

Factors Affecting the Electrofusion of Mouse and Ferret Oocytes With Ferret Somatic Cells

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ABSTRACT The domestic ferret, *Mustela putorius furo*, holds great promise as a genetic model for human lung disease, provided that key technologies for somatic cell nuclear transfer (SCNT) are developed. In this report, we extend our understanding of SCNT in this species by defining conditions for efficient cell fusion by electrical pulse. Two experimental systems were employed in this study. First, in vivo-matured mouse oocytes and ferret somatic cells were used to establish general parameters for fusion. One fibroblast, or cumulus cell, was agglutinated to nucleate, zona pellucida-free, mouse oocytes, and subjected to an electrical pulse. Similar electrical pulse conditions were also tested with 1 or 2 somatic cells inserted into the perivitelline space (PVS) of intact mouse oocytes. The fusion rate for a single fibroblast with a zona-free oocyte was 80.2%, significantly higher ($P < 0.05$) than that observed for 1, or 2, fibroblasts placed in the PVS (52.0% and 63.8%, respectively). The fusion rate (44.1%) following insertion of two cumulus cells was significantly higher ($P < 0.05$) than that following insertion of one cumulus cell (25.1%). Second, in vitro-matured ferret oocytes were enucleated, and one to three fibroblasts or cumulus cells were inserted into the PVS. Zona pellucida-free ferret oocytes were fragile and excluded from the study. The fusion rates with two or three fibroblasts were 71.4% and 76.8%, respectively; significantly higher ($P < 0.05$) than that for one fibroblast (48.6%). This cell number-dependent difference in fusion efficiency was also observed with cumulus cells. Fusion-derived (ferret–ferret) NT embryos cleaved, formed blastocysts in vitro, and underwent early-stage fetal development following embryo transfer. The rate of development was cell type-independent, in contrast to the cell type-dependent differences observed in fusion efficiency. In conclusion, fibroblasts fused more efficiently than cumulus cells and the efficiency of single cell fusions was improved when two or more cells were inserted into the PVS. These studies define conditions for efficient cell fusion with ferret oocytes and should facilitate SCNT and the development of genetically defined animal models in this species. *Mol. Reprod. Dev.* 72: 40–47, 2005.

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Key Words: electrofusion; cell-oocyte couplet; fetal fibroblast cell; cumulus cell; mouse; ferret

INTRODUCTION

The domestic ferret is an excellent model system for the study of human lung disease associated with influenza virus infection (Husseini et al., 1983; Fenton et al., 1999). Indeed, the ferret also holds tremendous promise as a genetic model for human lung diseases, such as cystic fibrosis (CF). A CF ferret model would be attractive for several reasons. First, ferrets and humans share a remarkably similar airway cytoarchitecture (Leigh et al., 1986; Mercer et al., 1994), a feature not shared between humans and mice. Second, the expression pattern of the CF transmembrane conductance regulator (CFTR) protein, the defective protein that causes CF, is extremely similar in ferret and human airways (Engelhardt et al., 1992; Sehgal et al., 1996). In contrast to mice, ferrets and human tracheobronchial airways contain abundant submucosal glands that express high levels of CFTR (Engelhardt et al., 1992; Sehgal et al., 1996; Choi et al., 2000). Moreover, the ferret has a 42-day gestation time and reaches sexual maturity in 4 to 5 months (Fox and Bell, 1998), making it one of the more rapidly reproducing species for animal modeling by SCNT.

A key requirement for genetic modeling using the ferret is the optimization of somatic cell nuclear transfer (SCNT) methodologies capable of producing viable ferret clones. To this end, we previously defined conditions for superovulation in the ferret, for in vitro

Grant sponsor: National Institutes of Health (J.F.E.); Grant number: DK47967; Grant sponsor: National Institutes of Health (M.J.W.); Grant number: HL61234.

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Received 10 March 2005; Accepted 16 April 2005
Published online 9 June 2005 in Wiley InterScience
(www.interscience.wiley.com).
DOI 10.1002/mrd.20321

culture of ferret embryos (Li et al., 2001) and for in vitro maturation and artificial activation of ferret oocytes (Li et al., 2002b). Furthermore, we recently demonstrated that direct injection of G₀/G₁-phase ferret fibroblast nuclei into enucleated oocytes results in blastocyst development in vitro, and fetus production following embryo transfer (Li et al., 2003), demonstrating that cultured cells can be used to drive early-stage fetal development of nuclear transfer (NT) ferret embryos. However, maturation of late-stage NT ferret embryos remains an inefficient process and further optimization of SCNT procedures is needed to produce viable clones and to enable genetic modeling with this species.

Electrofusion is a powerful approach for genome delivery in SCNT and we now describe conditions that promote efficient fusion of ferret fibroblasts or cumulus cells with mouse and ferret oocytes using an electrical pulse. Our data demonstrate the feasibility of this approach and reveal cell number- and cell type-dependent differences in fusion efficiency that should increase the likelihood of generating normal ferret clones.

MATERIALS AND METHODS

Chemicals and Animals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Invitrogen Co. (Grand Island, NY) unless otherwise noted. Mice (female, B6SJL1/J, 4–6 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at four animals per cage under controlled temperature (70°F) and light cycle (12L:12D) conditions. All ferrets were purchased from Marshall Farms (North Rose, NY). Six-month old virgin female ferrets, with sable-colored coats were in estrus when received. Ten-month old vasectomized albino male ferrets were used for mating female ferrets to induce follicular oocyte maturation in oocyte donor ferrets and to induce pseudopregnancy in recipient females. All ferrets were housed individually under controlled temperature (65°F–66°F) and long day light cycle (16L:8D) conditions. Animal use was carried out according to a protocol approved by the University of Iowa Institutional Animal Care and Use Committee and conformed to or exceeded standards set by the National Institutes of Health.

Oocyte Preparation

In vivo matured mouse oocytes, and in vitro matured ferret oocytes, were used in this study. Mouse superovulation was performed by an i.p. injection of 5 IU eCG (Sigma, G-4877), followed by an i.p. injection of 5 IU hCG (Sigma, C-1063) at an interval of 48 hr. Eighteen hours after hCG injection, cumulus–oocyte complexes (COCs) were collected from oviducts and treated for 1–3 min in M2 medium (Sigma, M-7167) containing 0.1% (w/v) hyaluronidase (Sigma, H-4272) to remove cumulus cells. These cumulus cell-free oocytes were either used directly in fusion experiments or were stripped of their zona pellucida before somatic cell agglutination and fusion (see below).

Immature ferret oocytes were harvested from ovaries derived from estrous females mated with vasectomized males 24 hr prior to the collection. For ferret oocyte collection, 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 pyruvate and 0.4% (w/v) BSA) to release COCs. COCs with uniform cytoplasm and several layers of cumulus cells were selected, washed with mPBS, and cultured in TCM-199 medium (Gibco, Invitrogen Co., Cat No. 12340-030) + 10% (v/v) FBS (Sigma, F-4135) + 10 IU/ml eCG (Sigma, G-4527) + 5 IU/ml of hCG (Sigma, C-8554), covered with mineral oil (Sigma, M-8410) at 38.5°C in 5% CO₂, 95% air for 22 hr (Li et al., 2002b). After maturation, expanded cumulus cells were removed by pipetting in mPBS containing 0.2% (w/v) hyaluronidase (Sigma, H-4272) for 1–3 min. Only oocytes with normal morphology, uniform cytoplasm and containing a first polar body were selected for this study.

Preparation of Fetal Fibroblasts and Cumulus Cells

Ferret fetal fibroblast cells were obtained from 28 dpc (days post copulation) fetuses derived from a Sable (female) × Cinnamon (male) mating (Marshall Farms, North Rose, NY) (Li et al., 2003). Briefly, the heads, and all internal organs of 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, 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 of 2-mercaptoethanol (Sigma, M-7522)]. Finally, cells from each fetus were plated in PEF medium onto 2–3 dishes (100 mm) and incubated at 37°C, 5% CO₂, 95% air for overnight. Once cells reached confluence, they were frozen as “zero passage” cells (2 vials per 100 mm dish) in DMEM containing 10% dimethyl sulfoxide (DMSO, Sigma D-2650), and 10% FBS, and thawed as needed. Thawed fibroblasts were passaged between one and three times before NT.

For cumulus cell preparation, the ferret COCs cultured in vitro for 22 hr were collected, and treated with mPBS containing 0.2% (w/v) hyaluronidase for 1–3 min to disperse cumulus cells. These cells were transferred to 10% FBS/DMEM medium, centrifuged at 1,500 rpm for 5 min, and then cultured in 10% FBS/DMEM medium for 3–7 days before use. Both fibroblast and cumulus cell cultures reached ~70%–80% confluence when cells were harvested and used for NT.

Cell-Oocyte Couplet Construction and Electrofusion

Somatic cell-oocyte couplets were constructed using two procedures; (1) zona pellucida-free agglutination, and (2) perivitelline space (PVS) insertion. In the first

method, zonae pellucidae were removed from mouse oocytes using Tyrode's solution (acidic, pH 2.5, Sigma, T-1788). Cultured ferret fibroblasts or cumulus cells (15–20 μm in diameter) were then placed in M2 medium containing 200 $\mu\text{g}/\text{ml}$ of phytohemagglutinin PHA-P (Sigma, L-9132) with the zona-free oocytes. Using a stereoscope (Leica S6E) with magnification 40 \times , one oocyte was agglutinated to one fibroblast cell or cumulus cell by gently pressing the two cells together with a fine blunt glass needle. In the second method, in vitro matured ferret oocytes were enucleated by aspirating the first polar body (PB1), and the metaphase chromosomes, with a small amount of surrounding cytoplasm in mPBS containing 7.5 $\mu\text{g}/\text{ml}$ cytochalasin B (CB, Sigma C-6762). One to three individual somatic cells were then inserted into the PVS. In mouse oocytes, the PB1 was removed and one to two somatic cells were inserted into the PVS. Mouse oocytes were not enucleated so that pulse-induced fusion (and activation) could be monitored by endogenous nuclear migration.

The homologous (ferret–ferret) and heterologous (mouse–ferret) couplets were transferred to electrical fusion medium [0.28M mannitol, 0.1 mM MgCl_2 , 0.1 mM CaCl_2 , 0.5 mM HEPES, 0.01% (w/v) BSA], 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 of 1 DC of 180 V/mm for 30 μsec using a BTX ECM 2001 (BTX, San Diego, CA). Homologous couplets were then cultured in TCM-199 + 10% FBS medium and the heterologous couplets in M16 medium (Sigma, M-7292) for 0.5–1 hr before evaluation. Some of the heterologous couplets were cultured for 6 hr in M16 medium containing 5 $\mu\text{g}/\text{ml}$ CB to prevent exclusion of the polar body from activated oocytes before Hoechst 33342 (Sigma B-2261) staining.

Assessment of Cell Fusion

Couplets receiving an electrical pulse were examined microscopically for fusion. Breakdown of the flat membrane diaphragm, loss of the “fusion cone” from the PVS, and cell mixing were used as evidence for successful fusion. When fused nuclei within the oocyte cytoplasm

was inconclusive, couplets were also stained with 10 $\mu\text{g}/\text{ml}$ Hoechst 33342 for 5 min and examined by UV fluorescence. Only fusion events that met at least one of the above criteria were scored as positive.

In Vitro and In Vivo Development of Homologous Nuclear Transfer (NT) Embryos

The majority of homologous (i.e., ferret–ferret) NT embryos fused by electrical pulse were incubated in TCM-199 medium containing 5 $\mu\text{g}/\text{ml}$ cyclohexamide and 2 mM 6-dimethylaminopurine for 1 hr to facilitate activation. The embryos were then either transferred to TCM-199+10% FBS and cultured at 38.5°C, 5% CO_2 , and 95% air for 1–7 days, or to pseudopregnant recipient ferrets and examined for mid-term development (21 days post copulation) after recipients were euthanized.

Statistical Analysis

The mean percentage (\pm SEM) of fusion events was calculated for each experimental group (Tables 1 and 2). The normality assumption of the percentages for each data set was checked by the Kolmogorov–Smirnov test, and the Bartlett test was performed to justify the equal variance assumption of the percentages for each data set. Both tests were conducted using Minitab 13 software (Minitab, Inc., State College, PA). When these two tests indicated that the two assumptions were valid, two-way ANOVA was used for statistical analysis with two independent variables (i.e., evaluating effects of cell number, and somatic cell type, on electrofusion rate). 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. For all statistical analysis including ANOVA and Tukey, a difference was considered to be significant when the *P*-value was <0.05 . All statistical analysis was performed using the SAS 8.0 statistical software (SAS Institute, Inc., Cary, NC).

RESULTS

Fusion of Ferret Somatic Cells With Mouse Oocytes

Mouse oocytes were matured in vivo and subsequently fused with ferret somatic cells in our pilot experiments

TABLE 1. Electroporation of Mouse Oocyte With Cultured Ferret Fetal Fibroblasts and Cumulus Cells

Group ^a	Cell type	Cell number ^c		Number of oocytes used	Number of oocytes fused	Percent of oocytes fused (Mean \pm SEM) ^b
		A	I			
1	Fibroblast	1	—	87	70	80.2 \pm 3.1 ^a
2	Fibroblast	—	1	81	42	52.0 \pm 4.1 ^{bc}
3	Fibroblast	—	2	86	55	63.8 \pm 3.7 ^{ab}
4	Cumulus	1	—	83	31	38.8 \pm 5.9 ^{cd}
5	Cumulus	—	1	94	23	25.1 \pm 4.2 ^d
6	Cumulus	—	2	87	38	44.1 \pm 4.1 ^c

^aFusions with each experimental group was carried out in triplicate.

^bTwo-way ANOVA and the follow-up Tukey multiple comparison test were used to assess significant difference among groups. Differences among percentages containing the different superscripted letter are significant ($P < 0.05$).

^cA, agglutination with zona-free oocytes; I, insertion into PVS of intact oocytes.

TABLE 2. Electrofusion of Ferret Oocyte With Cultured Ferret Fetal Fibroblasts and Cumulus Cells

Group ^a	Cell type	Number of cells inserted ^c	Number of oocytes used	Number of oocytes fused	Percent of oocytes fused (Mean \pm SEM) ^b
1	Fibroblast	1	116	56	48.6 \pm 4.1 ^b
2	Fibroblast	2	103	73	71.4 \pm 4.4 ^a
3	Fibroblast	3	105	81	76.8 \pm 5.2 ^a
4	Cumulus	1	115	36	30.8 \pm 2.8 ^c
5	Cumulus	2	111	65	58.8 \pm 2.9 ^{ab}
6	Cumulus	3	109	72	66.7 \pm 3.8 ^{ab}

^aFusions with each experimental group was carried out in triplicate.

^bTwo-way ANOVA and the follow-up Tukey multiple comparison test were used to assess significant difference among groups. Differences among percentages containing the different superscripted letter are significant ($P < 0.05$).

^cCells inserted into PVS of intact oocytes.

(Fig. 1). Oocytes were used directly after removal of cumulus cells or after the additional removal of the zona pellucida. In the “zona-free” group, a single ferret fibroblast (Fig. 1A), or cumulus cell (not shown), was agglutinated to the oocyte and given an electrical pulse.

Fusion was monitored microscopically after 30 min to 1 hr (Fig. 1B). Alternatively, one (Fig. 1C) or two (Fig. 1E) ferret fibroblasts, or cumulus cells (not shown), were inserted into the PVS of oocytes retaining the zona, given an electrical pulse, and monitored for fusion

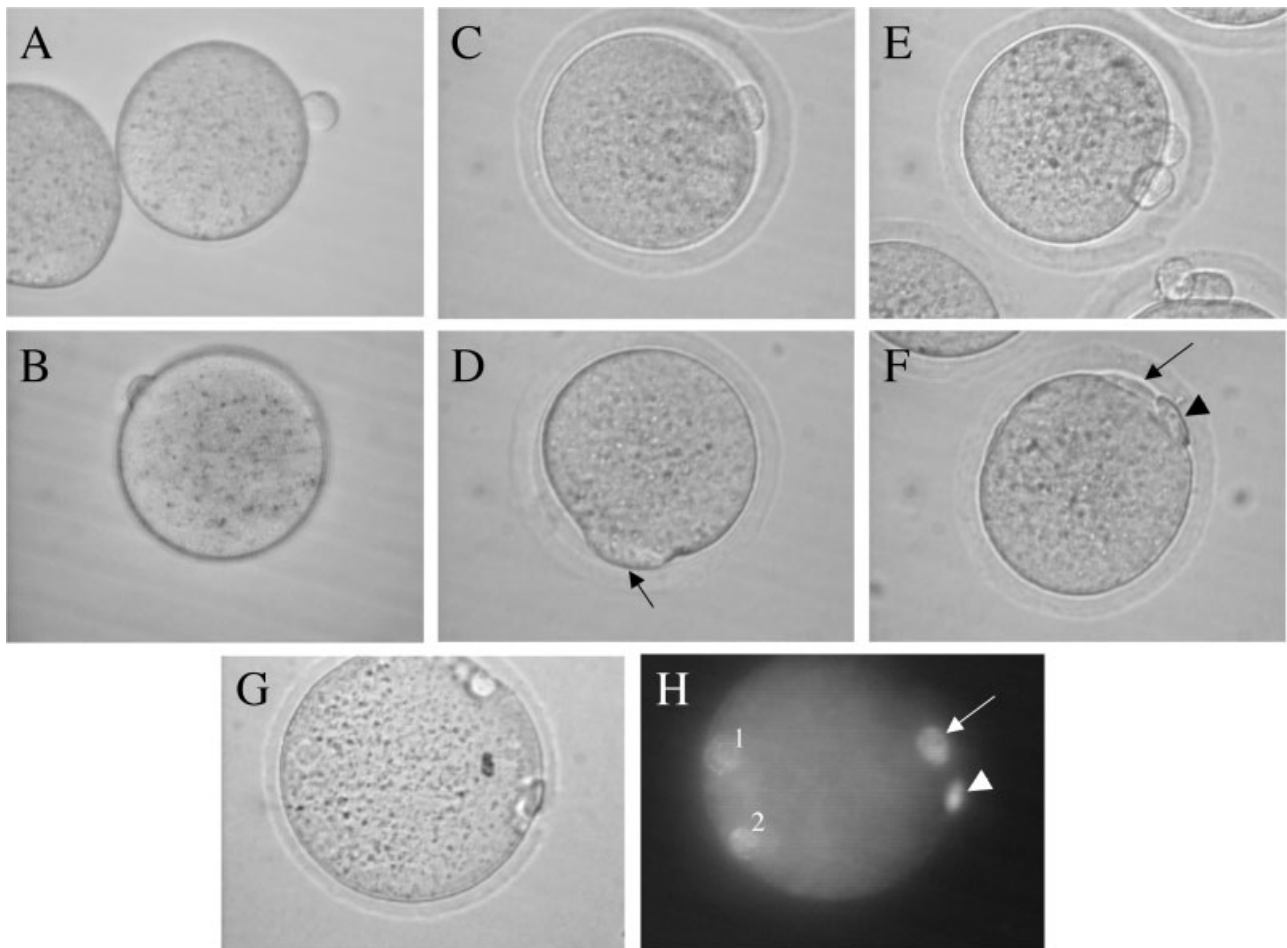


Fig. 1. Electrofusion of cultured ferret fetal fibroblasts with nucleate mouse oocytes. **A:** A zona-pellucida-free mouse oocyte agglutinated to a single ferret fibroblast. **B:** The couplet in “A” undergoing fusion following an electrical pulse. **C:** A single fibroblast inserted into the PVS. **D:** The couplet in “C” undergoing fusion. The arrow indicates the fusion ridge. **E:** Two fibroblasts inserted in the PVS. **F:** The couplet in “E” undergoing fusion. One fibroblast is fusing (arrow) the other is not (arrowhead). **G:** A two-fibroblast couplet undergoing fusion.

Couplet stained with Hoechst 33342 and viewed by phase-contrast microscopy. **H:** The couplet in “G” viewed by UV fluorescence. The electrical pulse has induced fusion of one fibroblast (arrow), but not the other (arrowhead). The pulse also induces oocyte activation that results in release from MII arrest, and the formation of two swollen mouse nuclei (1 and 2). Nuclear swelling in the fused fibroblast (arrow), but not the unfused fibroblast (arrowhead), confirms cytoplasmic entry. Magnification 400 \times .

(Fig. 1D,F, respectively). In some cases, the cell couplets were stained with Hoechst 33342 to monitor nuclear location, and fusion-associated changes in nuclear structure, following the electrical pulse (Fig. 1G,H). In $\geq 95\%$ of the cases involving insertion of two cells into the PVS, only one cell fused following electrical pulse (e.g., Fig. 1F–H).

The cumulative results of these heterologous fusions are shown in Table 1. Two-way ANOVA, and the Tukey multiple comparison test, were used to determine the significance of differences observed among experimental groups. The rate of fusion between a single fibroblast agglutinated to a zona-free oocyte (80.2%) was significantly higher ($P < 0.05$) than that following insertion of a single fibroblast (52.0%) or two fibroblasts (63.8%) into the PVS of intact oocytes. However, the slightly greater rate of fusion observed with two fibroblasts (63.8%), as compared to one (52.0%), was not significant. In general, fusion with cumulus cells followed a pattern similar to that of fibroblasts. However, in this case, the rate of fusion with two cells (44.1%) was significantly higher than with one cell (25.1%, $P < 0.05$), but not significantly higher than that (38.8%, $P > 0.05$) of agglutination of a single cell with a zona-free oocyte. Notably, fusion of agglutinated fibroblasts (80.2%) was significantly higher than similarly treated cumulus cells (38.8%, $P < 0.05$) and fusion with PVS-inserted fibroblasts was higher than inserted cumulus cells. When two cells were used, differences in fusion with PVS-inserted fibroblasts and cumulus cells were statistically significant (63.8% vs. 44.1%, $P < 0.05$). Taken together, these data demonstrate that fusion with fibroblasts is considerably more efficient than fusion with cumulus cells and that this pattern holds true whether cell couplets are generated by agglutination or PVS insertion. Furthermore, this cell type-based difference in fusion is observed virtually irrespective of cell number. In only one case did cell number-dependent differences between fibroblast and cumulus cell fusions lack statistical significance (compare Group 2, 52% and Group 6, 44.1%; $P > 0.05$).

Fusion of Ferret Somatic Cells With Ferret Oocytes

Ferret oocytes were matured in vitro and subsequently fused with ferret fibroblasts or cumulus cells following insertion of these cells into the PVS. The results are shown in Figure 2. Zona-free ferret oocytes are extremely fragile and therefore, could not be adequately evaluated in our homologous fusion studies. One (Fig. 2A–C), two (Fig. 2D–F), or three (not shown) ferret cumulus cells, or fibroblasts (not shown), were inserted in the PVS, given an electrical pulse, and monitored for fusion. In some cases, the cell couplets were stained with Hoechst 33342 to monitor fusion following the electrical pulse (Fig. 2G,H). In two- and three-cell couplets, only one cell fused following electrical pulse (e.g., Fig. 2F).

The results (Table 2) of these homologous fusions reveal several important points. First, fusion of fibroblasts was generally more efficient than fusion of cumulus cells, especially when equal numbers of cells

were inserted in the PVS (48.6% vs. 30.8%, singlet; 71.4% vs. 58.8%, doublet; and 76.8% vs. 66.7%, triplet). However, only single-cell fusions showed a statistically significant difference between these cell types (48.6% for fibroblast vs. 30.8% for cumulus, $P < 0.05$). Second, fusion rates with fibroblast triplets (76.8%) or doublets (71.4%) were significantly greater ($P < 0.05$) than the rate seen with a single fibroblast cell (48.6%). This same trend was observed with fusion of cumulus cells (66.7% for triplets, 58.8% for doublets, vs. 30.8% for singlet, $P < 0.05$). However, no significant differences in fusion rates were seen between doublets and triplets of either cell type. Thus, fusion efficiency was highest when more than one somatic cell was inserted in the PVS, but did not improve with increasing cell numbers. As with mouse oocytes, inclusion of multiple cells in the PVS of ferret oocytes most often ($\geq 95\%$) resulted in the fusion of only one of the two, or three, cells present.

In Vitro and In Vivo Development of Homologous Nuclear Transfer (NT) Embryos

One hundred fifty-three ferret NT embryos (86 produced with fibroblasts and 67 with cumulus cells) were cultured in vitro for 1–7 days following fusion. The rates of cleavage (9.3%), and of morula/blastocyst development (3.5%), with fibroblast-derived embryos were similar to those observed with cumulus cell-derived embryos (i.e., 9.0% cleavage rate and 3.0% morula/blastocyst development) (Table 3). In addition, 170 NT embryos (94 produced with fibroblasts and 76 with cumulus cells) were transferred into the oviducts of recipient ferrets (three recipients for each cell type) and examined at 21 dpc after recipients were euthanized. The rates of implantation and fetal formation were similar between both cell types (Table 3).

DISCUSSION

Membrane fusion between the plasma membranes of a recipient cytoplasm, and a donor cell, can be achieved by Sendai virus infection (Robl et al., 1987), treatment with polyethylene glycol (PEG) (Sims and First, 1994; Tesarik et al., 2000), or with a high-field-strength electrical DC current (Willadsen, 1986; Prather et al., 1989). Due to the unreliability of Sendai virus- and PEG-induced fusion, electrical fusion has become the method of choice for mammalian SCNT. Using this approach, several different laboratories have produced cloned animals including, sheep (Wilmot et al., 1997), cattle (Kato et al., 1998), goats (Baguisi et al., 1999), pigs (Polejaeva et al., 2000), a cat (Shin et al., 2002), rabbits (Chesne et al., 2002), a mule (Woods et al., 2003), a horse (Galli et al., 2003), and mice (Ogura et al., 2000). To our knowledge, however, the electrofusion conditions for ferret SCNT have not been reported.

In general, electrofusion efficiency may be affected by a number of factors including the cell cycle phase of the donor cell (Cheong et al., 1993), the donor cell type (Prather et al., 1987; Collas and Robl, 1991), the age of the recipient oocytes (Lagutina et al., 2002), specific

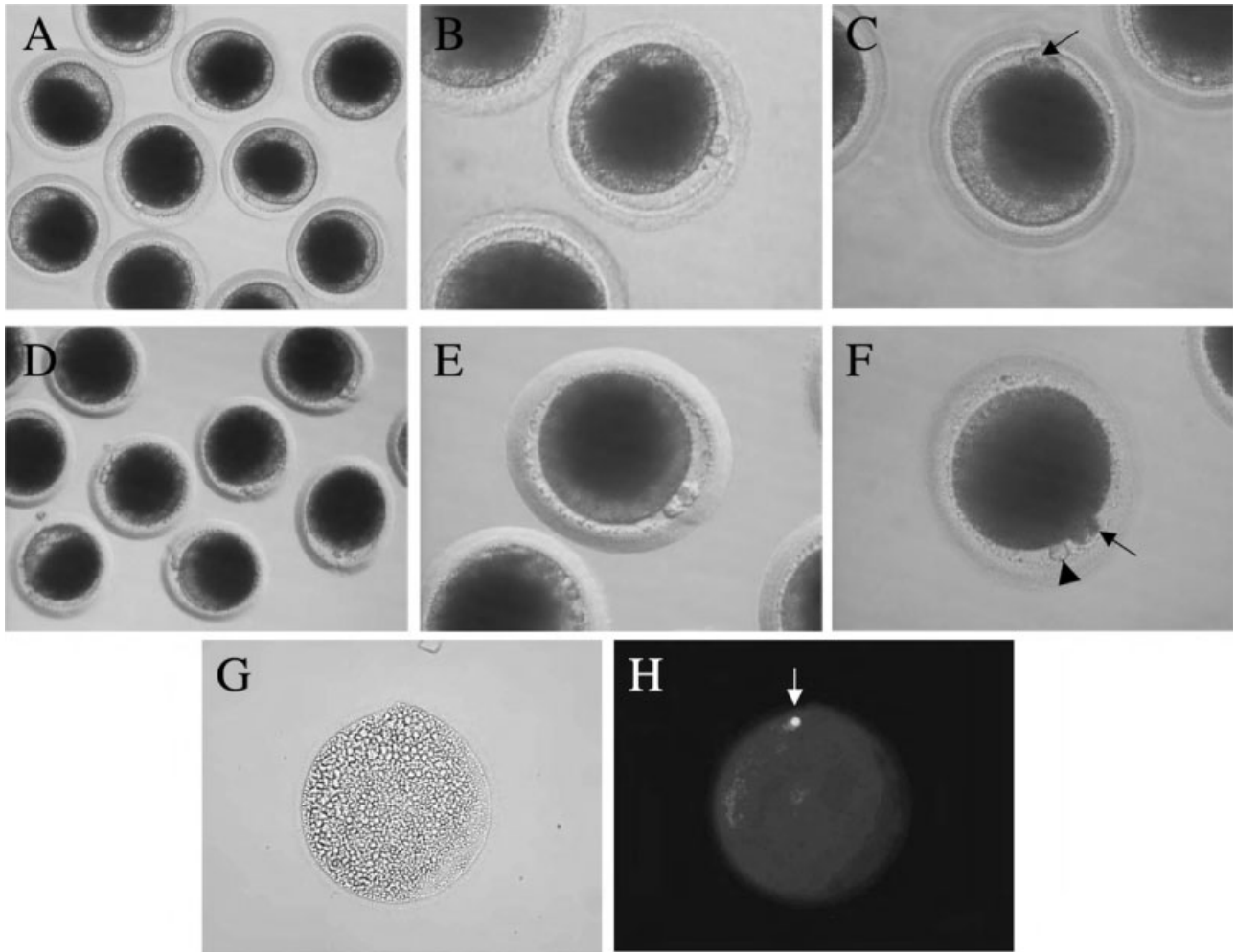


Fig. 2. Electrofusion of cultured ferret cumulus cells with enucleate ferret oocytes. **A:** Ferret oocytes, each with one cumulus cell inserted into the PVS. **B:** Enlarged from “A.” **C:** A couplet undergoing fusion (arrow). **D:** Ferret oocytes, each with two cumulus cells inserted into the PVS. **E:** Enlarged from “D”. **F:** A two-cumulus cell couplet undergoing fusion. One cell is fusing (arrow) the other is not (arrowhead). **G:** A

single-cumulus cell couplet undergoing fusion. The couplet stained with Hoechst 33342 and viewed by phase-contrast microscopy. **H:** The couplet in “G” viewed by UV fluorescence. The nucleus of the cumulus cell is visible within the oocyte cytoplasm (arrow), Magnification 100× (A, D); 200× (B, C, E–H).

chemicals (e.g., CB, PEG, and DMSO) (Li et al., 2002a; Kishi et al., 2003), and the oocyte’s post-enucleation cytoplasmic volume (Peura et al., 1998). In this light, we sought to develop an efficient fusion approach for ferret by exploring the effects of donor cell type and donor cell number on NT to the oocyte. To efficiently optimize these protocols for ferret oocytes, we chose to first explore the effects of these parameters on fusion between ferret

somatic cells and mouse oocytes. Low fusion rates often occur when the somatic cell and oocyte are disoriented in the electric field and/or when there is little or no cell-to-cell contact. To circumvent these problems, we removed the zona pellucida from mouse oocytes and agglutinated a single ferret fibroblast, or cumulus cell, to each zona-free oocyte. Alternatively, we inserted one or two fibroblasts, or cumulus cells, into the PVS of intact mouse

TABLE 3. In Vitro and In Vivo Development of Homologous Nuclear Transfer Embryos

Development	Donor cell type	Number of NT embryos incubated or transferred	Percent of NT embryos developing to indicated stage			
			Cleavage	M/B ^a	Implantation	Fetus
In vitro	Fibroblast	86	9.3 (8/86)	3.5 (3/86)	—	—
	Cumulus cell	67	9.0 (6/67)	3.0 (2/67)	—	—
In vivo	Fibroblast	94	—	—	5.3 (5/94)	3.2 (3/94)
	Cumulus cell	76	—	—	4.0 (3/76)	4.0 (3/76)

^aM/B, morula and blastocyst.

oocytes. The couplets were then manually aligned between electrodes before administration of the electrical pulse.

The fusion rate following agglutination with a single fibroblast was significantly higher than that following insertion of a single fibroblast into the PVS (Table 1). This difference appeared to be cell-type independent as agglutinated cumulus cells also had a higher rate of fusion than inserted cumulus cells. This agglutination-dependent difference in fibroblast fusion was no longer significant when two cells were inserted into the PVS, despite the fact that in most cases only one cell fused with the oocyte (Figs. 1F–H and 2F). A similar improvement was observed with cumulus cells (Table 1). These data suggest that agglutination may provide better cell-to-cell contact than PVS insertion, but that this advantage is partially overcome by including a second cell in the PVS. This latter observation, coupled with the fact that zona-free ferret oocytes are extremely fragile and difficult to fuse, prompted us to compare fusion rates with single and multiple somatic cells in the PVS. Indeed, insertion of two or three fibroblasts, or cumulus cells, significantly improved fusion efficiency with ferret oocytes (Table 2). The presence of multiple cells in the PVS allows more reliable positioning of the doublets or triplets in the electrical field, which may promote more efficient fusion following the electrical pulse. Importantly, in most cases, only a single fusion event occurred when multiple cells were inserted in the PVS indicating the technical feasibility of this approach.

Second, fusion of fibroblasts was more efficient than fusion of cumulus cells. A significant difference in fusion rate was observed when directly comparing all equivalent groups in the heterologous fusions, that is, Table 1: Groups 1 versus 4, 2 versus 5, and 3 versus 6. A similar trend was also observed in homologous fusions between ferret oocytes and ferret somatic cells although, not all of these differences were statistically significant (Table 2). Cell type-dependent differences in fusion rates have been reported previously. For example, it was found that fusion differences in bovine cells were related to relative cell size or the stage of the developing embryo from which they were derived (Prather et al., 1987). Interestingly, rabbit morula blastomeres fuse more efficiently than fetal fibroblasts, with fusion in the latter dependent, in part, on the extent of serum starvation in culture (Lagutina et al., 2002). These differences may also relate to differences in cell size as starved fibroblasts are often smaller than serum-fed cells, both of which being smaller than morula blastomeres. The ferret fibroblasts and cumulus cells used in this study were of similar size, and were grown and maintained under similar culture conditions, arguing that the fusion differences we observe here are related to other parameters.

Fusion-derived NT embryos cleaved, formed blastocysts in vitro, and underwent early stage fetal development following embryo transfer, demonstrating the potential viability of this approach for producing cloned ferrets. The rate of development was cell type-independent, in contrast to the cell type-dependent differences

we observed in fusion efficiency. Defining the conditions for efficient NT in the ferret is an important step forward in generating cloned animals and the development of this species as a genetic model for human disease.

ACKNOWLEDGMENTS

The authors want to thank Dr. R. Scipioni Ball and her staff at Marshall Farms for their kind assistance with ferret care and reproduction. The authors also thank the University of Iowa Statistical Analysis Center for their help in statistical analysis, as well as personnel in the University of Iowa Animal Facility for their efforts in maintenance of the ferret colony.

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