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Nuclear Transfer of Synchronized African Wild Cat Somatic Cells into Enucleated Domestic Cat Oocytes[1]

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ABSTRACT

The African wild cat is one of the smallest wild cats and its future is threatened by hybridization with domestic cats. Nuclear transfer, a valuable tool for retaining genetic variability, offers the possibility of species continuation rather than extinction. The aim of this study was to investigate the ability of somatic cell nuclei of the African wild cat (AWC) to dedifferentiate within domestic cat (DSH) cytoplasts and to support early development after nuclear transplantation. In experiment 1, distributions of AWC and DSH fibroblasts in each cell-cycle phase were assessed by flow cytometry using cells cultured to confluency and disaggregated with pronase, trypsin, or mechanical separation. Trypsin (89.0%) and pronase (93.0%) yielded higher proportions of AWC nuclei in the G_0/G_1 phase than mechanical separation (82.0%). In contrast, mechanical separation yielded higher percentages of DSH nuclei in the G_0/G_1 phase (86.6%) than pronase (79.7%) or trypsin (74.2%) treatments. In both species, pronase induced less DNA damage than trypsin. In experiment 2, the effects of serum starvation, culture to confluency, and exposure to roscovitine on the distribution of AWC and DSH fibroblasts in various phases of the cell cycle were determined. Flow cytometry analyses revealed that the dynamics of the cell cycle varied as culture conditions were modified. Specifically, a higher percentage of AWC and DSH nuclei were in the G_0/G_1 phase after cells were serum starved (83% vs. 96%) than were present in cycling cells (50% vs. 64%), after contact inhibition (61% vs. 88%), or after roscovitine (56% vs. 84%) treatment, respectively. In experiment 3, we evaluated the effects of cell synchronization and oocyte maturation (in vivo vs. in vitro) on the reconstruction and development of AWC-DSH- and DSH-DSH-cloned embryos. The method of cell synchronization did not affect the fusion and cleavage rate because only a slightly higher percentage of fused couplets cleaved when donor nuclei were synchronized by serum starvation (83.0%) than after roscovitine (80.0%) or contact-inhibition (80.0%). The fusion efficiency of in vivo and in vitro matured oocytes used as recipient cytoplasts of AWC donor nuclei (86.6% vs. 85.2%) was similar to the rates obtained with DSH donor nuclei, 83.7% vs. 73.0%, respectively. The only significant effect of source of donor nucleus (AWC vs. DSH) was on the rate of blastocyst formation in vitro. A higher percentage of the embryos derived from AWC nuclei developed to the blastocyst stage than did embryos produced from DSH nuclei, 24.2% vs. 3.3%, respectively ($P < 0.05$). In experiment 4, the effect of calcium in the fusion medium on induction of oocyte activation and development of AWC-DSH-cloned embryos was determined. The presence of calcium in the fusion medium induced a high incidence of cleavage of DSH oocytes (54.3%), while oocyte cleavage frequency was much lower in the absence of calcium (16.6%). The presence or absence of calcium in the fusion medium did not affect the fusion, cleavage, and blastocyst development of AWC-DSH-cloned embryos. In experiment 5, AWC-DSH-cloned embryos were transferred to the uteri of 11 synchronized domestic cat recipients on Day 6 or 7 after oocyte aspiration. Recipients were assessed by ultrasonography on Day 21 postovulation, but no pregnancies were observed. In the present study, after NT, AWC donor nuclei were able to dedifferentiate in DSH cytoplasts and support high rates of blastocyst development in vitro. Incomplete reprogramming of the differentiated nucleus may be a major constraint to the in vivo developmental potential of the embryos.

assisted reproductive technology, early development, gamete biology

INTRODUCTION

Most of the 36 species of wild felids are classified as threatened, vulnerable or endangered [1]. As an example, the African wild cat (*Felis silvestris libica*) is one of the smallest wild cats, whose future is threatened by hybridization with domestic cats (*Felis silvestris catus*). Much progress has been made toward applying assisted reproductive technologies for aiding in the conservation of endangered felids. In particular, nuclear transfer (NT) is a valuable tool for retaining genetic variability [2, 3] and offers the possibility of species continuation rather than extinction. Interspecies NT into bovine, rabbit, and sheep cytoplasts has been shown to support development of somatic cell nuclei from various mammalian species [4–8]. Accordingly, ooplasm of the domestic cat is a potential host for somatic cell nuclei from endangered felid species.

During normal fertilization and embryo development, mitochondrial DNA (mtDNA) is maternally inherited [9]. Animals produced by NT are genetic duplicates of the donor cell nuclear genome. Because foreign mitochondria are introduced into the oocyte, some degree of heteroplasmy can occur after NT [10], which, according to speculation, may be detrimental. There are some species-specific epigenetic factors present in the oocyte cytoplasm that may lead to nucleo:cytoplasmic incompatibilities either immediately after NT or at later stages of development [11].

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These potential incompatibilities will affect, to some degree, the ultimate utility of NT technology. It has been demonstrated that mtDNA present in the oocyte cytoplasm plays an important role in nuclear cytoplasmic incompatibilities [12] and affects embryo implantation [13]. Even so, offspring have been produced from embryos reconstructed by interspecies [5, 7, 8], cross-subspecies not expressing heteroplasmy [14], and cross-subspecies expressing heteroplasmy [15] nuclear transfer. These results demonstrated the possibility that, through nuclear transfer techniques, the ooplasm of domestic species could be used to preserve genetic diversity of closely related endangered species. Recently, the feasibility of producing viable felid offspring by cloning has been demonstrated [16]. Although the ratio of NT kittens produced to total embryos transferred was similar to that reported in other species, the importance of increasing the efficiency of felid NT is obvious.

The cell-cycle phase of the donor nucleus plays a major role in the effectiveness of the NT process and has multiple influences on embryo reconstruction [17]. In the first report on production of a live offspring from somatic cells, donor nuclei were in a quiescent stage (G_0) [18], where the cells were presumably arrested after having exited the normal cell cycle. Although, somatic cell cloning has been achieved with donor cells in G_1 and G_2/M phase [19, 20], for NT, it is generally accepted that better efficiency is obtained when the donor nucleus is in the G_1 or G_0 phase. To achieve cell-cycle synchronization at this desired phase, cultured cells may be subjected to a period of serum starvation before NT. A disadvantage of this method is the increase in DNA fragmentation that occurs after prolonged culture of donor cells [21], where the extent of the damage increases with the duration of the starvation period [22]. Alternatively, donor cells can be arrested in the G_0/G_1 phase by contact inhibition [23] or by exposure to reversible cycle inhibitors such as roscovitine [24].

Fibroblasts and cumulus cells of the domestic cat have an inherently long G_0/G_1 phase [25]. Serum starvation has been found to increase the percentage of both of these cell types at the desired stage; however, a notable portion undergoes apoptosis [25]. Therefore, additional stimuli would increase the percentage of properly synchronized cells. Furthermore, the dislodging process should be optimized to reduce effects on cell characteristics such as size and DNA integrity because viable single cells with intact DNA are essential for successful nuclear transfer.

In the present study, we determined the ability of somatic cell nuclei of the African wild cat to dedifferentiate in domestic cat cytoplasts and support early in vitro development after reconstruction. Accordingly, our objectives were (1) to compare the effect of different methods, enzymatic and mechanical, for obtaining single viable cells after dissociation; (2) to determine the effect of different cell-cycle inhibition treatments on the distribution of cells in the various phases of the cell cycle; (3) to evaluate the effect of different methods of cell-cycle synchronization and type of oocyte maturation (in vivo vs. in vitro) on reconstruction and in vitro development of domestic and African wild cat embryos derived by NT; (4) to determine if the presence of calcium in the fusion medium (a) induces concurrent oocyte activation and (b) affects in vitro development of African wild cat embryos derived by NT; and (5) to evaluate developmental competence after transfer into domestic cat recipients of NT embryos produced by fusion of African wild cat fibroblast cell nuclei with domestic cat cytoplasts.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise stated. Media were prepared weekly and filtered (0.22 μ, #4192 Acrodisc; Pall Corp., Ann Arbor, MI) into sterile 15-ml tubes. After filtration, bicarbonate-buffered culture media were gassed with 5% CO_2 in air before storage at 4°C and used within 7 days. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. government.

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Audubon Nature Institute for Research of Endangered Species as required by the Health Research Extension Act of 1985 (Public law 99-158). Domestic cat females were group housed in an environmentally controlled room with 14L:10D at 20–26°C. The African wild cat male was housed in a separate room under the same conditions. The rooms were cleaned, and cats were fed (Science Diet, Hill Pet Nutrition, Topeka, KS) daily. Fresh water was available at all times.

Establishment and Culture of Donor Fibroblasts

Cell lines were generated from skin tissue collected by biopsy from a male African wild cat (AWC) and a female domestic shorthaired cat (DSH). The tissue was washed twice in Ca^{2+} and Mg^{2+}-free Dulbecco PBS (PBS; Invitrogen, Grand Island, NY) and finely cut into 1-mm^2 pieces. Tissue pieces were plated in 75-cm^2 tissue-culture flasks (Nunc, Denmark) containing 7 ml of Dulbecco modified Eagles medium (DMEM) supplemented with 50 μg/ml gentamicin and 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, Utah), and cultured at 38°C in 5% CO_2/air. After 7–10 days of incubation, monolayer outgrowths with fibroblastic-like morphology were disaggregated with either 2.5 mg/ml of pronase or 2.5 mg/ml of trypsin, then regrown and passaged 2–3 times before being resuspended in DMEM with 10% FBS and 10% (w/v) dimethyl sulfoxide (DMSO) and cooled at 1.0°C/min to −80°C (Mr. Frosty; Nalgene, Rochester, NY) before storage in liquid nitrogen.

Cell Treatments and Flow Cytometric Analysis

Distributions of AWC and DSH fibroblasts in the cell-cycle phases were assessed by flow cytometric analysis between cells disaggregated with pronase, trypsin, or mechanical separation (experiment 1). Also, comparisons of the cell-cycle distribution of AWC and DSH fibroblasts were made between cycling cells, after serum starvation, after being cultured to confluence, and after exposure to roscovitine (experiment 2). Flat-sided tissue culture tubes (Nunc, Denmark) were plated with frozen/thawed AWC or DSH fibroblasts at a concentration of 1×10^5 cells.

Experiment 1. AWC and DSH cells were cultured until reaching 100% confluence (2–3 days) and were disaggregated with 2.5 mg/ml trypsin, 2.5 mg/ml pronase, or by mechanical separation before fixation. Cell viabilities in each treatment were determined by staining with 0.4% trypan blue [26].

Experiment 2. AWC and DSH cells were cultured for 1 day and allocated to one of the following treatments in which culture continued until (1) confluency to 60%–70% (cycling cells); (2) confluency to 100%, followed by an additional 5 days in culture, during which time the culture medium was replaced every other day (contact inhibition); (3) confluency to 60%–80%, replacement of tissue culture medium with DMEM + 0.5% FBS and 5 days additional culture (serum starved); and (4) confluency to 100%, culture medium replaced with DMEM containing 15 μM roscovitine and culture for an additional 24 h (roscovitine). Cells from each treatment were disaggregated with 2.5 mg/ml pronase prior to fixation.

Cells from each treatment were pelleted by centrifugation, then washed once with PBS, and stained with equal volumes of 0.112% sodium citrate containing 50 μg/ml propidium iodide (PI), 100 μg/ml RNAse A, and 0.1% (v/v) Triton X-100 for 20 min at 38°C. Stained cells were filtered through a 30-μm nylon mesh (Small Parts, Miami Lakes, FL) and the distribution of nuclei in the various phases of the cell cycle was analyzed with a flow cytometer (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, CA [BDIS]). Nuclei were analyzed at a rate of 300 per sec, and 5–10 K events collected in listmode files with FL2H linear set at 340, at 1024 channels, using linear size and scatter parameters and doublet discrimination mode. Histograms, dot plots, and density plots were generated by using CellQuest software (BDIS). Percentages of cells oc-

curring in the G_0/G_1, S, and G_2/M phase of the cell cycle were calculated through the ModFit LT (Verity Software House, Topsham, ME) and estimated with CellQuest software (BDIS).

Oocyte Maturation

Cat ovaries obtained after ovariohysterectomy from local veterinary clinics were transported to the laboratory at ambient temperature in Hepes buffered saline containing 50 μg/ml gentamicin. Within 2–6 h, the ovaries were minced and cumulus-oocyte complexes (COCs) collected. Oocytes with uniformly dark, finely granulated ooplasm surrounded by several layers of compact cumulus cells [27] were cultured in modified TCM-199 containing 1 IU/ml human chorionic gonadotropin (hCG), 0.5 IU/ml equine chorionic gonadotropin (eCG), 10 μg/ml epidermal growth factor (EGF), and 3 mg/ml BSA for 24 h in 5% CO_2, 5% O_2, and 90% N_2 at 38°C [28].

The protocol for ovarian stimulation and laparoscopic oocyte recovery to obtain in vivo-matured oocytes from donor females has been described previously [29]. Briefly, domestic cats 1–7 yr of age were treated with a total of 3–5 IU of porcine-follicle stimulating hormone (FSH; Sioux Biochemical, Sioux Center, IA) administered daily (s.c.) for 4 days, followed by 3 IU of porcine-luteinizing hormone (LH, Sioux Biochemical) on the fifth day. Twenty-four to 26 h after LH injection (i.m.), oocytes were collected by laparoscopic aspiration of mature ovarian follicles into TL Hepes medium supplemented with 10 IU/ml heparin (sodium salt, from porcine intestinal mucosa; Steris Laboratories, Phoenix, AZ) and 50 μg/ml gentamicin.

Cumulus cells of in vivo- and in vitro-matured oocytes were removed by vortexing in 1 mg/ml of hyaluronidase for 5 min, followed by gentle mechanical pipetting. Denuded oocytes were placed in Tyrode solution containing 1% MEM nonessential amino acids (NEAA), 3 mg/ml BSA (Fraction V, very low endotoxin; Serologicals, Norcross, GA) and supplemented with 15 mM $NaHCO_3$, 0.36 mM pyruvate, 2.2 mM calcium lactate, 1 mM glutamine, and 50 μg/ml gentamicin (IVC-1 medium) at 38°C in 5% CO_2 until further use.

Nuclear Transfer and Embryo Culture

African wild cat and domestic cat fibroblasts were synchronized by one of the following treatments: (1) contact inhibition, (2) serum starvation, or (3) roscovitine. A single cell, presumably synchronized in G_0/G_1, was introduced into the perivitelline space of each enucleated in vivo- or in vitro-matured oocyte to evaluate the effect of donor nucleus, cell synchronization, and oocyte maturation on the reconstruction and in vitro development of AWC-DSH- and DSH-DSH-cloned embryos (experiment 3).

The NT procedure was performed by using a micromanipulator (Model MMO-202D; Narishige Instrument, Tokyo, Japan) attached to an inverted microscope (Olympus IX-70, Olympus, Tokyo, Japan) equipped with Hoffman modulation contrast optics and a temperature-controlled stage set at 37°C (Olympus, Tokai Hit, Japan). Before enucleation, denuded M-II oocytes were incubated for 15 min in Ca^{2+}- and Mg^{2+}-free modified Tyrodes salt solution supplemented with 1% MEM nonessential amino acids, 3 mg/ml BSA, 30 mM $NaHCO_3$, 0.36 mM pyruvate, 1 mM glutamine, and 50 μg/ml gentamicin (ECM medium), 20 μg/ml Hoechst 33342 and 20 μg/ml cytochalasin B (CCB). After incubation, oocytes were enucleated in ECM medium, in which $NaHCO_3$ was reduced to 15 mM, and 15 mM Hepes was added, along with 20 μg/ml of CCB and 2 mg/ml of sucrose (330–340 mOsm, enucleation medium). The first polar body and approximately 10% of the underlying cytoplasm were drawn into an enucleation pipette (outer diameter, 20 μM), with subsequent confirmation of removal of the metaphase spindle by epifluorescence microscopy. The time of exposure to ultraviolet light was restricted to 1 sec.

A single AWC or DSH fibroblast cell (<20 μm in diameter), presumably synchronized in G_0/G_1 by one of the synchronization treatments, was introduced into the perivitelline space of the enucleated in vivo- or in vitro-matured oocyte. Fusion took place in fusion medium (0.3 M mannitol, 0.1 mM Mg^{2+}, and 0.05 M Ca^{2+}) by placing each NT couplet between two stainless-steel electrodes attached to micromanipulators (LF-101; Nepa Gene, Tokyo, Japan). The distance between electrodes was 120 μm and membrane fusion was induced by applying a 3-sec AC prepulse of 20V, 1 MHz; followed by two 30-μsec DC pulses of 240V/mm at intervals of 0.5 sec. Pulses were delivered perpendicularly to the shared membrane space of the donor cell/cytoplasm. Following the fusion pulses, NT couplets were washed and cultured in IVC-1 medium, and after 30 min, fusion was evaluated visually by confirming the presence or absence of the donor cell in the perivitelline space. To determine if the cell fusion method induces concurrent oocyte activation, in vitro-matured DSH oocytes were electrically pulsed in fusion medium with the presence or absence of calcium by the same procedure as described above, and cultured for 2 days to determine cleavage frequency. Also, AWC-DSH couplets reconstructed with a single AWC fibroblast cell, presumably synchronized in G_0/G_1 by serum starvation treatment and introduced into the perivitelline space of enucleated in vivo- or in vitro-matured oocytes, were electrically fused in fusion medium with or without calcium (experiment 4).

Activation of the fused couplets (experiments 3 and 4) was performed 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-μsec DC pulses of 120 V/mm. Then couplets were incubated in 30-μl drops of IVC-1 medium supplemented with 10 μg/ml cycloheximide and 5 μg/ml CCB at 38°C in 5% CO_2 under mineral oil (4008; Sage BioPharma, Bedminster, NJ) for 4 h. As controls for the activation protocol, some denuded oocytes were parthenogenetically activated by the same procedure as described for fused NT couplets.

Following activation, reconstructed couplets and parthenogenetically activated oocytes were cultured in 500 μl of IVC-1 medium until Day 4, when reconstructed and parthenogenetic embryos were moved into 500 μl of Tyrode solution containing 1% NEAA, 2% MEM essential amino acids (EAA), 10% FBS, and supplemented as for IVC-1 medium (IVC-2 medium), and cultured in 5% CO_2, 5% O_2, 90% N_2 at 38°C up to the day of embryo transfer or until Day 8.

Assessment of embryos

The number of cybrids and activated oocytes that cleaved was assessed on Day 2 and on Day 4, further development (morula) was assessed visually (light microscopy). The number of embryos developing to the blastocyst stage was determined on Days 7 and 8. Blastocyst cell numbers, both total and inner cell mass/trophectoderm cell (ICM/TPD) ratios, were determined by using a modification of a protocol previously described by Wells [30]. Blastocysts were incubated for 1 h at 38°C in 1 mg/ml of Hoechst 33342 stain solution. After incubation, blastocysts were exposed to 0.04% of Triton-X100 in PBS for 45–60 sec, and then cultured for 15 min at 38°C in 25 μg/ml of PI stain in water. Stained embryos were placed in a drop of mounting medium (glycerol; Hoechst 33342) on a clean glass microslide and cells were counted by epifluorescence microscopy [31]. The ratio of ICM cells to TPD cells per embryo was determined by counting the number of blue (ICM) and red (TPD) cells stained by Hoechst and PI, respectively.

Embryo Transfer

Experiment 5. To evaluate the in vivo competence of the cloned embryos, on Day 6 (n = 3 recipients) and Day 7 or 8 (n = 8 recipients), AWC-DSH-cloned embryos were transferred into the uteri of 11 gonadotropin-treated (FSH/LH) domestic cat recipients on Day 6 or 7 after oocyte aspiration, as previously described [32]. For transfer, one uterine horn was exteriorized through a 1.5-cm midventral incision and punctured with a sterile 16-gauge round-tipped, short bevel trocar. Embryos were aspirated in ~50 μl of IVC-2 medium into a 14.5-cm tom-cat catheter (3.5 Fr.; Sherwood Medical, St Louis, MO), which was then threaded 4–5 cm into the uterine lumen before depositing the embryos. To assess pregnancy status, abdominal ultrasonography was done on recipient cats between Days 21 and 23 after ovulation (i.e., oocyte recovery).

Statistical Analyses

Two and three-way ANOVA was used to analyze the data on cell-cycle comparisons, one-way ANOVA to analyze the data on the number of embryos that developed to premorula and morula stages, and three-way ANOVA to analyze the data for fusion, cleavage rate, and blastocyst development. Numbers of nuclei in blastocysts and in the ICM and TPD were subjected to an arcsine transformation for each replication. The transformed values were analyzed by using one-way ANOVA on ranks. The Tukey multiple-comparison test was used to determine any differences between two means after ANOVA. Values are reported as mean % ± standard error of the mean.

RESULTS

Experiment 1

Effect of disaggregation treatments on African wild cat and domestic cat fibroblasts. The distribution of AWC nuclei

TABLE 1. Percentages of African wild cat fibroblast cells in various phases of the cell cycle after culturing cells to confluence and cell separation by enzymatic or mechanical methods.

Dissagregation method	N[a]	Cell cycle phase (mean % ± SEM)			% Aggregates
		G_0/G_1	S	G_2/M	
Trypsin	10 000	89 ± 1^b	4 ± 0.2	7 ± 1^b	10^b
Pronase	10 000	93 ± 0.4^b	2 ± 0.3	5 ± 0.1^b	2^b
Mechanical	10 000	82 ± 3^c	0 ± 0	18 ± 3^c	37^c

[a] The total number of cells in each treatment was derived from three replications.
[b,c] Different superscripts within the same column indicate significant differences ($P < 0.0001$).

TABLE 2. Percentages of domestic cat fibroblast cells in various phases of the cell cycle after culturing cells to confluence and cell separation by enzymatic or mechanical methods.

Dissagregation method	N[a]	Cell cycle phase (mean % ± SEM)			% Aggregates
		G_0/G_1	S	G_2/M	
Trypsin	11 336	74 ± 3^b	14 ± 2	12 ± 3	2^b
Pronase	27 247	80 ± 4^c	8 ± 1	12 ± 2	5^b
Mechanical	41 931	87 ± 2^d	9 ± 2	4 ± 2	15^c

[a] The total number of cells in each treatment was derived from at least three replications.
[b,c,d] Different superscripts within the same column indicate significant differences ($P < 0.0001$).

in the various phases of the cell cycle indicated that trypsin and pronase (89.0%, 93.0%) yielded higher proportions of cells in the G_0/G_1 phase than did mechanical separation (82.0%; Table 1). The proportions were reversed for DSH nuclei, as the mechanical separation method yielded higher proportions of cells in the G_0/G_1 phase (87.0%) compared with that from trypsin (74.2%) and pronase (79.7%; Table 2) treatment. However, in both AWC and DSH cells, cell aggregates were more prevalent after mechanical disaggregation (37.3%, 15.0%) than after trypsin (10.5%, 1.7%) or pronase (1.8%, 4.8%, respectively, $P < 0.05$; Tables 1–2) treatment, respectively. Cell-viability testing indicated that trypsin and pronase treatments yielded higher percentages of viable cells for AWC (85%–90%) and DSH (90%–95%) cells compared with mechanical separation (AWC = 35%, DSH = 50%; $P < 0.05$). Overall, these findings suggest that pronase treatment yielded cells with higher viability and less cellular and DNA damage (data not shown) and gave a higher percentage of cells in the G_0/G_1 phase. Based on these results, pronase disaggregation was chosen as the preferred method for cell preparation prior to flow cytometric analysis or nuclear transfer in all further experiments.

Experiment 2

Effect of cell-cycle inhibitor treatments on the distribution of cells in the various phases of the cell cycle. Flow cytometry analyses revealed that most AWC and DSH fibroblast cells were in the G_0/G_1 phase, as shown by the prominent G_0/G_1 DNA peaks (Figs. 1, 2), and that the dynamics of the cell cycle varied as culture conditions were modified (Fig. 3). Higher percentages of AWC and DSH cells were in the G_0/G_1 phase after serum starvation compared with cycling cells, or after contact inhibition and roscovitine treatments ($P < 0.05$; Figure 3). In contrast, cycling cultures contained higher percentages of cells in the S and G_2/M phases than the other treatments. For both species, serum starvation resulted in lower percentages of cells in the S and G_2/M phases compared with contact inhibition and roscovitine. Cycling AWC cells had a lower proportion of cells in the G_0/G_1 phase (50%) compared with cycling DSH cells (64%; $P < 0.05$). Although AWC cells were synchronized in the G_0/G_1 phase by all synchronization treatments, proportions of cells in G_0/G_1 were lower than the proportion of DSH cells synchronized in G_0/G_1 in each treatment ($P < 0.05$).

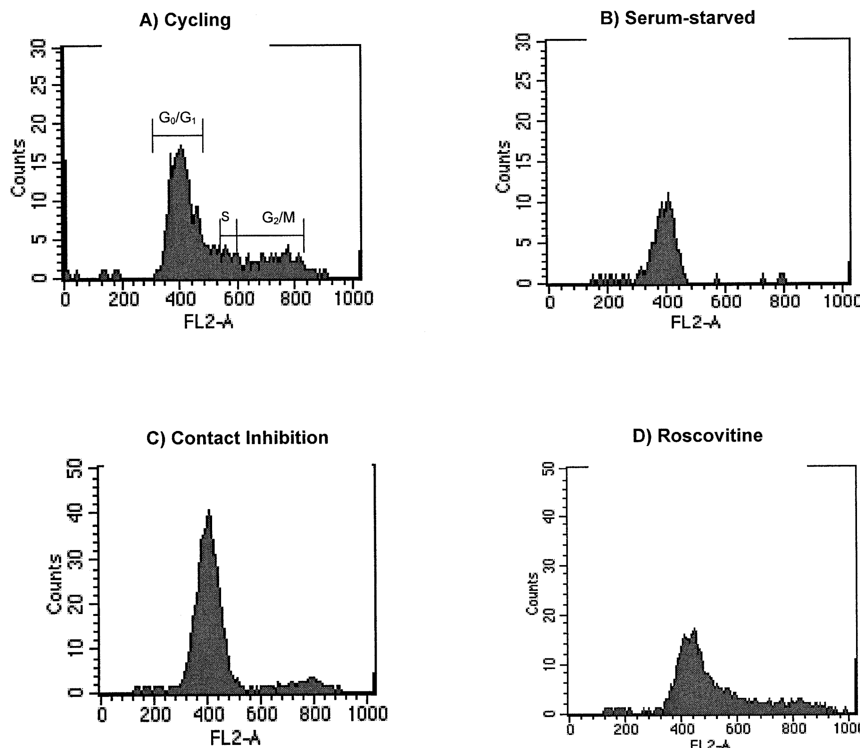


FIG. 1. Typical histograms of DNA, obtained by using flow cytometry of domestic cat fibroblast cells cultured under a variety of cell-cycle inhibitors.

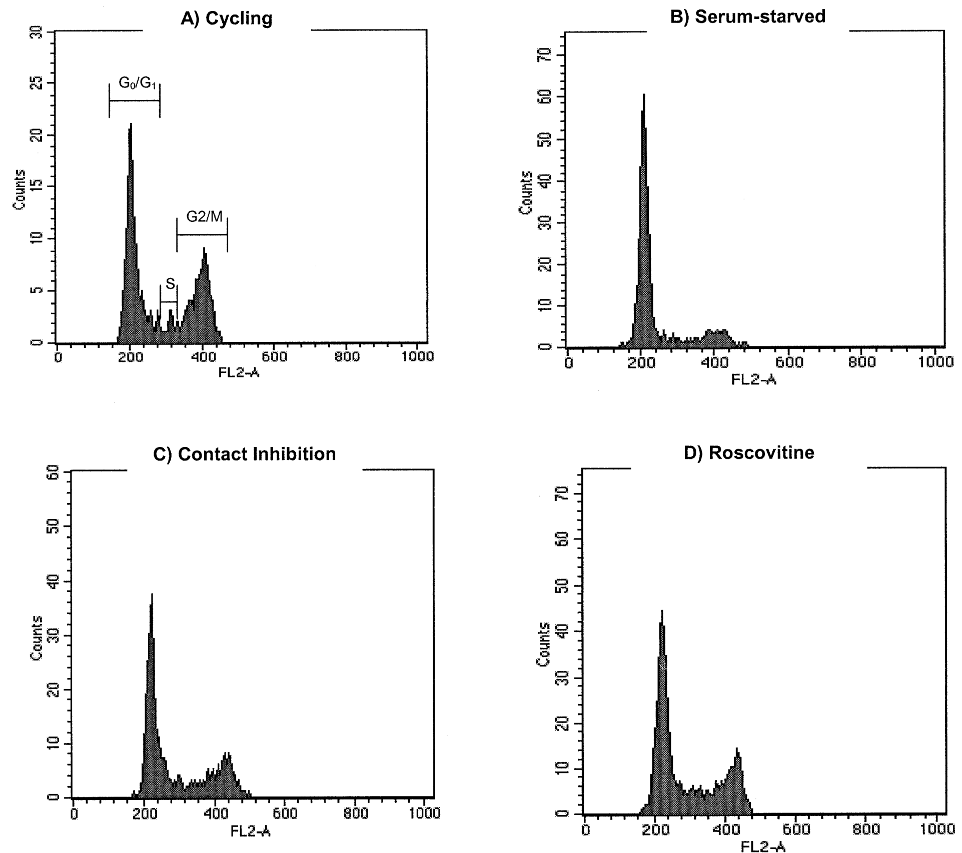


FIG. 2. Typical histograms of DNA, obtained by using flow cytometry of African wild cat fibroblast cells cultured under a variety of cell-cycle inhibitors.

Experiment 3

Effect of donor nucleus, cell synchronization, and oocyte maturation on the reconstruction and development of AWC-DSH- and DSH-DSH-cloned embryos. In this experiment, evaluations were made on the potential for in vitro development of NT embryos produced from in vitro- and in vivo-matured oocytes reconstructed with DSH or AWC cells from each cell-cycle synchronization treatment. Blastocyst development was influenced by the donor nucleus but was not affected by the cell-cycle synchronization method (Table 3). Higher percentages of blastocysts were observed (assessed as a percentage of cleaved couplets) when AWC donor cells were used compared with DSH cells (24.2% vs. 3.3%, respectively; $P < 0.05$; Table 3). The method of cell synchronization did not affect the fusion and cleavage rates. A slightly higher percentage of oocytes cleaved when cells synchronized by serum starvation (83.0%) were used as the donor nucleus compared with roscovitine (80.0%) or contact-inhibition (80.0%), although there were no statistical differences between the treatments. Despite the high percentage of embryos cleaving after NT in all treatments, 18% of AWC-DSH and 40% of DSH-DSH 'presumptive' embryos had undergone fragmentation and an intact donor nucleus was still present. The remainder of the 'presumptive' embryos did cleave (2–4 cells), with most NT embryos arresting at the premorula stage (2–10 cells) stage. There was not a statistically significant difference between treatments. Indeed, only 14% of embryos reconstructed with DSH cells reached the morula/blastocyst stage compared with 44% of embryos reconstructed with AWC cells ($P < 0.05$).

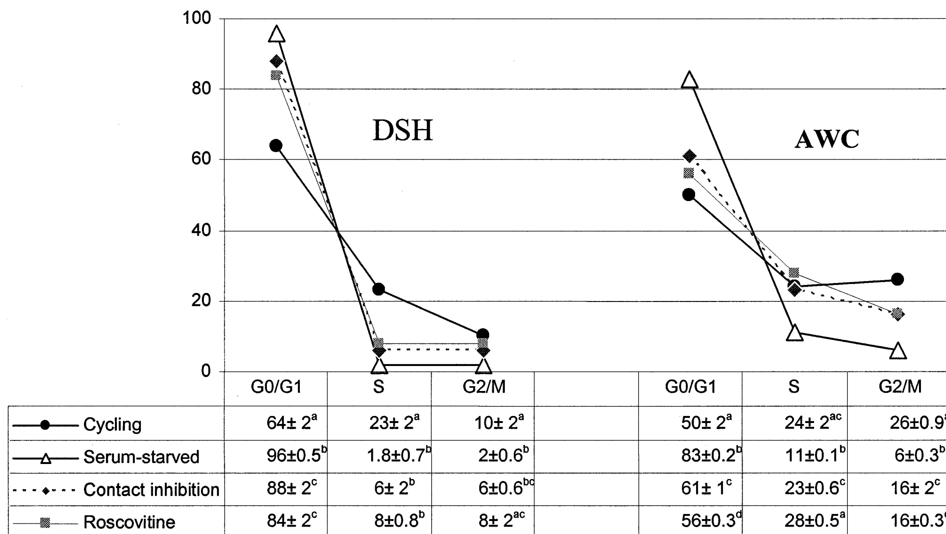
The fusion efficiency of in vivo- and in vitro-matured oocytes used as recipient cytoplasts with AWC cells as donor nuclei was not different (86.6% vs. 85.2%, respectively). Domestic cat cells (DSH) transferred into in vivo-matured oocytes showed a similar fusion efficiency (83.7%) to AWC cells, but fusion efficiency of NT couplets using in vitro-matured oocytes was slightly lower (78.0%), but not statistically different (Table 3). Likewise, the cleavage rate of embryos reconstituted with DSH cells and in vitro-matured oocytes was numerically lower (73.0%) but not significantly different from the other groups.

The percentage of blastocysts developing after parthenogenetic activation of in vivo- and in vitro-matured oocytes was 23.3 ± 2 and 27.4 ± 5, respectively. The rate of blastocyst development in embryos reconstructed with AWC cells and in vivo- (27.2 ± 3) or in vitro- (23.1 ±3.2) matured oocytes was higher than that of embryos reconstructed with DSH cells and in vivo- (3.0 ± 4) or in vitro- (2.3 ± 3) matured oocytes ($P < 0.05$).

The total cell number in NT blastocysts was not affected by the cell-cycle synchronization method or by type of oocyte,- and was similar to the cell number of the parthenotes (Table 4). The ICM cell numbers and the ratios of ICM/TPD cells were similar between treatments. We were not able to compare the total cell number of blastocysts derived from the DSH cell line because of the low number of blastocysts obtained.

Experiment 4

Effect of calcium in the fusion medium on domestic cat oocyte activation and development of AWC-DSH-cloned embryos. In this experiment, we evaluated the effect of calcium in the fusion medium on a) activation of domestic cat oocytes and b) embryo development of AWC-DSH-cloned embryos.



	DSH G0/G1	DSH S	DSH G2/M	AWC G0/G1	AWC S	AWC G2/M
Cycling	64± 2[a]	23± 2[a]	10± 2[a]	50± 2[a]	24± 2[ac]	26±0.9[a]
Serum-starved	96±0.5[b]	1.8±0.7[b]	2±0.6[b]	83±0.2[b]	11±0.1[b]	6±0.3[b]
Contact inhibition	88± 2[c]	6± 2[b]	6±0.6[bc]	61± 1[c]	23±0.6[c]	16± 2[c]
Roscovitine	84± 2[c]	8±0.8[b]	8± 2[ac]	56±0.3[d]	28±0.5[a]	16±0.3[c]

Values are reported as mean ± SEM.
[a,b,c,d] Different superscripts within the same column indicate significant differences ($P<0.05$).
The total number of cells in each treatment was derived from at least two replications.

FIG. 3. Percentages of African wild cat and domestic cat fibroblasts existing in the various phases of the cell cycle after treatment with different cell-cycle inhibitors.

The presence of calcium in the fusion medium induced cleavage in slightly more than half of the domestic cat oocytes (25/46= 54.3%), while the percentage of cleaved oocytes in the absence of calcium was much lower (4/26= 16.6%; $P < 0.05$). Although the presence of calcium in the fusion medium induced activation of domestic cat oocytes, the fusion rate, cleavage rate, and blastocyst development of AWC-DSH-cloned embryos were not affected by the presence of calcium in the fusion medium (Table 5).

Experiment 5

Embryo transfer of AWC-DSH-cloned embryos into domestic cat recipients. Derived AWC-DSH-cloned embryos were transferred into 11 domestic cat recipients. Fifty-three morulae (Day 6) were transferred to three of the recipients on Day 6 after oocyte recovery (mean = 17.6), and 57 blastocysts (Days 7 and 8), and 66 morulae (Day 7) were transferred to eight of the recipients on Day 7 after oocyte recovery (mean = 17.5 embryos). No pregnancies were detected after abdominal ultrasonography on Days 21–23 after oocyte recovery.

DISCUSSION

In the present study, we compared the ability of somatic cell nuclei of the African wild cat and the domestic cat to dedifferentiate in domestic cat cytoplasts and to support early development after reconstruction. Specifically, we examined the effect of type of oocyte maturation (in vivo vs. in vitro), different methods (mechanical and enzymatic) for donor cell isolation, and cell synchronization treatments (serum starvation, contact inhibition, and roscovitine) on the distribution of cells in the various phases of the cell cycle and the effect of calcium in the cell fusion medium on embryo reconstruction after NT.

The cell dislodging process can affect cell viability and DNA integrity. Both mechanical and enzymatic disaggregation have been used to dislodge cells cultured in vitro. Mechanical separation re-suspends cells more quickly than enzymatic digestion but may cause mechanical damage [33]. Enzymatic disaggregation using trypsin is the most widely used enzyme for tissue disaggregation, but can induce DNA damage [33], and is less effective at disaggregating fibroblastic cell lines than is pronase [34]. Similar

TABLE 3. Effect of donor nucleus, cell synchronization, and oocyte maturation of the recipient cytoplast on in vitro development of DSH-DSH and AWC-DSH cloned embryos.[a]

Donor nucleus	Oocyte maturation	Donor nucleus treatment	NT	Fused couplets	Cleavage	Premorulae	Morulae	Blastocysts
AWC	In vivo	Serum-starved	143	89 ± 2	89 ± 3	85 ± 8	38 ± 6[ab]	28 ± 3[a]
		Contact inhibition	52	89 ± 10	82 ± 9	69 ± 9	35 ± 7[ab]	17 ± 6[a]
		Roscovitine	55	81 ± 8	80 ± 9	62 ± 22	47 ± 17[ab]	33 ± 14[a]
	In vitro	Serum-starved	78	79 ± 8	79 ± 9	75 ± 19	46 ± 13[ab]	21 ± 9[a]
		Contact inhibition	77	80 ± 4	87 ± 7	78 ± 4	42 ± 3[ab]	28 ± 4[a]
		Roscovitine	79	97 ± 2	88 ± 6	83 ± 9	51 ± 9[a]	20 ± 4[a]
DSH	In vivo	Serum-starved	35	88 ± 2	83 ± 9	40 ± 4	4 ± 2[b]	3 ± 5[b]
		Contact inhibition	48	67 ± 2	86 ± 8	69 ± 19	10 ± 5[ab]	2 ± 1[b]
		Roscovitine	56	96 ± 3	84 ± 8	76 ± 12	11 ± 2[ab]	4 ± 3[b]
	In vitro	Serum-starved	60	78 ± 10	79 ± 7	44 ± 3	8 ± 5[b]	0 ± 0[b]
		Contact inhibition	104	70 ± 5	74 ± 5	39 ± 12	9 ± 3[b]	3 ± 2[b]
		Roscovitine	49	86 ± 7	67 ± 17	93 ± 6	22 ± 13[ab]	3 ± 3[b]

[a] The total number of NT couplets in each treatment was derived from at least three replications. Results are expressed as mean % ± SEM. Development rate of embryos at different stages, number of embryos/number of fused couplets.
[a,b] Different superscripts within the same column indicate significant differences ($P < 0.05$). DSH, Domestic cat; AWC, African wild cat.

TABLE 4. Numbers of cells in blastocysts and in inner cell mass (ICM) and trophodectoderm (TPD) cells of African wild cat cloned embryos.[a]

Oocyte maturation	Cell synchronization	n	Cell/blastocyst	n	ICM (%)	TPD (%)	ICM (#)	Ratio ICM : TPD
In vivo	Serum-starved	6	105 ± 24	2	25 ± 14	107 ± 43	19	1:4.2
	Contact inhibition	7	99 ± 18	5	23 ± 5	55 ± 7	30	1:2.3
	Roscovitine	4	155 ± 35	4	54 ± 21	100 ± 14	35	1:1.8
	Parthenotes	14	65 ± 9	7	24 ± 5	52 ± 8	32	1:2.1
In vitro	Serum-starved	2	218 ± 163	1	12 ± 0	43 ± 0	22	1:3.5
	Contact inhibition	4	128 ± 28	1	24 ± 0	49 ± 0	33	1:2.0
	Roscovitine	9	175 ± 34	4	34 ± 12	101 ± 28	25	1:2.9
	Parthenotes	11	129 ± 28	8	33 ± 7	113 ± 31	22	1:3.4

[a] N = total of blastocysts stained to determine the cell numbers. Results are expressed as mean % ± SEM.

results were found in our experiments, where mechanical separation yielded higher percentages of cell damage compared with enzymatic disaggregation. Also, flow cytometry analysis revealed that cells dissociated with pronase had less DNA damage. These results indicate that pronase is a reliable treatment to disaggregate fibroblastic cells before their use for NT. It is not clear how enzymatic disaggregation could affect the cell cycle of the somatic cells, but our results demonstrated that the nuclei of AWC and DSH cells dislodged with pronase had a higher percentage of cells in the G_0/G_1 phase than did those treated with trypsin or mechanically separated. Girard and Fernandes [35] reported that dislodging cells with proteases, such as trypsin and pronase, can moderately stimulate spontaneous deoxynucleic acid synthesis of lymphocyte cells and that the amount of stimulation was increased when the cells were exposed to proteases for periods longer than 1 min. Accordingly, we suggest that the exposure time used (2–3 min) to dislodge the cells in our experiments allowed the enzymes to permeabilize the membranes and, in fact, affected the cell-cycle phase. The possible mechanisms of action of these enzymes should be investigated.

Flow cytometry analysis revealed that AWC and DSH fibroblasts can be synchronized in the G_0/G_1 phase by using different cell-cycle inhibitor treatments. However, the AWC cells were synchronized in lower proportions than DSH cells ($P < 0.05$). One factor contributing to the difference may have been that AWC cells had a lower percentage of G_0/G_1 cells before exposure to the different synchronization treatments, and also there may be differences between the species in the way they react to each cell-synchronization treatment.

In both species, serum-starvation treatment elicited a higher percentage of cells in G_0/G_1, but induced higher rates of DNA fragmentation (data not shown). Similar studies have demonstrated that serum starvation significantly increases the proportion of cells at the G_0/G_1 phase [21, 23, 36], but prolonged culture in serum-deprived medium induced massive DNA fragmentation [22]. Effective arrest of pig fibroblast cells in the G_0/G_1 phase of the cell cycle using roscovitine has been demonstrated with higher percentages of G_0/G_1 phase cells (82.4%) compared with serum starvation (75.0%) [24]. In contrast, our results have shown that roscovitine and contact inhibition yielded lower percentages of cells synchronized in the G_0/G_1 phase of either AWC (56% and 61%) or DSH cells (84% and 88%, respectively) compared with serum-starved treatment of AWC (83%) or DSH cells (96%). Also, a slightly higher percentage of cybrids cleaved when cells synchronized by serum starvation (83.0%) were used as the donor nucleus compared with roscovitine (80.0%) or contact inhibition (80.0%). Therefore, the serum-starvation treatment is our current method of choice for synchronizing African wild cat or domestic cat somatic cells prior to NT. Further experiments will examine whether the addition of apoptosis inhibitors, such as proteases or antioxidants, as described by Lee and Piedrahita [37] can reduce DNA fragmentation after serum starvation without changing the proportion of G_0/G_1 cells.

TABLE 5. Development of African wild cat-domestic cat (AWC-DSH) nuclear transfer couplets after 2 and 7 days of culture when AWC cells were fused in the presence or absence of calcium.*

Oocyte maturation	Fusion treatment	NT[a]	Fused couplets	Cleavage (D2)	Blastocysts[b] (D7)
In vivo	Calcium present	85	89 ± 3	92 ± 1	25 ± 3
	Calcium absent	82	99 ± 1	93 ± 7	16 ± 2
In vitro	Calcium present	86	84 ± 8	90 ± 5	28 ± 7
	Calcium absent	51	100 ± 0	77 ± 8	22 ± 8

* Results are expressed as mean % ± SEM.
[a] The total number of NT couplets in each treatment was derived from three replications.
[b] Blastocysts = number of blastocysts/number of fused couplets.

The NT experiments showed that AWC fibroblasts can dedifferentiate in enucleated domestic cat oocytes at higher rates than DSH fibroblasts. Surprisingly, 43.8% and 24.2% of the AWC-DSH-cloned embryos reached the morula and blastocyst stage compared with 13.9% and 3.3% of DSH-DSH-cloned embryos, respectively. One of the possible reasons for the contrasting results in embryo development may be because each cell line was derived from individuals of different gender.

Few studies have compared the cloning competence of male and female somatic donors. In this study, we used a somatic cell line of AWC fibroblasts that were derived from a 2-yr-old male and a somatic cell line of DSH fibroblasts derived from a 2-yr-old female. Heyman et al. [38] compared percentages of blastocyst formation and full-term development between different donor animals, after using fibroblasts derived from skin biopsies of different adult bulls and cows. No differences were found when comparisons were made between the two genders. Instead, development and competence of the cloned embryos were affected by the genotype of the donor cells and the cell culture conditions. Similar observations were mentioned by Oback and Wells (Wells, unpublished results) [39], where no significant differences in embryo development were observed using donor cells derived from full-sib brother-sister cattle, although they mentioned that male clones tended to developed better. In our case, we did not use fibroblasts derived from female AWC and male DSH cats; therefore, we cannot conclude if the differences in embryo development between the species were affected by differences in gender. In the

present study, because the type of recipient cytoplasts, the cell synchronization, and the nuclear transfer method were essentially identical, the observed differences in development may be considered to be due in part to the origin of the donor nucleus. Underscoring this statement is that no conclusive reports have yet been published indicating that male somatic cells are influencing better embryo development after NT.

Significant effects have been reported on source of recipient cytoplast used for NT with lower rates of blastocyst production and fetal survival from in vitro-matured cytoplasts compared with in vivo-matured cytoplasts [40]. Instead, in our NT experiments, the fusion efficiency, embryo cleavage, blastocyst development, and total cell number in NT blastocysts were not affected by the source of oocytes used as recipient cytoplasts. We had previously demonstrated in our laboratory a reduction in cleavage frequency and in vitro development of cat oocytes matured in vitro and fertilized by intracytoplasmic sperm injection compared with that of in vivo-matured oocytes [21]. However, modification of the IVM medium by the addition of epidermal growth factor has improved both the number and quality of embryos produced [28, 41]. We can suggest that, although the IVM system is not fully optimized, improvements made in recent years to our IVM medium have improved the quality of in vitro-matured cytoplasts, making them comparable with in vivo-matured cytoplasts in terms of embryo development. The influence of source of recipient cytoplasts needs consideration with regard to pregnancy rate and embryo survival following the transfer of NT-derived embryos.

Direct comparisons of NT success between laboratories are not possible because of variations in the type of cells, embryo culture system, and NT procedures. The rates of blastocyst development of domestic cat embryos reconstructed with cumulus cells (2.6%), granulose cells (3.0%), and domestic cat fetal fibroblasts (5.2%) [42, 43] were similar to the present results using DSH somatic cells (3.3%). A possible explanation for the early developmental failure of most of our reconstructed embryos may be inadequate or incomplete reprogramming of donor nuclei.

Kim et al. [44] suggest that failure of activated oocytes before NT to remodel somatic nuclei is caused by the absence of cytoplasmic factors, such as maturation promoting factor (MPF), that are needed to induce nuclear envelope breakdown (NEBD). Because the electrical pulse necessary to induce fusion of the donor cell to the recipient cytoplast during NT can induce concurrent oocyte activation and a transient decrease of the maturation promoting factor activity, chromatin remodeling may be affected. In fact, pig cybrids that were fused in medium containing 0.1 mM of calcium and not receiving further chemical activation stimulus were activated and cleaved at higher rates (69%) than their counterparts fused under calcium-free conditions (10%) [45]. Similarly, in our study, a higher percentage of oocytes cleaved after receiving electrical pulses in the presence of calcium (54.3%) compared with those electrically pulsed in the absence of calcium (16.6%). These results indicate that domestic cat oocytes are susceptible to activation during electrical fusion in the presence of calcium. However, the fusion of AWC-DSH couplets in the presence or absence of calcium did not affect the fusion rates nor embryo development to the blastocyst stage. Several studies have shown that the level of active MPF declines significantly in oocytes within 2 h after electrical activation and remains depleted for at least 8–10 h, directly affecting chromatin remodeling [46, 47]. We did not measure levels of histone H1 kinase activity to determine the level of active MPF, but the proportions of couplets undergoing chromatin remodeling after fusion in medium containing calcium is similar to those couplets fused in the absence of calcium. Therefore, we suggest that the levels of MPF after fusion in the presence of calcium do not decline drastically within the next 2–3 h, which was the delayed activation interval we used before activating the reconstructed couplets. Because we do not know the extent to which the calcium concentration (0.05 mM) in the fusion medium is affecting the level of MPF activity during fusion, further studies should be done to investigate the level of histone H1 kinase activity.

In our NT experiments, we used oocytes of similar type and source that were at the same meiotic stage (M-II, nonactivated) when reconstructed. Only 18.0% of the AWC-DSH presumptive embryos were fragmented and were unable to remodel the chromatin compared with 40.0% of DSH-DSH presumptive embryos. From these results, it appears that chromatin remodeling failure may be partially due to the recipient cytoplast, but there are likely other factors involved as well. Such unknown factors may have contributed to the lower rate of chromatin remodeling in the DSH somatic cell line relative to the remodeling rate in the AWC somatic cell line. Differences in the epigenetic status of the DSH somatic nuclei may be a cause of the low rate of chromatin remodeling after NT [48]. A better understanding of the molecular basis for epigenetic variation within the donor cells will help to understand its influence during cell reprogramming.

During the initial stages of development, embryos are under the control of maternally derived proteins and transcripts accumulated in the oocyte. The timing of the transition from maternal to embryonic control of development (MET) and the beginning of the transcription period is species specific [49]. For example, in the mouse, embryonic genome activation and transcription begin at the 1-cell stage [50], whereas in domestic cats, the transition from maternal to embryonic control of development and embryonic transition occurs by the 5–8-cell stage [51]. Therefore, the possibility of a relationship between the high rate of development failure of both DSH-DSH (40.0%) and AWC-DSH (37.5%) NT embryos at the 8–10-cell stage and the transition from maternal to embryonic control of development cannot be overlooked. In spite of a similar rate of early development failure in embryos reconstructed from each of the two somatic cell lines, the embryos derived from AWC somatic cells developed in vitro to the blastocyst stage (24.2%) at rates comparable with domestic cat embryos produced by parthenogenetic activation of in vivo- (23.3%) and in vitro-matured (27.4%) oocytes or by introcytoplasmic sperm injection (ICSI) from in vivo- (29.5%) and in vitro-matured (19.0%) oocytes [29].

Although the AWC-DSH-cloned embryos developed to the blastocyst stage in vitro, we were not able to produce a pregnancy after embryo transfer. Interspecies and subspecies embryo transfer of nondomestic cat embryos into domestic cat recipients has resulted in the birth of Indian desert cat [52] and African wild cat kittens [53] and an early pregnancy of interspecies panda-rabbit cloned embryos [6], demonstrating that the domestic cat is not only a model for development of in vitro assisted reproductive technologies, but it can also serve as a successful recipient of embryos from closely related small non-domestic cats. Therefore, we do not think that species incompatibility contributed to the

developmental failure of the AWC-DSH embryos transferred into the domestic cat. More likely, an incomplete reprogramming of the differentiated nucleus was a major constraint to the in vivo developmental potential of these embryos [54]. Also, the influence of possible mitochondrial heteroplasmy and/or the deleterious effect of mtDNA of the derived AWC cloned embryos on preimplantation development should not be discounted. Nagao et al. [55] have demonstrated that mouse embryos derived from nuclear transfer of interspecific species (*M. musculus* and *M. spretus*) had reduced development in vitro and decreased physical performances after birth. Conversely, the birth of normal *Bos indicus* calves derived from nuclear transfer of *Bos indicus* donor cells into *Bos taurus* oocyte recipients demonstrated that cross-subspecies nuclear transfer is a viable approach to rescue closely related endangered species [14, 15]. Meirelles et al. [14] demonstrated that donor cell-derived mtDNA (*Bos indicus*) was reduced during early embryo development and completely eliminated by the end of the gestation. However, Steinborn et al. [15] found coexisting mtDNAs of both subspecies (heteroplasmy) in healthy cloned *Bos indicus* calves. Both studies indicated that the presence of mtDNA derived from the recipient oocyte or mtDNAs derived from both recipient oocyte and donor-cell (heteroplasmy) does not inhibit normal development. Although in our study we did not evaluate the mitochondrial inheritance of the derived AWC cloned embryos, we suggest that it should be possible to establish a pregnancy with cross-species- (DSH and AWC) derived cloned embryos after transfer to surrogate domestic cats. Indeed, further research is needed to evaluate the precise mitochondrial inheritance pattern of AWC-cloned embryos derived by cross-subspecies nuclear transfer and its influence on embryo implantation and survival rate.

In conclusion, the results of the current study indicate that (1) African wild cat and domestic cat cells can be synchronized in the G_0/G_1 phase by different methods, (2) a distinct difference in in vitro developmental competence of NT embryos produced from each of the two cat fibroblast cell lines, and (3) incomplete reprogramming of the differentiated nucleus may be a major constraint to the in vivo developmental potential of these embryos.

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