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Nuclear Transfer of Sand Cat Cells into Enucleated Domestic Cat Oocytes is Affected by Cryopreservation of Donor Cells

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Abstract

In the present study, we used the sand cat (*Felis margarita*) as a somatic cell donor to evaluate whether cryopreservation of donor cells alters viability and epigenetic events in donor cells and affects *in vitro* and *in vivo* developmental competence of derived embryos. In Experiment 1, flow cytometry analysis revealed that the percentage of necrosis and apoptosis in cells analyzed immediately after freezing/thawing (61 vs. 8.1%, respectively) was higher than that observed in frozen/thawed cells cultured for 18 h (6.9 vs. 3.3%, respectively) or 5 days (38 vs. 2.6%; respectively). The relative acetylation level of H3K9 was lower in frozen/thawed cells (5.4%) compared to that found in cultured cells (60.1%). In Experiment 2, embryos reconstructed with frozen/thawed cells had a lower cleavage rate (85%; day 2) than did embryos reconstructed with cultured cells (95%), while development to the blastocyst stage (day 8) was not affected by cell treatment (17.0% with frozen/thawed cells vs. 16.5% with cultured cells). In Experiment 3, pregnancy rates were similar between both cell treatments (32% with frozen/thawed cells vs. 30% with cultured cells), but the number of embryos that were implanted, and the number of fetuses that developed to term was lower for embryos reconstructed with frozen/thawed cells (1.2 and 0.3%, respectively) than those reconstructed with cultured cells (2.6 and 1.8%, respectively), while the number of fetuses reabsorbed by day 30 was higher (75%) for embryos reconstructed with frozen/thawed cells than those reconstructed with cultured cells (31%). A total of 11 kittens from cultured cells and three kittens from frozen/thawed cells were born between days 60 to 64 of gestation. Most kittens died within a few days after birth, although one kitten did survive for 2 months. In Experiment 4, *POU5F1* mRNA expression was detected in 25% of blastocysts derived from frozen/thawed cells, whereas 88 and 87% of blastocysts derived from cultured cells and by in vitro fertilization, respectively, expressed *POU5F1*. We have shown that cell cryopreservation increased the incidence of necrosis and apoptosis and altered epigenetic events in donor cells. Consequently, the number of embryos that cleaved, implanted, and developed to term-gestation and *POU5F1* expression in derived blastocysts indirectly was affected.

Introduction

The sand cat (*Felis margarita*), one of the smallest species of felids, inhabits the deserts of North Africa and the Arabian Peninsula, west of the Caspian Sea. Their future is threatened by habitat destruction and human encroachment, which results in a smaller, fragmented population. A potentially important approach to maintaining the genetic variability of the species is by the use of assisted reproductive technologies, in particular, somatic cell nuclear transfer (SCNT). The viability of preserving endangered felids by using interspecies SCNT has been demonstrated with the production of live male and female African wildcat (*Felis silvestris lybica*; AWC) kittens after the transfer of cloned embryos into domestic cat recipients (Gómez et al., 2004, 2005, 2006). Also, pregnancies after the transfer of interspecies black-footed cat (*Felis nigripes*) and intergeneric Leopard cat (*Prionailurus bengalensis*)-cloned embryos into do-

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mestic cats have been established (Gómez et al., 2005; Yin et al., 2006). However, many of the implantation sacs formed by the cloned embryos do not contain a normal fetus and are reabsorbed by day 30 to 45 of gestation (Gómez and Pope, 2006; Gómez et al., 2006). Incomplete and/or abnormal reprogramming of the differentiated nucleus is a major constraint to the *in vivo* developmental potential of interspecies felid embryos (Gómez et al., 2004, 2005). Some evidence indicates that successful nuclear reprogramming is dependent largely on partial erasure of preexisting epigenetic marks of donor cells (Enright et al., 2003b). Each cell type has its own epigenetic characteristics, and it is possible that epigenetic differences between donor cells may affect embryo development and implantation. In fact, development of bovine and monkey-cloned embryos to the blastocyst stage was associated with specific epigenetic markers observed in donor cells (Santos et al., 2003; Yang et al., 2007b), and certain epigenetic markers on donor cells are modified by *in vitro* culture conditions (Enright et al., 2003a). It is common practice to reconstruct cloned embryos with either fresh donor cells disaggregated from a monolayer, or with donor cells thawed just before SCNT. Several live offspring of different species, including AWC clones, have been produced from donor cells that were thawed immediately before SCNT. Even so, there is evidence indicating that development to the blastocyst stage of cloned embryos reconstructed from such donor cells is reduced (Zhang et al., 2006), and that repeated cell freezing increases fetal losses of bovine-cloned embryos (Marfil et al., 2005). In the present study, we determined (1) whether cryopreservation of donor cells increased the levels of apoptosis and necrosis and modified histone acetylation levels of sand cat fibroblast cells, and (2) if the use of donor cells thawed immediately before SCNT (a) affects embryo development and *in vivo* viability after transfer of reconstructed cloned embryos into domestic cat recipients and, (b) influences expression of the POU transcription factor *POU5F1* (previously known as OCT-4) gene in cloned embryos.

Materials and Methods

Subjects

Domestic cats (DSH) used as oocyte donors and embryo recipients were group-housed in environmentally controlled rooms with a 14 h:10 h light:dark cycle at 20–26°C at the Audubon Center for Research of Endangered Species (ACRES). The rooms were cleaned and cats fed once daily (Science Diet, Hill Pet Nutrition, Shawnee Mission, KS). Fresh water was available at all times. All animal procedures were approved by the Institutional Animal Care and Use Committee of ACRES as required by the Health Research Extension Act of 1985 (Public Law 99-1580).

Chemicals

All chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise stated.

Establishment and culture of donor fibroblasts

Fibroblast cell cultures were generated from skin tissue collected by biopsy of two adult sand cat males (cell cultures 1 and 2) located at the Birmingham Zoo, Birmingham, Alabama, and one male sand cat-cloned kitten (5 days of age; cell culture 3) produced from embryos reconstructed with fibroblasts derived from cell culture 1. Skin samples of adult males were sent to ACRES by overnight shipping at 4°C in HEPES-199 medium. The skin sample from the cloned kitten was taken within 5 h of death and processed after 3 days of refrigeration (4°C). All samples were processed as previously described (Gómez et al., 2003a) and cultured in Glasgow Minimal Essential Medium (GMEM) supplemented with 50 μg/mL of gentamicin, 2.4 mM glutamine, 2.4 mM sodium pyruvate, 2.5 μg/mL amphotericin B, and 15% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT) at 38°C in 5% CO_2/air. When monolayer outgrowths with fibroblastic-like morphology reached 100% confluence cells were disaggregated with 2.5 mg/mL of trypsin and resuspended in GMEM with 10% FBS and 10% (v/v) dimethyl sulphoxide and cooled at 1.0°C/min to −80°C (Mr. Frosty; Nalgene, Rochester, NY) before storage in liquid nitrogen (LN_2). Donor cells for all experiments were used at passage 2.

Analysis of apoptosis, necrosis, and histone acetylation levels by flow cytometry

The relative levels of histone acetylation of frozen/thawed and cultured cells was assessed by flow cytometry after incubation with primary rabbit anti-acetyl-histone 3 lysine 9 antibody (H3K9; Upstate Cell Signalling Solutions; Lake Placid, NY) according to the method previously reported by Enright et al. (2003b). Briefly, cell pellets collected after centrifugation of frozen/thawed and cultured cells from the three sand cat cell cultures were resuspended in cold "saline GM" solution and fixed in 100% ethanol for 12 h at 4°C. Then, cells were incubated with the primary antibody (1:100 dilution) for 30 min at 22°C and washed with PBS + 5% FBS. Finally, cells were incubated with secondary antirabbbit goat IgG conjugated with fluorescein isothiocyanate (1:50; Chemicon International; Billerica, MA) for 30 min at 38°C and counterstained with 30 μg/mL of propidium iodide (PI). Controls for nonspecific autofluorescence were performed by replacing the primary antibody for sheep IgG, while nonspecific binding was performed by incubating cells with the secondary conjugated antibody only, but not primary antibody. The relative level of histone acetylation in the sand cat cells was detected by the intensity of green fluorescence displayed.

For determining the percentage of cells undergoing necrosis and apoptosis, pellets collected after centrifugation of cultured cells (cells that were cultured *in vitro* for 18 h or 5 days after freezing and thawing) or frozen/thawed (cells that were not cultured after freezing and thawing) were resuspended in 500 μL of GMEM and incubated at 38°C for 2 h (mimicking the period of time usually required to do SCNT). Then, 5 μL of Caspase-3 substrate stock solution (5 μM; NucView™, Biotium, Inc. Hayward, CA) was added to each cell pellet. After 15 min of culture at 22°C with caspase-3 substrate, cells were counterstained with 30 μg/mL of PI and cultured for an additional 10 min before analysis by flow cytometry. As a control for apoptosis, caspase 3 was induced by incubation of cells with 1 μM staurosporine. For inhibition of apoptosis, cells were incubated with 1 μM staurosporine and subsequently exposed to 50 μM of caspase-3 inhibitor Ac-DEVD-CHO (NucView™ 488 caspase-3 substrate, Biotium, Inc.). Cells were analyzed by flow cytome-

try and unstained fibroblasts were classified as live, and cells that displayed green fluorescence were classified as apoptotic, while cells that displayed both red and green fluorescence were classified as necrotic.

For each treatment, 10,000–20,000 cells per sample were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Percentages of cells in apoptosis, necrosis and the relative levels of histone acetylation were calculated using Cell Quest software (BDIS).

For determining the morphological appearance of live/apoptotic cells, cultured cells (cells that were cultured *in vitro* after freezing and thawing) and frozen/thawed (cells that were not cultured after freezing and thawing) were centrifuged and resuspended in 500 μL of PBS. Then, 5 μL of caspase-3 substrate stock solution was added to each cell treatment. After 15 min culture at 22°C culture cells were counterstained with 30 μg/mL of PI, cultured for an additional 10 min, and analyzed by fluorescence microscopy. Unstained fibroblast cells were classified as live, cells that displayed green fluorescence were classified as apoptotic and cells that displayed red fluorescence were classified as necrotic.

Oocyte maturation

For collecting *in vivo* matured oocytes, domestic cats were treated with a total of 3 to 5 IU of porcine-follicle stimulating hormone (p-FSH, #915 Sioux Biochemical, Sioux Center, IA) given in decreasing daily doses (s.q.) for 4 days (day 1 = 1.0–1.50 IU, day 2 = 0.75–1.30 IU, day 3 = 0.75–1.10 IU, day 4 = 0.50–1.10 IU) followed by 3 IU of porcine-luteinizing hormone (p-LH, #925 Sioux Biochemical) on the fifth day. At 24 to 26 h after LH injection (i.m.), oocytes were collected by laparoscopic aspiration of preovulatory ovarian follicles (Gómez et al., 2000).

Embryo production by SCNT and IVF

SCNT was conducted according to the method previously reported by Gómez et al. (2003a). Briefly, at 1–2 h after oocyte retrieval, mature oocytes were denuded of cumulus and corona cells and enucleated (metaphase spindle and first polar body were visualized after exposing oocytes to 25 μg/mL Hoechst 33342). After enucleation, single frozen/thawed or cultured fibroblast cells from one of the three cell cultures was introduced into the perivitelline space and fusion was induced by applying two electrical pulses (3-sec AC of 19 V, 1 MHz; followed by a 30-μsec DC of 21 V) delivered with two stainless steel electrodes (LF-101; Nepa Gene, Tokyo, Japan) attached to micromanipulators. After 30 min of culture, fusion was confirmed visually. Fused couplets were cultured for a further 2 h before activation was induced by exposure to two 60-μsec DC pulses of 120 V/mm. Afterward, couplets were cultured for 4 h at 38°C in a gas atmosphere of 5% CO_2 and air under mineral oil, in a 30-μL droplet of Tyrode's solution (T2397) containing 1% MEM nonessential amino acids, 3 mg/mL BSA, 15 mM $NaHCO_3$, 0.36 mM pyruvate, 2.2 mM calcium lactate, 1 mM glutamine, and 50 μg/mL gentamicin (IVC-1 medium) that was further supplemented with 7.8 mM calcium lactate, 10 μg/mL cycloheximide, and 5 μg/mL cytochalasin B. After activation, reconstructed couplets were cultured in 500 μL of IVC-1 medium for 12–13 h and transferred to the oviducts of domestic cat females on day 1 or cultured *in vitro* until day 8, at which time development to the blastocyst stage was recorded and blastocysts were frozen for *POU5F1* analysis.

For production of IVF embryos, semen was collected from one of two adult cats using an artificial vagina and oocytes were coincubated with 5×10^5 motile spermatozoa/mL in IVF medium. After 5 to 6 h, oocytes were washed in IVF medium to remove spermatozoa and cultured for 8 days according to a previous protocol (Gómez et al., 2003b). On day 8, blastocysts were frozen for later *POU5F1* analysis (control group).

Embryo transfer, pregnancy detection and parturition

On day 1, sand cat embryos reconstructed with frozen/thawed (n = 25 recipients) and cultured (n = 20 recipients) donor cells were transferred by laparoscopy into the oviducts of gonadotropin-treated domestic cat recipients after induced ovulation or oocyte aspiration (Gómez et al., 2004).

Recipients were examined by ultrasonography on day 21 after embryo transfer to determine pregnancy status. A recipient was classified as pregnant if one or more gestational sacs were observed. Pregnant cats were examined weekly to monitor fetal status, including the presence of a heart beat. We had previously demonstrated that the administration of altrenogest and betamethasone to pregnant cats significantly reduced premature vaginal bleeding and perinatal mortality of AWC-cloned kittens, respectively (Gómez et al., 2004). Therefore, in the present study, altrenogest (0.08 mg/kg, Regu-Mate, DPI Laboratories, San Antonio, Texas) was administered orally to pregnant cats (n = 8) daily from days 52–55 to days 60–62 of gestation. Betamethasone (0.1 mg/kg, CelestoneSoluspan, Schering, New Jersey) was administered in four doses (i.m.); the first two injections were given 12 h apart on days 53–54 and the last two injections were given 5 days after the first doses on days 58–59 of gestation.

To reduce the risk of early labor, pregnant cats were monitored for uterine contractions by using an electronic monitoring system (system 37; Veterinary Perinatal Specialties Inc., Wheat Ridge, CO) starting on day 54 of gestation. Terbutaline, a beta-2 receptor agonist, was given to pregnant cats that had early, irregular uterine contractions to inhibit premature labor. Pregnant recipients were allowed to complete gestation, at which time kittens were delivered by Caesarean section, either (1) as an intervention after onset of vaginal bleeding or (2) after the detection of frequent and strong uterine contractions in association with fetal heart rates of $<$120 bpm. Fetal heart rates were monitored with a heart rate doppler (summit Doppler; Life Dop). All kittens received 24 h per day perinatal supportive care to maximize survival rates.

Microsatellite analysis

To determine the clonal status of kittens, DNA was extracted from blood of the sand cat donor, blood of the recipient dam, or spleen of dead kittens by using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Sterile cytological brushes were used to noninvasively obtain buccal (cheek) cells noninvasively from live kittens. DNA was isolated using the QIAmp DNA mini kit (Qiagen). Feline microsatellite loci were amplified as reported previously by Gómez et al. (2004) using standard ABI fluorescent chemistries on an ABI

3730 DNA Analyzer (Applied Biosystems, Foster City, CA). The results were analyzed using STRand (Hughes) software.

Analysis of POU5F1 *expresion by qRT-PCR*

To quantify feline *POU5F1* and *GAPDH* relative mRNA levels, standard curves were produced by fivefold serial dilution of genomic feline DNA (1.67E4–2.66E1 copies) in nuclease free water with 10 ng/mL tRNA to produce standards for analysis. Single cloned blastocysts obtained from each cell line and domestic cat IVF-derived blastocysts (control group) were washed in PBS supplemented with 0.1% PVP (PBS-PVP) and placed into RNAse free tubes containing 10 μL of PBS-PVP, frozen in liquid nitrogen, and stored at -80°C. mRNA was isolated from blastocysts using a ChargeSwitch® Total RNA Cell Kit (Invitrogen, Carlsbad, CA) with a final elution volume of 20 μL. The isolated RNA was subjected to two-step quantitative reverse transcriptase PCR. The first step involved reverse transcription of RNA into cDNA by using the iScript™ Select cDNA synthesis kit (BioRad, Hercules, CA) with a 20-μL reaction carried out as follows: 10 μL of RNA sample was conditioned with 2 μL GSP enhancer solution, 100 nM of forward primer 5′ AGTCTGGATGCAGATACTCCCACA 3′ for *POU5F1*, 100 nM of reverse primer 5′ GTGATCCTCTTCTGCTTCAGGAGT 3′ for *POU5F1*, 100 nM of forward primer 5′ GCAAAGTGGACATTGTCGCCATCA 3′ for *GAPDH*, 100 nM of reverse primer 5′ AGTTTCCCGTTCTCAGCCTTGACT 3′ for *GAPDH*. The sample was incubated at 65°C for 5 min, immediately transferred to 42°C for the addition of 4 μL of 5× iScript select reaction mix and 0 μL or 1 μL iScript reverse transcriptase (RT) for minus RT or plus RT, respectively, maintained at 42°C for a further 45 min, and then incubated at 85°C for 5 min to heat-inactivate reverse transciptase. Fifty microliters of qRT-PCR reactions were achieved by the addition of 20 µL of cDNA equivalent to half of an embryo to 30 μL of qRT-PCR mix containing 25 μL 2× TaqMan universal mastermix (ABI), 100 nM of forward primer for Oct4, 100 nM of reverse primer for *POU5F1*, 100 nM of probe 5′/56-FAM/ATGCAGTCCCAGGACATCAAAGCTCT/3BHQ_1 3′ for *POU5F1*, 100 nM forward primer for GAPDH, 100 nM reverse primer for GAPDH, and 100 nM of probe 5′/5Cy5/TCCAGTATGATTCCACCCACGGCAAA/3IAbRQSp/ 3′ for GAPDH.

The cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec, and 60°C for 60 sec using a Stratagene Mx3000P real-time PCR system. Analysis was performed using Mx3000P version 2.00 (Stratagene, La-Jolla, CA). Baselines were set using the Adaptive baseline MX4000 v1.00 to v3.00 algorithm setting and threshold value was determined by setting threshold bar within linear data phase.

Statistical analysis

Two-way ANOVA was used to analyze the data on percentage of necrotic, live, and apoptotic cells, and levels of histone acetylation of cultured and frozen/thawed cells. One-way ANOVA was used to analyze data on birth weight of cloned kittens. Chi-square was used to analyze data on embryo cleavage, blastocyst development, embryo implantation, and total number of live offspring. The Holm-Sidak multiple-comparison test was used to determine any differences between two means after ANOVA.

Experimental design

Experiment 1. Rate of apoptosis, necrosis, and relative levels of acetylation of H3K9 in frozen/thawed and cultured sand cat fibroblasts. Fibroblast cells from each of the three cell cultures that were frozen at passage 1 (P1) were thawed, plated equally into tissue culture flasks, and when cells reached 100% confluence, the culture medium was replaced every other day and cells were cultured for an additional 5 days. Then, flasks containing synchronized cells from each cell culture at passage 2 (P2) were dissociated with 2.5 mg/mL of pronase and divided in two treatments: (1) cultured cells (18 h or 5 days and analyzed immediately after dissociation) and, (2) frozen/thawed cells (after dissociation, cells were frozen/stored in LN_2 and thawed just before analysis). Cells from each treatment were distributed in two groups to analyze (1) the percentage of necrosis and apoptosis and (2) relative levels of histone acetylation. Two replicates per treatment were performed.

Experiment 2. *In vitro* development of sand cat cloned embryos reconstructed with frozen/thawed or cultured sand cat donor cells. Fibroblast cells from each of the three cell cultures that were frozen at passage 1 (P1) were thawed, plated equally into two flat-sided tissue culture tubes (Leighton) and passaged once (P2). When cells reached 100% confluence, culture medium was replaced every other day, and cells were cultured for an additional 3 to 5 days (Gómez et al., 2003a). Then, synchronized fibroblast cells were dissociated from culture tube with 2.5 mg/mL of pronase and either (1) frozen/stored in LN_2 and thawed just before SCNT (frozen/thawed cells) or (2) used immediately for SCNT (cultured cells). Embryo reconstruction was performed by using the protocol described in Materials and Methods. Cleavage and development to blastocyst stage was evaluated on days 2 and 8, respectively. The number of embryos from each treatment was derived from two to four replicates.

Experiment 3. *In vivo* developmental competence of sand cat-cloned embryos reconstructed with frozen/thawed or cultured donor cells. Cells from each of the three cell cultures that were frozen at passage 1 (P1) were thawed, plated equally into two flat-sided tissue culture tubes, and passaged once (P2). After reaching confluence, cells were cultured 3 to 5 days and medium was changed every other day (Gómez et al., 2003a). Then, synchronized fibroblast cells were dissociated from the culture tube with 2.5 mg/mL of pronase and either (1) frozen/stored in LN2 and thawed just before SCNT (frozen/thawed cells) or (2) used immediately for SCNT (cultured cells). Embryo reconstruction was performed by the same protocol as described above. Sand cat-cloned embryos (D1–2) reconstructed with frozen/thawed or cultured cells from each of the three cell cultures were transferred into the oviduct of synchronized domestic cat recipients. Three to 17 recipient cats received cloned embryos from each of the three cell cultures and from each of the cell treatments.

Experiment 4. *POU5F1* expression of cloned embryos reconstructed with frozen/thawed or cultured donor cells. The *POU5F1* gene is considered as a candidate marker for determining the potential of cloned embryos to develop into

live offspring (Li et al., 2006). Therefore, in this experiment, we evaluated whether cryopreservation influenced expression of the *POU5F1* gene in cloned embryos reconstructed with donor cells that were either (1) frozen/stored in LN2 and thawed just before SCNT (frozen/thawed cells), or (2) used immediately for SCNT (cultured cells). Domestic cat IVF-derived blastocysts were used as controls for *POU5F1* expression.

Results

Experiment 1

Flow cytometric analysis of cell cultures 1, 2, and 3 revealed that in frozen/thawed cells the percentage of necrotic (58 vs. 67 vs. 57%; respectively) and apoptotic cells (16.4 vs. 12.6 vs. 9.5%; respectively) were higher than the percentages of necrotic (1.8 vs. 2.0 vs. 5.2%; respectively) and apoptotic (0.8 vs. 2.1 vs. 6.2%; respectively) cells cultured for 18 h, and the percentage of necrotic (39 vs. 35 vs. 41%, respectively) and apoptotic (2.1 vs. 0.5 vs. 5.4%, respectively) cells cultured for 5 days ($p < 0.001$; Fig. 1 and Fig. 2). However, there were no differences in the percentages of apoptosis between frozen/thawed cells (9.5%) and those cultured for 18 h or 5 days (6.2 vs. 5.4%, respectively) within cell culture 3 (Fig. 2). The relative levels of acetyl H3K9 are presented in Figure 3. The relative acetylation levels of H3K9 were lower in frozen/thawed cells (cell culture 1 = 4.4% vs. cell culture 2 = 3.9%) compared to the levels in cells cultured for 5 days (cell culture 1 = 79% vs. cell culture 2 = 85%; $p < 0.001$; Fig. 4). However, there were no differences in the level of histone acetylation between cultured cells for 5 days (18%) and frozen/thawed cells (8.4%) within cell culture 3 (Fig. 4). Fluorescence was not detected in the nonspecific binding control in which cells were incubated only with the secondary conjugated antibody. However, some fluorescence was detected in the nonspecific autofluorescence control, which was deducted from the relative fluorescence level in each treatment group (0.9%, cultured cells; 4.0%, frozen/thawed cells).

Analysis by light microscopy of live/apoptotic cells revealed that cells at early apoptosis had a similar morphological appearance to that of live nonapoptotic cells (live/intact; Fig. 5).

Experiment 2

Nuclear transfer results are presented in Table 1. Cleavage of reconstructed embryos was affected by the cell treatment, but was not influenced by the genetic source of the donor nucleus. Higher percentages of cleavage were observed when embryos were reconstructed with cells cultured for 5 days from cell cultures 1, 2, and 3 (95 vs. 96 vs. 94%, respectively) compared with frozen/thawed cells from cell cultures 1 and 2 (83 vs. 84%; $p < 0.001$; Table 1.). However, cleavage rate of embryos reconstructed with frozen/thawed cells from donor cell culture 3 (97%) was similar to the rates observed with embryos reconstructed with cells cultured for 5 days from cell cultures 1, 2, and 3 (95 vs. 96 vs. 94%, respectively). Development to the blastocyst stage was not influenced by cell treatment (overall mean = 17.0% with frozen/thawed cells vs. 16.5% with cultured cells), but was affected by genetic source of the donor nucleus. A lower number of cleaved embryos developed to the blastocyst stage when embryos were reconstructed with cultured cells from cell culture 1 (6.0%) compared to embryos reconstructed with cultured cells from cell culture 2 (43.0%; $p < 0.05$; Table 1).

Experiment 3

To determine the influence of cell cryopreservation on pregnancy rate, embryo implantation, and rate of live offspring, we transferred sand cat cloned cybrids (D1; fused couplets that contain cytoplasm from the oocyte and the somatic cell donor) reconstructed with frozen/thawed (n = 984) or cultured (n = 616) donor cells into the oviduct of 25 (mean = 39.6 ± 10.3 cybrids) and 20 (mean = 32.1 ± 9.8 cybrids) domestic cat recipients, respectively. Because we did not observe an influence of genetic source on pregnancy rate, number of embryos implanting or developing to term, we

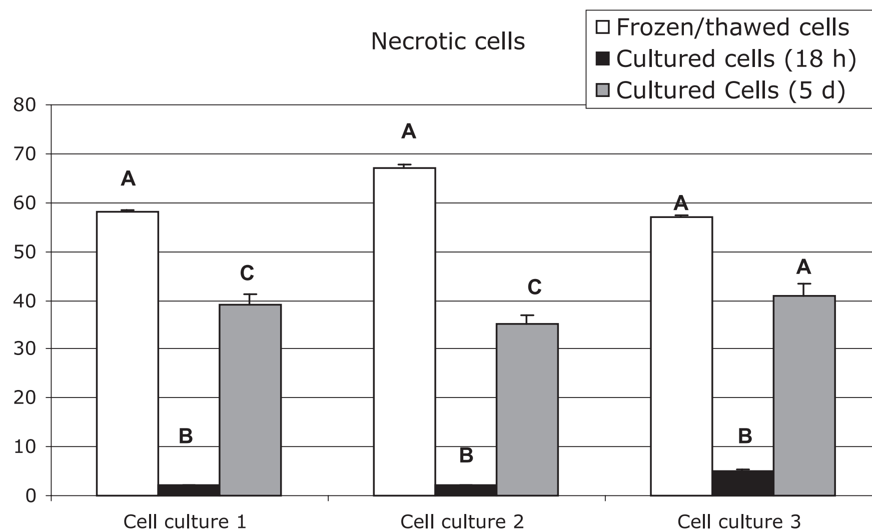
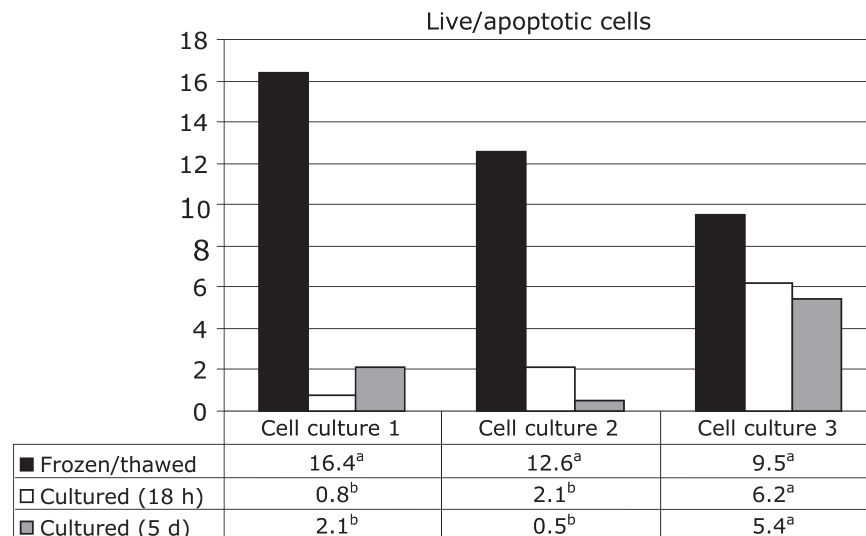


FIG. 1. Percentages of necrotic sand cat fibroblast cells after being frozen/thawed and cultured for 18 h or 5 days.



Columns with different superscripts within each cell culture differ significantly (P<0.001).

FIG. 2. Percentages of live/apoptotic sand cat fibroblast cells after being frozen/thawed and cultured for 18 h or 5 days.

pooled the data of the three cell cultures within the same cell treatment for statistical analysis.

Eight (32%) and six (30%) recipient cats receiving embryos reconstructed with frozen/thawed and cultured donor cells, respectively, were diagnosed to be pregnant when examined by ultrasonography at 22–23 days after induction of ovulation or oocyte aspiration (Table 2). Although pregnancy rate was not different between the two cell treatments, the overall mean number of embryos that implanted after transfer of cybrids reconstructed with frozen/thawed cells ($n = 12$; 1.2%) versus cultured cells ($n = 16$, 2.6%; Table 2) was different ($p < 0.05$). Similarly, the number of fetuses reabsorbed by day 30 was higher for the frozen/thawed ($n = 9$, 75%) than the cultured ($n = 5$, 31%; $p < 0.05$) cell treatment. The rate of term fetal development (>61 days) as a percentage of the total number of embryos that implanted or of the total number of embryos transferred, respectively, was lower in the frozen/thawed (25%, 0.3%) than the cultured cell treatment (69%, 1.8%; $p < 0.05$; Table 2).

Of the eight pregnant recipients carrying cloned kittens derived from frozen/thawed cells, three (37.5%) delivered a total of three kittens, while, of the six pregnant recipients carrying cloned kittens derived from cultured cells, five (83.3%) delivered a total of 11 kittens. The average birth weight of kittens derived from frozen/thawed cells was lower (39.5 g, range = 22–52 g) than the average weight of kittens derived from cultured cells (60.9 g, range = 46–82 g; $p < 0.05$). Of the 11 kittens from the cultured cell treatment, only one had physical abnormalities (cardiomyophathy), while the others died by either (a) respiratory failure (five) within 0 to 24 h after birth, (b) moderate to acute chronic pneumonia induced by aspiration of formula into lungs (four) within 5 to 60 days after birth (Fig. 6) or (c) intrauterine death immediately before birth (one; Table 3). All three kittens derived from the frozen/thawed cell treatment had incomplete closure of the ventral body wall musculature with abdominal organ exteriorization and one of the kittens died *in utero* on day 61 of gestation (Table 3).

Phenotypically, all cloned kittens ($n = 14$) were sand cats. Subsequent DNA analysis of 21 cat-specific microsatellite loci performed with "blind" testing at the School of Veterinary Medicine, University of California Davis, confirmed that they were identical to DNA of the three cell donor males (Table 4).

Experiment 4

RT-PCR was performed with nine and four single sand cat-cloned blastocysts reconstructed with frozen/thawed and cultured donor cells, respectively. As controls, 15 domestic cat IVF-derived blastocysts were analyzed. Expression of *POU5F1* in blastocysts was reported either scored (detectable) or unscored (nondetectable) after qRT-PCR. *POU5F1* mRNA expression was detectable in eight of nine (88%) blastocysts from the cultured donor cell treatment, whereas only one of four (25%) blastocysts from the frozen/thawed cell treatment expressed *POU5F1*. IVF-derived blastocysts showed *POU5F1* expression at a similar rate (13/15; 87%) to that observed in cloned blastocysts reconstructed with cultured donor cells.

Discussion

SCNT represents a supportive approach for preserving genetic variability in endangered mammalian species. As a step toward that goal, in the present study, we demonstrated that sand cat-cloned kittens can be produced by using both cultured or frozen/thawed donor cells derived from adult sand cats and from cloned sand cats of the first generation. However, all live cloned kittens died between the ages of 2 days to 2 months. Genetic source of cultured cells did not influence *in vivo* development of transplanted embryos, but it had a significant effect on the number of embryos that developed to the blastocyst stage *in vitro*. In addition, we observed that environmental experience of the somatic cell before use in SCNT strongly influenced its subsequent response. Indeed, the numbers of fused cybrids that cleaved by day 2 and em-

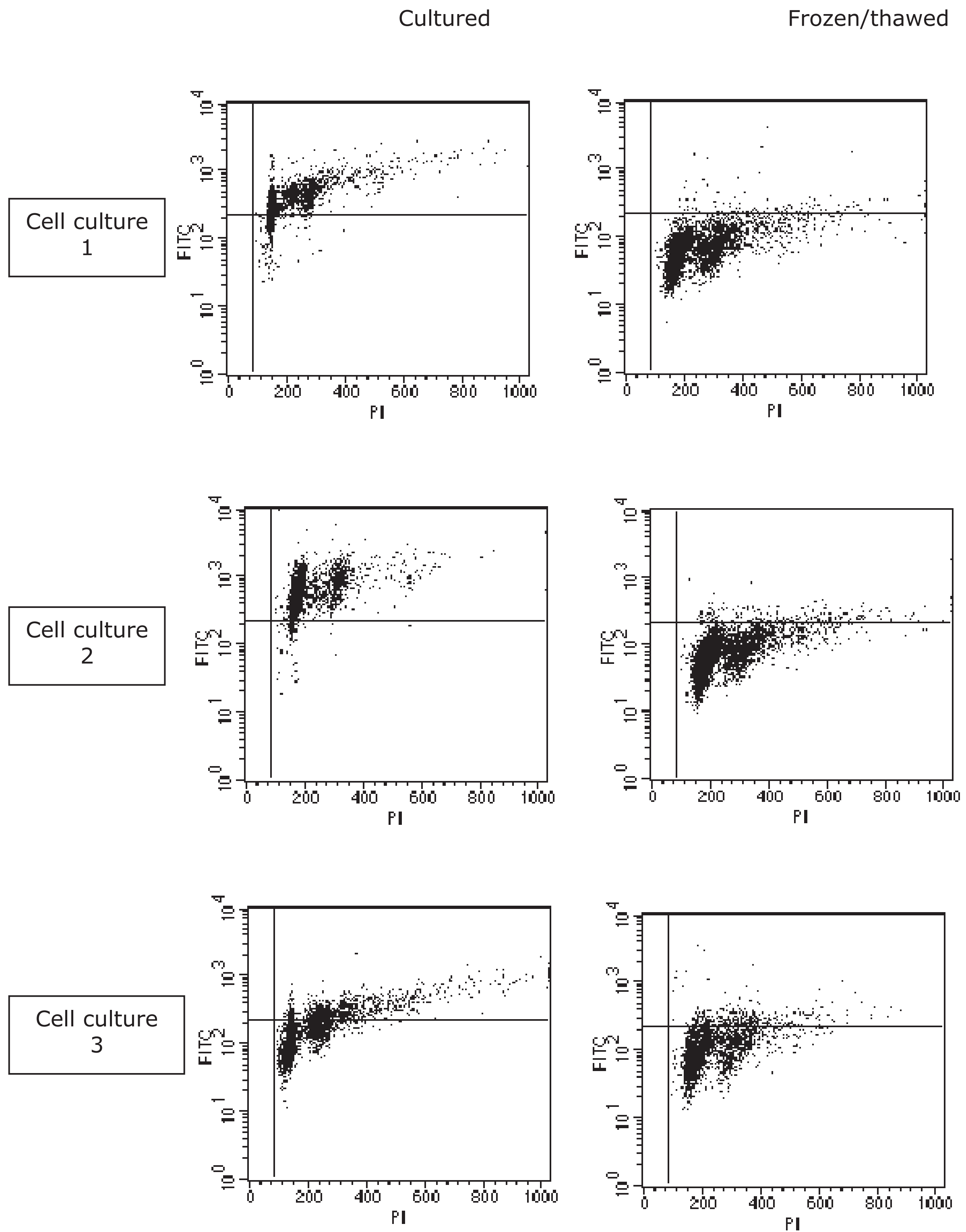
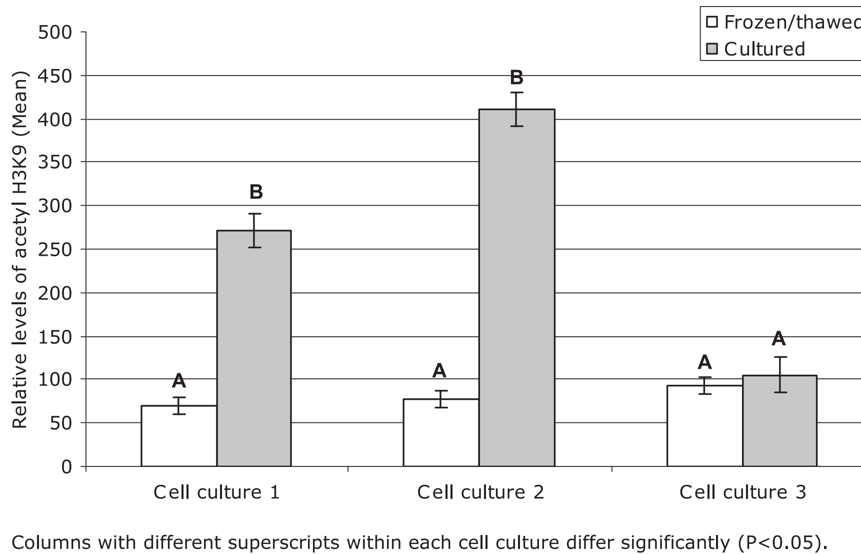


FIG. 3. Relative levels of acetyl H3K9 of frozen/thawed and cultured cells for 5 days were quantified by flow cytometry. Relative fluorescence values of $>10.^{25}$ in the FITC channel indicate cells having high levels of acetyl H3K9.

bryos that implanted and produced live offspring were lower when embryos were reconstructed with frozen cells that had been thawed immediately before SCNT. Analysis of donor cells after thawing revealed that cryopreservation increased the number of necrotic and apoptotic cells and altered epigenetic events.

Apoptosis, a regulated form of cell death, is also referred to as programmed cell death and is responsible for eliminating damaged cells within living tissues (Kerr et al., 1972). During apoptosis, the cell is terminated by a class of proteases called caspases. A member of this family, caspase-3 (CPP32, apopain, YAMA) has been identified as being a key



Columns with different superscripts within each cell culture differ significantly (P<0.05).

FIG. 4. Relative levels of acetyl H3K9 of cultured (5 days) and frozen/thawed sand cat cells.

mediator of apoptosis in mammalian cells (Kothakota et al., 1997). One of the earliest events of apoptosis is the release of cytochrome *c* from mitochondria, which is thought to be modulated by anti-apoptotic blc-2 protein, and triggers caspases activity and other downstream apoptotic effectors (Kluck et al., 1997; Yang et al., 1997). Consequently, during late apoptosis, the cell shrinks in size, DNA cleavage occurs and apoptotic bodies are formed (Darzynkiewicz et al., 1997). In our study, we cultured sand cat cells with a Caspase-3 Assay Kit (NucView™) which actually detects caspase-3 activity within live cells in real time without the need for cell fixation or alteration of membrane integrity before analysis. We identified cells that were considered to be live and at early stages of apoptosis (live/apoptotic) by (1) evaluating cell membrane integrity by exclusion of PI (also used for evaluating necrotic cells which had loss of structural integrity of the plasma membrane; Belloc et al., 1994), and (2) detecting the presence of active caspase-3 in the cytoplasm of live cells.

Several physiological, pathological (see review by Kam and Ferch, 2000) and mechanical stimuli, including cold exposure (Soloff et al., 1987) can induce apoptosis. The process of freezing and thawing bovine fibroblasts and spermatozoa results in an increased number of apoptotic cells, as measured by expression of anexin V and caspase-3 activity (Chacón et al., 2007; Martin et al., 2004, 2007). Similarly, in the current study, we observed that early apoptosis and necrosis of sand cat cells immediately after freezing and thawing (frozen/thawed treatment) was higher than that observed in cells that underwent a period of *in vitro* culture after freezing and thawing (cultured cell treatment). How the freezing process induces apoptosis is not clear, but studies indicate that freezing activates the apoptotic cascades by altering the balance of the antiapoptotic Bcl-2 protein and the pro-apoptotic Bax protein (Clarke et al., 2004). Therefore, to reduce the number of nonviable cells potentially selected during SCNT, mechanisms for inhibiting apoptosis during cell cryopreservation should be investigated.

In a recent study (Skrzyszowska et al., 2006) apoptosis and necrosis of cultured donor pig cells prior to SCNT were evaluated by detecting phosphatidylserine residues on the surface of cytoplasmic membrane and by membrane permeability. The authors reported that a higher proportion of cells were in late apoptosis and necrosis (10–40%), compared to the percentages of cells that were at early apoptosis ($<2\%$) and morphologically normal. After evaluating development of embryos reconstructed with "morphologically normal" cells, the authors concluded that morphological evaluation is an adequate criterion for selection of donor cells before SCNT (Skrzyszowska et al., 2006). Similarly, in our study, we detected a low percentage of early apoptosis in cultured cells; nevertheless, early apoptosis was higher in frozen/thawed cells. Also, analysis by light microscopy of live/apoptotic cells revealed that cells at early apoptosis had a similar morphological appearance to that of live nonapoptotic cells (live/intact). Consequently, the possibility that some of our cybrids were reconstructed with live/apoptotic cells cannot be discounted. During SCNT, live/intact cells were morphologically undistinguishable from live/apoptotic cells, and apoptotic cells can fuse successfully to donor cytoplasts during embryo reconstruction. In fact, it has been previously demonstrated that apoptotic cells (at least in the initial phase of cell death) maintain structural integrity and the capacity for plasma membrane transport (Belloc et al., 1994). For that reason, contrary to the results reported in the pig, we conclude that the morphological appearance of sand cat cells is not an acceptable criterion for selecting nonapoptotic donor cells for SCNT, and cell fusion during embryo reconstruction is not affected.

We found that a lower percentage of cybrids reconstructed with frozen/thawed cells cleaved by day 2 compared to cybrids reconstructed with cultured cells. A possible reason for the lower cleavage rate of frozen/thawed derived cybrids may be that live/apoptotic cells were used for reconstruction. Additionally, we observed that the percentages of cybrids that did not cleave from the frozen/thawed (15%) and cultured (5%) cell treatments were proportional to the percentages of apoptotic cells observed in each cell treatment (8.1 and 2.6%, respectively). We, therefore, suggest that, al-

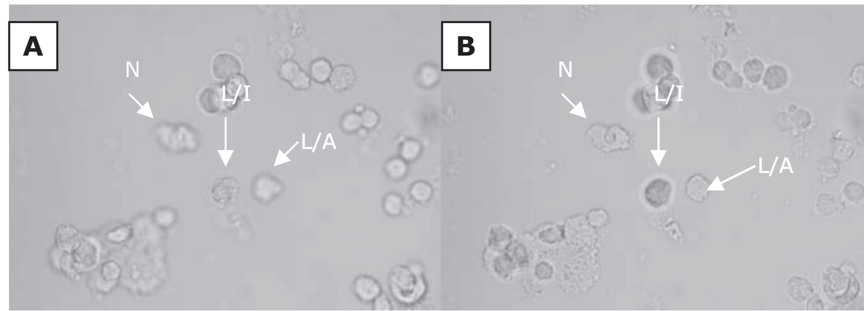


FIG. 5. Fluorescence and phase contrast microphotographs of live, apoptotic, and necrotic sand cat fibroblast cells. (A/B = phase contrast, same view, different focal points). Unstained cells were classified as live/intact (L/I), cells that displayed green fluorescence were classified as live/apoptotic (L/A) and cells that showed red fluorescence were considered as necrotic (N).

though the experiment was not specifically designed for selecting donor cells at an early stage of apoptosis before SCNT, the lower cleavage rate of cybrids reconstructed with frozen/thawed cells may be a consequence of reconstructing cybrids with cells that were undergoing apoptosis.

Alternatively, the lower cleavage rate of cybrids derived from frozen/thawed donor cells may have been due to inappropriate oocyte activation. Oocyte activation is a crucial step for initiation and regulation of events during the first cell cycle (Fissore et al., 1999). However, the type of recipient cytoplasts, the nuclear transfer method, activation protocol, and *in vitro* embryo culture used in our experiments were essentially identical for both cell treatments. Then, the difference in cleavage rate may be considered to be due partially to the treatment of the donor cell before SCNT, and not due to inappropriate oocyte activation.

Apoptotic cells that have active caspase-3 in the cytoplasm are considered to be irreversibly advancing toward cellular death. Caspase-3 is a major effector protease that eventually degrades crucial cellular proteins, and its presence indicates that cell death is proceeding irreversibly (Thornberry and Lazebnik, 1998). Although, we did not measure DNA replication of the transferred nucleus in cybrids that did not cleave, it is possible that replication did not occur. Thus, we conclude that cryopreservation increased the percentage of apoptosis and, consequently, reduced the cleavage of reconstructed cybrids. Accordingly, we recommend that donor cells be allowed to undergo a period of *in vitro* culture after freezing/thawing to induce a reentry into logarithmic growth and, thereby, reduce the percentage of apoptotic cells.

Thawing donor cells immediately before SCNT can have deleterious effects on *in vitro* development of cloned embryos (Zhang et al., 2006), and repeated freezing of donor cells can reduce fetal survival of bovine cloned embryos (Marfil et al., 2005). In our study, blastocyst development was not affected

FIG. 6. Sand cat-cloned kittens derived from cells cultured for 5 days from cell culture 1 at 5 days (**A**) and 2 months of age (**B**).

Table 1. Embryo Cleavage (Day 2) and Development to Blastocyst Stage (Day 8) of Sand Cat-Cloned Embryos Reconstructed with Frozen/Thawed or Cultured Cells

Cell culture	*Cell Treatment*	*Fused couplets* n^a	*Cleavage n/total fused (%)*	*Blastocysts n/total cleaved (%)*
1	Frozen/thawed	129	107 (83)[b]	22 (20.5)[b]
	Cultured	91	86 (94.5)[c]	5 (6)[c]
2	Frozen/thawed	93	78 (84)[b]	10 (13)[bc]
	Cultured	53	51 (96)[c]	22 (43)[d]
3	Frozen/thawed	39	38 (97)[c]	6 (16)[b]
	Cultured	80	75 (94)[c]	9 (12)[bc]

[a]The total number of couplets in each treatment was derived from two to four replicas.
[b,c,d]Different superscripts within the same column indicate significant differences ($p < 0.05$).

by the cell treatment before SCNT; however, fewer embryos derived from the frozen/thawed cell treatment implanted and developed to term. The developmental failure was possibly caused by epigenetic differences between cultured and frozen/thawed cells. In fact, the relative levels of histone acetylation in frozen/thawed cells were lower than the levels observed in cultured cells. There is evidence indicating that successful nuclear reprogramming is largely dependent on the partial erasure of preexisting epigenetic marks of donor cells (Enright et al., 2003b). Actually, development of bovine and monkey cloned embryos to the blastocyst stage (Santos et al., 2003; Yang et al., 2007b) and pregnancy rates after the transfer of rabbit cloned embryos (Yang et al., 2007a) were associated with specific epigenetic marks observed in donor cells. Monkey donor cells that showed a higher level of acetylation on lysine 9 of histone 3 (H3K9) supported a higher percentage of blastocyst development after SCNT than cells with a relatively low level of acetylation (Yang et al., 2007b). In contrast, rabbit cumulus cells that showed higher acetylation levels of H3K9/K14 supported similar development to blastocyst stage as did rabbit fetal fibroblasts that showed lower histone acetylation levels. Nevertheless, establishment of pregnancies was higher with rabbit cumulus cells that had higher histone acetylation levels. In our experiments, development to the blastocyst stage was not affected by differences in histone acetylation levels among different cell cultures. However, higher embryo implantation rates, lower fetal losses, and a higher number of live offspring were obtained when cultured donor cells with higher relative levels of acetylation on H3K9 were used as donor cells. Then, we can conclude that cultured donor cells containing higher levels of acetyl H3K9 improves *in vivo* viability of sand cat-cloned embryos.

Certain epigenetic marks on donor cells are modified by *in vitro* culture conditions (Enright et al., 2003a) and cryopreservation (Chacón et al., 2008). Bovine fibroblast cells that were analyzed immediately after freezing and thawing had lower histone acetylation levels (H3K9) than the levels observed in fresh cultured cells. Similarly, in our study sand cat frozen/thawed donor cells had significantly lower acetylation levels (H3K9) than cultured cells. Therefore, we suggest that differences in epigenetic events between frozen/thawed and cultured cells are affecting implantation and survival rates of cloned embryos following transfer.

It is not clear why cloned blastocysts reconstructed with frozen cells thawed before SCNT had reduced *in vivo* viability, but we consider that the lower histone acetylation levels observed in frozen/thawed cells may be one of the factors influencing survival rate. Although DNA methylation plays an important role in the regulation of gene expression in mammals and is essential for normal embryonic development, numerous reports have shown the important role of histone acetylation at lysine residues in the regulation of gene expression (Tse et al., 1998). In fact, histones H3 and H4 are hyperacetylated in active genes of mitotic cells (Clarke et al., 1993; Grunstein, 1997; O'Neill and Turner, 1995) and specifically, histone H3 is acetylated at lysine 9 and 14 when the gene is activated (Agalioti et al., 2002). Recently, it was demonstrated that chromatin of mouse spermatozoa and oocytes contains very few acetylated histones,

Table 2. Numbers of Pregnancies, Embryo Implantations, Fetuses Reabsorbed, and Term Sand Cat-Cloned Kittens Derived from Embryos Reconstructed with Frozen/Thawed or Cultured (5 Days) Donor Cells

	DSH recipients		*Embryos transferred*				*Live offspring*	
Cell treatment	*Total,* n	*Pregnant, n, (%)*	*Total,* n	*Average per recipient ± SD*	*Implanted* n, *(%)*	*Fetuses reabsorbed day 30* n, *(%)*	*From total embryos implanted* n, *(%)*	*From total embryos transferred* n, *(%)*
Cultured	20	6 (30)[a]	616	32.1 ± 9.8^a	16 (2.6)[a]	5 (31)[a]	11 (69)a	11 (1.8)[a]
Frozen/thawed	25	8 (32)[a]	984	39.6 ± 10.3^b	12 (1.2)[b]	9 (75)[b]	3 (25)[b]	3 (0.3)[b]

[a,b,c]Different superscripts within the same column indicate significant differences ($p < 0.05$).

Table 3. In Vivo Development of Sand Cat-Cloned Embryos Reconstructed with Cultured (5 Days) or Frozen/Thawed Cells and Transfer into the Oviduct of Domestic Cat Recipients

Cell treatment	*Cell culture*	*Pregnant recipients* n	*Total fetuses days 22–23*	*Fetuses reabsorbed days 30–35*	*Sand cat kittens > day 60*				
					Clone, n	*Stillborn* n	*Weight* *g*	*Kitten dead after birth, day*	*Pathology*
Cultured					1	1	46	0	Did not breath after birth
	1	1	4	1	2		49	5	Respiratory trauma, milk aspiration into lungs (Fig. 6A)
					3		61	5	Respiratory trauma, milk aspiration into lungs
	1	2	1	—	4	1	60	0	Died in uterus at day 60 gestation
					5		54	1	Never normalize respiration
	3	3	4	1	6		59	5	Cardiomyopathy
					7		82	29	Chronic pneumonia, milk aspiration into lungs
					8		54.4	0	Did not breath after birth
	2	4	3	—	9		54.5	0	Did not breath after birth
					10		68	1	Respiratory distress
	1	5	1	—	11		82.5	60	Supurative myocarditis secondary to chronic aspiration pneumonia (Fig. 6B)
Frozen/thawed	1	1	3	2	12	1	52	0	Died in uterus day 61 gestation, incomplete closure of body wall with organ exteriorization.
	1	2	1	—	13		22	0	Incomplete closure of body wall with organ exteriorization, respiratory distress
	1	3	1	—	14	1	44.7	0	Undeveloped, severe brain, and liver aplasia

TABLE 4. ANALYSIS OF FELINE GENETIC MARKERS (FCA) OF CLONED SAND CATS

Feline markers	*Cell Line 1*	*Cloned kittens (n = 8)*	*Cell Line 2*	*Cloned kittens (n = 3)*	*Cell Line 3*	*Cloned kittens (n = 3)*
FCA069	108/112	108/112	108/112	108/112	108/112	108/112
FCA075	124/138	124/138	138/138	138/138	124/138	124/138
FCA097	158/160	158/160	158/162	158/162	158/160	158/160
FCA105	186/192	186/192	186/196	186/196	186/192	186/192
FCA123	139/145	139/145	139/145	139/145	139/145	139/145
FCA145	176/176	176/176	176/176	176/176	176/176	176/176
FCA201	157/159	157/159	157/159	157/159	157/159	157/159
FCA210	166/168	166/168	166/168	166/168	166/168	166/168
FCA220	202/212	202/212	202/212	202/212	202/212	202/212
FCA224	166/166	166/166	—	—	166/166	166/166
FCA229	160/162	160/162	160/162	160/162	160/162	160/162
FCA240	160/160	160/160	160/160	160/160	160/160	160/160
FCA293	189/189	189/189	185/189	185/189	189/189	189/189
FCA305	205/205	205/205	205/205	205/205	205/205	205/205
FCA310	118/118	118/118	—	—	118/118	118/118
FCA441	137/141	137/141	137/141	137/141	137/141	137/141
FCA478	220/220	220/220	220/220	220/220	220/220	220/220
FCA649	134/134	134/134	130/134	130/134	134/134	134/134
FCA651	149/149	149/149	—	—	149/149	149/149
FCA674	150/150	150/150	152/152	152/152	150/150	150/150
FCA678	226/226	226/226	—	—	226/226	226/226

but, after fertilization, chromatin of both gametes became highly acetylated. Similarly, levels of acetylation of lysine residues of histone H3 and H4 were drastically reduced after injecting mouse somatic cells into enucleated mouse oocytes and reacetylated after oocyte activation of SCNT reconstructed embryos (Kim et al., 2003; Rybouchkin et al., 2006). Based on these observations, Kim et al. (2003) suggested that oocyte cytoplasm initializes programmed gene expression by deacetylating histones. However, in more recent studies, embryo development to the blastocyst stage (Kishigami et al., 2006; Li et al., 2008; Rybouchkin et al., 2006; Zhang et al., 2007) and full-term development after transferring cloned embryos to foster mothers (Rybouchkin et al., 2006) were increased after exposing reconstructed embryos to a specific histone deacetylase inhibitor, trichostatin A (TSA), before and after activation. In contrast to previous studies, these studies clearly demonstrated that inhibition of deacetylation of somatic histones after SCNT that mimicked the early stage of fertilization was not important for nuclear reprogramming and, actually, hyperacetylation improved nuclear reprogramming.

How hyperacetylation may improve nuclear reprogramming has not been elucidated. However, increased histone acetylation levels in amino acid residues are known to result in looser binding of the nucleosome to DNA and, consequent relaxation of chromatin structure (Zlatanova et al., 2000). Relaxation of the chromatin structure has been confirmed and visualized *in vivo* after exposing HeLa cells to TSA (Tóth et al., 2004). In that study, HeLa cells treated with TSA increased acetylation levels on histone H2A, which then caused dense chromatin to increase (relax) from an initial length of 0.2 μm up to 1 μm. Such data strongly indicate that exposure of a somatic cell nucleus with relaxed chromatin structure to nonactivated oocyte cytoplasmic factors after SCNT may be effective for improving the efficiency of nuclear reprogramming (Tani et al., 2001). Thus, we hypothesized that cultured donor cells containing higher levels of histone acetylation and relaxed chromatin were able to interact with transcription factors from the oocyte, thereby improving the nuclear reprogramming of donor cells. Nonetheless, an explanation for the reduction in histone acetylation after cryopreservation is still unclear. Analysis of the chromatin structure of frozen/thawed and cultured donor cells may clarify some of these questions.

Several reports indicate that fetal losses are associated with incomplete reactivation down regulation and nonexpression of the Oct–4 gene (Arat et al., 2003; Bortvin et al., 2003; Li et al., 2006). Boiani et al. (2002) demonstrated that mouse blastocysts derived from clones had abnormal *POU5F1* expression, and that the failure of mouse cloned embryos to develop beyond the blastocyst stage was related to incorrect lineage determination caused by inappropriate expression of *POU5F1*. Although we analyzed the expression of POU5F1 in a low number of cloned embryos, our results suggest that 25% of cloned blastocysts reconstructed with frozen/thawed cells expressed *POU5F1*, in comparison to 88% of cloned blastocysts reconstructed with cultured cells. An increase in the numbers of analyzed embryos will give a better indication of differences in POU5F1 expression between cloned embryos reconstructed with cultured or frozen/thawed cells. Additionally, a higher percentage of implanted cloned embryos reconstructed with frozen/thawed cells were reabsorbed by day 30 of gestation (75%) compared to that observed in embryos reconstructed with cultured cells (31%; $p < 0.05$). Thus, we concluded that early fetal losses of sand cat-cloned embryos are associated with abnormal or non-*POU5F1* expression and, possibly, to other unknown factors.

Apparently, the main function of *POU5F1* in ICM is to prevent differentiation into trophectoderm cells and to activate

Fgf4 production to maintain and induce proliferation of a trophoblast stem-cell population in the adjacent polar trophectoderm (Camus et al., 2004). Mouse embryos with low *POU5F1* expression were unable to form a mature ICM and died during the peri-implantation period (Nichols et al., 1998), and the absence of *POU5F1* induces ICM cells to form nonproliferating trophoblast cells (Nichols et al., 1998). Interestingly, ultrasound analyses of the implanted sand cat-cloned embryos that started reabsorption by day 30 of gestation showed a small, amorphous mass of fetal tissue with no heartbeat inside the gestational vesicle. By day 35 of gestation, the fetal mass had largely disappeared and the volume of the vesicle was greatly reduced. In contrast, embryos that resulted in live offspring contained an organized fetal mass with visible heartbeats at each weekly ultrasound examination. We speculate that the ICM of cloned embryos that did not express or had a low level of *POU5F1* expression could differentiate into trophodectoderm cells, but were not able to develop into a viable fetus.

In the present study, we also found that some of the physical abnormalities and perinatal deaths from respiratory failure were similar to that observed in AWC-cloned kittens (Gómez et al., 2004). Interestingly, incomplete closure of the ventral body wall musculature with abdominal organ exteriorization was found only in cloned kittens derived from frozen/thawed cells that had lower levels of histone acetylation, while perinatal death of kittens derived from cultured cells was mainly associated with respiratory failure. It has been suggested that phenotypic abnormalities in cloned animals might be associated with incomplete or inappropriate epigenetic reprogramming during early embryonic development (Wolffe and Matzke, 1999), and physiological problems, such as respiratory competence, were a function of the genetic makeup of the donor cell (Eggan et al., 2001). The authors in the latter study also concluded that the survival of mouse clones was influenced by the genetic heterozygosis of the cell donors. Their results are supportive of such a suggestion, because a lower number of pups respired after Caesarean section and died earlier by respiratory distress when mouse-cloned embryos were reconstructed with donor cells derived from inbred strains compared to the number of pups that developed normally when they were derived from cells of hybrid (F1) mice. Although, we do not know the exact reason(s) of the perinatal death of sand cat-cloned kittens, and because of the low number of kittens produced, we cannot disregard the possibility that the epigenetic status of frozen/thawed cells influenced the phenotypic abnormalities observed in kittens derived from them. In fact, similar abnormalities were observed in AWC-cloned kittens derived from frozen/thawed cells. Nonetheless, four kittens derived from the cultured cell treatment died between 5 and 60 days of age due to moderate to chronic pneumonia as a consequence of milk aspiration during bottle or tube feeding. Thus, the pneumonia-induced respiratory failure was different mechanistically from the respiratory distress observed in the other kittens. Another potentially important factor that requires evaluation is the influence of homozygozity of donor cells on the health of cloned kittens, because most captive wild cats are derived from a few individuals.

In summary, we demonstrated that cell cryopreservation not only increased the percentage of necrotic and apoptotic cells but also altered epigenetic events in donor cells, which in turn, indirectly affected the number of embryos that cleaved, implanted, and developed to term-gestation and the *POU5F1* expression in derived blastocysts. The epigenetic changes induced during cell cryopreservation may be the direct cause of the reduced viability of sand cat-cloned embryos; however, *in vitro* culture of somatic cells before SCNT modified epigenetic events in donor cells and improved the efficiency of SCNT. Although we were able to produce several cloned kittens, viability of offspring was not improved, and most of the deaths were associated with pulmonary difficulties and respiratory failure. Thus, there are still severe problems probably associated with epigenetic strengths placed on donor cells and embryos, and/or abnormal nuclear reprogramming/gene expression of cloned embryos. These experiments clearly demonstrated the importance of epigenetic status of donor cells and its influence on producing viable offspring. Thus, it is important to further examine the epigenetic changes of donor cells and oocytes and their impact on the viability and health of cloned animals.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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