

## Developmental Capacity of Ferret Embryos by Nuclear Transfer Using G0/G1-Phase Fetal Fibroblasts<sup>1</sup>

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### ABSTRACT

With the ultimate goal of establishing experimental protocols necessary for cloning ferrets, the present study has established parameters for the reconstruction of ferret embryos by nuclear transfer (NT) using G0/G1-phase donor fetal fibroblasts. Cumulus-oocyte complexes were harvested from superovulated ferrets and cultured in maturation medium for 24 h. Matured oocytes were then enucleated and injected with the fibroblast nuclei derived from 14–16-h serum-starved cells. Reconstructed embryos were then activated by a combination of electric pulses and chemical stimulations. Subsequently, the reconstructed and activated embryos were either cultured *in vitro* or transferred to pseudopregnant ferrets to evaluate their developmental capacity *in vitro* and *in vivo*. Our results demonstrated that 56.3% of reconstructed embryos ( $n = 187$ ) cleaved, while 26.0% and 17.6% developed to morula and blastocyst phases *in vitro*, respectively. The blastocysts derived from NT embryos demonstrated normal morphology by differentially staining as compared to normal blastocysts developed *in vivo* following fertilization. *In vivo* developmental studies at 21 days posttransplantation demonstrated 8.8% of reconstructed embryos ( $n = 91$ ) implanted into the uterine lining of recipients, while 3.3% formed fetuses. However, reconstructed embryos ( $n = 387$ ) failed to develop to term (42 days). These results demonstrate donor nuclei of G0/G1-phase fetal fibroblast cells can be reprogrammed to support the development of reconstructed ferret embryos *in vitro* and *in vivo*; however, a significant third-trimester block occurs preventing full-term development.

early development, embryo

### INTRODUCTION

The domestic ferret, *Mustela putorius furo*, has been considered one of the best animal models for studying lung diseases such as cystic fibrosis (CF). In an effort to rationally develop ferret models of CF, a better understanding of embryology and nuclear transfer cloning techniques will

set the stage for genetic manipulation in this species. CF is a recessively inherited genetic disease caused by a defect in an epithelial chloride channel called the cystic fibrosis transmembrane conductance regulator (CFTR) [1]. CF patients suffer from recurrent bacterial infection in the lung leading to bronchiectasis, compromised lung function, and ultimately death. Substantial efforts have been made to generate mouse models capable of reproducing the lung pathology seen in CF patients. However, due to differences in lung biology between mice and humans, CFTR-deficient and mutant mice do not develop spontaneous lung disease as seen in humans [2, 3]. This lack of appropriate animal models of CF lung disease has hindered progress in the development and testing of therapies for this disease.

Several aspects of ferret lung biology make this species a potentially attractive model for CF lung disease. First, in contrast to mice, the ferret has marked similarities to humans in lung physiology, airway morphology, and cell types [4–9]. Second, the expression of CFTR in the ferret airway epithelium and submucosal glands is also identical to that in humans [10, 11]. Third, amino acid identity between ferret and human nucleotide binding domain 1 (NBD1) of CFTR is a striking 97% [10], which is just as high as nonhuman primates (96%, *Macaca nemestrina*) and significantly greater than rodents (80%, rat and mouse). Finally, the domestic ferret has proven useful for the study of human infectious lung disease due to the similarity between ferret and human lung biology [12–19].

Of small animal species, the ferret, with a gestation period of 42 days and 6 mo to sexual maturity, has obvious advantages over larger species, such as sheep and nonhuman primates, for animal modeling. Recent successes in creating cloned animals by somatic cell nuclear transfer [20–26] have made animal modeling in less studied species, such as the ferret, more feasible. To this end, we have previously reported conditions for superovulation in ferrets and *in vitro* culture of ferret embryos [27]. Subsequently, we established the optimal conditions for *in vitro* maturation and artificial activation of ferret oocytes [28]. These protocols have paved the way for genetic manipulation of ferret oocytes and embryos using genetically altered somatic cells and nuclear transfer technologies. However, procedures for reconstruction of ferret embryos using somatic cell nuclear donors have not been previously reported. In this study, we describe procedures for nuclear transfer of G0/G1-phase ferret fetal fibroblasts into enucleated oocytes and the subsequent development of reconstructed embryos *in vitro* and *in vivo*.

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## MATERIALS AND METHODS

### Reagents and Animals

All chemicals used in this study were purchased from Sigma Chemical (St. Louis, MO) and Invitrogen (Grand Island, NY) unless otherwise noted. Ferrets (6–18 mo of age) with sable-, cinnamon-, and albino-colored coats were purchased from Marshall Farms (North Rose, NY). Female ferrets were in estrous when delivered. The vasectomized male ferrets were also purchased from Marshall Farms and were used to induce pseudopregnancy in females. Vasectomized males were confirmed as sterile by mating at least five times to Jills and by the lack of sperm in ejaculates. All ferrets were housed in separate cages under controlled temperature (20–22°C) and long daylight cycle (16L:8D).

### Preparation of Nuclear Recipient Oocytes

The nuclear recipient oocytes used in this study were in vitro-matured ferret oocytes obtained from ovaries of superovulated sable ferrets. Superovulation of ferrets was performed by an i.p. injection of 100 IU/kg of eCG (Sigma, G-4877), followed by an i.p. injection of 75–100 IU/kg of hCG (Sigma, C-1063) at an interval of 72 h [27]. To retrieve oocytes, ferrets were killed by administration of sodium pentobarbital (50–100 mg/kg, i.p.) 12–24 h after hCG injection. The ovaries were excised and washed three times with 0.9% (w/v) saline supplemented with 1% (v/v) penicillin and streptomycin at 37–38.5°C. The small vesicular follicles (0.5–2.0 mm in diameter) on the ovary surface were incised with a scalpel in a Petri dish containing mPBS (Dulbecco PBS supplemented with 0.1% [w/v] D-glucose, 36 mg/L of pyruvate, and 0.4% [w/v] BSA) to release the cumulus-oocyte complexes (COCs). COCs with uniform cytoplasm and several layers of cumulus cells were selected, washed three times with mPBS, and cultured in the medium of TCM-199 (Gibco, Invitrogen, catalog no. 12340-030) + 10% (v/v) FBS (Sigma, F-4135) + 10 IU/ml of eCG (Sigma, G-4527) + 5 IU/ml of hCG (Sigma, C-8554). The COCs cultures were covered with mineral oil (Sigma, M-8410) and incubated at 38.5°C in 5% CO<sub>2</sub>, 95% air for 24 h [28]. After maturation, expanded cumulus cells of oocytes were removed by pipetting in mPBS containing 0.2% (w/v) hyaluronidase (Sigma, H-4272) for 1–3 min. Only oocytes with normal morphology and uniform cytoplasm and containing a first polar body were selected for enucleation and nuclear transfer.

### Establishment of Fetal Fibroblast Cell Line

Ferret fetal fibroblasts were obtained from 28 dpc (days postcopulation) fetuses derived from a Sable × Cinnamon mating (Marshall Farms, North Rose, NY). The heads and all internal organs of fetuses were removed, and each remaining carcass was minced individually in a Petri dish. Each minced carcass was then transferred into a 50-ml tube and digested with 25 ml of Trypsin:EDTA (Invitrogen, catalog no. 25200-056) for 1 h at 37°C. Twenty milliliters of fresh Trypsin:EDTA was then added, and cells were dispersed by gentle agitation and incubated for an additional 30 min at 37°C. Following trypsinization, cells were pipetted several times, and large chunks of tissue were allowed to settle for 1–2 min before removing the suspension cells to a new tube. Suspended cells were collected by centrifugation at 1500 rpm for 5 min. Cells were then resuspended and washed once in 25 ml PEF media (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 were plated into PEF media on two to three dishes (100 mm) per fetus and incubated at 37°C, 5% CO<sub>2</sub> and 95% air overnight. On the following day, cells were washed with 1× PBS and replaced with fresh PEF medium. Once cells reached confluency, they were frozen down as zero passage (two vials/100-mm dish) in 10% dimethyl sulfoxide (DMSO, Sigma D-2650)/10% FBS in DMEM and thawed as needed.

### Synchronization of Fibroblast Cell Line

After the birth of Dolly from presumed quiescent (G0) nuclei [20], serum-starved cells are commonly used for NT [22–24]. To this end, our initial studies were designed to examine the effect of serum starvation treatment on the cell cycle of ferret fetal fibroblasts at different time points in order to achieve synchronous populations of cells in G0/G1 phase. Fetal fibroblast cells 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 incubated at 37°C, 5% CO<sub>2</sub> and 95% air for 1–30 days. Cells were passaged (1:5) every 3–5 days when they reached confluency for a period of

30 days. For cell cycle synchronization experiments (serum starvation treatment), 50% confluent monolayers of fibroblast were passaged for 4, 9, 14, 19, 24, and 29 days, washed with 1× PBS, and treated with DMEM supplemented with 0.2% FBS overnight (14–16 h). Cells were then trypsinized and fixed in cooled 80% ethanol (–20°C) for 15 min at 4°C. The cell suspension was centrifuged, and the supernatant was carefully aspirated to avoid losing cells. The cells were incubated in a PBS solution containing 1 mg/ml RNase (Sigma, R-5125), 50 μg/ml propidium iodide (PI, Sigma P-4170), and 0.1% saponin (catalog 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 10000 cells were analyzed for each experimental condition by fluorescence-activated cell sorting (FACS) for DNA content (Becton Dickinson FACScan). Percentages of cells existing within the different phases of the cell cycle were calculated by gating on G0/G1, S, and G2/M cell populations. The gates were held constant for control and serum-starved samples. All experiments were performed in duplicate with three different cell lines.

For nuclear transfer experiments, fetal fibroblasts were serum starved for 14–16 h prior to harvesting. Cells were then washed three times with 1× PBS, trypsinized, and centrifuged at 1500 rpm for 5 min. The pelleted cells were then resuspended in 0.5–1.0 ml mPBS. Approximately 1 μl of the cell suspension was transferred into the micromanipulation chamber for use as the nuclear donors.

### Enucleation Efficiency and Nuclear Transfer

Prior to performing nuclear transfer experiments, enucleation efficiencies were evaluated in a separate set of experiments aimed at determining the extent of chromosomal DNA removal using fluorescent dyes. Oocytes matured in vitro were stained with 10 μg/ml of Hoechst 33342 for 5 min. Oocytes showing blue chromosomes and a first polar body (PB1) by fluorescent microscopy were then transferred to mPBS medium containing 7.5 μg/ml of cytochalasin B (Sigma, C-6762) in the micromanipulation chamber. Under microscopic Nomarski visualization, the PB1 and one third to one half of the cytoplasm close to the PB1 was removed from oocytes with a 25-μm (inside diameter, ID) glass pipette. The oocytes subjected to enucleation were checked for removal of chromosomal DNA by fluorescent microscopy.

Oocytes were not stained in Hoechst 33342 at any time for nuclear transfer experiments. After enucleation of the oocytes, the enucleation pipette was replaced by PiezoDrill pipette (flat opening, 10-μm ID, Humagen Fertility Diagnostics, Inc., Charlottesville, VA) for nuclear transfer. Under microscopic visualization, fibroblast cells (diameter 10–15 μm), cultured in vitro for 5, 10, 15, 20, and 25 days with passaging every 5 days, were aspirated into the pipette and transferred into the cytoplasm of enucleated oocyte using the PiezoDrill. The cell membranes of fibroblast donors were broken during the process of aspiration while leaving the nuclei intact.

### Activation of Reconstructed Embryos

The reconstructed/nuclear-transferred embryos were removed to electronic activation medium (0.3 M Mannitol, 0.1 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 0.5 mM Hepes, 0.01% [w/v] BSA) and placed between two parallel electrodes (1-mm spacing) in a BTX Electro-cell Manipulator 2001 chamber (BTX, San Diego, CA). Electrical stimulation consisted of an electrical pulse of 3 V AC for 5 sec followed by 1 DC pulse of 180 V/mm for 30 μsec. Following electrical stimulation, the reconstructed embryos were incubated in TCM-199 medium containing 5 μg/ml of cyclohexamide (Sigma, C-7689) for 5 min and 2 mM/ml 6-dimethylaminopurine (6-DMAP, Sigma, D-2629) for 4 h to facilitate chemical activation. These methods were previously shown to be optimal for in vitro activation of ferret oocytes [28].

### In Vitro Culture and Embryo Transfer of Reconstructed Embryos

For in vitro developmental studies, reconstructed embryos subjected to electronic and chemical stimuli were transferred to TCM-199 medium containing 10% (v/v) FBS and cultured at 38.5°C, 5% CO<sub>2</sub>, 95% air for 1–6 days as previously optimized for ferret embryos [27]. For in vivo developmental studies, reconstructed embryos subjected to electronic and chemical stimuli were transferred directly into the oviducts of pseudopregnant ferrets immediately following activation. A pseudopregnant state was achieved in recipient albino females through mating with vasectomized albino male 24 h prior to embryo transfer. Embryo transfer (ET) into albino recipient ferrets was performed following anesthetization by i.p. injection of 20–30 mg/kg each of ketamine HCl (Abbott Laboratories,

TABLE 1. Percentage (mean ± SEM) of cycling cells following serum starvation at increasing passage number.<sup>a</sup>

Cell cycles/groups	Percentage (mean ± SEM) of cycling cells following serum starvation					
	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
<b>G0/G1-phase</b>						
Control	68.4 ± 4.1 <sup>a</sup>	59.2 ± 1.6 <sup>a</sup>	57.6 ± 2.4 <sup>a</sup>	69.2 ± 1.5 <sup>a</sup>	74.6 ± 1.3 <sup>a</sup>	85.9 ± 3.7 <sup>a</sup>
Starvation	86.3 ± 2.3 <sup>b</sup>	85.9 ± 5.6 <sup>b</sup>	89.3 ± 6.1 <sup>b</sup>	90.7 ± 3.9 <sup>b</sup>	93.3 ± 0.7 <sup>b</sup>	93.5 ± 1.2 <sup>a</sup>
<b>G2/M-phase</b>						
Control	8.5 ± 1.0 <sup>a</sup>	12.4 ± 2.0 <sup>a</sup>	16.5 ± 2.2 <sup>a</sup>	9.5 ± 0.3 <sup>a</sup>	9.5 ± 0.4 <sup>a</sup>	5.8 ± 1.7 <sup>a</sup>
Starvation	3.2 ± 0.5 <sup>b</sup>	4.5 ± 1.4 <sup>b</sup>	5.7 ± 2.8 <sup>b</sup>	3.9 ± 1.0 <sup>b</sup>	4.6 ± 0.6 <sup>b</sup>	4.2 ± 0.7 <sup>a</sup>
<b>S-phase</b>						
Control	23.1 ± 3.6 <sup>a</sup>	28.4 ± 4.6 <sup>a</sup>	25.9 ± 0.4 <sup>a</sup>	21.3 ± 1.8 <sup>a</sup>	15.9 ± 1.6 <sup>a</sup>	8.3 ± 2.1 <sup>a</sup>
Starvation	10.5 ± 2.8 <sup>b</sup>	9.6 ± 4.3 <sup>b</sup>	5.0 ± 3.3 <sup>b</sup>	5.4 ± 2.9 <sup>b</sup>	2.1 ± 0.3 <sup>b</sup>	2.3 ± 0.5 <sup>a</sup>

<sup>a</sup> Differences among percentages containing different superscripted letters are significant when comparing the same time point and same cell cycle phase between control and serum-starved samples ( $P < 0.05$ ).

North Chicago, IL) and xylazine (Phoenix Pharmaceutical Inc., St. Joseph, MO). A 3–4-cm incision was made along the midline of the abdomen to expose the ovaries and oviducts. Between 20 and 40 nuclear transfer (NT) embryos were delivered into oviducts using a fine glass pipette. After the surgical wound was sutured, the ferret was allowed to wake up in its own cage under close monitoring.

*Assessment of the Embryonic Development*

For evaluation of embryonic development in vitro, reconstructed embryos were evaluated for various developmental stages (from two cell to blastocysts) at 24-h intervals postactivation by staining with Hoechst 33342 (Sigma, B-2261) and examination by fluorescent microscopy. The development of reconstructed embryos was compared to those harvested from in vivo fertilization by mating [28]. The nuclei of cleaved embryos showed blue fluorescence using a DAPI filter. Selected blastocysts were differentially stained with 10 µg/ml each of Hoechst 33342 and propidium iodide (PI), using a previously described method capable of differentiating nuclei from the inner cell mass (ICM) and trophectoderm (TE) [28]. This method allows for fluorescent microscopic examination of embryo morphology. Using this procedure, both nuclei of the ICM and TE fluoresced blue under a DAPI filter. Nuclei of the TE, but not ICM, fluoresced red using a N2.1 filter. When the two fluorescent channels were merged, nuclei of ICM fluoresced blue, and nuclei of TE fluoresced pink.

For evaluation of embryonic development in vivo, the recipient ferrets were either killed at 3 wk following ET (half-gestational time of ferret) or at full term (6 wk following ET) when they failed to give birth. The uterus was then evaluated for implantation plaques and fetuses. The in vivo development of 3-wk NT reconstructed fetuses was compared to that of normal 3-wk ferret fetuses developed from in vivo fertilization by normal mating.

*Statistical Analysis*

For statistical analysis of cell cycle synchronization data (Table 1) and data of in vitro development of reconstructed embryos (Table 2), the mean percentage (±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 analysis of variance (ANOVA) was used for statistic analysis with one independent variable (i.e., evaluating the effects of time comparing control and starvation groups in each cell cycle phase and effects of cell culture time on in vitro development of reconstructed embryos). When ANOVA demonstrated a significant difference, the follow-up Tukey multiple comparison test was performed to determine  $P$ -values for all possible two-group comparisons within the data set. The Student  $t$ -test was applied to data sets with only two groups (Table 1, same time point and same cell cycle phase, and Table 3, same type of cell). A significant difference was determined when the  $P$ -value was  $< 0.05$ .

**RESULTS**

*Cell Cycle Synchronization of Fibroblasts*

Results from cell cycle analysis demonstrated that the percentages of G0/G1-phase cells following serum starvation (85.9%–93.5%, from Day 5 to Day 25) were significantly higher ( $P < 0.05$ ) than those of control group (57.6%–74.6%). In contrast, the percentages of G2/M- and S-phase cells following serum starvation (G2/M: 3.2%–5.7%; S: 2.1%–10.5%; from Day 5 to Day 25) were significantly lower ( $P < 0.05$ ) than those of control group (G2/M: 8.5%–16.5%; S: 15.9%–28.4%). Passage number significantly ( $P < 0.05$ ) reduced the percentage of cells in control groups of S-phase (compared Days 5, 10, and 15 with Day 30) and G2/M-phase (compared Day 15 with Day 30) and significantly ( $P < 0.05$ ) produced a trend toward elevation in G0/G1-phase (compared Days 5, 10, 15, and 20 with Day 30) in a time-dependent fashion. Although similar trends were also seen in starvation groups, no statistical differences were observed (Table 1).

*Enucleation Efficiency, Nuclear Transfer, and In Vitro Development of Reconstructed Embryos*

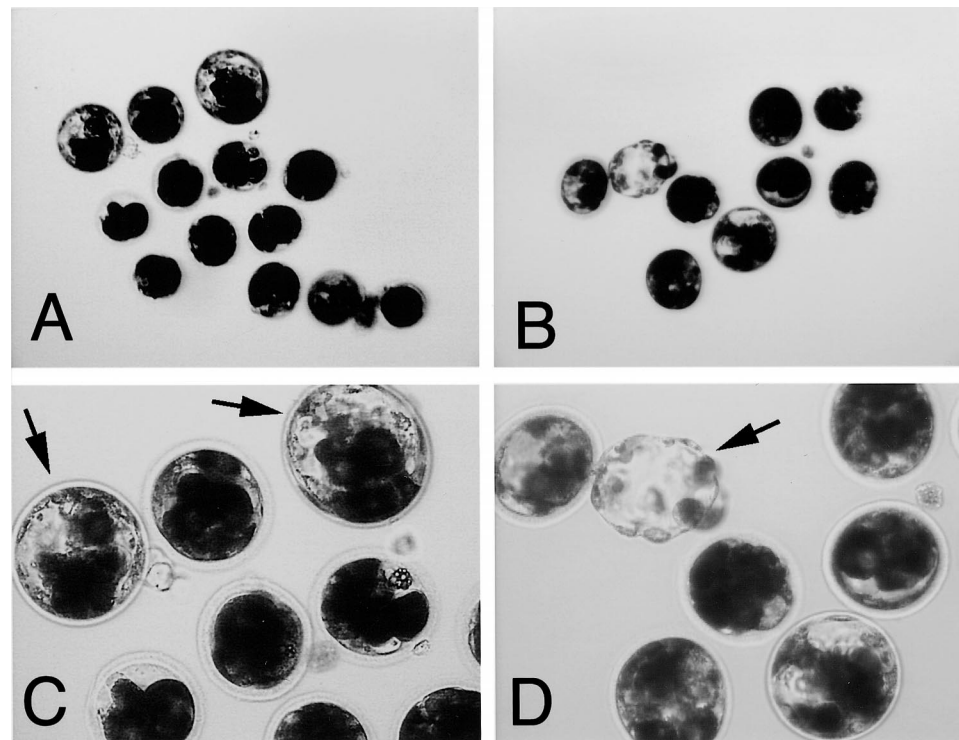
Following serum starvation of ferret fetal fibroblast cells, G0/G1-phase cells are the dominant population (85.9%–

TABLE 2. In vitro development of reconstructed embryos by nuclear transfer of ferret fetal fibroblasts.<sup>a</sup>

Groups	No. of ferrets	No. of oocytes used	Days of donor cells cultured in vitro	Percentage of the embryos developed to (mean ± SEM)		
				Cleavage	Morula	Blastocyst
1	3	36	5	38.7 ± 3.4 <sup>a</sup>	22.0 ± 4.3 <sup>a</sup>	13.8 ± 2.0 <sup>a</sup>
2	3	41	10	45.6 ± 4.4 <sup>a</sup>	25.1 ± 4.3 <sup>a</sup>	14.2 ± 2.5 <sup>a</sup>
3	3	35	15	48.7 ± 1.3 <sup>a</sup>	26.1 ± 5.1 <sup>a</sup>	14.6 ± 4.1 <sup>ab</sup>
4	3	37	20	64.4 ± 5.6 <sup>ab</sup>	27.4 ± 1.6 <sup>a</sup>	18.8 ± 1.2 <sup>ab</sup>
5	3	38	25	84.2 ± 0.8 <sup>b</sup>	29.2 ± 4.2 <sup>a</sup>	26.4 ± 1.4 <sup>b</sup>
Total	15	187	5–25	56.3 ± 5.6	26.0 ± 1.6	17.6 ± 1.8

<sup>a</sup> Differences among percentages containing different superscripted letters are significant ( $P < 0.05$ ).

FIG. 1. In vitro development of NT embryos cultured in TCM-199 + 10% FBS medium. **A**) Blastocysts derived from NT embryos. **B**) Hatched blastocysts derived from NT embryos. **C**) Enlarged from **A**. Arrows indicate blastocysts. **D**) Enlarged from **B**. Arrows indicate hatched blastocysts. Magnification  $\times 40$  (**A** and **B**) and  $\times 100$  (**C** and **D**).



93.5%). These synchronized G0/G1-phase fibroblasts were used for nuclear donors in subsequent nuclear transfer experiments. The results demonstrated that the enucleation rate of oocytes was 80.8% ( $n = 82$  oocytes from seven independent experiments). The results also indicated that the time of nuclear donor cell culture also significantly affected developmental potential of reconstructed embryos to cleavage and blastocysts (Table 2,  $P < 0.05$ ). Day 25 cultures of starved fetal fibroblast cells were the most efficient nuclear donors affording 84.2% cleavage and 26.4% blastocysts. On average for all the donor cell cultures (Days 5–25), 56.3% of reconstructed embryos ( $n = 187$ ) cleaved, and 26.0% and 17.6% developed to morula and blastocyst phases, respectively (Fig. 1 and Table 2). The time course for development of NT reconstructed embryos to cleavage (two-cell stage), morula, and blastocyst stages was typically 24, 120, and 144 h postactivation, respectively.

#### Cellular Counts of ICM and TE in Normal and NT Blastocysts

The reconstructed embryos that developed to blastocyst phase were differentially stained and examined under fluorescent microscopy for analysis of embryonic development (Fig. 2). These embryos were compared to those harvested from in vivo fertilization by mating. The results demonstrated that the blastocysts derived from reconstructed embryos appeared to have normal morphology and were composed of appropriate cell numbers of both ICM and TE as compared to normal blastocysts developed in vivo (Table 3).

#### In Vivo Development of Reconstructed Embryos by Nuclear Transfer of Ferret Fetal Fibroblasts

Four hundred seventy-eight ferret NT reconstructed embryos were transferred into 19 recipient albino pseudopregnant females. Among the recipients, 16 were allowed to go to full term (6 wk) and failed to give birth ( $n = 387$  NT

embryos). At that time the animals were killed, and the uterus was evaluated for implanted embryos; however, none were found (Table 4). In addition, three recipients were evaluated at an intermediate time point of half gestation (3 wk). Results of this analysis demonstrated that 8.8% of NT embryos ( $n = 91$ ) implanted into the uterine lining of recipients, while 3.3% formed fetuses (Fig. 3 and Table 4). One of the three NT reconstructed fetuses that developed to 3 wk had identical size and structure to that of in vivo-conceived 3-wk fetuses (Fig. 3D), while the remaining two NT reconstructed fetuses had blunted growth.

#### DISCUSSION

The successful cloning of sheep using cells synchronized in G0-phase of the cell cycle has demonstrated the capacity for reprogramming the DNA of differentiated cells [20, 29, 30]. Following that success, serum-starved cells are commonly used for NT [22–24], despite studies indicating that nuclei in other phases of the cell cycle may support embryo development and the birth of live offspring. For example, Cibelli et al. [31] reported that cycling cells, which may contain cells in different cycle phases, could be successfully used for nuclear transfer in cattle. However, the process of nuclear reprogramming after cloning is still poorly understood. It is thought to involve the replacement of factors that control chromatin structure and gene expression [32]

TABLE 3. Cellular counts of inner cell mass (ICM) and trophectoderm (TE) in native and transfer blastocysts.<sup>a</sup>

Blastocyst group	No. of blastocysts examined	Total cells (mean $\pm$ SEM)	ICM cells (mean $\pm$ SEM)	TE cells (mean $\pm$ SEM)
NT	11	72.2 $\pm$ 6.4 <sup>a</sup>	12.4 $\pm$ 1.9 <sup>a</sup>	59.8 $\pm$ 4.6 <sup>a</sup>
Native	5	78.2 $\pm$ 3.9 <sup>a</sup>	15.6 $\pm$ 1.7 <sup>a</sup>	62.6 $\pm$ 4.9 <sup>a</sup>

<sup>a</sup> In the same column, differences among these groups containing same superscripted letters are not significant ( $P > 0.05$ ) as determined using the Student *t*-test.

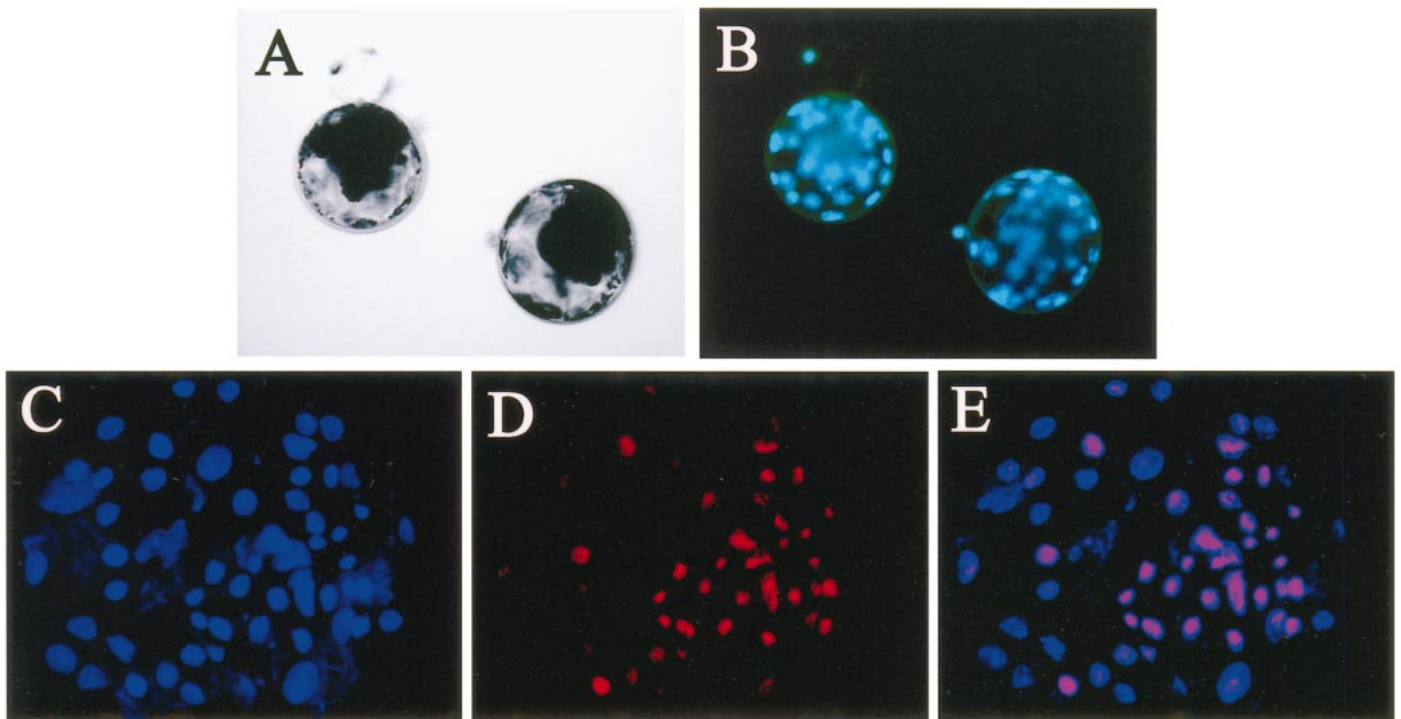


FIG. 2. Differential staining of ferret NT embryos with nuclear dyes. The blastocysts derived from NT embryos before (A) and after (B–E) Hoechst 33342 and PI differential staining are showing as phase-contrast (A) and fluorescent (B–E) photomicrographs. A) The blastocysts before staining. B) Total cells of the blastocyst appear blue using a DAPI filter (without coverslip). C) Total cells of the blastocyst appear blue using a DAPI filter (note embryo is flattened with coverslip). D) TE cells appear red using a N2.1 filter (note embryo is flattened with coverslip). E) Merged DAPI and N2.1 images. The ICM cells appear blue, whereas the TE cells appear pink. Magnification  $\times 100$  (A and B) and  $\times 200$  (C and D).

with oocyte-derived factors that can redirect chromatin structure and function to the requirements of the oocyte and embryo [33, 34]. Such parameters of NT donor cells have not been established for ferret cloning. In this study, we described the production of ferret NT reconstructed embryos using G0/G1-phase fetal fibroblast cells as donors and their subsequent developmental capacity in vitro and in vivo. Our results demonstrate that decondensed chromatin from G0/G1-phase fetal fibroblast cells can be reprogrammed to support the development of reconstructed ferret embryos in vitro and in vivo.

As shown in Table 1, we first evaluated the optimal parameters to synchronize ferret fetal fibroblasts in the G0/G1-phase of the cell cycle using serum starvation. Our results indicated that G0/G1-phase cells are the dominant population (85.9–93.5%) following serum starvation. Furthermore, passage number also significantly ( $P < 0.05$ ) increased the percentage of fibroblasts in G0/G1-phase in a time-dependent fashion. Similar passage-dependent trends

were also evident in serum-starved groups, though no statistical differences were observed. When fibroblasts were cultured for 30 days, no statistical differences were observed between control and serum starvation groups at any cell cycle phase. This results suggested that serum starvation treatment on ferret fetal fibroblasts cultured for 30 days might not be necessary when G0/G1-phase fibroblasts were needed for ferret NT. Since prolonged passage can adversely affect the capacity of a somatic cell to be reprogrammed in oocytes, we selected serum starvation of 5–25-day cultures as the optimal synchronization procedure for NT. Although choosing this condition for preparing nuclear donors suggests that cells in our study were derived from G0/G1-phase fibroblast, it is still plausible that G2/M- and/or S-phase cells may have contributed to the successful nuclear donor pool.

In our previous study, we demonstrated the feasibility of in vitro culture of ferret embryos [27] and conditions for in vitro maturation and artificial activation of ferret oocytes

TABLE 4. In vivo development of reconstructed embryos by nuclear transfer (NT) of ferret fetal fibroblasts.

Experimental animal number of recipient	No. of NT embryos transferred	Days of donor cells cultured in vitro	Week of uterus evaluation after ET <sup>a</sup>	No. of fetuses/embryo implantations <sup>b,c</sup>
1	24	10	3	3 fetuses/3 implantations
2	33	15	3	0 fetus/5 implantations
3	34	10	3	None
4–19	387 (25.2 $\pm$ 1.4 <sup>d</sup> )	5–25	6	None

<sup>a</sup> ET = embryo transfer.

<sup>b</sup> Fetus formation rate for experiments 1–3 was 3.3% (3/91).

<sup>c</sup> Implantation rate for experiments 1–3 was 8.8% (8/91).

<sup>d</sup> Mean  $\pm$  SEM.

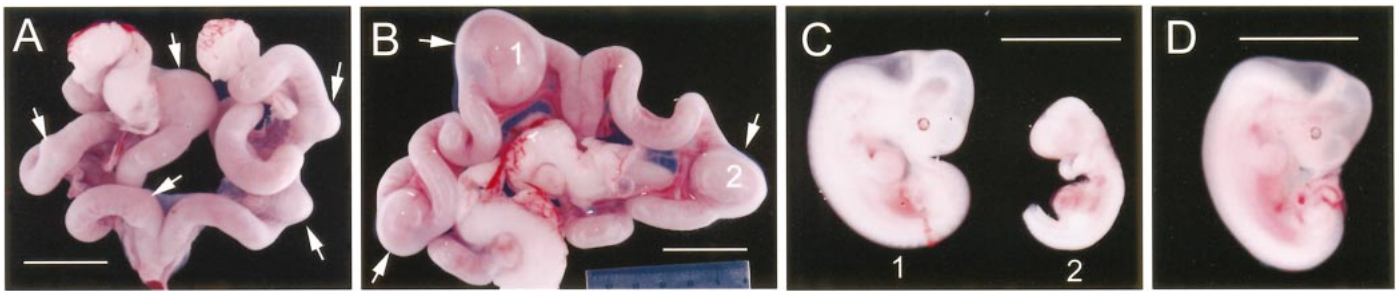


FIG. 3. In vivo development of NT embryos dissected from the recipient ferret at 3 wk post-embryo transplantation. **A** and **B**) Ovaries, oviducts, and the uterus were dissected from two recipient albino ferrets 21 days following transfer of 24–33 NT reconstructed embryos each. The swollen regions of the uterus (denoted by arrows) are regions of implanted NT reconstructed embryos. **C**) Fetuses marked 1 and 2 were dissected from the swollen regions of the uterus in **B**. **D**) Normal 3-wk ferret fetus developed from in vivo fertilization by natural mating. Scale marker: **A** and **B**, 1.5 cm; **C** and **D**, 0.5 cm.

[28]. These protocols were used in this study for 1) in vitro maturation of nuclear recipient oocytes, 2) electrical and chemical activation on nuclear transfer embryos, and 3) in vitro culture of nuclear transfer embryos. When combined with the methods of nuclear transfer described in this study, we observed 56.3% of NT embryos ( $n = 187$ ) progressed to cleavage, while 17.6% of them developed to a blastocyst cell phase in vitro. These blastocysts retained normal morphology and distribution of TE and ICM nuclei as seen in native embryos generated by in vivo fertilization. However, the level of blastocyst development remains lower than that previously observed with parthenogenetic-derived embryos (43.8%,  $n = 58$ ) [28]. Although none of NT embryos ( $n = 387$ ) developed to term in 16 recipient ferrets, the results of 8.8% implantation and 3.3% fetal development of NT embryos ( $n = 91$ ) are encouraging. These findings suggest that although our protocols for NT reconstruction are sufficient to retain some developmental potential of embryos in vivo, they still require further optimization.

Many factors involved in the NT procedure could explain this lower developmental competence of ferret NT embryos. First, it may stem from an inappropriate or partial synchronization of donor cells, though any effect of confluence or serum starvation on slowing or arresting the cell cycle was readily reversible [35]. Second, inadequate recipient cytoplasts may result in low development of NT embryos. In this study, in vitro-matured ferret oocytes were used as sources of recipient cytoplasts. Therefore, synchronization of recipient oocytes prior to the enucleation may be an important factor in the subsequent development of NT embryos. Third, the reduced cytoplasmic volume removed during the enucleation procedure may be another reason of lower blastocyst development. Finally, matching the developmental stage of NT reconstructed embryos with the optimal implantation time of recipient ferrets could be another reason for the lower NT embryo development in vivo. For example, the rapid developmental window for implantation in the rabbit has necessitated the need for stringent asynchronous breeding schedules of recipients to compensate for the lag in differentiation of NT reconstructed embryos and allow for efficient implantation [26]. It is presently unclear why implanted NT reconstructed embryos often abort during the third trimester [36]. Further optimization of the previously mentioned parameters may help to increase in vivo development of NT embryos in the ferret to full term.

In conclusion, the results of this study demonstrate that the donor nuclei of G0/G1-phase fetal fibroblast cells can be reprogrammed to support the development of recon-

structed ferret embryos in vitro and in vivo. However, cumulatively our experience to date suggests that a significant third-trimester block in NT embryo development remains a significant obstacle for cloning ferrets and requires further optimization.

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#### REFERENCES

1. Welsh MJ, Tsui L-C, Boat TF, Beaudet AL. Cystic fibrosis. In: Scriver CL, Beaudet AL, Sly WS, Valle D (eds.), *The Metabolic Basis of Inherited Disease*, 7th ed. New York: McGraw-Hill; 1995:3799–3876.
2. Davidson DJ, Rolfe M. Mouse models of cystic fibrosis. *Trends Genet* 2001; 17:S29–S37.
3. Grubb BR, Boucher RC. Pathophysiology of gene-targeted mouse models for cystic fibrosis. *Physiol Rev* 1999; 79:S193–S214.
4. Oldham MJ, Phalen RF, Huxtable RF. Growth of the ferret tracheo-bronchial tree. *Lab Anim Sci* 1990; 40:186–191.
5. Leigh MW, Gambling TM, Carson JL, Collier AM, Wood RE, Boat TF. Postnatal development of tracheal surface epithelium and submucosal glands in the ferret. *Exp Lung Res* 1986; 10:153–169.
6. Plopper CG, Hill LH, Mariassy AT. Ultrastructure of the nonciliated bronchiolar epithelial (Clara) cell of mammalian lung. III. A study of man with comparison of 15 mammalian species. *Exp Lung Res* 1980; 1:171–180.
7. Duan D, Sehgal A, Yao J, Engelhardt JF. Lef1 transcription factor expression defines airway progenitor cell targets for in utero gene therapy of submucosal gland in cystic fibrosis. *Am J Respir Cell Mol Biol* 1998; 18:750–758.
8. Kishioka C, Okamoto K, Kim J, Rubin BK. Regulation of secretion from mucous and serous cells in the excised ferret trachea. *Respir Physiol* 2001; 126:163–171.
9. Wang X, Zhang Y, Amberson A, Engelhardt JF. New models of the tracheal airway define the glandular contribution to airway surface fluid and electrolyte composition. *Am J Respir Cell Mol Biol* 2001; 24:195–202.
10. Sehgal A, Presente A, Engelhardt JF. Developmental expression patterns of CFTR in ferret tracheal surface airway and submucosal gland epithelia. *Am J Respir Cell Mol Biol* 1996; 15:122–131.
11. Engelhardt JF, Yankaskas JR, Ernst SA, Yang Y, Marino CR, Boucher RC, Cohn JA, Wilson JM. Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nat Genet* 1992; 2: 240–248.
12. Sweet C, Jakeman KJ, Bush K, Wagaman PC, McKown LA, Streeter AJ, Desai-Krieger D, Chand P, Babu YS. Oral administration of cyclopentane neuraminidase inhibitors protects ferrets against influenza virus infection. *Antimicrob Agents Chemother* 2002; 46:996–1004.

13. Fenton RJ, Morley PJ, Owens IJ, Gower D, Parry S, Crossman L, Wong T. Chemoprophylaxis of influenza A virus infections, with single doses of zanamivir, demonstrates that zanamivir is cleared slowly from the respiratory tract. *Antimicrob Agents Chemother* 1999; 43:2642–2647.
14. Leigh MW, Connor RJ, Kelm S, Baum LG, Paulson JC. Receptor specificity of influenza virus influences severity of illness in ferrets. *Vaccine* 1995; 13:1468–1473.
15. Collie MH, Rushton DI, Sweet C, Smith H. Studies of influenza virus infection in newborn ferrets. *J Med Microbiol* 1980; 13:561–571.
16. Jakeman KJ, Rushton DI, Smith H, Sweet C. Exacerbation of bacterial toxicity to infant ferrets by influenza virus: possible role in sudden infant death syndrome [published erratum appears in *J Infect Dis* 1991 Jul;164(1):232]. *J Infect Dis* 1991; 163:35–40.
17. Hussein RH, Collie MH, Rushton DI, Sweet C, Smith H. The role of naturally-acquired bacterial infection in influenza-related death in neonatal ferrets. *Br J Exp Pathol* 1983; 64:559–569.
18. Durchfeld B, Baumgartner W, Krakowka S. Intranasal infection of ferrets (*Mustela putorius furo*) with canine parainfluenza virus. *Zentralbl Veterinarmed B* 1991; 38:505–512.
19. Kishioka C, Okamoto K, Hassett DJ, de Mello D, Rubin BK. *Pseudomonas aeruginosa* alginate is a potent secretagogue in the isolated ferret trachea. *Pediatr Pulmonol* 1999; 27:174–179.
20. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997; 385:810–813.
21. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 1998; 394:369–374.
22. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of a single adult. *Science* 1998; 282:2095–2098.
23. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW, Echelard Y. Production of goats by somatic cell nuclear transfer. *Nat Biotechnol* 1999; 17:456–461.
24. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH. Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* 2000; 407:86–90.
25. Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L, Westhusin M. A cat cloned by nuclear transplantation. *Nature* 2002; 415:859.
26. Chesne P, Adenot PG, Viglietta C, Baratte M, Boulanger L, Renard JP. Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nat Biotechnol* 2002; 20:366–369.
27. Li ZY, Jiang QS, Zhang YL, Liu XM, Engelhardt JF. Successful production of offspring after superovulation and in vitro culture of embryos from domestic ferrets (*Mustela putorius furo*). *Reproduction* 2001; 122:611–618.
28. Li Z, Jiang Q, Rezaei Sabet M, Zhang Y, Ritchie TC, Engelhardt JF. Conditions for in vitro maturation and artificial activation of ferret oocytes. *Biol Reprod* 2002; 66:1380–1386.
29. Campbell KH, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 1996; 380:64–66.
30. Campbell KH, Loi P, Otaegui PJ, Wilmut I. Cell cycle co-ordination in embryo cloning by nuclear transfer. *Rev Reprod* 1996; 1:40–46.
31. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM. Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. *Nat Biotechnol* 1998; 16:642–646.
32. Wolffe AP, Khochbin S, Dimitrov S. What do linker histones do in chromatin? *Bioessays* 1997; 19:249–255.
33. Kubiak JZ, Prather RS, Maul GG, Schatten G. Cytoplasmic modification of the nuclear lamina during pronuclear-like transformation of mouse blastomere nuclei. *Mech Dev* 1991; 35:103–111.
34. Prather RS, Rickords LE. Developmental regulation of an snRNP core protein epitope during pig embryogenesis and after nuclear transfer for cloning. *Mol Reprod Dev* 1992; 33:119–123.
35. Kasinathan P, Knott JG, Wang Z, Jerry DJ, Robl JM. Production of calves from G1 fibroblasts. *Nat Biotechnol* 2001; 19:1176–1178.
36. Renard JP, Zhou Q, LeBourhis D, Chavatte-Palmer P, Hue I, Heyman Y, Vignon X. Nuclear transfer technologies: between successes and doubts. *Theriogenology* 2002; 57:203–222.