lost between Days 25 and 45 of gestation. Several laboratories have noted aberrant placental development of NT embryos and fetuses; one factor that may contribute to these defects is faulty maternal or embryonic angiogenesis during placentation. The aim of this study was to determine if angiogenesis in maternal caruncular tissues differs in bovine NT- and IVF-derived pregnancies at the time of placentation. Cloned embryos were produced using cultured ear skin fibroblasts from a 15-year-old Hereford cow; in other experiments, these donor cells resulted in a term live calf and 67% of viable Day-30 embryos dying by Day 51 of gestation. For IVF-derived embryos, IVM oocytes were fertilized with semen from Hereford or Angus bulls. All embryos were cultured in vivo in ligated sheep oviducts, and at Day 8 blastocysts were transferred to synchronized recipient heifers. Whenever possible, one embryo was transferred to each uterine horn. A total of 41 NT and 41 IVF grade 1, 2 and 3 embryos were transferred to 48 recipients. Viable NT ($n = 9$; 22.0% of transferred) and IVF ($n = 9$; 22.0%) embryos were recovered at Day 30, and caruncular tissue adjacent to each embryo was sampled. Expression of genes implicated in angiogenesis was measured by real-time quantitative RT-PCR and normalized to histone 2A expression. In addition, paraformaldehyde-fixed sections were stained by the Periodic Acid-Schiffs method to identify blood vessels. Microvascular density was determined as percentage of total tissue area composed of blood vessels in the luminal caruncular stroma as measured by two observers. Data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, Cary, NC, USA). Quantification of gene expression revealed no differences between NT and IVF pregnancies for angiogenesis-promoting growth factors and their receptors: vascular endothelial growth factor-A (VEGF-A), VEGF-C, placental growth factor, VEGF receptor-1 (VEGFR-1/flt-1), VEGFR-2 (flk-1/KDR), VEGFR-3 (flt-4), angiopoietin-1 (Ang-1), and Ang receptor-1 (tie-1). Likewise, NT and IVF pregnancies demonstrated similar mean microvascular densities in the caruncles; however, density differed between individual pregnancies across both groups ($P < 0.05$). For these experiments, the timing of NT and IVF, embryo transfer, and tissue collection on Day 30 was as consistent as possible. Despite these efforts, embryo morphology and developmental stage were highly variable even within the IVF group. It follows that the progress of placentation, and hence the signaling between each embryo and the maternal tissues, may have differed as well, accounting for the variation observed. Our results suggest that failure of maternal tissue to increase angiogenesis during placentation is not a primary cause of aberrant placental development in cloned cattle.

43 IMPROVED IN VITRO DEVELOPMENT OF PORCINE NUCLEAR TRANSFER EMBRYOS WITH 6-DMAP FOLLOWING FUSION

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Although nuclear transfer (NT) has successfully produced cloned piglets, the development to blastocyst and to term is still low. Activation of the NT embryos is one of the key factors to improve the developmental ability of porcine NT embryos. Electric pulses as well as chemicals have been used to activate porcine NT embryos. This study was conducted to investigate the effect of continued activation following fusion pulses on in vitro development of porcine NT Embryos. Oocytes derived from a local abattoir were matured for 42 to 44 h and enucleated. Ear skin cells were obtained from a 4-day-old transgenic pig transduced with eGFP recombinant retrovirus. Enucleated oocytes were reconstructed and cultured in PZM-3 in a gas atmosphere of 5% CO_2 in air. Cleavage and blastocyst developmental rates were assessed under a stereomicroscope on Day 3 or 6. Blastocysts were stained with 5 μg of Hoechst 33342 and total cell number was determined with an epifluorescent microscope. In Experiment 1, oocytes were activated with two 1.2 kV/cm for 30 μs (E) in 0.3 M mannitol supplemented with either 0.1 or 1.0 mM Ca^{2+}. In each treatment, activated oocytes were divided into three groups. The first group was control (E). Other two groups were exposed to either ionomycin and 6-DMAP (E + I + D) or 6-DMAP (E + D) immediately after the electric pulses. In Experiment 2, fusion was conducted by using 1.0 mM Ca^{2+} in the fusion medium. Fused NT embryos were divided into three treatments. NT embryos were fused and activated simultaneously with electric pulse as a control (C); the second group was treated with 6-DMAP immediately after fusion treatment (D0); and the third group was treated with 6-DMAP at 20 min (D20) after fusion. In experiment 1, for 0.1 mM Ca^{2+}, developmental rates to the blastocyst stage for E, E + I + D or E + D were 12.5, 26.7 and 22.5%, respectively. For 1.0 mM Ca^{2+}, developmental rates to the blastocyst stage were 11.4, 28.3 and 35.6%, respectively. The activated oocytes treated with 6-DMAP following the electric pulses by using 1.0 mM Ca^{2+} in fusion medium had higher ($P < 0.05$) developmental rates to the blastocyst stage. In Experiment 2, developmental rates to the blastocyst stage for C, D0 or D20 were 10.0, 12.3, and 19.9%, respectively. Developmental rate to the blastocyst stage was higher ($P < 0.05$) in D20. Fragmentation rates were 19.9, 10.8, and 9.0%, respectively. Regardless of Ca^{2+} concentration in fusion medium, continued treatments with chemicals following electric pulses supported more development of porcine activated oocytes. Treating NT embryos with 6-DMAP alone after fusion was completed by using 1.0 mM Ca^{2+} in fusion medium improved the developmental rates to the blastocyst stage and prevented fragmentation accompanied by electric fusion. This study was supported by NIH NCRR 13438 and Food for the 21st Century.

44 POSSIBILITY OF PRODUCTION OF A TRANSGENIC CLONED COW USING THE SOMATIC CELL NUCLEAR TRANSFER OF HUMAN ALPHA 1-ANTITRYPSIN GENE-TRANSFECTED CELLS

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The present study was designed to produce, using a somatic cell nuclear transfer (SCNT), transgenic cloned cows that secreted a therapeutic protein into milk. Bovine cumulus cells were transfected with a plasmid that contained the alpha 1-antytrypsin DNA and green fluorescent protein (GFP) reporter gene using a Fugene 6 as a lipid carrier. In vitro-matured bovine oocytes were enucleated followed by confirmation of enucleation via nuclear staining. The GFP-expressing donor cells with the transgene were selected under blue fluorescent light just before injection of cells. The SCNT was performed according to the established system in our laboratory (Jang G *et al.*, 2003 Reprod. Fertil. Dev. 15, 179–185). The couplets were fused, chemically activated, and cultured in modified synthetic oviduct fluid (mSOF) at 39°C in a humidified atmosphere of 5% CO_2 in air for up to 7 days. The developmental ability (in Experiment 1) and number of inner cell mass (ICM) and trophectoderm (TE) cells (in Experiment 2) in transgenic and non-transgenic cloned embryos determined by differential staining as described by Thouas *et al*., (2001 Reprod. Biomed Online 3, 35–29) were compared. In Experiment 3, only GFP-expressing blastocysts were transferred to the uteri of recipient cows. A general linear model consisting of ANOVA and a least squares method in the SAS program was used for statical analysis. The percentage of blastocysts was lower ($P < 0.05$) in transgenic cloned embryos compared to non-transgenic embryos (34% v. 20%). No difference ($P < 0.05$) in the cell number of ICM and TE cells between two groups of embryos was observed. Out of 47 recipient cows, three pregnancies were detected by non-return to estrus and by rectal palpation. However, the pregnancies were not maintained to term; 2 fetuses were aborted at Days 60, and 150, and one fetus was aborted at Day 240. The genomic DNA from the fetus aborted at Day 240 was prepared and analyzed for the expression of GFP and alpha 1-antitrypsin gene by polymerase chain reaction. The expected PCR products (710 bp for alpha 1-antytrypsin DNA and 431 bp for GFP) were obtained and confirmed by sequence analysis. In conclusion, the present study demonstrated that developmental competence of cloned embryos derived from transgenic donor cells was lower compared with cloned embryos derived from non-transfected donor cells. Although we failed to produce a viable transgenic cloned calf, expression of alpha 1-antitrypsin in fetal tissue represented the possibility of production of a transgenic cloned cow by SCNT. This study was supported by Biogreen 21-1000520030100000.

45 PRODUCTION OF CHIMERIC TRANSCHROMOSOMIC CALVES

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Chimeras have been used for investigating fundamental aspects of early embryonic development, and differentiation, and for introducing foreign genes into mammals (Robertson *et al*., 1986 Nature 323, 445–448; Cibelli *et al*., 1998 Science 280, 1256–1258). The main objective of this study was

to determine if the transfer of blastomeres from in vitro-produced (IVP) embryos into cloned, transchromosomic embryos improved the efficiency of producing transchromosomic calves. Cloned embryos were produced using in vitro-matured bovine oocytes and bovine fetal fibroblasts containing a human artificial chromosome (HAC) (Kuroiwa *et al*., 2002 Nat Biotechnol 20, 889–894). IVP embryos were produced using standard procedures and blastomeres were harvested at the 8–16 cell stage by removing the zona pellucida with protease. Cloned embryos were randomly divided on Day 4 into two groups. One group received 3–4 IVP blastomeres while a second group served as a control (nonmanipulated cloned embryos). After transferring the blastomeres, the chimeric and cloned embryos were placed in culture (Kasinathan *et al*., 2001 Biol. Reprod. 64, 1487–1493) and on Day 7 development to the blastocyst stage was evaluated. Grades 1 and 2 embryos were transferred; two each per synchronized recipient. Pregnancy maintenance, calving, and calf survival were evaluated in both groups. Presence of a HAC in live calves was evaluated in both fibroblasts and peripheral blood lymphocytes (PBLs) using FISH analysis. Embryo development to the blastocyst stage, maintenance of pregnancy and number of calves born were analyzed using Chi-square. There were no differences in the rate of blastocyst development at day 7 or establishment of pregnancy at 40 d ($P > 0.05$). However, pregnancy rate at 120 d, and number of calves that developed to term and were alive at birth (chimera 14/54 and clone 4/90), and at 1 month of age (chimera 13/54 and clone 1/90) were lower ($P < 0.01$) for cloned embryos. The proportion of cells containing an HAC in PBLs, was higher in cloned calves (100%) compared to chimeric calves (26%). The HAC retension rates in PBLs in HAC-positive chimeric and cloned calves were 84% and 95%, respectively. These data indicate that, although the proportion of calves retaining an HAC was lower in chimeras compared to clones, more HAC-positive calves were produced in the chimeric treatment from fewer cloned embryos. We speculate that higher rates of development in the chimeras may be related to the normality of the placenta. Future studies will be required to determine the contribution of the IVP blastomeres to both the inner cell mass and trophectoderm. Therefore, a chimeric approach may be useful for improving the efficiency of producing cloned transchromosomic calves.

46 DIFFERENTIAL PATTERNS OF PROTEIN SYNTHESIS BETWEEN NORMAL AND CLONED PLACENTAE

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Practical application of animal cloning by somatic cell nuclear transfer (SCNT) has been hampered by extremely low success rate. Most clones die before birth and survivors frequently display abnormalities. It is speculated that epigenetic reprogramming is somehow defective in reconstituted embryos (Reik W *et al*., 2003 Theriogenology 59 21–32; Han YM *et al*., 2003 Theriogenology 59, 33–44). It is likely that placental anomalies are directly or indirectly responsible for the death of cloned fetus and neonates. To address this question, we analyzed protein patterns of two placentae obtained after postnatal death of fetuses from SCNT of Korean Native Cattle and two normal placentae obtained after birth of AI fetuses. Global proteomics approach was employed by using 2-D gel electrophoresis and mass spectrometry to separate the different placenta proteins. Proteins within an isoelectric point range of 4.0 to 7.0 and a molecular weight range of 20–100 kDa were analyzed by means of 2-D gel electrophoresis with three replications of each sample. The stained gels were scanned and calibrated at an optical resolution of 63.5 μm/pixel using a GS-710 (Bio-Rad Laboratories, Hercules, CA, USA). Approximately 480 spots were detected in placental 2-D gel stained with coomassie-blue. Then, image analysis by Malanie III (Swiss Institute for Bioinformatics, Geneva, Switzerland) was performed to detect variations in protein spots between normal and SCNT placentae. In the comparison of normal and SCNT samples, at least 15 protein spots were identified as regulated differentially. Using MALDI-TOF-MS (PerSeptive Biosystems, Framinham, MA, USA), 10 spots were identified as up-regulated proteins in SCNT placentae including BPLP-I, Rho GDI 2, osteoclast stimulating factors, SM22, 60S Acidic Ribosomal and Protein P2, whereas five spots were down-regulated proteins such as Peroxiredoxin 2. Mass spectrometry with sequencing was used to further analyze the uncharacterized proteins. Most identified proteins in this analysis appeared to be related to cell proliferation and differentiation, fetal growth and development or metabolism. Further, specific functions of proteins in placenta have been investigated at the molecular levels during pregnancy.

47 BOVINE OOCYTE CYTOPLASM SUPPORTS NUCLEAR REMODELING BUT NOT REPROGRAMMING OF MURINE FIBROBLASTS

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Nuclear transfer (NT) is used to elucidate fundamental biological functions such as cell differentiation reversibility and interactions between the cytoplasm and nucleus. In the present study, we compared nuclear and microtubule dynamics in bovine oocytes following NT of bovine and murine fibroblast cells. To clarify the nuclear reprogramming procedures, we additionally examined the expression of development-related genes (Octamer-binding transcription factors, Oct-4; E-cadherin, E-cad) and housekeeping genes (Heat shock protein 70.1, Hsp; *Bos taurus* apoptosis regulator box-a, Bax; Glucose transporter 1, Glut-1) in bovine embryos that had received nuclei from bovine and murine fibroblast cells. Bovine oocytes were matured in vitro and enucleated after 22 h. The oocytes reconstructed with mouse embryonic fibroblast cells or bovine somatic fibroblast cells were cultured in CR1aa media. While the embryos that received nuclei from bovine fibroblast cells developed into blastocysts, those that received nuclei from murine fibroblasts did not develop beyond the 8-cell stage. Similar nuclear and microtubule dynamics were observed in oocytes reconstructed with murine and bovine fibroblast cells. A small microtubule aster-containing γ-tubulin spot was observed in association with decondensed chromatin following NT of mouse fibroblasts. Within 1 h of fusion of enucleated, non-activated cytoplasm, most mouse fibroblast nuclei were transformed to premature chromosome condensation (PCC). Randomly arrayed microtubules were tightly associated with PCC and formed meiotic-like microtubular spindles in all cases. Condensed chromosomes were divided into two or three chromatin masses and developed into multiple pronuclear-like structures. Microtubule asters were observed near the pronuclear-like structures during apposition in the cytoplasm. Two poles of the γ-tubulin spot evident at the mitotic metaphase stage are involved in the formation of the astral microtubule spindle for initial mitosis. A number of housekeeping mouse genes

(hsp70, bax and glt-1) were abnormally expressed in embryos that had received nuclei from mouse fibroblast cells. However, development-related genes, such as Oct-4 and E-cad, were not expressed. The results collectively suggest that the bovine oocyte cytoplasm supports nuclear remodeling, but not reprogramming of mouse fibroblast cells.

Table 1. Relative abundance of mRNA expression (mean ± SEM) in mouse and xenonuclear-transferred (X-NT) embryos

Gene names	Embryos	2-cell	4-cell	8-cell
Oct-4	mouse	0.36 ± 0.2	1.73 ± 0.43	1.84 ± 0.53
	X-NT	0	0	0
E-cad	mouse	0.06 ± 0.02	0.23 ± 0.11	0.22 ± 0.12
	X-NT	0	0	0
HSP	mouse	0.12 ± 0.02	0.52 ± 0.06	0.85 ± 0.30
	X-NT	0.08 ± 0.06	0.59 ± 0.19	0.04 ± 0.01
Glut-1	mouse	0.44 ± 0.21	0.35 ± 0.12	0.34 ± 0.11
	X-NT	2.56 ± 0.52	1.58 ± 0.24	1.86 ± 0.38
Bax	mouse	0.58 ± 0.39	0.68 ± 0.19	0.76 ± 0.19
	X-NT	0.97 ± 0.08	0.95 ± 0.03	0.72 ± 0.01

48 EFFECT OF INSULIN-LIKE GROWTH FACTOR-1 SUPPLEMENT TO NCSU-23 MEDIUM ON PREIMPLANTATION DEVELOPMENT OF PORCINE EMBRYOS DERIVED FROM IN VITRO FERTILIZATION AND SOMATIC CELL NUCLEAR TRANSFER

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The developmental potential of in vitro production of embryos is affected by various factors, including the culture system, oocyte quality, the presence of serum, and embryo paracrine and autocrine growth factors. Insulin-like growth factor is a good stimulator of oocyte maturation and embryo development. The present study investigated the effect of insulin-like growth factor-I (IGF-I) supplement on the preimplantation development of porcine embryos derived from in vitro fertilization (IVF) and somatic cell nuclear transfer (SCNT). Developmental competence was evaluated by monitoring the numbers of 2-cell embryos and blastocysts at Days 2 and 7, respectively. The number of total cells and inner cell mass (ICM) cells in blastocysts were counted after differential staining at Day 7. All data were analyzed by ANOVA using a Generalized Linear Model (SAS). In Experiment 1, a total of 2,462 in vitro-matured oocytes (527, 458, 498, 481 and 498, respectively) were inseminated with frozen-thawed boar semen and subsequently cultured in North Carolina State University (NCSU)-23 medium supplemented with various concentrations of IGF-1 (0, 1, 10, 50 and 100 ng mL^{-1}). As a result, significant model effects on the development to the 2-cell stage ($P = 0.033$) and to the blastocyst stage ($P = 0.0067$) were found, and more blastocysts (16.9, 16.6, 17.5, 21.8 and 14.7 %, respectively) were obtained in medium supplemented with 50 ng mL^{-1} of IGF-I. Moreover, increase in the total cell number (56.5, 53.2, 74.0, 76.4 and 58.4) and ICM (6.6, 5.8, 9.3, 9.4 and 6.1) cells was observed in IVF embryos cultured in NCSU-23 medium supplemented with 50 ng mL^{-1} IGF-1. In Experiment 2, porcine cloned embryos were produced by our standard protocol using fetal fibroblasts as donor cells (Hyun SH *et al.*, 2003 Theriogenology 59, 1641–1649) and cultured in NCSU-23 supplemented with the same concentration of IGF-1 as Experiment 1. As a result, a total of 501 reconstructed oocytes (99, 98, 102, 99 and 96, respectively) were cultured and significant model effects on the development to the 2-cell stage ($P = 0.0179$) were found. More blastocysts (10.5, 11.2, 11.8, 20.8 and 10.1%) were produced when embryos were cultured in NCSU-23 medium supplemented with 50 ng mL^{-1}, even though no statistical significance was found ($P = 0.1182$). Increases in the total cell number (42.7, 46.0, 45.9, 51.1 and 38.2) and ICM cells (3.8, 3.8, 5.6, 6.6 and 4.8, respectively) were observed in cloned embryos cultured in NCSU-23 medium supplemented with 50 ng mL^{-1} of IGF-I. In conclusion, the present study demonstrated that IGF-1 at the concentration of 50 ng mL^{-1} improves the development of preimplantion embryos derived from IVF and SCNT. This study was supported by the Advanced Backbone IT Technology Development (IMT 2000-C1-1).

49 IN VITRO DEVELOPMENT OF PORCINE CLONED EMBRYOS FOLLOWING DIFFERENT ACTIVATION TREATMENTS

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The present study compared the development of cloned porcine embryos following different activation treatments. Cumulus-oocyte complexes (COCs) were aspirated from slaughterhouse ovaries and cultured for 22 h in NCSU#23 medium supplemented with 10% porcine follicular fluid,

0.57 mM cysteine, 0.5 μg mL^{-1} LH, 0.5 μg mL^{-1} FSH and 10 ng mL^{-1} EGF. The COCs were further cultured for an additional 22 h in the same medium at 39°C in an atmosphere of 5% CO_2 in air, without hormonal supplements. Primary cultures of fibroblasts from a female fetus on Day 40 of gestation were established in DMEM + 15% FCS. For nuclear donation, cells at the 5th-6th passage were cultured in DMEM + 0.5% FCS for 5 days in order to arrest the cells in G0/G1. Following enucleation, oocytes were reconstructed by transfer of donor cells and fusion by means of three DC pulses (1.4 kV cm^{-1}, 30 μs) delivered by a BTX 200, in 0.28 M mannitol containing 0.01 mM $CaCl_2$ and 0.01 mM $MgCl_2$. Eggs were then divided into three treatment groups; control (without further treatment, Group 1), eggs cultured in 10 μg mL^{-1} cycloheximide (CHX) for 5 h (Group 2), and eggs cultured in 1.9 mM 6-dimethylaminopurine (6-DMAP) for 5 h (Group 3). The eggs were then cultured in sets of 30 in 60 μL drops of NCSU#23 supplemented with 4 μg mL BSA (essentially fatty acid free) until Day 7 at 39°C in a humidified atmosphere of 5% CO_2. On Day 4 the culture were fed by adding 20 μL NCSU#23 supplemented with 10% FBS. All experiments were performed as 4 replicates and statistical analysis was performed by one-way ANOVA ($P < 0.05$). Blastocyst development rates were significantly higher ($P < 0.05$) in Group 3 embryos compared to Group 1 controls (27.6 ± 2.7% v. 20.1 ± 4.1%, respectively), but rates did not differ in Group 2 (23.8 ± 5.7%) compared to control. Total cell number in Group 3 blastocysts was, however, significantly higher ($P < 0.05$) than in Groups 1 and 2 (44.6 ± 2.4 v. 19.9 ± 1.9 and 21.9 ± 2.1, respectively). These results suggest that 6-DMAP is more efficient than cycloheximide in the activation of electrically fused NT oocytes during in vitro production of cloned porcine embryos. [Supported by High Technology Development Project for Agriculture and Forestry Korea, MAF-SGRP, 300012-05-3-SB010 and Cho-A Pharm. Co. LTD.]

50 SPATIAL EXPRESSION OF OCT4 IS NORMALLY REGULATED IN PREIMPLANTATION STAGE BOVINE SOMATIC CELL CLONES

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Oct4 is a member of the POU family of transcription factors and is expressed in blastomeres, pluripotent embryonic cells and the germ cell lineage. In the mouse blastocyst, expression of Oct4 becomes restricted to the inner cell mass (ICM). In bovine, Oct4 has not been considered to be a marker for pluripotency because Oct4 protein is expressed in both the ICM and the trophectoderm (TE) of bovine blastocysts (van Eijk MJT *et al.*, 1999 Biol. Reprod. 60, 1093–1103; Kirchhof N *et al.*, 2000 Biol. Reprod. 63, 1698–1705). Oct4 has been used as a marker gene for nuclear reprogramming in cloned embryos. Aberrant spatial distribution and levels of Oct4 have been observed in the majority of mouse clone blastocysts and blastocyst outgrowths (Boiani M *et al.*, 2002 Genes Dev. 16, 1209–1219), indicating reprogramming failure of mouse clones. Lack of or abnormal Oct4 protein expression was also observed in cloned monkey embryos (Mitalipov SM *et al.*, 2003 Biol. Reprod. 68 (suppl 1), 159). The spatial distribution of Oct4 mRNA and protein in bovine clones has not been reported. Bovine oocytes were obtained from a commercial supplier (BOMED, Inc., Madison, WI, USA), and were matured in vitro. Enucleated oocytes were fused with fibroblasts from ear skin and then treated with 10 μg mL^{-1} cycloheximide and 1.25 μg mL^{-1} cytochalasin D for 6 h. Embryos were cultured in SOF supplemented with 1% fetal calf serum (FCS) at 39°C under 5% CO_2, 5% O_2 and 90% N_2 for 2 days. At Day 2, embryos were transferred to SOF supplemented with 5% FCS and cultured under the same conditions until Day 7. Blastocysts were analyzed at Day 7. Oct4 mRNA expression was visualized by whole-mount in situ hybridization using a bovine Oct4-specific antisense riboprobe. Oct4 protein was detected by immunocytochemistry. Control embryos were produced by IVF and were cultured under the same conditions to the blastocyst stage (Day 7). We found that Oct4 mRNA signal was restricted to the ICM in bovine blastocysts. Bovine clones were not different from control embryos in that distribution of Oct4 mRNA signal was typically restricted to the ICM (14 of 16). In contrast to our previous report on mouse clones (Boiani M *et al.*, 2002 Genes Dev. 16, 1209–1219), ectopic expression of Oct4 mRNA in the TE was rarely detected in bovine clones (2 of 16). Distribution of Oct4 protein was also similar between clones and controls with distribution in both the ICM and TE (clones: 9 of 9; controls: 9 of 9). It is unclear why defects in Oct4 distribution should differ between bovine and other species tested including monkey (Mitalipov SM *et al.*, 2003 Biol. Reprod. 68 (suppl 1), 159–160); however, the higher rate of normal Oct4 distribution is consistent with the generally higher rates of postimplantation development of bovine clones (Shi W *et al.*, 2003 Differentiation 71, 91–113).

51 DEVELOPMENT OF BOVINE CLONES CONSTRUCTED WITH CYTOPLASM ORIGINATING FROM OOCYTES CULTURED IN RETINOL

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Incomplete reprogramming of a somatic nucleus by the oocyte cytoplasm may contribute in large part to inefficiencies of cloning procedures using somatic cell nuclear transfer. Predominant use of cytoplasts derived from in vitro-matured oocytes may further exacerbate problems. Addition of all-trans-retinol (Livingston TL *et al*. 2002 Biol. Reprod. 66, 104–105 abst) or 9-cis-retinoic acid (Duque P *et al*. 2002 Human Reprod. 17, 2706–2714) to culture medium improved developmental competence of oocytes to blastocyst stage. The objective of this study was to evaluate effects of retinol for improving developmental competence of cloned embryos constructed with retinol-treated cytoplasts. After removal from 3–8 mm follicles, COC were matured in the presence of 0 ($n = 1005$; diluent only) or 5 μM all trans-retinol ($n = 1017$). Beginning approximately 18 h after placement into culture, oocytes with extruded polar bodies were enucleated of maternal chromatin. Ovarian/granulosa cells were aspirated from adult Jersey cows ($n = 2$) using an ultrasound-guided transvaginal probe. Primary cell lines were established and, before nuclear transfer, cultured in the presence of 0.5% serum (5–8 days; passage = 3). Ovarian/granulosa cells were fused with cytoplasts originating from control or retinol-treated oocytes using two electrical pulses of 2.2 kV cm^{-1} for 20 μs between 24–27 h post-maturation (hpm). Cloned embryos were activated between 26.5 and 30 hpm and then cultured in an atmosphere of 7% O_2 and 5.5% CO_2 in KSOMaa + BSA. Development of cloned embryos to morula and blastocyst stages was assessed on Days 6–7 post-activation. In 5 replicates, compact morulae and blastocysts were transferred to synchronous recipients. Establishment

of pregnancy was confirmed 28 days post-estrus by presence of an embryonic heartbeat using ultrasound. With the exception of pregnancy, data were analyzed as a randomized block design using mixed models of SAS (2000) after testing for normality. Proportion of oocytes recovered after denuding (94.5 and 88.9%; SEM = 2.2), those that had visibly lysed (6.4 and 7.3%; SEM = 1.5), and extruded a polar body by 20 hpm (61.7 and 62.5%; SEM = 7.0) was similar for control and retinol-treated oocytes, respectively. In addition, ability of ovarian/granulosa cells to fuse with control or retinol-treated cytoplasts was similar (80.6 and 73.1%; SEM = 4.5). Lysis of cytoplasts after electrical pulses (8.2 v. 13.6%; SEM = 3.3) and activation (11.6 v. 5.0%; SEM = 2.9) did not differ for control and retinol-treated cytoplasts. Development to morula and blastocyst stages was lower in cloned embryos constructed with retinol-treated cytoplasts (Table). However, ability of morulae and blastocysts to establish a pregnancy was comparable. One clone from each treatment developed to term and was born alive. Culture of oocytes in medium containing retinol during maturation did not improve developmental competence of clones.

Oocyte cytoplasm	No. clones	M/B[1] (%)	ET[2]	No. recips.	Day 28 preg. (%)	No. term	Preg. loss (%)
Control	173	35.5	46	46	41	1	94.7
Retinol	138	18.9	24	24	41.6	1	90.0
P-value		0.04					
SEM		5.0					

[1]M/B = Proportion of embryos developing to morulae and blastocysts. [2]Total number of clones transferred to synchronous recipients. Experiment was replicated 6 times; however, embryos were transferred for only 5 replicates.

52 GREATER PHENOTYPIC VARIABILITY IN CLONED CATTLE FETUSES FROM ONE CELL LINE THAN CONTEMPORARY HALF-SIBS GENERATED BY ARTIFICIAL INSEMINATION OR IN VITRO EMBRYO PRODUCTION

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The cloning of cattle by somatic cell nuclear transfer (NT) is associated with considerable variation in developmental abnormalities. Some of this variation may be due to the cell types/lines used and the specific production and culture methods for NT embryos. Fetal and placental development were studied in 24 pregnancies generated by NT (Wells *et al.*, 2003 Theriogenology 59, 45–59) from a granulosa cell line. The controls consisted of 11 and 14 pregnancies resulting from in vitro-produced embryos (IVP) and artificial insemination (AI), respectively. All fetuses shared the same Friesian sire; oocytes for the derivation of NT or IVP embryos were obtained from abattoir-derived ovaries of Friesian cows. Morphometric measurements were made on the fetuses, fetal membranes, fluid volumes and placentomes at Days 50, 100 and 150 of gestation after slaughter. Pairwise comparisons of within group variances between the treatment groups were made using the *F*-test. The pregnancy loss from AI or embryo transfer to Day 150 was ~40% for the AI and IVP groups. However, 60% of NT embryos transferred had been lost by Day 150. NT fetal weights at Day 100 were more variable than for the AI ($P < 0.001$) and IVP ($P < 0.05$) groups. The NT crown-rump lengths were also more variable compared with the IVP ($P < 0.05$) but not the AI group. At Day 100, NT heart ($P < 0.01$), kidney ($P < 0.01$) and liver ($P < 0.05$) weights were more variable compared with the AI group; both liver ($P < 0.05$) and kidney ($P < 0.01$) weights were similarly more variable in the Day 150 NT group. Part of this variability may be due to disproportionate organ growth in NT fetuses. Increased variability was most evident in the utero-placental tissues. At Day 50, the total fetal membrane weights were more variable in the NT compared with the AI ($P < 0.001$) but not the IVP group. The Day 50 IVP membrane weights were also more variable ($P < 0.05$) than for the AI group. The greater variability of the Day 150 NT membrane weights, when compared with AI ($P < 0.05$) or IVP ($P < 0.01$), could be due partly to greater variation in the allantoic and amniotic fluid volumes. Placentome numbers were significantly lower in the surviving Day 100 NT pregnancies but the weights of the total maternal caruncular tissue of the placentomes were significantly higher and more variable in the NT group ($P < 0.05$ for both AI and IVP). Placentome numbers in surviving NT pregnancies at Day 150 were similar to the controls. However, the total caruncle weights in both the NT and IVP groups were more variable when compared with the AI group ($P < 0.05$ for both). Thus, there is greater variability in fetal membrane and placental development in NT and, to a certain extent, IVP fetuses, when compared with the AI group. The increased variability within this NT group suggests that epigenetic effects arising from incomplete reprogramming of the donor genome and embryo culture can override genetic traits to a certain extent. Supported by FRST C10X0018.

53 MODIFIED AIR-DRY METHOD REVEALS THAT DIPLOID CELLS ARE PREDOMINANT IN CLONED BOVINE EMBRYOS

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Chromosomal analysis is important for evaluating embryo quality. Reports on the cytogenetic analyses of bovine nuclear transfer (NT) embryos, however, have varied according to methods used (Mohamed NMS *et al*. 2000 J. Vet. Med. Sci. 62, 339–342; Arat S *et al*. 2002 Biol. Reprod. 66, 1768–1774; Booth PJ *et al*. 2003 Biol. Reprod. 68, 922–928). An improved air-dry method was developed in this study to specifically investigate the chromosomal composition of bovine NT embryos. NT embryos were obtained by transferring cumulus cells into enucleated oocytes, the fused embryos were then co-cultured in CR1aa with bovine cumulus cells. Day 5 embryos were cultured in the presence of 0.01μg mL^{-1} colcemid for

18 h (classical method is 5 h) and then in 1% sodium citrate for 3 to 5 min (classical method is 15 to 30 min), and fixed on slides, total of 55 embryos were analyzed. The categories for the chromosome composition were classified as follows: diploid (2n), triploid (3n), tetraploid (4n), mixoploid (including 2n/3n, 2n/4n, and 2n/3n/4n). Any embryo that did not show an interpretable metaphase spread due to gross overspreading or clumped chromosomes was not classified. The mitotic index (number of metaphase plates/total cell number of analyzed embryo) was 41.1% (700/1701), which is significantly higher ($P < 0.001$) than that obtained by the classical method (11.9%, 50/419). Of 700 metaphase plates observed, 608 (86.9%) were diploid. Triploidy and tetraploidy were 49 (7%) and 42 (6%), respectively. There were 72.7% (40/55) of the embryos diploid, and 27.3% (15/55) polyploid or mixploid, which included 3n (1/15), 4n (1/15), 2n/3n (5/15), 2n/4n (3/15), and 2n/3n/4n (4/15). Of the 15 non-diploid embryos, 64% of the total cells were diploid. The results of this study indicate that diploidy is the predominant state in bovine Day 5 NT embryos.

54 THE DEVELOPMENT AND CHARACTERIZATION OF MITOCHONDRIAL DNA (MTDNA)-DEPLETED *CAPRA HIRCUS* FETAL FIBROBLASTS: CANDIDATE DONORS FOR SOMATIC CELL NUCLEAR TRANSFER (SCNT)

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Mammalian mtDNA is approximately 16.6 kb in size. It has 37 genes, 13 of which encode protein subunits of the oxidative phosphorylation (OXPHOS) system, the major ATP-generating pathway of the cell. Normally, mammals inherit a single mtDNA genome (homoplasmy) from their mother. Somatic cell nuclear transfer (SCNT) violates this strict maternal, homoplasmic inheritance of mtDNA as cytoplasm is transferred along with the nucleus, which often results in an oocyte harboring both donor and recipient mtDNA genomes (heteroplasmy). This been previously reported (reviewed St. John JC 2002 Theriogenology 57, 109–123). To overcome the problem of donor mtDNA transmission, we have developed and characterized mtDNA-depleted *C. hircus* (goat) cells for use as donors in SCNT. *C. hircus* primary foetal fibroblast cells were established in culture and depleted of their mtDNA by supplementing their growth medium with a low concentration, 50 ng mL^{-1}, of ethidium bromide (EthBr). Conventional PCR, using a series of primers designed specifically for goat mtDNA, was used to screen for the presence of mtDNA during the EthBr treatment. In addition, mitochondrial organization, activity and morphology in the cells were analyzed using the mitochondrial specific fluoroprobe JC1. mtDNA-encoded and mitochondrial transcription factor A (mtTFA) transcript levels were analysed using RTPCR. Furthermore, both mtDNA depleted and non-depleted cells were characterised using immunocytochemistry to detect the expression of specific protein subunits of the OXPHOS system. Progressive mtDNA depletion was observed, using conventional PCR, in cells treated for 3 to 25 days with EthBr, while 42 days of culture resulted in complete depletion. RTPCR showed a progressive reduction followed by complete elimination of the mtDNA-encoded ND1, ND2, ND3, COX I and mtTFA transcripts. In addition, the expression of mtDNA-encoded protein subunits, e.g. COXI, of the OXPHOS system were reduced following mtDNA depletion whereas the expression of nuclear-DNA encoded protein subunits, e.g. COXVic, were unaltered. We hypothesize that the elimination of mtDNA and mtDNA transcripts from the donor cells will facilitate normal mtDNA replication and transcription in SCNT embryos, thus maintaining the strict unimaternal transmission of mtDNA to the offspring. Consequently, genetically identical offspring will be generated which have identical nuclear and mitochondrial DNA content, assuming oocytes from the same ovary are used. This technique is important for the generation of offspring for the livestock industry and animal models for the analysis of single gene disorders as well as the propagation of endangered species.

55 IN VITRO DEVELOPMENT OF ENUCLEATED DOMESTIC CAT OOCYTES RECONSTRUCTED WITH SKIN FIBROBLASTS OF DOMESTIC AND LEOPARD CATS

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The domestic cat is a valuable model for studies in assisted reproductive technology in felid species. Therefore, in this experiment we evaluated the in vitro developmental potential of enucleated domestic cat oocytes reconstructed with somatic cells from domestic and leopard cats. Skin fibroblasts were isolated from female domestic and leopard cats. The oocytes were collected by aspiration of follicles from ovaries that were superovulated with 200 IU PMSG. In vitro-matured oocytes were enucleated and individual donor cells (diameter 14–16 μm) were inserted into the perivitelline space of the enucleated oocyte. Fusion was performed at 26–27 h post-maturation by placing a cell-oocyte couplet between both tips of the needle electrode and electrostimulating with a 2-DC pulse (30 V, 30 μs) in fusion medium containing 0.3 M Mannitol + 0.1 mM $MgCl_2$. Activation was performed 1 to 2 h post-fusion by incubation in 7% ethanol at room temperature for 5 min followed by cultured in 10 μg mL^{-1} cycloheximide and 1.25 μg mL^{-1} cytochalasin D at 38°C in 5% O_2, 5% CO_2, 90% N_2 conditions. After activation, the reconstructed embryos were cultured in 100-μL droplets of Tyrode's medium (Gomez *et al.*, 2003 Theriogenology 60, 239–251.) supplemented with 0.3% BSA at 38°C in a 5% O_2, 5% CO_2, 90% N_2 environment for 2 d. Then, 8-cell embryos were cultured in 100-μL droplets of Tyrode's medium supplemented with 10% FCS at 38°C in a 5% O_2, 5% CO_2, 90% N_2environment for 5 d. The cleavage rates of oocytes reconstructed with either donor cell types were not different. The percentages of blastocyst formation from parthenogenotes and nuclear transfer embryos derived from domestic cat fibroblasts (8/56, 14.3% and 7/51, 13.7%, respectively) were significantly higher than that for nuclear transfer embryos constructed with leopard cat fibroblasts (3/45, 6.7%). These results indicate that enucleated domestic cat oocytes reconstructed with skin fibroblasts of leopard cats can develop to the blastocyst stage. This experiment was supported by Suranaree University of Technology.

Table 1. In vitro development of domestic cat oocytes reconstructed with domestic and leopard skin fibroblasts and parthenogenetic activation

Donor cell types	Fused (%)	Cultured	Cleaved (%)	8-cell (%)	Mor. (%)	Blast (%)
Parthenogenote	–	62	56/62 (90.3)	45/62 (72.6)	37/56 (66.1)	8/56 (14.3)[a]
Domestic Cat	60/90 (66.7)	60	51/60 (85.0)	33/60 (58.1)	24/51 (47.1)	7/51 (13.7)[a]
Leopard Cat	55/80 (68.7)	54	45/54 (83.3)	36/54 (65.9)	20/45 (44.4)	3/45 (6.7) [b]

[a,b]Values with different superscripts are significantly different; $P < 0.01$ (ANOVA).

56 CARDIOMYOCYTES FOR THE STUDY OF DEDIFFERENTIATION IN BOVINE NUCLEAR TRANSFER

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Nuclear transfer facilitates the study of the dedifferentiation process of differentiated somatic cells. Cardiomyocytes are a good model of terminally differentiated cells showing a unique gene expression pattern of cardiac marker genes. The purpose of this study was to test bovine cardiomyocytes as donor cells in nuclear transfer. Cardiomyocytes were isolated from fetal heart muscle (3–5 months of gestation), which were obtained at the abbatoir and immediately perfused with cold Custodiol (Dr. Franz Köhler Chemie, Germany) to reduce metabolism and protect the cells against ischaemia. Subsequently, hearts were perfused with collagenase in Krebs-Henseleit buffer (KHB) to dissociate the tissue and isolate single elongated, contractile cells. For nuclear transfer and fusion the cardiomyocytes were rounded up by exposure to increasing calcium concentrations (2.5–200 μM) in the culture medium before the cells were incubated in suspension for 46–48 hours in MEM medium plus 10% FCS. Nuclear transfer was performed as described earlier (Lucas-Hahn *et al.*, 2002, Theriogenology 57, 433). As a control, adult female fibroblasts were employed. Fusion rate, cleavage (day 3 of in vitro culture) and development up to the morula/blastocyst (day 7 of in vitro culture) were recorded and statistically analysed with Student's *t*-test. A total of 243 nuclear transfer complexes with cardiomyocytes and 127 with fibroblasts were produced. Fusion rates for cardiomyocyte complexes were significantly ($P < 0.001$) lower (28.8%) compared to fibroblasts (84.3%). Cleavage rates were 48.1% for cardiomyocytes and 62.8% for the fibroblast-derived embryos. The developmental capacity to the morula/blastocyst was significantly ($P < 0.01$) reduced for cardiomyocyte (9.4%) compared with the fibroblast-derived (32.4%) reconstructed embryos. Most of the Day 7 embryos were frozen for investigation of gene expression patterns of cardiac marker genes. Staining with Hoechst 33342 for counting total cell numbers revealed that 87.3 ± 20.9 blastocysts were derived from fibroblasts and 100 blastocysts from cardiomyocytes. These results indicate that nuclear transfer with terminally differentiated cardiomyocytes is possible, although with reduced rates. Studies are underway to analyze the gene expression of cardiac marker genes in reconstructed embryos to gain insight into dedifferentiation after nuclear transfer using cardiomyocytes as a model. This study was supported by Deutsche Forschungsgemeinschaft (DFG; Ni 256/16-1)

57 EMBRYO DEVELOPMENT FOLLOWING INTERSPECIES NUCLEAR TRANSFER OF AFRICAN BUFFALO (*SYNCERUS CAFFER*), BONTEBOK (*DAMALISCUS DORCUS DORCUS*) AND ELAND (*TAUROTRAGUS ORYX*) SOMATIC CELLS INTO BOVINE CYTOPLASTS

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Wildlife conservation requires traditional as well as innovative conservation strategies in order to preserve gene and species diversity. Interspecies nuclear transfer has the potential to conserve genes from critically endangered wildlife species where few or no oocytes are available from the endangered species, and where representative cell lines have been established for the wildlife population while numbers were still abundant. The purpose of this study was to investigate the developmental ability of embryos reconstructed with transfer of somatic cells from the African buffalo (*Syncerus caffer*), bontebok (*Damaliscus dorcus dorcus*) and eland (*Taurotragus oryx*) to enucleated domestic cattle (*Bos taurus*) oocytes. Skin tissue from the three wildlife species were collected by surgically removing approx. 1.0×1.0 cm ear skin notches from animals immobilized with a combination of etorphine hydrochloride (M99; South Africa) and azaperone (Stressnil, South Africa). The biopsies were placed into physiological saline and transported to the laboratory at 4°C within 2 h, cleaned with chlorohexidine gluconate and sliced finely in Minimal Essential Medium supplemented with 10% fetal calf serum. The resultant tissue explants were treated as previously described (Baumgarten and Harley 1995 Comp. Biochem. Physiol. 110B, 37–46) and actively growing fibroblast cultures made available for the nuclear transfer process. Nuclear transfer was performed using the HMC technique (Vajta *et al.*, 2003 Biol. Reprod. 68, 571–578) using slaughterhouse-derived bovine oocytes. Culture was performed in SOFaaci (Vajta *et al.*, 2003 Biol. Reprod. 68, 571–578) medium supplemented with 5% cattle serum using WOWs (Vajta *et al.*, Mol. Reprod. Dev. 50, 185–191). Two identical replicates were made with somatic cells of each species. After successful reconstruction, 57, 42 and 48 nuclear transferred and activated buffalo, bontebok and eland embryos were cultured, respectively. All except for 2 buffalo embryos cleaved; 22 (39%) developed to or over the 8-cell stage, and 2 (3.5%) of them to the blastocyst stage. All but 3 bontebok embryos cleaved, 17 (40%) developed to or over the 8-cell stage, but none of them reached the compacted morula or blastocyst stage. Sixteen (33%) of the eland embryos developed to or over the 8-cell stage with one (2%) reaching the blastocyst stage. In conclusion, buffalo, bontebok and eland embryos developed from reconstruction using their respective somatic cells combined with bovine cytoplasts, however, in vitro developmental ability to the blastocyst stage was limited.

Additional basic research that establishes the regulative mechanisms involved with early preimplantation development together with optimising nuclear transfer techniques may have the potential to one day play a role in the conservation of critically endangered wildlife species.

58 ESTRUS SYNCHRONIZATION OF DAIRY GOATS UTILIZED AS RECIPIENTS FOR CAPRINE NUCLEAR TRANSFER EMBRYOS

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The timing of estrus synchrony between donor and recipient does is an important consideration in an embryo transfer program. Experiments were conducted to determine the optimal time of estrus synchrony between donor and recipient dairy goats used in a commercial nuclear transfer (NT) program. Donor and recipient synchronization was achieved by implanting either a 3-mg norgestomet ear implant (Crestar®, Intervet Int. B.V., Boxmeer, Holland) or a 300-mg progesterone vaginal implant (CIDR-G®, Pharmacia and Upjohn Ltd. Co., Auckland, NZ) on Day 0. A single 5 mg intramuscular injection of prostaglandin (Lutalyse®, Pharmacia and Upjohn, Kalamazoo, MI, USA) was administered on Day 7. Recipients received a single 200–500 IU intramuscular injection of PMSG (Calbiochem, LaJolla, CA, USA) on Day 13. Alternatively, starting on Day 12 donors received twice daily intramuscular injection (64 mg/day) of FSH (Folltropin®, Vetrepharm, Ontario, Canada) over four consecutive days. On Day 15 the implants were removed from both donors and recipients and the animals were mated several times daily to vasectomized bucks over two consecutive days. In Experiment 1, estrus synchrony or asynchrony was achieved by removing the implant from recipients at the same time or 12 h later than donors, respectively. In Experiment 2, only estrus asynchrony was utilized and was achieved by removing the implant from recipients either 12 or 18 h later than donors. In vivo-ovulated MII oocytes surgically recovered from superovulated donors on Day 17 were enucleated and reconstructed with transfected caprine fetal or adult skin cells or transgenic adult skin cells. Couplets were simultaneously fused, activated, and then cultured in SOF/BSA for 48 h at 38°C. Two-to-eight-cell NT embryos at 48 h post-fusion and activation were surgically transferred to the oviducts of surrogate recipients with similar implant types and PMSG doses. Pregnancies were determined by ultrasonography starting at approximately Day 28 post-fusion and activation and then monitored weekly. In Experiment 1, there were significantly more pregnant asynchronous recipients compared with synchronous recipients (6 of 24 v. 12 of 124 does, respectively). While there were no significant differences, more offspring were produced per embryo transferred to asynchronous recipients compared with synchronous recipients (5 of 135 v. 11 of 690 offspring per embryo transferred, respectively). In Experiment 2, while not significant, there were more pregnant +12-h asynchronous recipients compared with +18-h asynchronous recipients (16 of 72 v. 5 of 36 does, respectively). Again, while there were no significant differences, more offspring were produced per embryo transferred to +12 h compared with +18 h asynchronous recipients (11 of 424 v. 3 of 224 offspring per embryo transferred, respectively). These results suggest that asynchrony of estrus between recipients and donors is more beneficial in a commercial caprine NT program, and that +12 h may be a more optimal period of asynchrony for recipient does receiving NT embryos.

Table 1. Summary of recipient estrus synchronization

	# Recipients/# Embryos	# Recipients pregnant (%)	# Term pregnancies (%)	# Offspring (% embryo)
Experiment 1				
Synchronous	124/690	12[a] (10)	10 (83)	11[a] (1.6)
Asynchronous (+12h)	24/135	6[b] (25)	5 (83)	5[a] (3.7)
Experiment 2				
Asynchronous (+12h)	72/424	16[a] (22)	10 (62)	11[a] (2.6)
Asynchronous (+18h)	36/224	5[a] (14)	3 (60)	3[a] (1.3)

Data were analyzed by Chi-square test. [a,b]within columns by Experiment differ significantly, $P < 0.05$.

59 IN VITRO AND IN VIVO SURVIVAL OF NELORE NUCLEAR TRANSFER EMBRYOS RECONSTRUCTED WITH ADULT AND FETAL FIBROBLASTS

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Adult skin and fetal fibroblasts are the most frequently used donor cell types for bovine cloning by nuclear transfer (NT) but there are few reports concerning Nelore cattle. The aim of this study was to evaluate in vitro and in vivo viability of Nelore nuclear transfer embryos reconstructed with Metaphase II oocytes and differentiated somatic cells (adult ear and fetal fibroblasts). Oocytes from ovaries collected at slaughterhouse were matured in vitro for 17 h. Enucleation was conducted by aspiration of the first polar body (PB) and small volume of cytoplasm containing metaphase plate. For NT, each nucleus donor cell was inserted under the zona pellucida of each enucleated oocyte and the enucleated oocyte-nuclei donor cell complexes were electrofused (2 pulses of 4 KV cm^{-1} for 20 s). After electrical activation, the couplets were incubated in TCM199 plus 7.5% FCS supplemented with cycloheximide (10 g mL^{-1}) and cytochalasin D (2.5 g mL^{-1}) for 1 h and ciclohexemide alone for 4 additional hours. Immediately after activation, reconstructed embryos were co-cultured with granulosa cells in SOF + 5% FCS for 7–9 days. At 7th day of culture, some blastocysts were fixed for counting cells and some transferred into recipients. A total of 377 couplets were reconstructed from fetal and 457 from adult fibroblasts. After electrofusion, 138 (fetal cells) and 166 (adult cells) embryos were incubated, and 24 (17.4%) and 26 (15.7%) reached blastocyst stage, respectively.

The blastocyst cell number means were 101.3, 120 and 114.3, respectively, for adult, fetal and IVF (control) embryos. There were no significant differences in the numbers of cells of blastocysts among the groups. After transferring 18 (fetal cells) and 21 (adult cells) blastocysts, pregnancy rates at day 90 were 16.7% (3) and 19% (4). There were no significant differences between pregnancy rates. The first pregnancy from fetal cells delivered a healthy male calf weighing 34 kg at Day 290. One of the remaining recipients died with hydrallantois at Day 229 and the other aborted at Day 252. There are four 5-month-pregnancies of adult fibroblast reconstructed embryos. These results indicate that NT embryos produced by fetal and adult fibroblasts of Nelore breed show similar rates of in vitro and in vivo developments. This work was supported by FAPESP (99/07377-3).

60 EPIGENETIC CHARACTERISTICS OF BOVINE AND CAPRINE EMBRYOS AND DONOR CELLS USED FOR NUCLEAR TRANSFER

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The molecular aspects of epigenetic events taking place in nuclear transfer (NT)-derived embryos are not well defined, but DNA methylation is known to be involved. One leading hypothesis is that the significant losses that occur during both pre- and post-implantation development are in great part due to improper epigenetic reprogramming. Aims of this study were to perform comparative quantitative analyses of the overall DNA methylation of bovine (both IVF- and NT-derived) and caprine IVF-derived preimplantation embryos as well as of donor cells used for NT. Caprine IVF was performed according to Blash S *et al.* (2001 Theriogenology 54, 899–905). Bovine and caprine 8- to 16-cell embryos were harvested on Day 4 post-insemination (dpi). Caprine donor cells (two adult fibroblast cell lines, T75-514 and F638) at the G1 phase of the cell cycle were prepared as previously described (Memili E *et al.* 2003 Theriogenology 59, 274 abst). Bovine cumulus cells were serum starved for four days prior to use and NT were performed as previously described (Echelard Y *et al.* 2002 Theriogenology 57, 779 abst). Embryos and the donor cells were vacuum-fixed onto 10-μm filters, and DNA methylation was determined by immunoassaying with a well-defined anti-5-methyl-cytosine antibody (Dean W *et al.* 2001 Prod Natl Acad Sci 98(24), 13734–13738) and fluorescent labeled anti-mouse IgG as secondary antibody following the protocol of Shi and Haaf (2002 Mol Reprod Dev 63, 329–334). Fluorescent imaging was performed by epifluorescence microscopy. The methylation-specific signal was recorded digitally with a high-resolution charge-coupled devise camera, followed by analysis with the MetaMorph™ imaging software (Universal Imaging Corporation, Downingtown, PA, USA). These experiments showed a high level of heterogeneity in the methylation levels of bovine and caprine donor cells. However, caprine 8- to 16-cell IVF embryos exhibited similar levels of DNA methylation to their bovine IVF counterparts. Conversely, when the DNA methylation level of 97 bovine IVF nuclei was compared to that of 55 bovine NT embryonic nuclei, significant differences were found. Levels of signal intensities per nucleus were almost 9-fold greater for NT embryos (133 v. 15), a significant difference ($P < 0.01$). In conclusion, donor cell populations with heterogeneous DNA methylation and incomplete reprogramming of DNA methylation in NT embryos are likely to be the underlying reasons for low level of successful NT embryonic and fetal development. Thus, designing NT protocols supporting better reprogramming may result in improvement since, in order for a successful embryonic development after NT, DNA methylation needs to be reprogrammed from a somatic cell pattern to an early embryonic pattern. Furthermore, similar levels of DNA methylation between caprine and bovine IVF embryos may be an indication of a similar pattern of embryonic gene expression between these species.

61 ISOLATION AND CULTURE OF SOMATIC CELLS OBTAINED FROM SEMEN AND MILK OF GULF COAST NATIVE SHEEP

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In addition to the sperm found in semen, somatic cells (SC) are frequently present. Furthermore, SC are also present in fresh milk. These SC could also be a potential source of biomaterial for the preservation of endangered species. In the present study, the possibility of isolating SC from fresh, cooled and frozen semen, as well as from fresh milk, was evaluated in the endangered Gulf Coast Native sheep. Semen from 7 mature rams was collected by electro-ejaculation and a 0.5-mL sample from each ejaculate was then diluted in PBS (22°C). The remainder of the ejaculate was cooled (4°C), extended in a TRIS-yolk-glycerol extender, loaded into 0.5-mL plastic straws and frozen. Semen from the same collection (2–5 straws) was also diluted in PBS and allotted to the cooled semen treatment group (4°C). Sheep milk samples ($n = 26$) from 5 Gulf Coast Native (GCN) ewes and 11 crossbred ewes (GCN × Suffolk) were collected and stored at 4°C. Samples of fresh (0.5 mL), cooled (2–5 straws) and frozen–thawed (2–5 straws) semen and refrigerated milk (15–50 mL) were washed with PBS, centrifuged at 500*g* for 10 min and the re-suspended pellet cultured in tissue culture dishes containing 2 mL of DMEM with 15% fetal bovine serum (FBS) plus 100 μM nonessential amino acids, supplemented with antibiotics and incubated at 38.5°C in 5% CO_2 in air. After 24 h, the semen sample culture dishes were rinsed twice with fresh culture medium to remove remaining sperm cells and then returned to the the incubator. After 3 to 7 d of incubation, groups of epithelial-like cell colonies were detected in 1 sample from the fresh semen group (1 of 7 rams, 14.3%) and 4 semen samples from the cooled group (4 of the 7 of rams, 57%). These cells subsequently proliferated until a monolayer was observed; the cells were then harvested and plated (in a single culture dish) for further passage. One cell line survived from the cooled semen group, which was frozen in DMEM medium with 15% FBS and 10% DMSO and then stored in LN_2. No cells were derived from the frozen-thawed semen group. Colonies of fibroblast-like or epithelial-like cells were detected after culture of the milk samples. Of the 26 colonies, 23 (88%) initially plated cells, 11 (41%) proliferated to the 1st passage, 5 (19%) resulted in cell lines that were frozen and 2 (8%) of these subsequently proliferated during post-thaw incubation. The genetic status of the cell lines that proliferated until cryopreservation was assessed by PCR amplification of microsatellite markers. Results confirmed that the cell populations derived from both the semen and milk samples originated from the appropriate donor animals. Also, immuno-histochemical analyses for vimentin and cytokeratin were performed on these cell populations to determine the morphological cell type. Cells derived from semen samples were identified as epithelial cells by the presence of cytokeratin, whereas the milk samples produced epithelial and fibroblast cells. In the present study, we demonstrated that it is possible to establish

a somatic cell line from cooled semen, as well as from fresh milk, in an endangered sheep. This approach could provide cell lines from valuable animals when other means of obtaining cells are not possible.

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62 SPINDLE MORPHOGENESIS AND THE MORPHOLOGY OF CHROMOSOMES IN MOUSE NUCLEAR TRANSFER: AN ABNORMAL START IN CLONING OF MICE

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Cloning of mammals by somatic nuclear transfer (NT) was achieved more than 6 years ago. Nonetheless, the success rate is very low. Although spindles and nuclear mitotic apparatus protein (NuMA) are thought to play important roles in the cell cycle and maybe reprogramming after NT, the spindle and NuMA morphogenesis after NT is not well understood. The aim of this study was to examine the spindle, NuMA morphogenesis and chromosome morphology in mouse NT. Maturated mouse oocytes were collected from mature B6D2F1 females 16 h after hCG injection. The donor nuclei used in this study were cumulus cells. A donor nucleus was injected into an oocyte by means of a piezo pulse system. For double labeling, DNA was stained with PI (red) or Hoechst 33342 (blue); tubulin, NuMA, and phosphorylated histone H3 were stained with FITC or alexa 488 (green). All samples were visualized with a BioRad Radiance 2100 confocal scanning laser microscope. In this study, the spindle, NuMA morphogenesis, chromosome morphology, and histone H3 phosphorylation were identified using more than 3000 maturated mouse oocytes in five experimental studies to examine (1) the morphological changes of spindle and NuMA in NT oocytes at 10, 30, 60, 90, 120, 180, and 360 min after injection of somatic cell nuclei into intact or enucleated maturated oocytes; (2) the localization and morphology of spindles and NuMA of NT embryo followed by activation at 1, 2, and 6 h after somatic nuclear injection; (3) the effects of the timing of activation and donor cell membrane on the spindle morphogenesis; (4) the effects of intact nuclear membrane or its breakdown by micropipette on spindle morphogenesis; and (5) the correlation between donor cell chromosome condensation and histone H3 phosphorylation after injection into enucleated maturated oocytes. As control, the NuMA morphogenesis during the early pronuclear stages of NT was compared with those of ICSI oocytes. We consistently observed that abnormal spindle morphogenesis occurred during the early stages of mouse NT. Most of the NT oocytes began with monopolar spindle and monopolar NuMA (90–95%), and this phenomenon occurred 10 min after NT. Only 5–10% of NT oocytes started with bipolar spindles. However, monopolar spindles were transformed into bipolar spindles during 30–60 min after NT by bipolarization of centrosomal NuMA. After activation, NuMA was localized in the perispindle region, and finally concentrated inside the pronuclei of embryo. This spindle morphogenesis was independent of the presence or absence of metaphase II [not like in rhesus monkeys, whereas meiotic spindle removal depletes the ooplasm of NuMA and HSET, both vital for mitotic spindle pole formation (Lanza R *et al.*, 2003 Science, 300, 297)]. The breakdown of the donor nuclear membrane by micropipette resulted in the increase of bipolar spindle formation in NT. The initiation of chromosome condensation and histone H3 phosphorylation were observed 30 min after NT and the maximum of histone H3 phosphorylation was occurred 60 to 90 min after NT. Future studies are required to elucidate the mechanism of monopolar spindle formation, chromosomal abnormalities in NT, and the relation of the monopolar phenomenon to the success rate in mammalian cloning.

63 INTERFERON-TAU EXPRESSION FROM PRIMARY TROPHECTODERM OUTGROWTHS OF BOVINE BLASTOCYSTS: COMPARISON BETWEEN IVP, NT, AND PARTHENOGENIC EMBRYOS

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Interferon-tau (IFN-tau) is expressed soon after bovine blastocyst formation and might be useful as a marker of appropriate biological function in embryos produced by nuclear transfer. To assess this possibility we have compared IFN-tau levels in the conditioned medium of primary trophectoderm cultures derived from IVP, nuclear transfer (NT), or parthenogenic bovine embryos. Embryos were produced from in vitro-matured cumulus-oocyte complexes processed from local slaughterhouse ovaries or obtained from Bomed, Inc. (Madison, WI, USA). In vitro fertilization, NT, and parthenogensis were as previously described (Talbot *et al.*, 2000 Tissue and Cell, 32, 9–27) except that embryo culture was in G1/G2 medium in 5% oxygen (Lane *et al.*, 2003 Theriogenology, 60, 407–419). Each 8–11-day embryo was cultured individually in a 4-well plate well (Nunc) with STO feeder cells using DMEM medium containing 10% fetal bovine serum as previously described (Talbot *et al.*, 2000 Biol. Reprod. 62, 235–247). Any contaminating epiblast or endoderm was physically dissected and discarded so as to produce pure trophectoderm outgrowths. The success/failure ratio for colony formation was similar for IVP and NT embryos (IVP = 155/29; NT = 104/25), but was significantly different ($P < 0.05$) for parthenogenic embryos (54/43). Trophectoderm colonies reached diameters of 1 to 1.5 cm in 3–4 wk, and, at this time, 72-h-conditioned cell culture medium was harvested, frozen, and measured for IFN-tau anti-viral activity as previously described (Talbot *et al.*, 2000 Biol. Reprod. 62, 235–247). From 313 observations, IFN-tau production was analyzed as a two-factor mixed linear model. Differences in IFN-tau production by type of embryo were statistically significant ($F = 42.61$; $P < 0.0001$; df = 2). Mean comparisons were done with Sidak adjusted P-values so that the experiment-wise error was 0.05. IFN-tau production means for IVP-, NT-, and parthenogenic-derived trophectoderm were 4311 IU mL^{-1} ($n = 155$), 626 IU mL^{-1} ($n = 104$), and 1595 IU mL^{-1} ($n = 54$), respectively. The results show that mean IFN-tau production from trophectoderm cultures derived from NT embryos is significantly reduced in comparison to IVP- and parthenogenote-derived cultures. Parthenogenote-derived cultures also produced significantly less IFN-tau than IVP embryos on average. IFN-tau production from trophectoderm outgrowths may be a useful measure of NT reprogramming success.

64 COMPARISON OF DEVELOPMENTAL POTENTIAL OF IN VIVO AND IN VITRO RECIPIENT OOCYTES AFTER NUCLEAR TRANSFER IN GOAT

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Recent techniques in somatic cell nuclear transfer (SCNT) have been widely used for animal research. In addition, SCNT techniques may allow for the rescue of endangered species. Despite efforts for wildlife preservation, however, some threatened or endangered wild animal species will likely become extinct. As a preliminary experiment of a series in wildlife research, we tried to identify an improved method for the production of more transferable NT embryos in goats. Mature donor animals of Korean native goats (20–25 kg) were synchronized with a CIDR (type G; InterAg, New Zealand) vaginal implant for 10 days followed by a total of 8 twice daily injections of 70 mg of FSH (Folltropine, London, Ontario, Canada) and 400 IU of hCG (Chorulon, Intervet, Moxmeer, The Netherlands). Oocytes were then collected surgically by retrograde oviduct flush or direct aspiration from ovarian follicles in vivo at 29–34 h after hCG. Oocytes collected from follicles were matured in TCM-199 containing 10% FBS and hormones. Prepared ear skin cells from the goat were cultured in TCM-199 containing 10% FBS at 39°C, 5% CO_2 in air, and confluent monolayers were obtained. Oocytes were enucleated and donor cells from serum starvation (0.5%) culture were fused through a single electric pulse (DC 2.36 kv cm^{-1}, 17 μs), and then activated by a single electric pulse (AC 5 v mm^{-1}, 5s + DC 1.56 kv cm^{-1}, 30 μs) or chemical treatment (5 μg mL^{-1} ionomycin 5 min^{-1}, 1.9 mM 6-DMAP/4h). Reconstructed oocytes were cultured in M16 medium with 10% goat serum (GS) for 6–7 days. Data were analyzed by chi-square test. In in vitro development, significantly ($P < 0.05$) more oocytes were cleaved (24/30, 80.0%) and developed (7/24, 29.2%) to morula or blastocyst stage, respectively, in NT oocytes activated by Iono + DMAP compared to electric stimulated oocytes (2/21, 40.0%; 0/2, 0%). There was a significant difference in in vitro development of NT embryos by the method of oocyte collection. Cleavage rate was higher ($P < 0.05$) in NT embryos from in vivo oocytes (23/28, 82.1%) than in in vitro matured oocytes (19/35, 54.3%), and further development to morula or blastocyst was also significantly ($P < 0.05$%) higher in NT embryos from in vivo oocytes (7/23, 30.4%) than in NT embryos from in vitro matured oocytes (0/19, 0%). When we compared NT embryos to parthenotes, developmental rate was not significantly different between NT embryos and parthenotes. These results strongly suggest that the in vivo oocytes will have superior developmental potential to oocytes matured in vitro.

Table 1. Effect of different oocyte source on in vitro development following caprine SCNT

Oocyte source	No. of oocytes cleaved (%)	No. of oocytes developing to (%)		
		4-cell stage	8-cell stage	Mor. & Bl.
In vivo	23/28 (82.1)[a]	13 (56.5)[a]	10 (43.5)[a]	7 (30.4)[a]
In vitro	19/35 (54.3)[b]	6 (31.6)[a]	1 (5.3)[b]	0 (0.0)[b]

*Values with different superscripts within a column are significantly different ($P < 0.05$).

65 RARE AND OFTEN UNRECOGNIZED CEREBROMENINGITIS AND HEPATOPNEUMONIC CONGESTION ARE MAJOR CAUSES OF SUDDEN DEATH IN CLONED MALE PIGLETS

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In this study, we investigated the relationship between the sex of cloned pigs and sudden infant death syndrome (SIDS). Three cell lines (2 male and 1 female) were obtained from F1 fetuses derived from 3 different dams (Yorkshire) inseminated by the same sire (Landrace); one female fibroblast cell line was obtained from a Duroc-strain fetus acquired from a slaughterhouse, the age of the fetus unknown. The fetal fibroblast cells were cultured in DMEM supplemented with 10% fetal bovine serum under 5% CO_2 in air at 37°C. For NT, we used the 4 cell lines described above. All 37 cloned piglets derived from 10 pregnant recipients were alive at term and began breathing readily. Of those piglets born, 18/22 males and 5/15 females died within 2 months of age. A total of 350 paraffin blocks from 18 deceased cloned male piglets and 90 paraffin blocks from six age-matched normal control piglets were prepared from the midbrain, medulla oblongata, liver, lung, kidney, spleen, small and large intestine, thymus, uterus, placenta, ovary, testis, skin, and skeletal muscle. We found that the birth weights of male clones were 57% lower than those of control age- and sex-matched piglets. Piglets with low birth weight (<900 g) had more than twice the risk of SIDS relative to those piglets weighing more than 1000 g. The low birth weights of the cloned male piglets (0.84 ± 0.05 kg) were not merely an artifact, as the average birth weights of cloned female piglets (1.47 ± 0.09 kg) were not lower than weights of piglets produced by AI-derived control female piglets (1.36 ± 0.12 kg). An initial examination of brain samples from 18 cloned male piglets that died soon after birth identified seven piglets with meningitis characterized by severe neutrophilic inflammation in the temporal brain lobes (38.8%, 7/18). We verified meningitis when more than 1000 neutrophils were counted per cubic millimeter of tissue. Next, we found hepatopneumonic congestion (16.6%, 3/18). The deceased male clones with meningitis showed extensive neuronal cell death and blood-brain barrier damage, whereas cloned piglets with congestion had fewer and larger alveolar air sacs. Extensive alveolar cell death, especially of pneumocytes and the bronchial epithelium, was confirmed by TUNEL assay. Lung and liver congestion may be caused by a slowly flowing blood stream from heart, resulting in CO_2 and O_2 exchange problems. Although the gross anatomy of the cloned male piglets was normal, they were associated with other severe handicaps, the commonest being leg abnormality which occurred in 33% (6/18) of dead male cloned piglets followed by Leydig cell hypoplasia and short face. Even though 4 of 41 AI-derived control piglets died within 1 week after birth, we could not find any anomalies in them. Thus, the present study suggests that our data might reflect sex difference but not cell type difference, and that death of the cloned male piglets might be caused by risk factors of sudden infant death syndrome such as low birth weight and multiple organ failure in conjunction with hepatopneumonic congestion and cerebromeningitis.

66 EFFECTS OF CONTACT INHIBITION INTERVALS V. SERUM DEPRIVATION ON DEVELOPMENT OF PORCINE NUCLEAR TRANSFER DERIVED EMBRYOS

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Contact inhibition and serum deprivation are commonly used to synchronize donor cells at the G0/G1 state of the cell cycle prior to use in nuclear transfer. Here we compared the effects of serum deprivation (SD) and different intervals of contact inhibition (CI) of the donor cells on the blastocyst rate. One batch from pooled porcine fetal fibroblasts (passage 3) was used in this study. The cells were thawed, seeded to a six-well plate and cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2 mM glutamine, 1% non-essential amino acids, 0.1 mM mercaptoethanol, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin containing 10% fetal calf serum (FCS). Serum deprivation was achieved by culturing cells in DMEM containing 0.5% FCS for 48 h. Cells of the CI groups were grown to 100% confluency and kept in that state for 24 h, 48 h and 72 h. Immediately after cell cycle synchronization, cells were used in nuclear transfer. Cell cycle state of the cells was evaluated by FACS analysis at 24 h after beginning of CI and prior to nuclear transfer. Blastocyst rate was determined 7 days after nuclear transfer. An average of 38-42 h in vitro matured oocytes were used in nuclear transfer (NT). NT was performed as described previously (Betthauser J *et al.*, 2000 Nat. Biotechnol. 17, 456–461). There were no differences in the proportion of cells in G0/G1 of the cell cycle in any of the treatment groups (85.0%, 85.8%, 85.5% and 86.3% for SD and CI at either 24 h, 48 h and 72 h, respectively). After nuclear transfer (for each CI group $n = 336$–384 reconstr. embryos; SD $n = 215$) there was a statistically significant difference in the fusion rate between 48 h CI and SD cells (74.8% v. 87.5%, *t*-test $P < 0.050$). Blastocyst rate (blastocysts/fused) differed significantly between SD, 24 h CI and 48 h CI (17.4%; 9.1%, 9.6%, *t*-test $P < 0.050$), there was no difference between SD and 72 h CI and within the CI groups (72 h CI 10.6%). Four transfers of reconstructed embryos (72 h CI, $n = 138$–163 embryos/gilt, 1-cell embryos) to prepuberal Landrace gilts led to 2 initial pregnancies determined at Day 25 by ultrasound. One pregnancy was lost at Day 35; the other recipient remained pregnant and farrowed 4 piglets. One piglet was stillborn and one died 7 h after birth; the remaining two piglets are healthy and now 4 months old. Four transfers of embryos ($n = 96$–110) reconstructed with SD cells revealed two initial pregnancies determined on Day 25 by ultrasound. Again, one was lost on Day 35, and the other one is now at Day 100. Our results show that, despite similar proportions of cells being in G0/G1 of the cell cycle, cells either contact-inhibited for 72 h or serum-deprived both show higher rates of blastocyst development compared to cells contact-inhibited for shorter time periods. Both donor cell preparations can lead to full term development of nuclear transfer-derived embryos. This work was funded by the Deutsche Forschungsgemeinschaft (DFG, SFB 265).

67 ORGAN WEIGHT VARIATION IN CLONED PIGS

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Previously, we compared adult clones and naturally bred control females using a series of physiological and genetic parameters and found considerable variation in certain traits in clones. In order to more fully understand this clonal variation we examined the organ weight differences among a group of clones from a single litter. Briefly, in vitro-matured oocytes were stripped of their cumulus cells at 46–48 h postmaturation by vortexing in TCM 199 containing 5% FCS with 0.1% hyaluronidase, and placed in Ca-free NCSU-23 with 4 mg mL^{-1} BSA. Oocytes were stained in Ca-free NCSU-23 containing 4 mg mL^{-1} BSA and 5 μg mL^{-1} Hoechst 3334 for 10 min, and enucleated in Ca-free, salt buffered, NCSU-23 containing 5% FCS and 7.5 μg mL^{-1} of cytochalasin B, Puromycin-resistant fetal fibroblasts grown for 35 days in D-MEM/F-12 containing 15% FCS and 1.65 μg mL^{-1} puromycin were synchromized by contact inhibition for 1-2 days. Cells were trypsinized, resuspended in salt buffered NCSU-23 with 10% FCS and placed into the perivitelline space. Couplets wer fused by 2 V AC for 2 s followed by 2 pulses DC of 1.4 kv cm^{-1} for 50 μs. Following fusion, the oocytes were placed in NCSU-23 containing 4 mg mL^{-1} BSA for 1.5–2 h. The oocytes were then electroactivated by 2 pulses of 1.2 kg cm^{-1} for 50 μs, allowed to recuperate for 10–15 min, and transferred into the oviduct of a naturally synchronized recipient. A total of 286 doublets were transferred into 4 recipients one of which became pregnant and gave birth to 9 clones (9/81). One clone died soon after birth. The remaining 8 clones were weaned at three weeks and kept as a group until Day 120 at which time the animals were euthanized for organ recovery. Weights were determined for major organs, and coefficients of variation (CV) calculated for both absolute weights, and corrected total weight. Corrected weights were calculated as weight of organ divided by weight of clone. CV for absolute and corrected weights, respectively, were: uterus, 15.1% and 5%; left kidney, 13.2% and 6.9%; right kidney, 11% and 6.8%; spleen, 29.7% and 27.1%; liver, 20.6% and 19.7%; lung, 37.5% and 32%; heart, 20.2% and 15.5%; and brain, 5.6% and 9.5%. Both absolute and corrected weights indicated that there is a high degree of variation in some of the organs among a group of identical clones. To determine whether the variation we observed is typical of non-cloned swine, we compared the variations in the clones to those observed in a group of 3 age-matched, sex-matched, breed-matched controls that had been kept with the clones for approximately 90 days. Relative coefficients of variation of corrected organ weights between clones and non-cloned animals were: uterus, 0.18; left kidney, 0.34; right kidney, 0.34; spleen, 2.58; liver, 1.08; lung, 1.06; heart, 0.59; and brain, 0.7. In summary, these results indicate that some organs in cloned animals have a high degree of weight variability, most likely due to epigenetic aberrations of overriding environmental effects.

68 EFFECT OF ROSCOVITINE ON FIBROBLASTS' ABILITY TO FORM BLASTOCYSTS AND ESTABLISH PREGNANCIES AFTER BOVINE NUCLEAR TRANSFER

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Cloning efficiency of fibroblast nuclear transfer is dependent on donor cell chromatin status. Chromatin status is commonly regulated by serum starvation or contact inhibition. We have tested 3 methods of synchronizing chromatin activity, roscovitine exposure (in MEM + 10% serum) for

24 h, with serum starvation (0.5% serum) for 5 days or growth to confluence in 10% serum prior to nuclear transfer. Roscovitine, a specific cyclin-dependent kinase (CDK)2 inhibitor, provides a means of precisely synchronizing bovine fetal fibroblasts (BFF) at G0/G1 cell cycle stage. Fibroblasts were from 100-day-old Jersey fetuses. Cells, frozen at passage 2, from fetus 10 are known to produce calves. Fetus 13 cells, frozen at passages 1 and 2, were compared for their ability to serve as nuclear donor cells. Oocytes, either purchased from Bomed or harvested from ovaries obtained from a local slaughterhouse and matured in Ham's F10, were enucleated between 18–21 h post-maturation initiation. Couplets were produced and fused by standard techniques. Embryos were activated 2 to 4 hours after fusion by exposure to ionomycin for 4 min and DMAP for 4 h. Embryos were then held in CR1aa for 12 h before being cultured in G1 media for 3 days and then G2 media for another 3 days (38.5°C and 5% O_2 + 5% CO_2 + 90% N). On Day 7, good quality blastocysts were transferred to synchronized recipient heifers. The remaining embryos were evaluated after another day in culture. Blastocyst development [(100) X (total blastocysts/fused couplets)] was not influenced by fetus (BFF10, 31 ± 3%; BFF13, 26 ± 2%, $P = 0.126$). However, a higher proportion of blastocysts were produced when fibroblasts were cultured in 0.5% serum (38 ± 3%) compared to culture in 10% serum (29 ± 3%) or in roscovitine (23 ± 2%, $P = 0.001$). Time in culture, as measured by passage, had a variable effect on the fibroblast's ability to product blastocysts from the three fibroblast culture conditions tested. Passage 1 and 2 fibroblasts responded similarly to the 0.5% and 10% serum treatments ($P > 0.80$). When cultured in roscovitine, passage 1 fibroblasts performed better then passage 2 fibroblasts (29 ± 4% v. 16 ± 3% blastocysts, $P = 0.010$). Embryos have been transferred to 51 recipients to date. Ten recipients have given birth or are still pregnant. The 60-day non-return rate for those animals was 29%, 50%, and 31% for serum starvation, 10% serum, and roscovitine treatments, respectively. BFF10 and BFF13 cells have generated the same non-return rates (33%). In this study, of the 3 methods of synchronizing fibroblast chromatin, serum-starvation had an in vitro advantage. Cells cultured for different lengths of time (passages) responded differently to synchronization treatments. This may reflect a heterogeneous population of cells at early passages. Current non-return rates seem to favor synchronization by contact inhibition. Any advantage roscovitine offers may not be revealed until calving.

69 DOLLY: A FINAL REPORT

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Dolly, the first animal to be derived by transfer of a nucleus from an adult somatic cell was euthanized on February 14, 2003, because of the presence of a virally-induced lung tumor resulting in progressive decline in respiratory function. We have carried out full gross and histopathological analysis of tissues and additionally re-examined the length of her telomeres. Dolly was derived from an oocyte recovered from a Scottish Blackface ewe and the nucleus of a cell cultured from mammary tissue of a 6-year-old Finn Dorset ewe. The culture had been through 27 population doublings. Dolly was 5 years and 7 months old at the time of her death. A detailed comparison was made between Dolly and four other ewes of similar breed and age produced by natural mating. Full post mortem examination was carried out and a range of tissues examined histologically. Major findings were the presence of extensive tumor infiltrates in both lungs and bilateral severe stifle arthritis in Dolly. Histopathology confirmed the presence of extensive sheep pulmonary adenomatosis/SPA (syn. ovine pulmonary adeoncarcinoma/OPA) lesions in the lung. This infection is caused by Jaagsiekte sheep retrovirus (JSRV) and is transmitted in respiratory droplets. Other animals in the barn had previously developed SPA. The risk of transmission of infection may have been increased by the fact that the animals were housed in close proximity in a barn. There is currently no reliable diagnostic test before the tumor develops and it is probable that Dolly was infected before we were aware of the presence of the infection in the group of sheep. There is no treatment for the disease. There is no reason to think that Dolly was more vulnerable to infection because she was a clone. The osteoarthritis was first observed during the autumn of 2001 when radiographs of the left stifle revealed osteophytes and osteophytosis associated with the patella, distal femur and proximal tibia. Radiographs of the right stifle were normal at that stage. A regime of anti-inflammatory drugs enabled the ewe to live a normal life. None of the other cloned sheep at the Institute have shown any symptoms of arthritis. Arthritis has been described in a cloned bull, but has not been described in any other cloned sheep. Telomeres in kidney tissue taken from Dolly were found to be shorter than those in kidney of the four control sheep (Dolly's = 15.6 kb v. controls = 17.9 ± 0.27 kb). This confirms the observation that her telomeres were shorter at 1 year of age than those of age-matched controls (Dolly 19.14 kb v. controls 24 kb). It is not appropriate to contrast these measurements, as they were not made in a single experiment. Dolly was fertile and delivered a total of 6 healthy lambs in three pregnancies. During routine husbandry there were no unusual findings apart from the development of the arthritis. Since the birth of Dolly, experiments in several species have revealed a range of abnormal phenotypes associated with unusual patterns of gene expression.

70 PRODUCTION OF CLONES BY FIBROBLAST NUCLEAR TRANSFER FROM AN X-AUTOSOME TRANSLOCATION CARRIER COW

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Poor reproductive outcome associated with chromosome anomalies is well documented in humans and domestic animals. In cattle, female carriers of Robertsonian and X-autosome translocations tend to be repeat breeders, probably due to synaptic difficulties during meiotic prophase of gametogenesis. Although viable offspring have been obtained through somatic cell nuclear transfer (NT) using adult or fetal cells from normal animals in various species, there have been no reports to date on the application of this technology to translocation carriers. Since NT circumvents meiotic problems encountered by translocation carriers, we used this approach to generate cloned embryos, fetuses and calves from a subfertile Limousin-Jersey crossbred cow previously identified as a carrier of an X-autosome translocation. Primary cultures were established from ear skin biopsies, and used at the 5th or 6th passage for NT. Recipient oocytes were enucleated at 19 h post-maturation (hpm), fused with individual fibroblasts by a single electrical pulse (1.4 KV cm^{-1}, 40 μs) and activated at 24 hpm with a combination of ionomycin (5 μM, 5 min) and cycloheximide (10 μg mL^{-1}).

Reconstructed eggs were cultured in SOF at 39°C in a humidified low oxygen atmosphere. In 33 runs involving 2470 oocyte-donor cell complexes, cleavage and blastocyst rates were 88% (2173/2470) and 36% (889/2470), respectively. Two or three blastocysts (Day 7 ± 1) were transferred into each recipient, previously synchronized with a combination of CIDR, GnRH and $PGF_2\alpha$. Ultrasonography was performed at Days 28 to 60 and at Days 90 and 150. Pregnancy was confirmed on Day 28 in 10 of a total of 22 recipients, 2 of which were later found to be carrying twin fetuses. Of 60 embryos transferred, 11 (18.3% of embryos) survived to Day 42, 6 (10%) to Day 60, and 4 (6.6%) to Day 90. A Day-94 fetus was surgically retrieved to examine the synaptic pattern of meiocytes in fetal ovaries. The fetus and internal organs were normal in appearance, and of normal size (16.5 cm C-R length). The X-autosome translocation was confirmed in blood cultures, and synaptic anomalies involving chromosome 23 and the X chromosome were detected in fetal ovaries. Another clone was delivered by C-section at 276 days but died within 1 h of delivery, while one singleton pregnancy is still ongoing at >200 days. These results demonstrate that NT can be used to produce embryos, fetuses and offspring from an X-autosome translocation carrier, with the potential to facilitate study of synaptic behaviour of female germ cells, and X-inactivation in different cell lineages of cloned blastocysts, and to generate individuals with otherwise poor reproductive prospects. [Supported by NSERC, Canada and OMAFRA]

71 EFFECT OF CALCIUM IONOPHORE AND CYTOCHALASIN D ON ACTIVATION AND IN VITRO DEVELOPMENT OF NUCLEAR TRANSFER BOVINE EMBRYOS

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The activation of oocytes is one of the most important steps for a successful cloning, and chemicals used for activation can affect the viability of cloned offspring. Therefore, some of them may be omitted for activation to eliminate their possible detrimental effect on nuclear transfer (NT) embryos. The objective of this study was to examine the effect of calcium ionophore (CaI, A23187, Sigma, St. Louis, MO, USA) and cytochalasin D (CD) on activation and in vitro development of nuclear transfer units derived from bovine granulosa cells (GCs) treated with the cell cycle inhibitor, roscovitine. Bovine oocytes isolated from slaughterhouse ovaries were matured in TCM199 supplemented with fetal bovine serum (FBS), sodium pyruvate, penicillin/streptomycin, rIGF-1, bFSH, and bLH. GCs were isolated from ovarian follicles and cultured in DMEM-F12 supplemented with 10% FBS at 37°C in 5% CO_2 in air. Prior to NT, donor cells were exposed to 15 mM roscovitine for 24 hours and small cells were used for NT. A single cell was inserted into the perivitelline space of the enucleated oocyte. Oocyte-cell couples were fused using a 20 μs DC pulse of 40V/150 μm. Two hours after fusion, NT units were assigned into four groups and activated by CaI (5 μM for 10 min.), and then incubated with cycloheximide (CHX, $10\,\mu g\,mL^{-1}$) + CD ($2.5\,\mu g\,mL^{-1}$) for 1 h, followed by CHX alone (without CD) for 5 h (Group I) or CaI (for 10 min.), followed by CHX alone for 6 h (Group II). In the Group III, NT units were activated by CHX ($10\,\mu g\,mL^{-1}$) + CD ($2.5\,\mu g\,mL^{-1}$) for 1 h, followed by CHX for 5 h, and Group IV, CHX alone for 6 h. The base activation medium was TCM199 with 1% FBS for CaI and 10% FBS for CHX and CD. After activation, NT units were cultured for 7 days in BARC medium. Differences in activation, cleavage and blastocyst formation rates among treatments were analyzed by one-way ANOVA after arcsin square transformation. The results are summarized in Table 1. Our data showed that CaI and CD did not affect the activation and in vitro development of NT embryos derived from roscovitine-treated GCs. It suggests that both chemicals may be redundant during cloning procedure. This study was supported by a grant from ProLinia, Inc and TUBITAK, Turkey (VHAG-1908-102V048).

Table 1. In vitro development of NT embryos in different activation treatments

Groups	NT units	NT units fused (%)	NT units cleaved (%)	NT units blastocyst (%)
I	88	69 (78.4)	46 (66.6)	9 (13.0)
II	96	68 (70.8)	44 (64.7)	6 (8.8)
III	96	76 (79.2)	46 (60.5)	13 (17.1)
IV	98	69 (70.4)	46 (66.6)	14 (20.3)

Values within each column are not significantly different ($P > 0.05$).

72 EFFECTS OF GENE EXPRESSION IN BOVINE EMBRYOS RECONSTRUCTED WITH FIBROBLASTS TRANSFECTED WITH LUCIFERASE GENE ON THE SUBSEQUENT DEVELOPMENT

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During embryo development, embryonic gene activation (EGA) is one of the first critical events. Inappropriate EGA results in failure of further development. We have reported that gene expression in bovine embryos reconstructed with fibroblasts begins at 48 hours postfusion (hpf) and reaches a maximum level at 60 hpf as detected by their bioluminescence following injection of chicken β-actin/firefly luciferase fusion gene (β-act/luc+) into their nuclei (Saeki *et al.*, 2001 Theriogenology 55, 289). In the present study, effects of gene expression in embryos reconstructed with bovine fibroblasts transfected with luciferase gene on their subsequent development to the blastocyst stage were examined. Cultured bovine fibroblasts taken from an ear of a female calf were transfected with plasmid containing β-act/luc+/IRES/EGFP and neo^r using GeneJammer (StrataGene, La Jolla, CA, USA). Neomycin-resistant cells were selected by culturing with G418. Then, EGFP-positive colonies were further selected under fluorescence microscopy to obtain stably transfected cells. The transfected cells were cultured for several passages. Growing (50 to 60% confluence, GCs) and

serum-starved cells (SCs) were 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 calcium ionophore and cycloheximide. Luminescence in the embryos was detected with an imaging photon counter at 0 and 60 hpf. Luminescence-positive (P) and -negative (N) embryos were cultured separately at each detection time. Embryos were cultured until 168 hpf, and examined for cleavage and blastocyst development. Experiments were repeated 3 times, and totals of 91 and 123 embryos were reconstructed with GCs and SCs, respectively. Data were analyzed with Fisher's PLSD test following ANOVA by Stat View software (Ver. 5.0). At 0 hpf, luminescence was detected in 55 and 4% of embryos reconstructed with GCs and SCs, respectively. At 60 hpf, luminescence was detected in 47 and 28% of P and N embryos with GCs, and 17 and 40% of P and N embryos with SCs at 0 hpf, respectively. Cleavage rates were not different among groups ($P > 0.05$). Blastocysts were obtained only from the groups of embryos that were N at 0 hpf and P at 60 hpf (8% with GCs and 17% with SCs). No embryos in the other groups developed to the blastocyst stage. These results suggest that appropriate gene expression in embryos reconstructed with somatic cells is important for their subsequent development and that detecting the reporter gene expression can be used for selection of viable cloned embryos.

73 EFFECT OF CALF RECLONING ON EMBRYO AND FETAL SURVIVAL

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In a large-scale cloning program destined to produce transgenic animals, it is very important to incorporate well-characterized transgene integration and gene expression. However, after non-homologous transfection, a wide variety of transgene copies are introduced, and these occur in different chromosome locations. Recloning a selected first-generation transgenic calf offers the opportunity to increase the homogeneity among transgenic animals. Calf recloning was performed in an experiment in which the survival rate was evaluated after a second round of cloning from transgenic umbilical cord and ear calf fibroblasts. The original genetically modified fetal cell line that produced the clones was used as control. Oocytes were aspirated from slaughterhouse ovaries and matured in TCM-199 + 5% FCS at 39°C for 24 h. Matured oocytes were denuded by vortexing for 3 min in TL HEPES with 1 mg mL^{-1} bovine testis hyaluronidase. Metaphases were assessed and oocytes were enucleated by visualization with Hoechst 33342 (5 μg mL^{-1}) under UV light (<6 s). A fetal fibroblast cell line was initially established from a 75-day old Jersey female fetus. Genetically modified cells were isolated after selection with geneticin for 10–15 days following liposome transfection with a DNA construct containing a selectable neomycin resistance gene. Following nuclear transfer with these transgenic cells, new cell lines were isolated from umbilical cord and ear fibroblasts obtained from one of the cloned-transgenic calves so-produced. Donor cells from all three sources were used for nuclear transfer at G0/G1 cell cycle stages and were fused to enucleated oocytes by an electrical pulse. After 3 h, activation was induced by incubation in TL-HEPES with 5 μM ionomycin for 4 min and then 2 mM 6-DMAP for 3 h. The oocytes were then washed with TL- HEPES and cultured in SOF medium with an atmosphere of 5% CO_2 + 5% O_2 + 90% N_2. Development to blastocyst stage (Days 7 to 9) was recorded. One or two blastocysts were transferred non-surgically per recipient cow, and pregnancies at 30 days or 60 days were determined by ultrasonography. All data were analyzed by chi-square test. Seven births were obtained from the original fetal cell line, one birth was obtained from recloned umbilical cord and four pregnancies are in the last third of gestation from recloned ear fibroblasts. Development to blastocyst stage was significantly different between transfected fetal fibroblast and both recloned treatment groups. Differences were observed in pregnancy rates between blastocysts generated by the different sources of donor cells. In spite of the lower blastocyst production, our results suggest that recloning provides an additional method to obtain transgenic animals, and that fibroblasts from umbilical cord could give better results for recloning than those obtained from young calf ear.

Effect of different sources of donor cells for calf recloning on embryo and fetal survival

Treatment	*n*	No. of blastocysts (%)	No. of implanted recipients	No. Pregnant at 30 days (%)	No. Pregnant at 60 days (%)
Transfected fetal fibroblast	966	213 (22.0)[a]	145	63 (43)[a]	36 (25)[a]
Recloned ear fibroblast	680	119 (17.5)[b]	101	24 (24)[b]	3 (3)[b]
Recloned umbilical cord	93	14 (15.1)[b]	7	3 (43)[a]	2 (28.6)[a]
Total	1739	346 (19.9)	253	90 (36)	46 (18)

Percentages within columns with different superscripts differ significantly ($P < 0.05$).

74 PREGNANCY ESTABLISHMENT OF NUCLEAR TRANSFERRED EMBRYOS AFTER VITRIFICATION AND EMBRYO TRANSFER IN CATTLE

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The success of cryopreservation of bovine cloned embryos is important to commercial application of somatic nuclear transfer (NT) in the dairy and beef industry, especially for solving the problem of embryo transfer (ET) between fresh NT embryos and recipients. This experiment was conducted

to test the possibility of establishing pregnancy by embryo transfer of vitrified bovine NT embryos. Oocytes were aspirated from antral follicles of slaughterhouse ovaries, and subsequently cultured in maturation medium for 20 h. Cumulus cells were then denuded from the oocytes by vigorous vortexing for later enucleation and donor nuclear transfer. Skin fibroblast cells used for NT were derived from cultured ear explants taken from an elite dairy cow, and cumulus cells were cultured from the cumulus-oocytes complexes collected by ultrasound-guided transvaginal retrieval. Fibroblasts and cumulus cells were cultured at passage 5 or 6 in 10% FBS DMEM at 37°C in 5% CO_2 humidified air, and used as nuclear donors. After donor cell transfer, somatic cell-cytoplast pairs were then fused by applying two direct current pulses at 2.0 kV cm^{-1} for a duration of 10 μs/pulse. Fused embryos were activated in 10 mg mL^{-1} cycloheximide in M199 + 7.5% FBS for 4 h. Embryos were cultured in CR1aa plus 3 mg mL^{-1} BSA for 2 days (initiation of activation = Day 0) at 39°C, 5% CO_2, 5% O_2 and 90% N_2, and then cultured on bovine cumulus monolayers in CR1aa medium supplemented with 7.5% FBS for 5 successive days. Expanding and hatching blastocysts on Day 7 were selected for cyropreservation via liquid nitrogen surface vitrification (LNSV). Vitrification solution contained HEPES-buffered TCM199 supplemented with 20% FBS, ethylene glycol and dimethylsulphoxide. A droplet of 1–2 μL vitrification solution containing two blastocysts was directly dropped onto a cooled surface within 30 s after 3 min incubation in equilibration solution. Vitrified NT embryos were kept at −150°C vapor phase until recipients were synchronized to Day 7 for ET. Embryos are warmed and subsequently washed several times in rehydration solutions and M199 + 7.5% FBS medium. The warmed embryos from initial trials were cultured for 2 h to evaluate their viability after cryopreservation. Non-surgical transfer was carried out to transfer two embryos to a synchronous recipient, and pregnancy was determined by palpation via rectum around Day 70 of transfer. After warming of vitrified embryos, similar high survival rates were achieved in NT embryos derived from either cumulus (93.8%, $n = 16$), or fibroblast cells (95.8%, $n = 48$) as nuclear donors, respectively ($P > 0.05$). The pregnancy data (Table 1) on Day 70 of gestation indicated that there were no significant differences among ET groups with fresh NT blastocysts, vitrified cumulus NT and fibroblast NT embryos ($P > 0.05$). This study demonstrates that cryopreserved bovine NT embryos via LNSV vitrification can maintain an in vivo developmental viability comparable to freshly produced NT counterparts. Further developmental potential of vitrified NT embryos to term is under investigation.

Table 1. Pregnancy outcomes of vitrified bovine NT embryos after embryo transfer

Treatment	No. recipients	Day 70 pregnancy (%)
Fresh NT-fibroblast	12	3 (25)[a]
Vitrified NT-cumulus	8	2 (25)[a]
Vitrified-NT-fibroblast	22	3 (14.4)[a]

[a]values within columns with same superscripts are not significantly different ($P > 0.05$).

75 HIGHLY EFFICIENT AND RELIABLE CHEMICALLY ASSISTED ENUCLEATION METHOD FOR HANDMADE CLONING IN CATTLE AND SWINE

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In bovine and porcine nuclear transfer, most traditional enucleation procedures require potentially harmful chromatin staining and UV illumination. The purpose of our work was to find an efficient and reliable chemically-assisted procedure for enucleation connected to the handmade cloning (HMC) technique without chromatin staining. Slaughterhouse-derived oocytes were collected and matured in vitro. At 21 (bovine) or 43 (porcine) h after the start of maturation, cumulus cells were removed with vortexing and oocytes were further incubated in the maturation medium supplemented with 0.5 μg mL^{-1} demecolcine for 2 h. Subsequently, zonae pellucidae were digested with 2 mg mL^{-1} pronase in the presence of 10% cattle serum (CS) for 6 to 8 min and washed in HEPES-buffered TCM-199 medium and 20% CS. Bisection was performed in the same medium by hand under a stereomicroscope by using a microblade. A small membrane protrusion observable on the surface of oocytes was used as an orientation point. One-third of the cytoplasm connected to this protrusion was removed, and the cytoplasts and karyoplasts were collected separately. Bovine cytoplasts were used as recipients for HMC experiments (Vajta *et al.*, 2003, Biol. Reprod. 68, 571–578) with fetal fibroblasts as donors, and reconstructed embryos were cultured for 7 days. In Experiment 1 (3 replicates), the possibility of oriented bisection at different time points was determined on a total of 225 bovine oocytes. At 5, 15, 25, 35 and 55 min after the end of pronase digestion 64, 91, 93, 72 and 59% of oocytes had membrane protrusions ($P < 0.05$ between all groups, SAS Genmod) illustrating the time-dependent manner of the protrusion. In Experiment 2, the efficiency and reliability of enucleation was measured. Bisection was performed between 5 and 35 min after pronase digestion. Subsequently both supposed cytoplasts and karyoplasts were stained with Hoechst and investigated under UV light. In cattle (9 replicates), bisection was successfully performed in 94% (519/552) of oocytes, and 98% (507/517) of those bisected were enucleated, i.e. the chromatin was entirely in the presumptive karyoplast. In swine (3 replicates), 91% (302/331) of oocytes were successfully bisected and 95% (280/296) were enucleated. In Experiment 3 (cattle; 4 replicates), blastocyst per reconstructed embryo rates were 47% (139/293), illustrating the high developmental ability in vitro. Considering that no oocyte selection based on the presence of polar body was performed, the above system seems to be more efficient and reliable than other enucleation methods. Moreover, expensive equipment (inverted fluorescent microscope) and a potentially harmful step (staining and UV illumination) can be eliminated from the HMC without compromising the high in vitro efficiency.

76 CLONED HORSE PREGNANCIES PRODUCED USING ADULT CUMULUS CELLS

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We recently reported the birth of the first clone of an equine species, a mule, which was produced using a fetal fibroblast cell line (Woods GL *et al.*, 2003 Science 301, 1063). Since the birth of the first foal, two more identical cloned mule foals have been born. All three foals were delivered spontaneously without assistance, and have been healthy and vigorous since birth. Even more recently, the birth of a horse foal cloned from an adult fibroblast cell line was reported (Touchette N, 2003 Nature 424, 635). Despite these successes, the efficiency of equine nuclear transfer (NT) continues to be very low. The objective of this study was to use NT to clone adult horses using cumulus cells. Cumulus-oocyte complexes used for NT were obtained using transvaginal ultrasound-guided follicle aspiration (TVA) 24 hrs after hCG treatment; oocytes were used as cytoplasts, while cumulus cells (from one of three different mares) were used as donor cells. Cumulus cells were recovered from TVA fluid, washed two times by suspension in PB1 medium (Whittingham DG, 1974 J. Reprod. Fertil. 37, 159–162), followed by centrifugation (200*g*) and placement in Glasgow MEM BHK-21 containing 10% FBS. Nuclear transfer procedures were performed as described (Woods GL *et al.*, 2003 Science 301, 1063). Immediately following NT and activation procedures, cloned embryos were surgically transferred to the oviduct of recipient mares ($n = 2$ to 5 embryos/recipient) that had ovulated within 24 hrs prior to the transfer. An initial pregnancy examination was performed between Days 14 and 16 (Day 0 = surgery); subsequent examinations were then performed at approximately weekly intervals. A total of 136 follicles were aspirated in 96 mares, from which 72 oocytes were recovered (53%). Sixty-two cloned embryos were subsequently transferred to recipient mares, which resulted in 7 (11.3%) ultrasonographically-detectable pregnancies. Cumulus cells from Mare 160 tended ($P = 0.08$) to result in more pregnancies than cumulus cells from Mare 221 (4/17 v. 1/25, respectively). All seven cloned pregnancies underwent spontaneous pregnancy loss between Days 16 and 80. An embryo-proper and heartbeat were detected in three conceptuses. Of four conceptuses in which an embryo-proper was not observed, three did not develop past Day 24; therefore, they were lost before the time at which an embryo-proper generally becomes readily apparent. One conceptus developed to Day 28, yet still failed to form an embryo-proper. There were no premonitory signs of impending embryonic loss in the conceptuses that did not develop an embryo-proper; the conceptus was simply not evident at the subsequent examination. Signs of impending embryonic loss were observed in the three conceptuses in which an embryo-proper was observed, and included: (1) loss of embryonic heartbeat, (2) disorganization of the conceptus membranes, and (3) increased echogenicity of conceptus fluids. One or more of these signs were observed in all three conceptuses prior to pregnancy loss. To our knowledge, this is the first report documenting the establishment of cloned horse pregnancies produced using adult cumulus cells.

77 CLONED EMBRYO DEVELOPMENT IN VITRO: COMPARISON OF CONVENTIONAL AND INDUCED ENUCLEATION PROCEDURES FOR BOVINE CYTOPLAST PREPARATION

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Induced enucleation (IE) of oocytes with demecolcine produces competent ooplasts for SCNT as demonstrated previously in mouse, goat, cow and pig. Whether bovine IE cytoplasts are more or less competent than conventionally enucleated MII oocytes to support nuclear reprogramming of somatic chromatin and embryo development in vitro is not known. This study compared in vitro development of cloned bovine embryos produced by conventional and IE enucleation methods. Three experimental groups were: (1) Parthenogenetic controls. In vitro-matured, MII-arrested bovine oocytes were activated by a single (1 × Act, 10 μM ionomycin in Tyrodes-HEPES, 5 min) or double activation (2× Act; 1× Act, wash 5 min, 10 μg mL^{-1} cycloheximide [CHX] 20 min, repeat 1× Act) followed by incubation in CHX and 5 μg mL^{-1} cytochalasin B (CB) for 6 h, and then culture (BARC medium) for 7 days. (2) Conventional SCNT. MII oocytes were enucleated by micromanipulation in HEPES-buffered enucleation medium (BARC containing 7.5 μg mL^{-1} CB, 5 μg mL^{-1} Hoechst 33342, 10% FBS) under UV illumination (3–5 s). Donor cells (fibroblasts, passage 7–9) were inserted into the perivitelline space, and the reconstructed couplets activated (1× Act). Reconstructed couplets were then electrofused, placed in BARC medium containing 10 μg mL^{-1} CHX and 5 μg mL^{-1} CB (6 h), and then cultured for 7 days. (3) IE SCNT. MII oocytes were activated (1× Act), placed into BARC-5% FBS containing 0.4 μg mL^{-1} demecolcine (DEME), 10 μg mL^{-1} CHX, 2 μg mL^{-1} cytochalasin D for 20 min, then 20 min without DEME, then returned to DEME. At 1–1.5 h post-activation, the extruding second polar body (PB2) containing nuclear chromatin was removed by micromanipulation, couplets were reconstructed and fused as above, and additionally activated (two pulses, 20–30 V/mm, 20 μs). Embryos were cultured in 10 μg mL^{-1} CHX and 5 μg mL^{-1} CB medium for 4–5 hour, then BARC for 7 days. The results (Table 1) reveal that 2× Act increases embryo development at Day 2, but not Day 7. Further, there are no significant differences in embryo development rates between conventional and IE SCNT protocols. Respectively, 46%, 32% and 21% of cleaved control (1× Act), conventional and IE embryos developed to 16 cells on Day 7. In vitro development of cleavage embryos to the blastocyst stage was greater in controls (25–32%) than in conventional (22%) and IE (17%) SCNT groups on Day 7. Further comparisons of in vivo development between conventional and IE SCNT methods following embryo transfer are warranted. Supported by ACT, Cyagra and USDA NRI #2001-35205-09966.

Table 1. Embryo development: Conventional v. induced enucleation

Treatment group	# Oocytes	Stage of Development (%)		
		Cleavage (2–8 cell, Day 2)	16-cell to Morula (Day 7)	Blastocyst (Day 7)
Parth-Control1× Act	1424	837 (58.8)^b	386 (27.1)^a	270 (19.0)^a
Parth-Control2× Act	122	94 (77.0)^a	27 (22.1)^a	24 (19.7)^a
Conventional NT	817	472 (57.8)^b	149 (18.2)^b	104 (12.7)^b
IE NT	684	426 (62.3)^b	89 (13.0)^b	74 (10.8)^b

Values within columns with different superscripts differ significantly ($P < 0.05$).

78 CHRONOLOGICAL EVENTS OF IN VITRO MATURATION IN CAMEL (*CAMELUS DROMEDARIES*) OOCYTES

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Experiments were conducted to investigate the chronological events and optimum time for in vitro oocyte maturation in the dromedary camel. Follicles measuring 3–12 mm were isolated from ovaries obtained from an abattoir and the oocytes harvested by teasing apart these follicles under a stereo microscope. Pooled oocytes were randomly distributed to 4-well dishes (20–25 per well) (Nunc, Denmark) containing 400 μL of the maturation medium (TCM-199 supplemented with 0.6 mg mL^{-1} calcium lactate, 0.1 mg mL^{-1} L-glutamine, 0.8 mg mL^{-1} sodium bicarbonate, 1.4 mg mL^{-1} HEPES, 0.25 mg mL^{-1} pyruvate, 50 μg mL^{-1} gentamicine, 10 μg mL^{-1} FSH, 10 μg mL^{-1} LH, 1 μg mL^{-1} estradiol and 10% heat-inactivated estrous camel serum) and incubated at 38.5°C under 5% CO_2 for 4 to 48 h. After every 4 h (starting from 0 to 48 h), oocytes were denuded by treating them with hyaluronidase (1 mg mL^{-1}) followed by repeated pipetting. Denuded oocytes were mounted on glass slides and fixed in 3 : 1 ethanol : acetic acid for 24 h. Oocytes were stained with 1% aceto-orcein and examined under a phase contrast microscope at 400×. For each experimental group, 3 to 7 replications were made. Based on the visualization of the chromatin, oocytes were categorized as at germinal vesicle (GV), diakinesis (DK), metaphase-I (M-I), anaphase (Ana), metaphase-II (M-II) stage and those with no visible chromatin as NVC. At the start of maturation, 75.4% (43/57) of oocytes were at GV stage; however, none of the oocytes revealed a GV at 28 h of maturation (0/97). At 8 h of maturation 49.3% (34/69) of oocytes were at DK stage, and after 16 h of maturation 50% (49/98) of oocytes were at M-I stage. At 24 h of maturation the maximum number of oocytes were in Ana (24.7%, 21/85) stage. At 44 h the maximum number of oocytes had reached M-II stage (52%, 103/198) whereas, 10.6% (21/198) of the oocytes were at Ana stage. After 48 h the proportion of oocytes with NCV increased to 52.9% (45/85) and the proportion of M-II stage oocytes decreased to 37.6% (32/85). It may be concluded that 40–44 h of in vitro maturation yields the highest proportion of matured (M-II stage) oocytes suitable for further use in assisted reproductive technologies in camel.

79 RELATIONSHIPS BETWEEN SURVIVAL RATE AND BIRTH WEIGHT IN CLONED KOREAN NATIVE CALVES

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Cloning of somatic cells has been investigated actively in cattle, but the cloned calves have been characterized by high birth weight and low survival rate. The present study was conducted to investigate the relationships between survival rate and birth weight in cloned and AI calves. The ear skin fibroblasts were obtained from 2- to 3-year-old Korean native cows (Hanwoo) and the cells were cultured in Dulbeccos Modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 38.5°C, 5% CO_2 in air. Bovine oocytes collected from ovaries obtained from a nearby slaughterhouse were cultured in vitro and then enucleated, injected with donor cells and fused, and cultured to produce cloned embryos at the blastocyst stage. Somatic cell cloning and in vitro culture of embryos were performed by the procedures described previously (Im *et al.*, 2001 AJAS 14, 759–764, and Im *et al.*, 2001 AJAS 14, 1260–1266). A total of 580 cloned embryos at blastocyst stage were transferred to 293 recipient cows; 32 female calves (5.5%) were born (2 of them were born dead). Thirty-four (15 female and 19 male) calves (57.6%) were born from 59 artificially inseminated Korean native cows as control. Fifteen of the 32 cloned calves were delivered by caesarean section. However, all the artificially inseminated cows delivered naturally. Birth weights of 30 live cloned calves averaged 31.08 kg (>15 kg: 3, 20 kg: 2, 25 kg: 2, 30 kg: 5, 35 kg: 9, 40 kg: 6, <45 kg: 3), while those of female AI calves averaged 23.67 kg (>15 kg: 0, 20 kg: 3, 25 kg: 6, 30 kg: 6, 35 kg: 0, 40 kg: 0, <45 kg: 0). After calving, 11 of 30 cloned calves survived for more than 365 days (birth weight of these calves averaged 28.25 kg), but 19 of 30 calves died within 175 days and their average birth weight was 32.80 kg (650 kg). Gestation length of cows that received cloned embryos was 287 (279–295) days on average (excluding the data of calves delivered by caesarean section) and that of cows artificially inseminated was 287 (255–293) days. In conclusion, the birth weight was significantly correlated ($P < 0.05$) with survival rate of cloned calves, and survival rates of calves with extremely high or low birth weights were significantly low. However, there was no relationship between gestation length and survival rate.

80 DEVELOPMENTAL POTENTIAL OF BOVINE NUCLEAR TRANSFER EMBRYOS DERIVED FROM FETAL FIBROBLASTS TRANSDUCED WITH SIMIAN VIRUS 40 LARGE T ANTIGEN

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Primary cultured cells with limited lifespan are generally used as donors for nuclear transfer (NT). Immortalization or even extension of their proliferative capacity would provide an additional time-window for various genetic manipulations prior to NT. Previously, we reported that a spontaneously immortalized mammary epithelial cell line failed to support development of reconstructed embryos into blastocysts [Zakhartchenko *et al.*, 1999 Mol. Reprod. Dev. 54, 264–272]. In contrast, telomerase-immortalized sheep fibroblasts could be substantially reprogrammed, although they were not fully competent for NT since no fetuses survived beyond 40 days of development [Cui *et al.*, 2003 Biol. Reprod. 69, 15–21]. Simian virus 40 large T antigen (SV40Tag), a viral oncoprotein, is known to immortalize human diploid fibroblasts by soaking up the cellular negative growth regulators, pRb, p53, and some related factors to enable cells to grow continuously [Kim *et al.*, 2001 Exp. Mol. Med. 33, 293–298]. In this study we examined the developmental competence of bovine NT embryos derived from SV40Tag-transduced fetal fibroblasts. Primary fetal fibroblasts (BFF) obtained from a 49-day-old fetus were first transduced with the green fluorescent protein (GFP) gene and then with SV40Tag using a replication-defective retrovirus. GFP-SV40Tag-positive cells (BFF-GFP-Tag), which can proliferate over 50 passages, were cultured until confluence and then used for nuclear transfer at passages 27–30. As control, confluent GFP-positive cells (BFF-GFP) and BFF, which stopped proliferation after 28 passages, were used at passages 4–6 and 1–2, respectively. Nuclear transfer and embryo culture procedures were essentially as described previously [Shi *et al.*, 2003 Biol. Reprod. 69, 301–309]. Data were compared using chi-square test; differences were considered significant for $P < 0.05$. There were no significant differences in the fusion and cleavage rates between all three groups [BFF-GFP-Tag: 233/248 (94%) and 167/233 (72%); BFF-GFP: 274/294 (93%) and 216/274 (79%); BFF: 129/136 (96%) and 97/129 (75%)]. However, development to the blastocyst stage was significantly lower ($P < 0.05$) in the BFF-GFP-Tag group [5/233 (2%)] than in the BFF-GFP and BFF groups [110/275 (40%) and 53/129 (41%), respectively)]. From our results we conclude that SV40Tag-transduced bovine fetal fibroblasts with high proliferative potential may have lost the ability to respond to the normal cell cycle controls and, as a consequence, have low developmental potential for nuclear transfer.