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Short communication

Dogs cloned from fetal fibroblasts by nuclear transfer

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ABSTRACT

Fetal fibroblasts have been considered as the prime candidate donor cells for the canine reproductive cloning by somatic cell nuclear transfer (SCNT) in regard to the future production of transgenic dogs, mainly due to their higher developmental competence and handling advantage in gene targeting. In this study, the cloning efficiency with canine fetal fibroblasts as donor cells was determined. A total of 50 presumptive cloned embryos were reconstructed, activated and transferred into the oviducts of naturally synchronous recipient bitches. While the fusion rate (76.9%) was similar to those of our earlier studies with adult fibroblasts as donor cells (73.9–77.1%), a high cloning efficiency (4.0%; 2 births/50 embryos transferred) was found compared to the previous success rate with adult fibroblasts (0.2–1.8%). The cloned beagles were healthy and genotypically identical to the donor fibroblast cells. This study shows that a fetal fibroblast cell would be an excellent donor for future production of transgenic dogs via gene targeting in this cell followed cloning using SCNT technology.

1. Introduction

While gene targeting using embryonic stem (ES) cells has been the predominant procedure for generating transgenic mice, application of this technology to the generation of large transgenic animals largely has not been available mostly due to the difficulty in getting ES cells to produce chimera by

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germ line transmission. Thus, cloning technology using somatic cell nuclear transfer (SCNT) has been regarded as an alternative approach for gene targeting in large animals (McCreath et al., 2000; Denning and Priddle, 2003) as is for preserving genetically valuable or endangered animals. In many species including sheep, cow, pig, goat, and rat (Wilmut et al., 1997; Cibelli et al., 1998; Baguisi et al., 1999; Boquest et al., 2002; Zhou et al., 2003), fetal fibroblasts have been primarily used for producing SCNT-derived offspring because of their high developmental competence (Wakayama and Yanagimachi, 2001; Forsberg et al., 2002). Moreover, fetal fibroblasts have been preferred especially in generating transgenic SCNT because of their nature to grow fast and ability to maintain its characteristics before going through cellular senescence (Schnieke et al., 1997; Cibelli et al., 1998; Baguisi et al., 1999).

Dogs are valuable models in drug discovery and basic research because they share multiple common genetic diseases with human (Vail and MacEwen, 2000; Sutter and Ostrander, 2004; Mack, 2005). Therefore, the need for generating transgenic dogs has grown sharply. A large amount of canine fetal fibroblasts can relatively easily obtained, and they have been considered as the prime candidate cells as the donor cells for generating transgenic dogs. Here, we demonstrate that canine fetal fibroblasts were successfully used as donor cells for generating dog clones with high cloning efficiency.

2. Materials and methods

2.1. Use of animals

In the study, mixed-breed female dogs between 1 and 5 years of age were used as oocyte donors and embryo transfer recipients. The study was conducted in accordance with recommendations described in "The Guide for the Care and Use of Laboratory Animals" published by Seoul National University.

2.2. Isolation and preparation of donor cells

Among the many breeds of dogs, we chose the beagle in that they are most often used in a range of research procedures: fundamental biological research, applied human medicine, and applied veterinary medicine. Fetal fibroblasts were isolated from the fetuses of a beagle bitch obtained at 28 days after artificial insemination. The head of the fetus was removed by iris scissors and soft tissues such as liver and intestine were discarded by scooping out with two watchmaker's forceps. After washing three times, the tissue was minced with a surgical blade and cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) FBS (Invitrogen), 1 mM glutamine (Invitrogen), 25 mM $NaHCO_3$ and 1% (v/v) minimal essential medium non-essential amino acid solution (Invitrogen) at 39 °C in a humidified atmosphere of 5% CO_2 and 95% air. Prior to SCNT, cells were thawed, cultured for 3–4 days until confluent and retrieved from the monolayer by trypsinization for 1 min. To determine the sex of fetal fibroblast, specific sequences of *SRY* (sex-determining region Y) gene were amplified by PCR as described previously (Jang et al., 2008a).

2.3. Recovery of in vivo matured oocytes

Recovery of in vivo matured oocytes was performed as described previously (Jang et al., 2007; Kim et al., 2007). Briefly, blood samples were collected and serum progesterone concentrations were analyzed for determining ovulation time. The day of ovulation was considered as the day when serum progesterone concentration reached 4.0–7.5 ng ml^{-1}. Post-ovulatory oocytes were retrieved using laparotomy by aseptic surgical procedures 70–76 h after ovulation. The fimbria of the oviduct was accessed through the bursal slit and cannulated using an inverted flanged bulb steel needle.

2.4. Somatic cell nuclear transfer, embryo transfer, and parental analysis

SCNT and embryo transfer were performed as described previously (Jang et al., 2008c). Briefly, a donor cell was deposited into the perivitelline space of each enucleated oocyte. Then, couplets were fused electrically and activated chemically. Within 4 h after reconstruction, presumptive cloned embryos were surgically transferred into the oviducts of naturally synchronous recipient dogs at

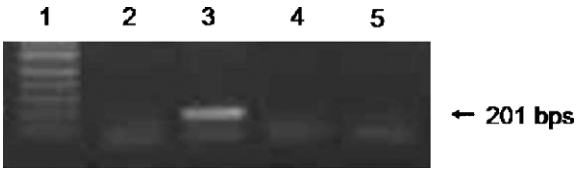

Fig. 1. Genomic DNA of female, male, and fetal fibroblasts was used as templates in PCR using canine specific primers for *SRY* gene. Lane 1: 1 kb DNA ladder; lane 2: female dog; lane 3: male dog; lane 4: donor fetal fibroblasts; lane 5: negative control.

Table 1
Microsatellite analysis of cloned beagles.

Marker	PEZ 01	PEZ 02	PEZ 05	PEZ 17	FH 2010	FH 2054	FH 2079
Donor fibroblasts	114/114	127/127	101/105	211/211	228/232	147/155	273/273
Cloned dog-1[a]	114/114	127/127	101/105	211/211	228/232	147/155	273/273
Cloned dog-2[a]	114/114	127/127	101/105	211/211	228/232	147/155	273/273
Surrogate mother	114/114	119/127	101/109	199/223	228/228	168/172	269/273

[a] Cloned dog-1 and -2 are same litter.

70–76 h after ovulation. Reconstructed embryos were placed in the ampullary portion of the oviduct using a 3.5-Fr Tom Cat Catheter. The day of transfer to the recipient is considered as Day 0. After cloned dogs were born, microsatellite and mitochondrial (mt) DNA analyses were performed as described previously (Jang et al., 2008b).

3. Results

From 5 oocyte donor dogs, 69 oocytes (range = 12–18, average = 13.8 oocytes per oocyte donor dog) were recovered. Cumulus cells from intact oocytes were removed and the status of oocytes was evaluated as previously described (Jang et al., 2007). Among them, 43 oocytes were in mature (from 3 oocyte donors), 14 oocytes were in moderate-aging (from 1 oocyte donor), and 12 oocytes were in severe-aging (from 1 oocyte donor) stages. Except two oocytes that were damaged, rest of the oocytes were used for SCNT. As donor cells, female fetal fibroblasts were used (Fig. 1). Total 65 enucleated oocytes were used for fusion with a donor fetal fibroblast cell, which resulted in the fusion rate of 76.9% (50 fused embryos). After manipulation, all of the reconstructed oocytes were surgically transferred into the oviducts of two recipient dogs. One pregnancy diagnosed on Day 23 was maintained to term and two healthy female beagles weighing 250 and 260 g were delivered by natural delivery on Day 60 post-embryo transfer. The cloned beagles were phenotypcially very similar (Fig. 2) and parentage analysis showed that they were genetically identical with the donor fetal fibroblasts (Table 1). Sequence analysis revealed that the two cloned dogs have identical DNA sequences in the hypervariable region of mtDNA (Table 2).

Table 2
Mitochondrial DNA sequences of oocyte donor dog, two cloned dogs, donor fibroblasts, and a surrogate mother.

	Nucleotide positions[a]					
	15526	15595	15612	15643	15652	15736
Reference[a]	C	C	T	A	G	G
Oocyte donor dog	C	C	T	A	G	G
Cloned dog-1	C	C	T	A	G	G
Cloned dog-2	C	C	T	A	G	G
Donor fibroblasts	T	T	C	G	A	A
Surrogate mother	T	C	T	A	A	G

[a] GenBank accession number: U96639 (Kim et al., 1998).

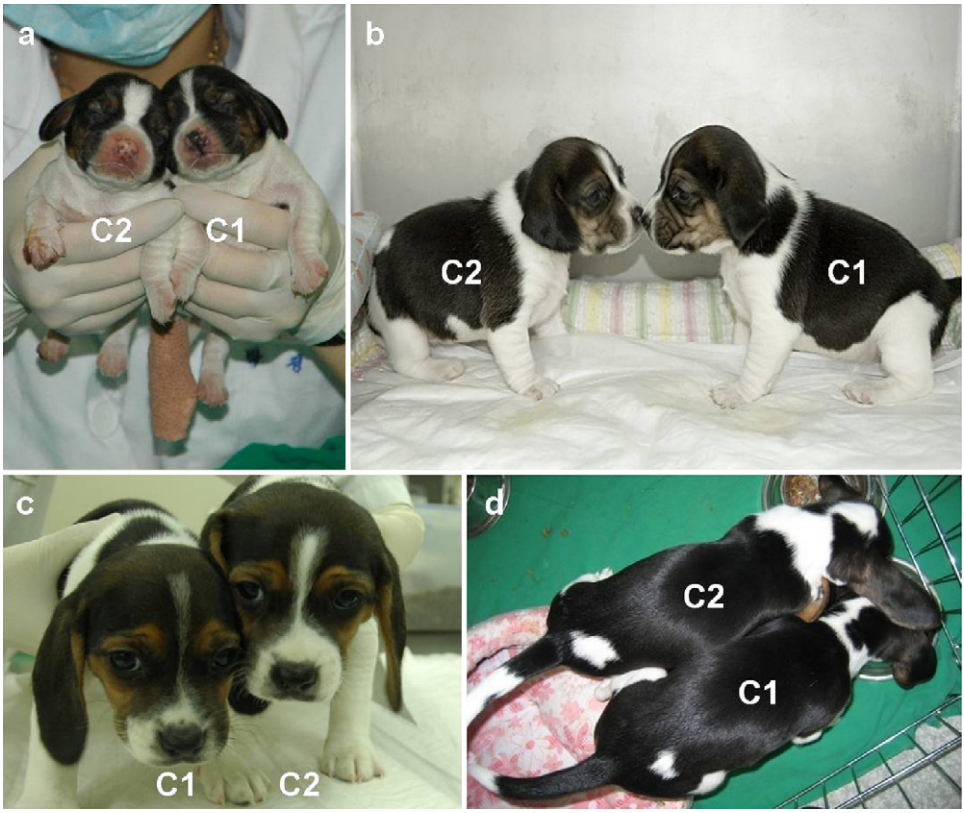

Fig. 2. Two cloned beagles (designated C1 and C2) derived from fetal fibroblasts. (a) C1 and C2 at 2 days after birth, prior to eye opening, (b) at 17 days, (c) at 45 days after birth, and (d) at 2 months old. Notice that overall black and white pattern in the back of two puppies are not exactly same, but similar.

4. Discussion

The dog has been proposed as an animal model for humans because they have many common genetic diseases (Starkey et al., 2005). Moreover, the genomes of dog and human show a higher level of nucleotide conservation than those of human and mouse (Sutter and Ostrander, 2004). Several reports on mammalian SCNT suggest that fetal fibroblasts are superior to adult fibroblasts as donor cells for animal cloning (Kato et al., 2000; Wakayama and Yanagimachi, 2001; Forsberg et al., 2002). In general, cultured fetal cells performed better with respect to pregnancy and delivery rates than adult cells (Forsberg et al., 2002). Indeed, fetal fibroblasts have been widely used for producing transgenic or non-transgenic cloned animals (Schnieke et al., 1997; Wilmut et al., 1997; Cibelli et al., 1998; Vignon et al., 1998; Baguisi et al., 1999; Zakhartchenko et al., 1999; Boquest et al., 2002; Hyun et al., 2003).

In the study, we investigated if the fetal donor cells have any beneficial factors in canine SCNT. First, fusion rate, one of the most important factors affecting the efficiency of SCNT, was 76.9% (fused embryos/cell transferred oocytes). This efficiency was comparable to our earlier studies. For instance, fusion rates of cloned afghan male, females, and toy poodle were 75.0% (Lee et al., 2005), 77.1%, and 73.9%, respectively (unpublished data), indicating that there is no significant difference between fetal fibroblasts and adult fibroblasts on fusion rate. Second is about developmental competence to cloned offspring. Over previous cloned canids research using adult fibroblasts, survival of transferred cloned embryos derived from adult fibroblasts into surrogate mothers was 0.2–1.8% (Lee et al., 2005; Jang et al., 2007, 2008b). In this study, survival of cloned embryos from fetal fibroblasts was 4.0%. Although

we cannot exactly compare the efficiency between adult and fetal fibroblasts, general percentage of efficiency on survival of transferred embryos from fetal fibroblasts to recipients was increased. It is believed that the increase may show that fetal fibroblasts are readily reprogrammed as reported in other species (Wakayama and Yanagimachi, 2001).

The cloned beagle puppies are genetically identical to the donor cell as was demonstrated by mtDNA analysis, which showed that their mtDNAs were all originated from one of the four oocyte donor dogs. Moreover, as the first cloned dogs has similar hair coat pattern of the somatic donor dog (Lee et al., 2005), two cloned beagles had similar overall black and white coat color pattern in the back as well (Fig. 2). To date, the cloned puppies are healthy and do not show any abnormalities and they have very similar growth patterns to the reference data (data not shown).

In conclusion, for the first time, we demonstrated the production of cloned dogs using fetal fibroblasts as donor cells. The cloned beagle dogs, which had identical nucleus and mtDNA, and were born from one recipient, will be provided for biomedical researches as bioresources. Moreover, canine SCNT technique using fetal fibroblasts will contribute to the production of transgenic dogs for the study of human diseases.

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