Cats cloned from fetal and adult somatic cells by nuclear transfer

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Abstract

This work was undertaken in order to study the developmental competence of nuclear transfer (NT) into cat embryos using fetal fibroblast and adult skin fibroblast cells as donor nuclei. Oocytes were recovered by mincing the ovaries in Hepes-buffered TCM199 and selecting the cumulus oocyte complexes (COCs) with compact cumulus cell mass and dark color. Homogenous ooplasm was cultured for maturation in TCM199+10% fetal bovine serum (FBS) for 12 h and used as a source of recipient cytoplasm for exogenous somatic nuclei. In experiment 1, we evaluated the effect of donor cell type on the reconstruction and development of cloned embryos. Fusion, first cleavage and blastocyst developmental rate were not different between fetal fibroblasts and adult skin cells (71.2 vs 66.8; 71.0 vs 57.6; 4.0 vs 6.1% respectively; $P < 0.05$). In experiment 2, cloned embryos were surgically transferred into the oviducts of recipient queens. One of the seven recipient queens was delivered naturally of 2 healthy cloned cats and 1 stillborn from fetal fibroblast cells of male origin 65 days after embryo transfer. One of three recipient queens was delivered naturally of 1 healthy cloned cat from adult skin cells of female origin 65 days after embryo transfer. The cloned cats showed genotypes identical to the donor cell lines, indicating that adult somatic cells can be used for feline cloning.

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Introduction

Many animal species including sheep (Wilmut *et al.* 1997), cattle (Cibelli *et al.* 1998, Kato *et al.* 1998), goat (Baguisi *et al.* 1999), mice (Wakayama *et al.* 1998), pig (Polejaeva *et al.* 2000, Yin *et al.* 2002a, 2003), rabbit (Chesne *et al.* 2002), mule (Woods *et al.* 2003), horse (Galli *et al.* 2003) and rat (Zhou *et al.* 2003) have been successfully cloned using somatic cells. Up to now, cats have been considered difficult to clone. Indeed, although one group reported the birth of a cloned cat with donor cumulus cells (Shin *et al.* 2002), no group has obtained feline cloning using fetal and skin fibroblasts as the nuclear donor (Skrzysowska *et al.* 2002, Gomez *et al.* 2003, Kitiyanant *et al.* 2003, Li *et al.* 2003, Wen *et al.* 2003). We report here the successful cloning of cats from male fetal fibroblasts and female adult somatic cells. Four cats were naturally delivered from the two cell types. Our results will contribute to the rescue of endangered feline species.

Materials and Methods

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animal care and use

Domestic hybrid female cats were used for this study. The cats were cared for in facilities and using procedures which exceed the standards established by the Suncheon National University Association for Accreditation of Laboratory Animal Care.

Ovary recovery and in vitro maturation

Healthy mature female cats were selected for superovulation. Each cat was injected i.m. with 200 IU pregnant mare serum gonadotropin (PMSG; Daesung, Kyungg-Do, Korea) and 100 IU human chorionic gonadotropin (hCG; Daesung) 4 days apart. Ovaries were removed from the tract and minced with a scalpel blade in TALP-Hepes to release cumulus oocyte complexes (COCs). The collected

oocytes were matured in TCM199 (M-7528) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin G/streptomycin (P/S; P-4333) for 12 h at 38°C, 5% CO₂ in air.

Cell culture and preparation of donor cells

Fetal fibroblast cells were isolated from a domestic cat fetus (2.5 cm in length) acquired by ovariohysterectomy. Adult fibroblast cells were cultured from ear skin tissue of a female cat (gold and tan spots over white coat color). The cells were cultured in DMEM+10% FBS at 38°C, 5% CO₂ in air. After confluence, the cells were passaged 1 to 9 times, collected, and stored in liquid nitrogen. To induce a quiescent state, semi-confluent fibroblast cells were cultured in DMEM+0.5% FBS for an additional 3 days before use.

Nuclear transfer

The cumulus cells from oocytes were removed by gently pipetting in TCM199 supplemented with 0.1% hyaluronidase. The denuded oocytes were cultured in TCM199 supplemented with 0.2 µg/ml demecolcine for 1 h and placed in TCM199 containing 5 µg/ml cytochalasin B and 0.2 µg/ml demecolcine. The protrusion of the polar body and chromatin plate was removed with a beveled pipette mounted on Narishige micromanipulators while viewing with a Nikon microscope, as previously described (Yin *et al.* 2002a, 2003). Donor cells were trypsinized using a 1% trypsin-EDTA solution and placed in Ca²⁺-, Mg²⁺-free D-PBS supplemented with 0.3% BSA. Micromanipulation was used to place a single nuclear donor cell into the perivitelline space of enucleated ova. The ovum/cell couplets were equilibrated in 0.3 M mannitol containing 0.1 mM Mg²⁺ and transferred to an electrofusion chamber containing the same medium. Cell fusion was induced by 2.0 kV/cm 60 µs DC pulses delivered by an Electro cell fusion generator (Nepagene, Chiba, Japan) applied 2 times. The couplets were removed from the fusion chamber, washed, and incubated in TCM199 supplemented with 0.3% BSA at 38°C, 5% CO₂ in air. One hour after electrofusion, fused couplets were removed from the incubator and equilibrated in 0.3 M mannitol containing 0.1 mM Ca²⁺ and 0.1 mM Mg²⁺, placed into a fusion chamber containing the same medium and electropulsed with 1.0 kV/cm 20 µs DC pulses, 0.1 seconds apart, applied 2 times. The fused couplets were washed and incubated for 4 h in TCM199 supplemented with 0.3% BSA and 5 µg/ml cytochalasin B at 38°C, 5% CO₂ in air. Cloned embryos were cultured in TCM199+0.3% BSA until embryo transfer.

In vitro culture of nuclear transfer embryos and cell count

Fused couplets were cultured in 50 µl droplets of TCM199 supplemented with 0.4% BSA under mineral oil in a

humidified atmosphere of 5% CO₂ for 6 days. Cleavage and blastocyst development of nuclear transfer embryos was evaluated on day 1 (day 0 = day of activation) and day 6. The number of cells in the blastocysts was evaluated by fluorescence microscopy. The embryos were stained with Hoechst 33342 and the cell nuclei were counted.

Synchronization of recipient females and embryo transfer

Cloned embryos were surgically transferred into the oviducts of recipient queens. Estrus synchronization of recipient queens was attained using the same hormone injection regimen described above. The cloned embryos at the one-cell stage after fusion or at the two- to four-cell stage after a day of culture were transferred into the oviducts of synchronized females and confirmed at the ovulated point on the ovary approximately 30 h after hCG injection. Pregnancy was determined by palpation 40 or 45 days after embryo transfer and confirmed by X-ray photography at 60 days.

Microsatellite analysis

Parentage analysis was performed on the cats obtained by nuclear transfer and the surrogate recipient females to confirm identity of the donor cells used for nuclear transfer. DNA was extracted from ear punches or tail clippings obtained from each newborn cat, recipients and donor cells. Five feline DNA microsatellite markers (FCA229, FCA290, FCA441, FCA201, and FCA224) were used to confirm the genetic identity of the cloned cats, the fetus and the skin cells as donor cells.

Statistical analysis

Data were analyzed by a General Linear Model technique (SAS, 1990). Statistical significance was established at the $P < 0.05$ level.

Results

Experiment 1. In vitro development of nuclear transfer embryos

When matured eggs with a first polar body were treated with demecolcine for 1 h, more than 70% of the eggs had a membrane protrusion and the chromosome mass migrated to a cortical location. Enucleation of the eggs with a small volume of cytoplasm was easy and the enucleation rate was high (123 out of 145; 85%).

The *in vitro* development of nuclear transfer embryos reconstructed with fetal and adult fibroblast cells is shown in Table 1. Fusion rates were not different between fetal and adult fibroblast cells (71.2 vs 66.8%; $P < 0.05$). The proportion of reconstructed embryos that cleaved was higher in the fetal group compared with the adult group

Table 1 *In vitro* development of nuclear transferred embryos from different cell lines.

Donor cells	NT units	Fused (%)	Cultured	First cleavage (%)	Blastocyst (%)	The average number of cells
Male fetal fibroblast	174	124 (71.2) ^a	124	88 (71.0) ^a	5 (4.0) ^a	22.2
Female skin fibroblast	151	101 (66.8) ^a	33*	19 (57.6) ^a	2 (6.1) ^a	23.5

Values with same superscripts were not significantly different ($P < 0.05$).

*Thirty three of 101 fused embryos were cultured *in vitro*, the rest of the fused embryos were transferred into a recipient.

(71.0 vs 57.6%; $P < 0.05$). Development to the blastocyst stage was not different using either type of karyoplast (4.0 vs 6.1%). Total cell numbers for the five blastocysts produced using fetal fibroblasts were 23, 31, 17, 12 and 28. The blastocysts produced using adult fibroblasts had a total cell number of 20 and 27.

Experiment 2. Cloned embryo transfer into recipient queens

A donor fibroblast cell line was established from a five-week-old male fetus and passaged one to nine times for expansion in culture. These cells were cultured in DMEM+0.5% serum for an additional three days before use of nuclear transfer and fusion with enucleated oocytes devoid of metaphase chromosomes by micromanipulation as described (Yin *et al.* 2002a, 2003). Cloned embryos at the one-cell stage (120 and 140 eggs) or at the two- to four-cell stage ($n=30-90$ eggs) were transferred into seven synchronized recipients approximately 30 h after hCG treatment (Table 2). Recipient 7 received 140 cloned embryos, which were reconstructed with 9 passaged cells and maintained the pregnancy to term. Pregnancy was confirmed by X-ray photography one day before parturition. Two live kittens and one stillborn fetus were delivered naturally 65 days after embryo transfer. The birth weights of the cloned live cats at delivery were 96 and 84 g and the stillborn offspring weighed 56 g. We suspected that the stillborn fetus was related to the palpation used to confirm pregnancy. Two cloned cats are still alive and healthy after 4 months (Fig. 1a). An adult somatic cell line was established from a 12-month-old female cat as described above. One hundred and forty-five cloned embryos at the two- to four-cell stage were transferred into three recipients. One recipient was confirmed pregnant after receiving 65 recon-

structed embryos derived from one passaged cells and then produced one kitten (Fig. 1b) by natural delivery 65 days after embryo transfer. The birth weight of the cloned kitten was 102 g. The kitten was vigorous at birth and appeared to be completely normal, but died three days post-parturition from starvation as it refused to suck colostrum. Pathological analysis of the cloned cat did not detect any abnormalities. Microsatellite analysis revealed that the donor cells were the source of the genetic material used to produce the newborn cats (Table 3).

Discussion

In the present study, we investigated the possibility of producing normal cloned cats by using fetal and adult somatic donor cells. This is the first time, to our knowledge, that normal cloned cats have been produced using fetal donor cells. The results of our experiment showed that fusion, cleavage and developmental rate to the blastocyst stage were similar to those reported previously (Skrzysowska *et al.* 2002, Gomez *et al.* 2003, Kitiyanant *et al.* 2003, Wen *et al.* 2003). However, the quality of the blastocysts, based on the cell number, was poor in comparison with other reported experiments (Skrzysowska *et al.* 2002, Gomez *et al.* 2003, Kitiyanant *et al.* 2003, Wen *et al.* 2003). In this study, we used a culture regime of TCM199 supplemented with 0.3% BSA for 6 days. It would seem that this *in vitro* culture system is not ideal for the culture of cloned cat embryos.

A major difference between the methods described here and those used to produce cloned kittens in a previous report (Shin *et al.* 2002) is the use of chemically assisted removal of maternal chromosomes, as in pig cloning (Yin *et al.* 2002a, 2003), and the transfer of large numbers of

Table 2 Production of cloned cats following nuclear transferred embryos into recipients.

Donor cells	Passage number	Recipient	No. of transferred NT units (cell stage)	Delivery	No. of kittens	Sex of kittens
Fetal fibroblast	P-9	1	30 (2,4-cell)	No	—	
	P-9	2	44 (2,4-cell)	No	—	
	P-9	3	34 (2,4-cell)	No	—	
	P-5	4	72 (2,4-cell)	No	—	
	P-9	5	90 (2,4-cell)	No	—	
	P-9	6	120 (1-cell)	No	—	
	P-9	7	140 (1-cell)	Yes	1 stillborn & 2 kittens	Male
Skin fibroblast	P-1	1	40 (2,4-cell)	No	—	
	P-1	2	65 (2,4-cell)	Yes	1 kittens	Female
	P-1	3	40 (2,4-cell)	No	—	

Figure 1 Cloned kittens with surrogate mother and nuclear donor (fetus or adult). (a) Surrogate mother and 2 cloned kittens; insert shows nuclear donor fetus. (b) Surrogate mother and a cloned kitten; insert shows nuclear donor cat.

Table 3 Analysis of feline genetic markers. Values represent the sizes (in base pairs) of the two versions of the amplified microsatellite DNA markers in each sample.

Feline markers	Experiment 1 (tail tissue)					Experiment 2 (tail tissue)		
	Fetal cell	Surrogate queen	Kitten 1	Kitten 2	Kitten 3	Skin cells	Surrogate queen	Kitten 1
FCA229	ND	ND	ND	ND	ND	170	163/170	170
FCA290	213/215	213	213/215	213/215	213/215	213	213	213
FCA441	150/154	150/159	150/154	150/154	150/154	150/154	ND	150/154
FCA201	151/155	151/153	151/155	151/155	151/155	151	138	151
FCA224	175	153/175	175	175	175	162/175	162	162/175

ND, not determined.

embryos. Matured cat eggs treated with demicolcine had a membrane protrusion in which the condensed chromosome mass was located. Although the mechanisms of action of demicolcine are not clear, the appearance of the protrusion might be related to the condensation of maternal chromosomes. Such protrusions are observed in demicolcine-treated pig (Yin *et al.* 2002a, 2003) and rabbit (Yin *et al.* 2002b) eggs. This simple, chemically assisted method to remove maternal chromosomes makes it possible to produce a large number of nuclear-transferred eggs and efficiently to produce cloned cats.

To verify the genetic overlap between donor and cloned kittens, we performed parentage analysis with feline microsatellite markers among the cloned kittens, surrogate mothers and control cats. Analysis of five unlinked, highly polymorphic, feline-specific microsatellite loci confirmed that the kittens were clonal.

Although the coat color of the two cloned males (gold on white) resembled each other, the coat color of a cloned female (gold on white coat) was different from the donor female cat (gold and tan spots on white coat). We attribute this to the fact that the pigmentation pattern in multicolored animals is related not only to genetic factors, but also to developmental factors that are not controlled by genotype. The mitochondria inherited from the recipient oocyte would have a major influence over functions that depend on mitochondria gene expression (Holt *et al.* 2004).

In conclusion, we produced cloned cats from fetal fibroblast cells of a male donor and from adult somatic cells of a female donor by natural delivery. These results

will have important applications in domestic cat breeding as well as in endangered felid species cloning, and will contribute to extending the use of cat models for biotechnological applications.

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