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Production of second-generation cloned cats by somatic cell nuclear transfer

X.J. Yin^a, H.S. Lee^a, X.F. Yu^a, L.H. Kim^b, H.D. Shin^b,
S.J. Cho^c, E.G. Choi^c, I.K. Kong^{c,d,*}

^aDepartment of Animal Science & Technology, Suncheon National University, Suncheon 540-742, South Korea

^bDepartment of Genetic Epidemiology, SNP Genetics, Inc., Geumcheon-Gu, Seoul 153-801, South Korea

^cDivision of Applied Life Science, Gyeongsang National University, Jinju 660-701, South Korea

^dInstitute of Agriculture and Life Science, Gyeongsang National University, Jinju 660-701, South Korea

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Abstract

We successfully produced second-generation cloned cats by somatic cell nuclear transfer (SCNT) using skin cells from a cloned cat. Skin cells from an odd-eyed, all-white male cat (G0 donor cat) were used to generate a cloned cat (G1 cloned cat). At 6 months of age, skin cells from the G1 cloned cat were used for SCNT to produce second-generation cloned cats. We compared the *in vitro* and *in vivo* development of SCNT embryos that were derived from the G0 donor and G1 cloned donor cat's skin fibroblasts. The nuclei from the G0 donor and G1 cloned donor cat's skin fibroblasts fused with enucleated oocytes with equal rates of fusion (60.7% vs. 58.8%, respectively) and cleavage (66.3% vs. 63.4%). The 2–4-cell SCNT embryos were then transferred into recipients. One of the five recipients of G0 donor derived NT embryos (20%) delivered one live male cloned kitten, whereas 4 of 15 recipients of the G1 cloned donor cat derived NT embryos (26%) delivered a total of seven male second-generation cloned kittens (four live kittens from one surrogate, plus two stillborn kittens, and one live kitten that died 2 d after birth from three other surrogate mothers). The four second-generation cloned kittens from the same surrogate all had a white coat color; three of the four second-generation cloned kittens had two blue eyes, and one of the second-generation cloned kittens had an odd-eye color. Despite low cloning efficiency, cloned cats can be used as donor cats to produce second-generation cloned cats.

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1. Introduction

Although somatic cell nuclear transfer (SCNT) has several limitations, including low cloning efficiency, it is still a promising method of producing transgenic

animals. We are striving to improve feline SCNT, with a long-term goal of efficiently producing genetically identical cats. Nuclear transfer has progressed from a novel technology to: (1) a widely used technique for generating identical individuals; (2) a model for understanding the cellular and molecular aspects of nuclear reprogramming; and most recently, (3) a means to produce embryonic stem cells for potential use in cell-based therapies [1]. Nuclear transfer in domestic cats (*Felis silvestris catus*) was first demonstrated by the birth of a cloned kitten following the transfer of embryos that were reconstructed by

* Corresponding author at: Division of Applied Life Science, Gyeongsang National University, 900 Gajwa-dong, Jinju 660-701, GyeongNam Province, South Korea. Tel.: +82 55 751 5512; fax: +82 55 756 7171.

E-mail address: ikong@gnu.kr (I.K. Kong).

inserting cumulus cells into enucleated oocytes [2]. Feline SCNT cloning has a potential application for preserving endangered felids [1,3]. Most wild felids are threatened, and the feasibility of using this technology to preserve endangered felids was demonstrated with the birth of African wildcat (*Felis silvestris lybica*) cloned kittens [1]. Moreover, despite the high rates of fetal and newborn mortality, surviving cloned cats are generally normal and healthy [2,4,5]. Serial cloning has been successful in several species, including pigs [6,7], mice [8], and bulls [9,10].

Domestic cats have several distinctive characteristics, including a close genetic relationship to the human genome [11–13] and they are phylogenetically close to humans. In that regard, the feline gene map has a higher level of systemic conservation with humans than that of rodents or other laboratory mammals [11–13]. The efficient use of nuclear transfer associated with genetic modification of donor cells in domestic cats could substantially improve the efficacy of producing genetically identical cats that may carry genes for the study of specific human disorders [1].

Serial cloning is an excellent way to study the effect of progressively accumulating somatic mutations on development, health, and reproductive performance. Moreover, second-generation cloning of existing cloned fetuses or animals has the distinct advantage of reproducing unique genetic characteristics. For example, transgenic cell populations that were produced by random gene insertion exhibit many different transgene integration sites and copy numbers. Using these transgenic cell populations in SCNT could facilitate production of fetuses or animals derived from a small subset of unique cells that would be difficult to isolate from the transgenic donor fetuses or animals. Although serial cloning of adult mice, cattle, and pigs has been reported [7,14,15], there are no reports on second-generation cloning of felid species. The objective of the present study was to determine the efficiency of second-generation cloning, namely, producing cloned cats using skin cells from an adult cloned cat.

2. Materials and methods

2.1. Source of reagents

All chemicals used in this study were purchased from the Sigma–Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise stated.

2.2. Animal care and use

The stray female cats that were used as oocyte donors were housed in stainless steel cages measuring 0.9 m × 0.7 m × 0.65 m with dry food and water *ad libitum*. They were maintained in a controlled room on a 14-h light:10-h dark photoperiod cycle, with light onset at 06:00. The experiments were approved by the Suncheon National University Association for the Accreditation of Laboratory Animal Care.

2.3. Ovary recovery and oocyte maturation

A total of 78 stray female cats were stimulated and 1496 matured oocytes were produced (average 20.2 oocytes/female cat). Ovarian follicular development of mature cats was stimulated with 200 IU of equine serum gonadotropin (eCG, Daesung, Seoul, South Korea) given i.m., and oocyte maturation was induced 4 d later, by treatment with 100 IU of human chorionic gonadotropin (hCG, Daesung) given i.m. Twenty-four hours later, ovaries were removed (routine ovariohysterectomy), placed in TALP Hepes [5], and minced with a scalpel blade to release cumulus oocyte complexes. The collected oocytes were matured in TCM199 (M-7528) supplemented with 10% fetal bovine serum (FBS, Gibco/Invitrogen, Carlsbad, CA, USA) and 1% penicillin G/streptomycin (P/S; P-4333) for 4 h at 38 °C in an atmosphere of 5% CO₂ in air.

2.4. Cell culture of donor cells

Several fibroblast cell sublines were previously established from single 6-mm skin biopsies recovered aseptically from a male, odd-eyed all-white cat (Turkish Angora, G0 donor cat), as well as a newly established 6-months old cloned cat (G1 cloned cat, Fig. 1a). The primary cultures were first obtained by mincing dermal tissue with sterile scissors in a 35-mm Petri dish (Nunc, Roskilde, Denmark) and culturing the fragments at 38 °C with 5% CO₂ in air, in medium consisting of DMEM (Lot no. 1267027, Gibco-BRL, USA) with 10% FBS until 90% confluence. The cells were then trypsinized and reconstituted (concentration, 1 × 10⁶ cells/mL), grown to confluence, and frozen as zero passage cells (1 vial/35 mm dish) in DMEM containing 10% dimethyl sulfoxide and 10% FBS. The cells were thawed as needed, cultured for up to four passages, and used for SCNT after a 3-d culture yielded confluent growth.



Fig. 1. The G0 donor and G1 cloned cats, recipient, and second-generation cloned cats. (a) Odd-eyed donor cat (left) and his cloned donor cat (right); (b) recipient and four second-generation cloned cats at 1 month of age; and (c) four second-generation cloned cats and cloned donor cat at 4 months (arrow indicates G1 cloned cat).

2.5. SCNT

The SCNT protocol was previously described [4]. Briefly, cumulus cells were removed from oocytes by gentle pipetting in TCM199 supplemented with 0.1% hyaluronidase. The denuded oocytes were then cultured for 1 h in TCM199 (supplemented with 0.2 $\mu\text{g}/\text{mL}$ demecolcine), and then placed in TCM199 containing 5 $\mu\text{g}/\text{mL}$ cytochalasin B and 0.2 $\mu\text{g}/\text{mL}$ demecolcine. The protruding first polar body and chromatin plate were removed with a beveled micropipette mounted on micromanipulators (Narishige, Tokyo, Japan), while being viewed with an inverted microscope (Nikon, Kanagawa, Japan) using Hoffman modulation contrast optics. Successful enucleation was confirmed by staining oocytes with 5 $\mu\text{g}/\text{mL}$ of Hoechst 33342 and observing them with a fluorescent microscope under ultraviolet light. Donor cells were then dissociated using 1% trypsin–EDTA and placed in Ca^{2+} - and Mg^{2+} -free D-PBS supplemented with 0.3% BSA (fatty acid free, A-6003). Micromanipulation was used to place a single donor cell nucleus into the perivitelline space of each enucleated oocyte. The cytoplasm/cell couples were equilibrated in 0.3 M mannitol containing 0.1 mM Mg^{2+} and transferred to an electrofusion chamber containing the same medium. Cell fusion was induced by applying 2.0 kV/cm and 20 μs DC pulses ($2\times$) delivered by an electro cell fusion generator (Nepagene, Chiba, Japan). The couples were removed from the fusion chamber, washed and incubated in TCM199 supplemented with 0.3% BSA at 38 °C in an atmosphere of 5% CO_2 in air. One hour after electrofusion, the fused couples were equilibrated in 0.3 M mannitol containing 0.1 mM Ca^{2+} and 0.1 mM Mg^{2+} , placed into a fusion chamber containing the same medium, and electropulsed by applying 1.0 kV/cm and 20 μs DC

pulses, 0.1 s apart ($2\times$). The activated couples were then washed and incubated for 4 h in TCM199 supplemented with 0.3% BSA and 2 mM 6-DMAP (D-2629) at 38 °C in an atmosphere of 5% CO_2 in air.

2.6. In vitro culture of SCNT-derived embryos and embryo transfer into synchronized recipients

Activated cytoplasm/cell couples were cultured for 24 h in 50 μL droplets of TCM-199 with 4 mg/mL BSA under mineral oil in a humidified atmosphere of 5% CO_2 in air. The number of embryos at the 2–4-cell stage was counted before they were transferred into the oviducts of healthy mature female domestic cats, synchronized with 100 IU of equine chorionic gonadotropin (eCG, Daesung) i.m., followed by 100 IU human chorionic gonadotropin (hCG, Daesung) i.m. 96 h later. A total of 28 female cats were stimulated; 20 exhibited synchrony and were used as recipient females. The cloned embryos were transferred approximately 30 h after the hCG injection. Each recipient was anesthetized with acepromazine maleate (0.025 mg/kg, Samwoo, ChoongNam, South Korea) and ketamine (5 mg/kg, Daesung) before laparotomy. Only cats that had fresh ovulation sites in their ovaries were used as recipients. Pregnancies were detected 40 or 45 d post-transfer, using a SONOACE 900 ultrasound scanner (Medison Co. LTD, Seoul, South Korea) with a 7.0-MHz linear-array transducer.

2.7. Statistical analysis

The frequency at which the reconstructed oocytes fused and cleaved was analyzed using the Chi square-test (SAS Institute, Cary, NC, USA) and $P < 0.05$ was considered significant.

Table 1

In vitro development of embryos cloned from fibroblast cells obtained from the G0 and G1 cloned donor cats

Skin cell donor	No. of oocytes	No. of fused (%)	No. of cultures	No. of cleaved (%)
G0 donor cat	334	203 (60.7)	190	126 (66.3)
G1 cloned donor cat	1162	683 (58.8)	678	430 (63.4)

Table 2

In vivo development of embryos cloned from fibroblast cells obtained from the G0 and G1 cloned donor cat

Skin cell donor	Surrogate mother (S)	No. of SCNT embryos transferred (mean)	Embryo transfer to parturition (d)	Eye color
G0 donor cat	S1	32	1 kitten (66)	Blue
	S2–S5	94 (23.5)	–	–
Total		126 (25.2)	(20.0%, 1/5)	
G1 cloned donor cat	S6	33	4 kittens (67)	3 blue and 1 odd-eye
	S7	23	1 born dead (67)	–
	S8	21	1 born dead (71)	–
	S9	32	1 kitten (60) ^a	–
	S10–S20	321 (29.2)	–	–
Total		430 (28.7)	(26.0%, 4/15)	

G0: generation 0 of odd-eyed all-white coat color cat. G1 cloned donor cat: generation 1 of cloned cat. S1 from G0 donor cat was delivered by C-section and S6–S9 from G1 cloned donor cat were delivered naturally.

^a Dead 2 d after birth.

3. Results

3.1. *In vitro* development of cloned embryos

Fibroblast cell lines were previously generated from skin biopsies that were taken from the odd-eyed all-white male G0 donor and a newly established 6-month-old cloned cat. When these lines achieved confluence, they were cultured for an additional 3 d in DMEM containing 0.5% FBS before being used as the SCNT source of nuclei. The SCNT embryos were compared to the *in vitro* developmental competence. The fusion frequency of the juxtaposed nucleus/enucleated oocyte couples that fused did not vary depending on the source of the nuclei (60.7% vs. 58.8%; Table 1). Moreover, the embryos that were generated using these lines had similar cleavage rates with nuclei from the G0 donor cat and the G1 cloned cat (66.3% vs. 63.4%; Table 1).

3.2. *In vivo* development of cloned and second-generation cloned embryos

In total, 126 G0 donor cat derived NT embryos were transferred into five surrogate mothers (mean = 25.2/recipient), and 430 G1 cloned cat derived NT embryos were transferred into 15 recipients (mean = 28.7/recipient; Table 2). One of the surrogates that received the G0 donor cat derived NT embryos (S1) delivered one cloned

kitten (success rate, 20%), and four of the surrogates that received the G1 cloned cat derived NT embryos delivered seven second-generation cloned kittens (success rate, 26%; Table 2). Of the seven second-generation cloned kittens, two (from S7 and S8) were stillborn and one (from S9) died a few days after birth because the surrogate mother refused to allow it to suckle. The remaining four kittens were from surrogate S6; photographs of these second-generation kittens at 1 and 4 months of age are shown (Fig. 1b and c). The S1 from the G0 donor cat was delivered by C-section 66 d after embryo transfer, whereas S6–S9 from the G1 cloned cat were delivered naturally after 60–71 d (Table 2). The pregnancy periods were not significantly different between the G0 and G1 cloned donor derived cloned cats. At 6 months of age, the G1 cloned cat had two blue eyes and an all-white coat. Although all of the second-generation cloned cats had an all-white coat at 4 months of age, their eye color varied, with three of the cats having two blue eyes, and one expressing the odd-eye phenotype. Microsatellite analysis revealed that all cloned and second-generation cloned kittens were genetically identical to the G0 donor cat (data not shown).

4. Discussion

The domestic cat has several distinctive characteristics, including a close genetic relationship to the

human genome [11,12]. Cats, like dogs, have had intensive veterinary medical surveillance that has described 200 genetic diseases analogous to human disorders [16–18]. Feline infectious agents offer powerful natural models of deadly human diseases, including feline immunodeficiency virus (FIV)-AIDS, feline coronavirus (FeCoV)-SARS, avian influenza, canine distemper virus (CDV)-neurotropic viruses, and feline leukemia and sarcoma virus (FeLV, FeSV)-leukemia and sarcoma [17,19].

Cloning cats may be useful for generating transgenic animals that can serve as models of human diseases. We previously reported that SCNT can be used to produce viable first generation cloned cats [4,5]. In the current study, we tested whether it was possible to produce viable second-generation cloned cats. In that regard, SCNT using skin cells that were collected from a 6-month-old cloned cat (G1 cloned cat) resulted in seven second-generation cloned cats. Their clonal status was confirmed by nine sets of microsatellite markers. Furthermore, the efficiency of the production of second-generation cloned cats was similar to the clones derived from the G0 donor cat (26 and 20% delivery rate in the cloned and second-generation cloned cats, respectively). Similarly, in previous second-generation cloning studies in mice, cumulus cells derived from donor mice and their adult clones produced clones equally as efficiently [8]; 2.8 and 2.0% of the transferred cloned and re-cloned embryos developed to term. There were no changes in the cloned mice that influenced the subsequent cloning efficiency [8]; however, the success rate dropped in successive cloned generations, such that re-cloning was no longer successful after the fifth generation [15]. Another re-cloning study using cattle examined embryos that were generated by SCNT using adult cells from a bull and fetal cells from the bull's clone [9]. Regardless of the nucleus donor, 28% of the fused embryos developed to the blastocyst stage. Moreover, based on DNA analysis and morphological observations, the re-cloned fetus that was removed at 56 d of pregnancy appeared to be normal [10]. Our data were consistent with these observations, and indicated that the efficiency of second-generation cloned cat production was similar to that of first-generation cloning.

In the present study, with SCNT and skin cells, we generated one cloned cat and seven second-generation cloned cats from 20 recipients. However, this yield was below that obtained using other reproductive technologies, including *in vitro* fertilization, AI, and embryo transfer [1]. Many hypotheses that seek to explain the

low efficiency of cloning have been proposed; they include the incomplete reprogramming of donor cell nuclei, improper imprinting, and poor quality oocytes [20,21].

As with other companion animal species, much of the interest in cloning cats has come from the desire of pet owners to replicate a particularly beloved cat. We found that the cloned cats that were derived from the odd-eyed cat were not carbon copies of the donor, as they usually lacked its odd-eye phenotype. However, the rationale was unclear. Perhaps the donor cells accumulated spontaneous mutations during cell division *in vivo*, or, more likely, accumulated mutations during their *in vitro* propagation [22], and the mutations happened to occur during the cloning process [23]. Additional studies are needed to elucidate mechanisms responsible for the aberrant eye color of the cloned offspring.

In conclusion, we produced seven second-generation cloned cats from a G1 cloned cat that was initially derived from an odd-eyed, all-white coat color cat (G0 donor cat). These second-generation cloned cats appeared to be healthy; they all expressed an all-white coat color and two blue eyes, with the exception of one with odd-eyes. Therefore, cloned cats can be used as donor cats for the production of second-generation cloned cats by SCNT, although the cloning efficiency was low.

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References

- [1] Gomez MC, Pope CE, Dresser BL. Nuclear transfer in cats and its application. *Theriogenology* 2006;66:72–81.
- [2] Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, et al. A cat cloned by nuclear transplantation. *Nature* 2002;415:859.
- [3] Wen DC, Yang CX, Cheng Y, Li JS, Liu ZH, Sun QY, et al. Comparison of developmental capacity for intra- and inter-species cloned cat (*Felis catus*) embryos. *Mol Reprod Dev* 2003;66:38–45.
- [4] Yin XJ, Lee HS, Lee YH, Seo YI, Jeon SJ, Choi EG, et al. Cats cloned from fetal and adult somatic cells by nuclear transfer. *Reproduction* 2005;129:245–9.
- [5] Yin X-J, Lee H-S, Kim L-H, Shin H-D, Kim N-H, Kong I-K. Effect of serum starvation on the efficiency of nuclear transfer using odd-eyed white cat fibroblasts. *Theriogenology* 2007;67: 816–23.

- [6] Cho SK, Kim JH, Park JY, Choi YJ, Bang JI, Hwang KC, et al. Serial cloning of pigs by somatic cell nuclear transfer: restoration of phenotypic normality during serial cloning. *Dev Dyn* 2007;236:3369–82.
- [7] Kurpme MTR, Ueno S, Hiruma K, Saito H, Nakamura K, Okumura K, et al. Production of third-generation clones of a pig by somatic cell nuclear transfer. *Reprod Fertil Dev* 2006;18:133–4.
- [8] 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;394:369–74.
- [9] Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, et al. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. *Biol Reprod* 2000;63:1787–94.
- [10] Hill JR, Winger QA, Long CR, Looney CR, Thompson JA, Westhusin ME. Development rates of male bovine nuclear transfer embryos derived from adult and fetal cells. *Biol Reprod* 2000;62:1135–40.
- [11] Menotti-Raymond M, David VA, Lyons LA, Schaffer AA, Tomlin JF, Hutton MK, et al. A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). *Genomics* 1999;57:9–23.
- [12] Murphy WJ, Sun S, Chen ZQ, Pecon-Slattery J, O'Brien SJ. Extensive conservation of sex chromosome organization between cat and human revealed by parallel radiation hybrid mapping. *Genome Res* 1999;9:1223–30.
- [13] Pontius JU, Mullikin JC, Smith DR, Agencourt Sequencing T, Lindblad-Toh K, Gnerre S, et al. Initial sequence and comparative analysis of the cat genome. *Genome Res* 2007;17:1675–1689.
- [14] Kubota C, Tian XC, Yang X. Serial bull cloning by somatic cell nuclear transfer. *Nat Biotechnol* 2004;22:693–4.
- [15] Wakayama T, Shinkai Y, Tamashiro KL, Niida H, Blanchard DC, Blanchard RJ, et al. Cloning of mice to six generations. *Nature* 2000;407:318–9.
- [16] Griffin B, Baker HJ, James GF, Lynn CA, Franklin ML, Fred WQ. Domestic cats as laboratory animals. In: *Laboratory animal medicine* 2nd ed., Burlington: Academic Press; 2002. p. 459–482.
- [17] O'Brien SJ. *Cats*. *Curr Biol* 2004;14:R988–9.
- [18] O'Brien SJ, Menotti-Raymond M, Murphy WJ, Yuhki N. The Feline Genome Project. *Ann Rev Genet* 2002;36:657–86.
- [19] Kuiken T, Rimmelzwaan G, van Riel D, van Amerongen G, Baars M, Fouchier R, et al. Avian H5N1 influenza in cats. *Science* 2004;306:241.
- [20] Kitiyanant Y, Saikhun J, Pavasuthipaisit K. Somatic cell nuclear transfer in domestic cat oocytes treated with IGF-I for *in vitro* maturation. *Theriogenology* 2003;59:1775–86.
- [21] Skrzyszowska M, Katska L, Rynska B, Kania G, Smorag Z, Pienkowski M. *In vitro* developmental competence of domestic cat embryos after somatic cloning: a preliminary report. *Theriogenology* 2002;58:1615–21.
- [22] Crow JF. Spontaneous mutation as a risk factor. *Exp Clin Immunogenet* 1995;12:121–8.
- [23] de Montera B, Boulanger L, Taourit S, Renard JP, Eggen A. Genetic identity of clones and methods to explore DNA. *Cloning Stem Cells* 2004;6:133–9.