

# Nuclear Transfer of M-phase Ferret Fibroblasts Synchronized with the Microtubule Inhibitor Demecolcine

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**ABSTRACT** The development of reconstructed embryos following nuclear transfer (NT) appears to be dependent upon a variety of factors, including cell cycle synchronization between the donor nucleus and recipient oocyte. Here we use the microtubule inhibitor, demecolcine, to synchronize ferret fibroblasts in metaphase (M-phase) in order to match their cell cycle position with that of the recipient oocyte at the time of NT. The fibroblasts were obtained from 28-day fetuses and cultured for 1–30 days prior to NT. Fibroblast cultures were treated with 0.05 µg/ml of demecolcine for 3 hr or overnight (14–16 hr) after various times in culture to determine the optimal conditions for M-phase synchronization. The percentage of G2/M-phase cells in demecolcine-treated cultures was significantly greater than that found in untreated cultures ( $P < 0.05$ ). Optimally synchronized M-phase fibroblasts were collected by mitotic shake-off and evaluated for their effectiveness in NT. M-phase somatic cell-derived NT embryos reconstituted by electrofusion or microinjection underwent implantation and formed fetuses at similar rates (5.4% vs. 3.4%, and 1.8% vs. 1.2%, respectively); however, no NT embryos developed to term. In summary, these data demonstrate two important points. First, demecolcine treatment effectively synchronizes ferret fibroblasts in M-phase of the cell cycle; and second, these somatic cells are capable of driving embryo development following NT. Our results should facilitate the development of cloned ferrets as an animal model for human lung disease such as influenza and cystic fibrosis. *J. Exp. Zool.* 303A:1126–1134, 2005.

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The domestic ferret, *Mustela putorius furo*, is considered an excellent animal model for studying human lung diseases such as influenza (Pearson and Gorham, '98; Maher and DeStefano, 2004), and cystic fibrosis (CF) (Welsh et al., '95). This is due, in part, to the remarkable similarity between ferret and human lung cell biology (Mercer et al., '94; Choi et al., 2000; Wang et al., 2001). Recent successes in creating cloned animals by somatic cell nuclear transfer (SCNT) have made animal modeling more feasible in species, such as the ferret, that may model specific human diseases more closely than currently used rodent model systems. In order to exploit the ferret as an animal

model for human lung disease, it is essential that key technologies are developed for the efficient production of ferret clones. To this end, we have defined conditions for superovulation in ferrets and for in vitro culture of ferret embryos (Li et al.,

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2001), established optimal conditions for in vitro maturation (IVM) and artificial activation of ferret oocytes (Li et al., 2002), developed technologies for nuclear transfer (NT) of G0/G1-phase ferret fetal fibroblasts into enucleated oocytes by direct cytoplasm injection (Li et al., 2003), and more recently, by electrofusion (Li et al., 2005).

The success of SCNT critically depends on the cell cycle phases of both the donor nucleus and the recipient oocyte. After the birth of Dolly from serum-starved adult cells (Wilmut et al., '97), the use of presumably quiescent (G0) nuclei for NT has become a relatively standard approach (Kato et al., '98; Baguisi et al., '99; Polejaeva et al., 2000; Galli et al., 2003; Woods et al., 2003). However, several reports suggest that somatic cells arrested in metaphase (M-phase) may represent a viable alternative to G0 cells in mammalian SCNT. In cattle, M-phase fetal fibroblasts transferred to MII oocytes resulted in 14% morula and blastocyst development (Korfiatis et al., 2001). In mice, 56–70% morula/blastocyst development and 1.5% live birth were achieved when M-phase embryonic stem cells were used in NT (Zhou et al., 2001). It is also reported that M-phase fetal fibroblasts transferred to MII oocytes resulted in 2.3% live birth in rats (Zhou et al., 2003). However, M-phase somatic cells have not been used for NT in the domestic ferret. Therefore, the present study was undertaken to: (1) establish an efficient method for the synchronization of ferret fibroblasts in M-phase using the microtubule inhibitor, demecolcine; and (2) evaluate the developmental capacity of ferret NT embryos reconstructed with these M-phase fibroblasts.

## METHODS

### *Chemicals and animals*

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Invitrogen Co. (Grand Island, NY) unless otherwise noted. All ferrets were purchased from Marshall Farms (North Rose, NY). Female sable coat-color ferrets (virgin, 6–7 months of age) and albino coat-color ferrets (primipara, 9–12 months of age) were in estrus when delivered. The vasectomized male ferrets (albino, 12 months of age) were used for mating to induce follicular oocyte maturation in oocyte donor ferrets and to induce pseudopregnancy in NT embryo surrogate ferrets. All ferrets were housed in separate cages under a controlled temperature (20–22°C) and long day light cycle (16 hr light, 8 hr dark). The use of animals in this

study was carried out according to a protocol approved by the University of Iowa animal care review committee and conformed to or exceeded National Institutes of Health standards.

### *Oocyte collection and IVM*

In vitro matured ferret oocytes, obtained from sable coat-color ferrets mated with vasectomized male ferrets 24 hr prior to collection, were used in this study. To retrieve immature oocytes, ferrets were euthanized by administration of sodium pentobarbital (50–100 mg/kg, i.p.). The ovaries were excised, and small vesicular follicles on the ovary surface were incised in mPBS to release the cumulus–oocyte complexes (COCs) (Li et al., 2002). COCs with uniform cytoplasm and several layers of cumulus cells were washed with mPBS, and were cultured in TCM-199+10% FBS+10 iu/ml of equine chorionic gonadotropin+5 iu/ml of human chorionic gonadotropin at 38.5°C in 5% CO<sub>2</sub>, 95% air for 22 hr. After maturation, expanded cumulus cells of oocytes were removed by pipetting in mPBS containing 0.2% (w/v) hyaluronidase. Only oocytes with normal morphology, uniform cytoplasm and a first polar body were considered matured and used for enucleation and NT.

### *Preparation of fetal fibroblast cells*

Ferret fetal fibroblast cells were obtained from 28 dpc (days post copulation) fetuses derived from a Sable (female) × Cinnamon (male) mating as reported previously (Li et al., 2003). Briefly, the heads and all internal organs of the fetuses were removed, and each remaining carcass was minced individually in a Petri dish. The minced carcass was digested with Trypsin:EDTA (Invitrogen Co., Cat. No. 25200-056) for 1–2 hr at 37°C. Cells were then collected by centrifugation at 1,500 rpm for 5 min, were resuspended, and washed in PEF medium [Dulbecco's modified Eagle medium (DMEM, high glucose) supplemented with 10% (v/v) FBS, 1% (v/v) L-Glutamine (Sigma, G-7513), and 7.0 µl/L of 2-mercaptoethanol (Sigma, M-7522)]. Finally, cells from each fetus were placed in PEF medium onto 2–3 dishes (100 mm) and incubated at 37°C, 5% CO<sub>2</sub> overnight. Once the cells reached confluence, they were frozen as “zero passage” cells (2 vials per 100 mm dish) in DMEM containing 10% FBS and 10% dimethyl sulfoxide (Sigma D-2650), and thawed as needed. Thawed fibroblasts were passaged between 1 and 30 days.

Three different male-derived fibroblast cell lines were used for NT and were grown and cultured in complete MEM medium (20% FBS, 1% L-glutamine/penicillin/streptomycin). Male fibroblast cell lines were selected by karyotyping using a standard chromosome harvesting procedure (Barch et al., '97). Briefly, 12  $\mu$ l Karyomax Colcemid<sup>®</sup> solution (Invitrogen) and 25  $\mu$ l ethidium bromide working solution (1mg/ml) were added to each culture and incubated for 2 hr. Cultures were then placed on a Tecan Miniprep robotic harvester programmed for two changes of hypotonic solution (3:1 0.075 M potassium chloride, 0.8% sodium citrate), followed by three changes of fixative (3:1 absolute methanol, glacial acetic acid). Fixative was removed and coverslips dried in a Thermotron slide-drying chamber to obtain well-spread metaphases. Slides were then aged (20 min at 60°C, 60 min at 90°C) and stained using the routine GTW banding method (Barch et al., '97). Digitized images of chromosomes from each cell were captured on a CCD camera with a Cytovision imaging system (Applied Imaging, Santa Clara, CA). Identification of each chromosome was made as described (Cavagna et al., 2000). Five cells per cell line were karyotyped and these cells were found to have normal karyotypes (40, XY, Fig. 1A and B).

#### ***Cell cycle synchronization of fibroblasts and flow cytometry analysis***

Ferret fetal fibroblasts at passage zero were thawed in a 37°C water bath and seeded onto 100 mm dishes with 10 ml DMEM containing 10% FBS, and were incubated at 37°C, 5% CO<sub>2</sub> and 95% air for 1–30 days. Cells were passaged (1:5 dilution) when they reached confluence every 3–5 days over a period of 30 days. For cell cycle synchronization, 50% confluent monolayers of fibroblasts were passaged for 4, 9, 14, 19, 24 and 29 days, washed with 1  $\times$  PBS, and treated with DMEM containing 10% FBS and 0.05  $\mu$ g/ml demecolcine (Sigma, D6165) for 3 hr or overnight (O/N, 14–16 hr). Cells were then trypsinized and fixed in cooled 80% methanol (–20°C) for 15 min at 4°C and centrifuged. The cells were incubated in a PBS solution containing 1 mg/ml of RNase (Sigma, R-5125), 50  $\mu$ g/ml of propidium iodide (PI, Sigma P-4170), and 0.1% of saponin (Cat. No. H277-57, Mallinckrodt Baker, Inc., Paris, KY) for 30 min at room temperature in the dark. The cells were subsequently transferred into PBS, and 10,000 cells were analyzed for each experimental condi-

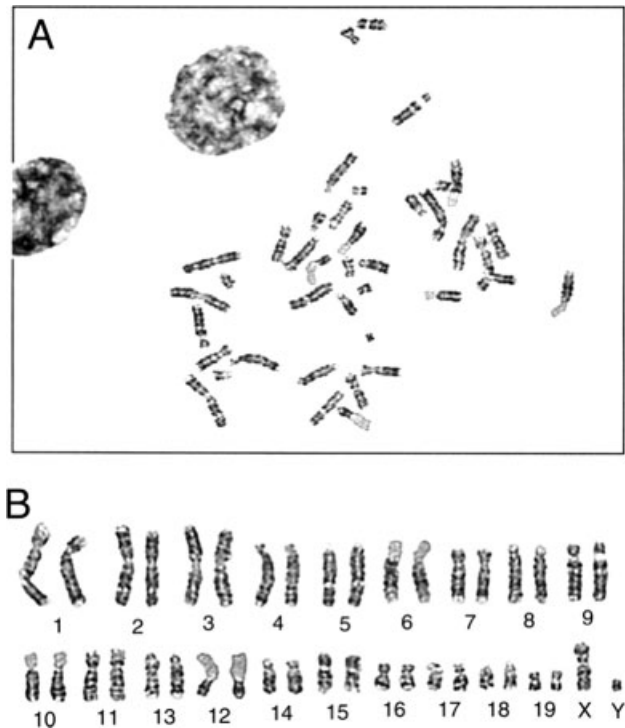


Fig. 1. Ferret fibroblast G-banding karyotype analysis. (A) A male fetal fibroblast chromosomal spread; (B) G-banding karyotype of the 40 chromosomes shown in (A).

tion by fluorescence-activated cell sorting (FACS) for DNA content (Becton Dickinson FACScan). The percentages of cells within the different phases of the cell cycle were calculated by gating on G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M cell populations. The gates were held constant for control (untreated) and demecolcine-treated samples. All experiments were performed in duplicate with three different cell lines.

For NT, fibroblasts within 3 passages were treated with 0.05  $\mu$ g/ml of demecolcine for 3 hr, and then dislodged from the culture dish by mitotic "shake-off". The cell suspension was centrifuged and samples of fibroblasts were stained by Hoechst 33342 (10  $\mu$ g/ml). Our analysis revealed that >90% of the "shake-off" fibroblasts were in M-phase of the cell cycle. The unstained M-phase fibroblasts were used as nuclear donors.

#### ***Nuclear transfer and activation***

In vitro matured oocytes were transferred to mPBS medium containing 7.5  $\mu$ g/ml of cytochalasin B (CB) in the micromanipulation chamber. Using Nomarski optics, the first polar body and chromosome spindle were aspirated into the

pipette with a minimal volume of oocyte cytoplasm with a 15  $\mu\text{m}$  (inside diameter, ID) PeizoDrill glass pipette. NTs were performed by two methods including microinjection of nuclei and electrofusion of intact cells as described below.

### Microinjection and activation

M-phase donor cells were transferred to micromanipulation chamber in mPBS medium containing 7.5  $\mu\text{g}/\text{ml}$  of CB and 0.05  $\mu\text{g}/\text{ml}$  of demecolcine. One fetal fibroblast (diameter  $\geq 15 \mu\text{m}$ ) was aspirated in and out of a 10  $\mu\text{m}$  (ID) PeizoDrill glass pipette to break the cell membrane. The ruptured cell was then microinjected into the oocyte cytoplasm. The NT embryos reconstructed by microinjection were transferred into electrical activation medium [0.3M Mannitol, 0.1mM  $\text{MgCl}_2$ , 0.1mM  $\text{CaCl}_2$ , 0.5mM Hepes, 0.01% (w/v) BSA], placed between two parallel electrodes and subjected to an electrical pulse of 1 DC of 180 V/mm for 30  $\mu\text{sec}$  from ECM 2001 (BTX, San Diego, CA). The NT embryos subjected to electrical pulse were kept in TCM-199+10%FBS medium for 1hr, then incubated in TCM-199 medium containing 5  $\mu\text{g}/\text{ml}$  of cyclohexamide and 2mM 6-dimethylaminopurine for 1hr to facilitate chemical activation.

### Electrofusion and activation

M-phase donor cells were transferred to a micromanipulation chamber in mPBS medium containing 7.5  $\mu\text{g}/\text{ml}$  of CB and 0.05  $\mu\text{g}/\text{ml}$  of demecolcine. One or two fibroblasts were aspirated into a 15  $\mu\text{m}$  (ID) PeizoDrill glass pipette and inserted into the perivitelline space (PVS) of enucleated oocytes. These injected cell-oocyte couplets were then transferred into electrical fusion medium (the same as the above activation medium), placed between two parallel electrodes and manually aligned with a fine pipette so that the contact surface between the oocyte and the donor cell was parallel to the electrodes. Cell fusion was induced with an electrical pulse (as above). The electrofusion efficiency of reconstructed embryos was examined within 20–30 min after electrical pulse. The fused NT embryos were kept in TCM-199+10% FBS medium for 30 min (1hr from electrical pulse), then subjected to 1hr of chemical activation, as above.

### Embryo transfer and in vivo development

After NT and activation, embryos were rinsed in mPBS and immediately transferred into pseudo-

pregnant albino primipara ferrets. A pseudopregnant state was achieved in surrogate albino primipara ferrets through mating with a vasectomized albino male 24 hr prior to embryo transfer (ET). ET into albino surrogate ferrets was performed following anesthetization by i.p. injection of 15–30 mg/kg each of ketamine HCl (Abbott Laboratories, N. Chicago, IL) and xylazine (Phoenix Pharmaceutical Inc., St. Joseph, MO 64503). The abdomen was shaved and disinfected with 10% povidone iodine and 70% ethanol. A 3–4 cm incision was made along the midline of the abdomen to expose the ovaries and oviducts. The 23-gauge needle was used to generate a hole on the surface of left oviduct. The NT embryos were delivered into the left oviduct through the hole using a fine glass pipette when NT embryo numbers were less than 30, or into both oviducts, when NT embryo numbers were over 30. After the surgical wound was sutured, the ferret was allowed to wake up in its own cage under close monitoring.

For assessment of embryonic development in vivo, the recipient ferrets were either euthanized at 3 weeks following ET (mid-term gestation of ferrets) or at 6 weeks post-ET for full-term examination when they failed to give birth. The uteri were then evaluated for implantation spots and fetus formation. The in vivo development of 3-week NT reconstructed fetuses was compared to that of normal 3-week ferret fetuses developed from in vivo fertilization by normal mating.

### Statistical analysis

For statistical analysis of cell cycle synchronization data (Tables 1 and 2), the mean percentage ( $\pm$ SEM) was calculated for each group. The normality assumption of the percentages for each data set was checked by the Kolmogorov–Smirnov test using statistical Minitab 13 software (Minitab Inc., State College, PA). In addition, the Bartlett test, also using Minitab 13 software, was performed to justify the equal variance assumption of the percentages for each data set. When these two tests suggested that the two assumptions were valid, one-way ANOVA (analysis of variance) was used for statistical analysis with one independent variable (e.g., evaluating the effects of time points on percentages of cell cycle phase in control and demecolcine groups). When ANOVA demonstrated a significant difference between samples, the Tukey multiple comparison test was performed to determine *P* values for all possible two-group

TABLE 1. Percentage (mean  $\pm$  SEM) of phase-specific cells following a 3 hr treatment with demecolcine (0.05  $\mu$ g/ml) at increasing passage numbers

Cell cycle phases	Percentage (mean $\pm$ SEM) of cells in specific cell cycle phases at increasing days in culture					
	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
<i>G0/G1-phase</i>						
Control	68.4 $\pm$ 4.1 <sup>a</sup>	59.2 $\pm$ 1.6 <sup>a</sup>	57.6 $\pm$ 2.4 <sup>a</sup>	69.2 $\pm$ 1.5 <sup>a</sup>	74.6 $\pm$ 1.3 <sup>a</sup>	85.9 $\pm$ 3.7 <sup>a</sup>
Demecolcine	55.7 $\pm$ 2.1 <sup>b</sup>	48.2 $\pm$ 0.1 <sup>b</sup>	46.4 $\pm$ 0.2 <sup>b</sup>	58.2 $\pm$ 0.7 <sup>b</sup>	69.3 $\pm$ 2.1 <sup>a</sup>	74.9 $\pm$ 2.7 <sup>a</sup>
<i>S-phase</i>						
Control	23.1 $\pm$ 3.6 <sup>a</sup>	28.4 $\pm$ 4.6 <sup>a</sup>	25.9 $\pm$ 0.4 <sup>a</sup>	21.3 $\pm$ 1.8 <sup>a</sup>	15.9 $\pm$ 1.6 <sup>a</sup>	8.3 $\pm$ 2.1 <sup>a</sup>
Demecolcine	22.3 $\pm$ 2.4 <sup>a</sup>	22.3 $\pm$ 1.0 <sup>a</sup>	21.0 $\pm$ 1.1 <sup>a</sup>	20.9 $\pm$ 0.1 <sup>a</sup>	17.5 $\pm$ 2.4 <sup>a</sup>	13.1 $\pm$ 2.9 <sup>a</sup>
<i>G2/M-phase</i>						
Control	8.5 $\pm$ 1.0 <sup>a</sup>	12.4 $\pm$ 2.0 <sup>a</sup>	16.5 $\pm$ 2.2 <sup>a</sup>	9.5 $\pm$ 0.3 <sup>a</sup>	9.5 $\pm$ 0.4 <sup>a</sup>	5.8 $\pm$ 1.7 <sup>a</sup>
Demecolcine	22.0 $\pm$ 1.9 <sup>b</sup>	29.6 $\pm$ 1.0 <sup>b</sup>	32.6 $\pm$ 1.3 <sup>b</sup>	21.0 $\pm$ 0.6 <sup>b</sup>	13.3 $\pm$ 2.5 <sup>a</sup>	12.0 $\pm$ 3.3 <sup>a</sup>

Note: Differences among percentages containing different superscripted letters are significant when comparing the same time point and same cell cycle phase between control and demecolcine treatment samples ( $P < 0.05$ ).

TABLE 2. Percentage (mean  $\pm$  SEM) of phase-specific cells following an O/N treatment with demecolcine (0.05  $\mu$ g/ml) at increasing passage numbers

Cell cycle phases	Percentage (mean $\pm$ SEM) of cells in specific cell cycle phases at increasing days in culture					
	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
<i>G0/G1-phase</i>						
Control	68.4 $\pm$ 4.1 <sup>a</sup>	59.2 $\pm$ 1.6 <sup>a</sup>	57.6 $\pm$ 2.4 <sup>a</sup>	69.2 $\pm$ 1.5 <sup>a</sup>	74.6 $\pm$ 1.3 <sup>a</sup>	85.9 $\pm$ 3.7 <sup>a</sup>
Demecolcine	24.7 $\pm$ 0.5 <sup>b</sup>	19.9 $\pm$ 2.7 <sup>b</sup>	37.6 $\pm$ 1.4 <sup>b</sup>	60.1 $\pm$ 2.7 <sup>a</sup>	69.2 $\pm$ 1.3 <sup>a</sup>	77.8 $\pm$ 1.6 <sup>a</sup>
<i>S-phase</i>						
Control	23.1 $\pm$ 3.6 <sup>a</sup>	28.4 $\pm$ 4.6 <sup>a</sup>	25.9 $\pm$ 0.4 <sup>a</sup>	21.3 $\pm$ 1.8 <sup>a</sup>	15.9 $\pm$ 1.6 <sup>a</sup>	8.3 $\pm$ 2.1 <sup>a</sup>
Demecolcine	35.3 $\pm$ 1.4 <sup>a</sup>	21.7 $\pm$ 0.7 <sup>a</sup>	14.5 $\pm$ 1.2 <sup>b</sup>	9.1 $\pm$ 0.8 <sup>b</sup>	6.3 $\pm$ 0.1 <sup>b</sup>	5.9 $\pm$ 1.0 <sup>a</sup>
<i>G2/M-phase</i>						
Control	8.5 $\pm$ 1.0 <sup>a</sup>	12.4 $\pm$ 2.0 <sup>a</sup>	16.5 $\pm$ 2.2 <sup>a</sup>	9.5 $\pm$ 0.3 <sup>a</sup>	9.5 $\pm$ 0.4 <sup>a</sup>	5.8 $\pm$ 1.7 <sup>a</sup>
Demecolcine	40.0 $\pm$ 1.9 <sup>b</sup>	58.4 $\pm$ 2.0 <sup>b</sup>	47.9 $\pm$ 0.2 <sup>b</sup>	30.8 $\pm$ 3.5 <sup>b</sup>	24.5 $\pm$ 1.4 <sup>b</sup>	16.3 $\pm$ 0.6 <sup>b</sup>

Note: Differences among percentages containing different superscripted letters are significant when comparing the same time point and same cell cycle phase between control and demecolcine treatment samples ( $P < 0.05$ ).

TABLE 3. Nuclear transfer and embryo development in vivo<sup>1</sup>

NT methods	No. repetitions	No. NT embryos transferred	Exam time after ET	% (No.) Embryo development	
				Implantation	Normal fetus formation
Microinjection	5	174 (34.8 $\pm$ 1.9)	Mid-term	3.4 (6/174) <sup>a</sup>	1.2 (2/174) <sup>a</sup>
Microinjection	3	106 (35.3 $\pm$ 1.8)	Full term	3.8 (4/106)	–
Electrofusion	5	222 (44.4 $\pm$ 4.1)	Mid-term	5.4 (12/222) <sup>a</sup>	1.8 (4/222) <sup>a</sup>
Electrofusion	3	137 (45.7 $\pm$ 2.9)	Full term	5.8 (8/137)	–

<sup>1</sup>Donor fibroblast cells were cultured in vitro from day 5 to day 10.

Note: Differences among percentages containing the same superscripted letters are not significant ( $P > 0.05$ ) when comparing total implantation and normal fetus formation between microinjection and electrofusion groups.

comparisons within the data set. The Student's *t*-test was applied to data sets with only two groups (the same time point and same cell cycle phase in Tables 1 and 2; the mid-term exam between microinjection and electrofusion groups in Table 3). For all statistical analysis including

ANOVA, Tukey, and Student's *t*-test, differences were considered to be significant when the *P*-value was  $<0.05$ . All statistical analyses were performed using SAS 8.0 statistical software (SAS Institute Inc., Cary, NC).

## RESULTS

### *Synchronization of ferret fibroblasts in M-phase*

FACS analysis demonstrated that the percentages of G2/M-phase cells, following 3 hr of demecolcine treatment, were significantly greater than those observed in the control (untreated) group (21–32.6% vs. 8.5–16.5% from Day 5 to Day 20 in culture) (Table 1, Fig. 2A and B). Correspondingly, the percentages of G0/G1-phase cells in treated cultures were significantly reduced as compared to those of the control group (46.4–58.2% vs. 57.6–69.2% from Day 5 to Day 20 in culture). No statistical differences were observed in the percentages of S-phase cells between demecolcine-treated and control groups over the entire period of analysis.

The passage number significantly ( $P < 0.05$ ) reduced the percentage of cells in control groups of S-phase (compare Days 5, 10 and 15 with Day 30) and G2/M-phase (compare Day 15 with Day 30). Additionally, there was a significant ( $P < 0.05$ ) trend toward elevation in G0/G1-phase (compare Days 5, 10, 15 and 20 with Day 30) in a time-dependent fashion. Similar trends were also observed in demecolcine-treated groups: the passage number significantly ( $P < 0.05$ ) reduced the percentage of cells in G2/M-phase (compared Days 10 and 15 with Days 25 and 30), and in turn significantly ( $P < 0.05$ ) produced a trend toward elevation in G0/G1-phase (compare Day 5 to Day 20 with Day 25 to Day 30) in a time-dependent fashion (Table 1).

Overnight treatment of fibroblasts with demecolcine (Fig. 2A and C, and Table 2) had passage-dependent effects on cell cycle progression that were generally similar to those observed in control cultures and to those cultures receiving a 3 hr drug treatment (Fig. 2A and B, and Table 1). However, prolonged drug treatment did increase the percentage of G2/M-phase cells, and reduce the percentage of G0/G1-phase cells, relative to a 3 hr drug treatment. These differences were most apparent up until Day 20 (compare Table 1 with Table 2) after which the G2/M-phase differences were reduced and the G0/G1-phase differences disappeared altogether.

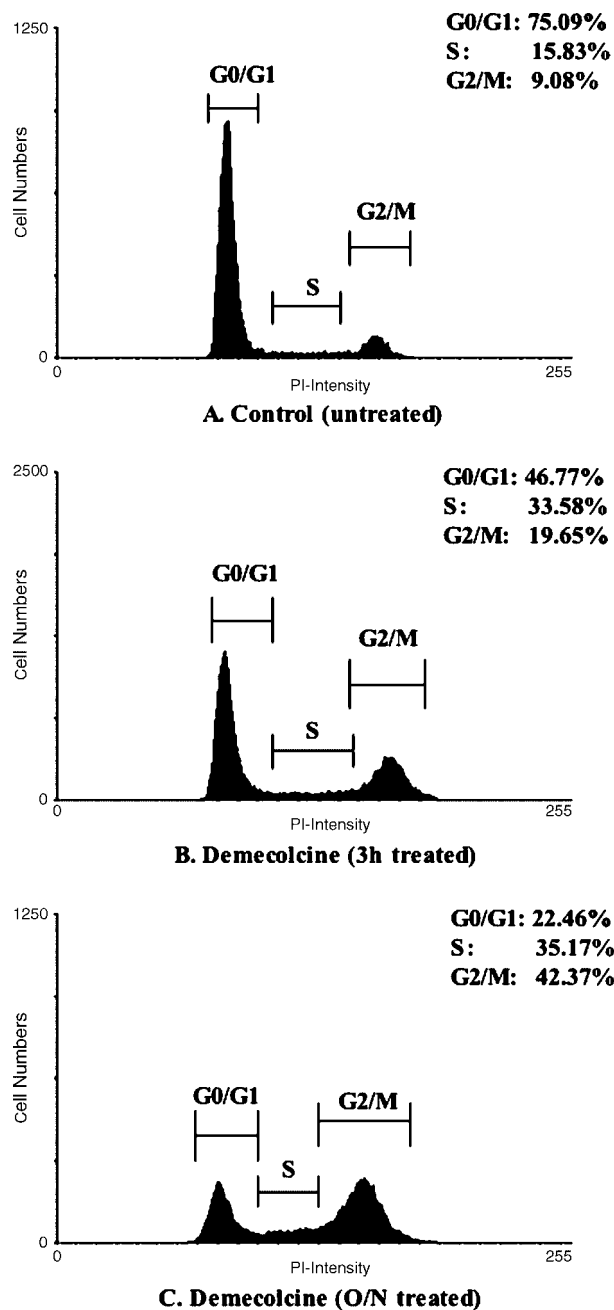


Fig. 2. Fluorescence-activated cell sorting (FACS) analysis of cellular DNA content in control and demecolcine-treated fibroblast cultures. (A) Control (untreated) group; (B) Cells treated with demecolcine for 3 hr; (C) Cells treated with demecolcine for overnight (14–16 hr).

### *Nuclear transfer and in vivo development*

Two methods of NT were employed in this study. First, demecolcine-treated (3 hr) fetal fibroblast nuclei were injected into the cytoplasm of enucleated oocytes using a PizeoDrill pipette. Following electrical and chemical activation,

270 NT embryos were transferred into eight pseudopregnant albino primipara ferrets (Table 3). The mid-term development of the 174 NT embryos in five surrogate ferrets was evaluated at 3 weeks post-ET by dissection from the surrogate females. Three surrogate females were allowed to carry any developing fetuses to term. The results showed that 3.4% of NT embryos underwent implantation in the uterine lining of recipients, while 1.2% formed fetuses (Table 3). The remaining 106 NT embryos in three surrogate females were allowed to develop to term (Table 3). No live births occurred with M-phase microinjection-derived, NT embryos.

Using a second method, one or two demecolcin-treated M-phase fibroblasts were inserted into the PVS of enucleated oocytes and fused via electrical pulse. The insertion of at least two cells in the PVS of each oocyte improved the efficiency of fusion of one cell of the pair (Li et al., 2005). Only those oocytes that underwent visible single cell fusion were used for activation with chemical stimulation. Following chemical activation, 359 NT embryos were transferred into eight surrogate females. The mid-term development of 222 NT embryos was assessed from five surrogate females at 3 weeks post-ET. Twelve NT embryos (5.4%) underwent implantation with four showing fetal development (1.8%). One of the three NT reconstructed fetuses that developed to 3 weeks (Fig. 3A and C) had identical size and morphology to that of in vivo conceived 3-week fetuses (Fig. 3D), while the remaining two NT reconstructed fetuses were smaller (Fig. 3B). The remaining 137 NT embryos in three surrogate females were allowed to develop to term (Table 3). No live births occurred with M-phase fusion-derived NT embryos.

## DISCUSSION

The phases of the somatic donor cell cycle and the recipient oocyte are of vital importance to ensure the normal development of embryos reconstructed by NT. The successful cloning of sheep using quiescent cells, synchronized in G0 by serum starvation, demonstrated that the nuclei from differentiated cells can be reprogrammed by the oocyte to direct the normal developmental program in mammals (Campbell et al., '96; Wilmut et al., '97). G0 cells are now commonly used for NT (Kato et al., '98; Baguisi et al., '99; Polejaeva et al., 2000; Galli et al., 2003; Woods et al., 2003), despite the uniformly low efficiency of live births resulting from this approach and data indicating that nuclei from cycling cells may support embryo development and the birth of live offspring (Cibelli et al., '98). Several reports have also suggested that M-phase cells may be viable chromatin donors and could provide an advantage over interphase or G0 cells because of the cell cycle similarity between these cells and the mature oocyte, which is arrested in metaphase of meiosis II (Alberio et al., 2000; Korfiatis et al., 2001; Zhou et al., 2001; Zhou et al., 2003). However, to our knowledge, no previous study has investigated the developmental potential of NT embryos from the ferret using M-phase cells.

In this study, we used demecolcine to synchronize ferret fetal fibroblasts in M-phase of the cell cycle, after varying lengths of time in culture, and evaluated their ability to direct development in surrogate females following NT. Demecolcine arrests cells in M-phase by virtue of its ability to depolymerize microtubules, thereby preventing spindle assembly and progression through

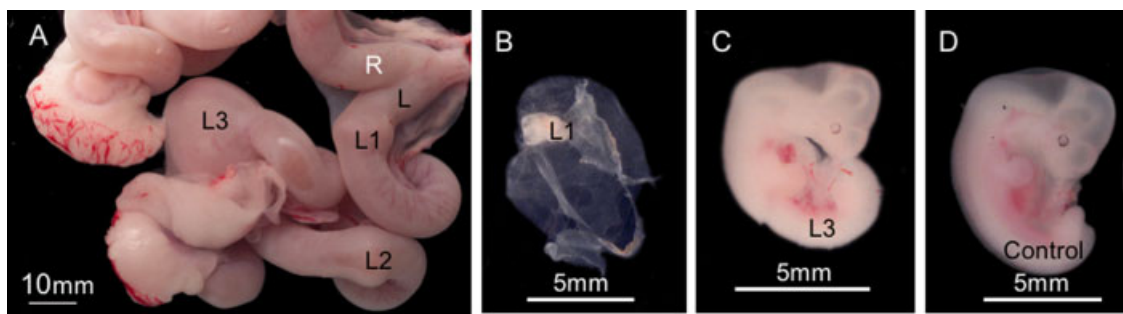


Fig. 3. In vivo development of NT embryos dissected from the recipient ferret at 3-week post-embryo transfer. (A) Ovaries, oviducts, and uterus were dissected from a recipient albino ferret 21 days following transfer of NT reconstructed embryos. The swollen regions (L1, L2 and L3) of the left uterus (L) are regions of implanted NT reconstructed embryos. R indicates the right uterus; (B and C) Fetuses marked L1 and L3 were dissected from the swollen regions of left uterus from panel (A). (D) Normal 3-week ferret fetus developed from in vivo fertilization by natural mating.

cytokinesis. Although mouse embryos treated with nocodazole showed a drug-induced delay in development (Kato and Tsunoda, '92), no remarkable deleterious drug-induced effects on treated ferret fibroblasts were observed in this study. The percentage of control cells in G0/G1-phase increased after prolonged passage of cells, while the percentage of control cells in S-phase and G2/M-phases decreased in a passage-dependent fashion. Similar trends were also observed in demecolcine-treated groups and in both cases, may reflect a general progression to cellular senescence. To collect M-phase cells from the synchronized populations, we used the mitotic "shake-off" technique (Korfiatis et al., 2001), which resulted in >90% M-phase-specific cells. Using M-phase fibroblast cells as "nuclear" donors, the NT embryos reconstructed by electrofusion showed a somewhat increased implantation rate (5.4% vs. 3.4%) and fetus formation rate (1.8% vs. 1.2%) relative to those reconstructed by microinjection. These rates using M-phase fibroblasts are lower than the 8.8% implantation rate, and the 3.3% fetus formation rate, using G0/G1-phase fibroblasts as nuclear donors in ferret NT (Li et al., 2003). Those lower developmental rates, although not statistically significant, when using M-phase donor nuclei compared to the G0/G1-phase nuclei, may suggest that G0/G1 nuclei are more compatible with embryonic development due to their diploid chromosome configuration.

Our results demonstrate that demecolcine treatment effectively synchronizes ferret fibroblasts in M-phase of the cell cycle, and that metaphase chromatin from these cells can be reprogrammed to support the development of ferret NT embryos. These results should facilitate the development of cloned ferrets as an animal model for human lung disease such as influenza and CF.

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