

ORIGINAL ARTICLE

Propagation of elite rescue dogs by somatic cell nuclear transfer

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ABSTRACT

The objective of the present study was to compare the efficiency of two oocyte activation culture media to produce cloned dogs from an elite rescue dog and to analyze their behavioral tendencies. In somatic cell nuclear transfer procedure, fused couplets were activated by calcium ionophore treatment for 4 min, cultured in two media: modified synthetic oviduct fluid (mSOF) with 1.9 mmol/L 6-dimethylaminopyridine (DMAP) (SOF-DMAP) or porcine zygote medium (PZM-5) with 1.9 mmol/L DMAP (PZM-DMAP) for 4 h, and then were transferred into recipients. After embryo transfer, pregnancy was detected in one out of three surrogate mothers that received cloned embryos from the PZM-DMAP group (33.3%), and one pregnancy (25%) was detected in four surrogate mothers receiving cloned embryos from the SOF-DMAP group. Each pregnant dog gave birth to one healthy cloned puppy by cesarean section. We conducted the puppy aptitude test with two cloned puppies; the two cloned puppies were classified as the same type, accepting humans and leaders easily. The present study indicated that the type of medium used in 6-DMAP culture did not increase in cloning efficiency and dogs cloned using donor cells derived from one elite dog have similar behavioral tendencies.

Key words: activation culture media, elite lifesaver dog, puppy aptitude test, somatic cell nuclear transfer.

INTRODUCTION

Dogs are man's best friends. Dogs and humans have had a close relationship for thousands of years and during that time their bonds have become greatly strengthened. The dog is one of the most intelligent and thoughtful animals. It also exhibits some outstanding abilities, for example, the sense of smell in dogs is on average 10,000 to 100,000 times better than in humans (Walker *et al.* 2003); even the best odor detection machines cannot outperform elite dogs. Because of such attributes, dogs have been bred for many useful purposes, such as: (i) assisting humans in hunting and driving livestock; (ii) guarding humans from dangerous predators; (iii) being a close companion; and (iv) being a valuable tool in society as service animals (Stafford 2006; Walsh 2009). Service dogs have special roles to provide security, detect drugs and explosives, assist the blind and save human lives (Maejima *et al.* 2007; Walsh 2009; McKeown 2012). However, it is difficult to produce dogs that are completely suitable as service animals because of the extensive training period and costs, so that the production efficiency of general service dogs is about 30% (Maejima *et al.* 2007).

In the Korean National Emergency Management Agency, there is a retired veteran rescue dog named Baekdu that performed lifesaving activities worldwide for 6 years. Baekdu demonstrated excellent discretion

in the International Rescue Dog Organization as well as in domestic/international disaster situations. Here, we considered canine somatic cell nuclear transfer (SCNT) for the preservation and propagation of Baekdu's abilities. Canine SCNT is a useful assisted reproductive technique for producing pet dogs (Jang *et al.* 2008; Park *et al.* 2011), endangered canids (Kim *et al.* 2007; Oh *et al.* 2008) and transgenic dogs (Hong *et al.* 2009; Kim *et al.* 2011; Oh *et al.* 2011), but the overall efficacy of the process is still low. In order to improve this low efficiency, two basic activation media used for reconstructed oocytes were compared in terms of *in vivo* development of cloned embryos. The first medium is modified synthetic oviduct fluid (mSOF) which is a culture medium containing BSA (bovine serum albumin) which a protein preparation that has variable, undefined functions and is used in our laboratory for dog cloning (Oh *et al.* 2011; Kim *et al.* 2014). The second medium is porcine zygote medium (PZM-5) which is a chemically defined medium with a synthetic polymer, PVA (polyvinyl alcohol) (Bavister 1981; Garcia-Mengual *et al.* 2008).

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Thus, the purpose of the present study was: (i) to clone Baekdu and to compare *in vivo* development of canine cloned embryos produced using two oocyte activation media and (ii) behavioral analysis of Baekdu clones through the puppy aptitude test which is performed primarily to evaluate basic aptitudes of puppies as service dogs (Pfaffenberger 1976; Weiss 2002).

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Animals

In the study, 30 kg mixed origin, large-breed bitches (12 oocyte donor dogs and 7 embryo transfer recipients) between 1 and 5 years of age were used. All animal care and experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals established by the Institutional Animal Care and Use Committee of Seoul National University (Approval number; SNU-121123-13).

Preparation of donor fibroblasts

Ear skin tissue was collected from a 10-year-old male German shepherd, a veteran rescue dog, Baekdu. The tissue was washed three times using PBS (Dulbecco's phosphate-buffered saline; Invitrogen, Carlsbad, CA, USA), minced, cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen) at 38°C in a humidified atmosphere of 5% CO₂ and 95% air. After 7 days of incubation, a fibroblast monolayer was established, passaged, cryopreserved in 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. Cells from passages 3 to 5 were used as donor cells for SCNT.

Somatic cell nuclear transfer

Canine *in vivo* matured oocytes were recovered by aseptic surgical procedures 70–76 h after the day of ovulation which was considered as the day when the serum progesterone concentration reached 4.0–9.9 ng/mL as previously described (Kim *et al.* 2012). Oocytes surrounded by cumulus cell layers were denuded by repeated pipetting in HEPES-buffered tissue culture medium (TCM)-199 supplemented with 0.1% (w/v) hyaluronidase. The first polar body and metaphase II spindle of denuded oocytes were removed under an inverted microscope equipped with fluorescence. One donor somatic cell was injected into the perivitelline space of each enucleated oocyte, then fused with electric stimulation using two pulses of direct current of 72 V for 15 μsec with an Electro-Cell Fusion apparatus (NEPA GENE Co., Chiba, Japan). The fused couplets were activated by calcium ionophore treatment for 4 min, and then cultured in mSOF medium supplemented with 1.9 mmol/L

6-dimethylaminopyridine (DMAP) (SOF-DMAP), or in PZM-5 supplemented with 1.9 mmol/L DMAP (PZM-DMAP) for 4 h.

Embryo transfer and pregnancy diagnosis

After activation, SCNT couplets were immediately surgically transferred using a 3.5-Fr Tom Cat Catheter (Sherwood, St. Louis, MO, USA) into the ampullary portion of the oviducts of naturally synchronous recipients. Recipients were prepared by predicting ovulation time based on serum progesterone concentrations. Pregnancy diagnoses were assessed with a SONOACE 9900 ultrasound machine (Medison, Seoul, Korea) approximately 31 days after embryo transfer.

Puppy aptitude test

The puppy aptitude test (PAT) of this experiment used Volhard PAT (The American Kennel Club AkC 1985) with modification. The PAT used a scoring system from 1–6 and consisted of 10 tests: Social attraction, Following, Restraint, Social Dominance, Elevation Dominance, Retrieving, Touch Sensitivity, Sound Sensitivity, Sight Sensitivity, and Stability. The tests were performed sequentially and in the order listed. The tests were carried out in an area (3 m x 3 m) unfamiliar to the puppy. At age 7 weeks, a puppy got each subtest for about 3 sec, counting only the first response. There were no other dogs or people, except the tester, in the testing area. The tester was a stranger to the puppy and four scorers evaluated the puppy from outside the test area through a window at the same time. Each test was scored separately, and interpreted on its own merits. In order to preclude the nursing and raising environment, the cloned puppies were brought up suckling breast milk of the same recipient (recipient 1) in the same condition. The puppies stayed with recipient 1 till 7 weeks of age when they were weaned.

Microsatellite analysis for genotyping

Parentage analysis was performed using genomic DNA from the oocyte donor dog, donor cells, cloned puppies and recipients. Genomic DNA was extracted from blood or tissue samples or cell pellets according to the instructions provided with the G-spin Genomic DNA Extraction Kit (Intron Biotechnology, Seongnam-si, Gyeonggi-do, Korea), and analyzed using microsatellite assays with canine-specific markers (Lee *et al.* 2005; Oh *et al.* 2009). Based on the complete nucleotide sequence of canine mitochondrial DNA (mtDNA; GenBank accession number U96639), oligonucleotide primers were synthesized for the hypervariable region as described in previous studies (Lee *et al.* 2005; Oh *et al.* 2009).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). Statistical significance of *in vivo* development of canine cloned

embryos was analyzed using Column Statistics. Dog behavior data was analyzed by two-way analysis of variance (ANOVA) with the Bonferroni post-test. Significance level was considered as $P < 0.05$.

RESULTS

In vivo development of canine cloned embryos using two different activation protocols

A total of 56 and 64 activated, cloned embryos in the PZM-DMAP and SOF-DMAP groups were transferred into three and four female recipient dogs, respectively. Pregnancy was detected in one out of the three

surrogate mothers for the PZM-DMAP group (33.3%), and one pregnancy (25%) was detected in the four surrogate mothers of the SOF-DMAP group (Table 1). The two pregnant dogs each gave birth to one healthy cloned puppy by cesarean section (Fig. 1). The cloned dogs and the donor cells had identical microsatellite patterns for all loci (Table 2), and the mtDNA sequences of the cloned dogs were identical to those of the oocyte donor dog (Table 3).

Puppy aptitude test of the two cloned puppies

PAT was conducted to assess each puppy's temperament. A score of 1 to 6 was used to evaluate the response of each

Table 1 *In vivo* development of canine cloned embryos using two different oocyte activation protocols

Group	No. of transferred embryos	No. of recipient females	No. of pregnancies (pregnancies/ recipients)	No. of cloned pups (births/ embryos transferred)	Clone ID	Status of cloned pups
SOF-DMAP	64	4	1 (25.0%)	1 (1.6%)	Clone 1	Live
PZM-DMAP	56	3	1 (33.0%)	1 (1.8%)	Clone 2	Live

SOF-DMAP, modified synthetic oviduct fluid with 1.9 mmol/L 6-DMAP; PZM-DMAP, porcine zygote medium with 1.9 mmol/L 6-DMAP.

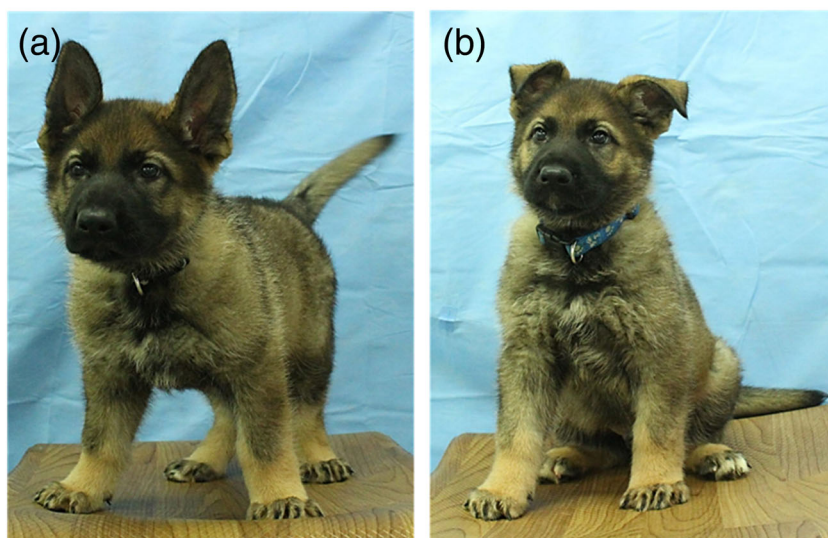


Figure 1 (a) 8-week-old first cloned dog, (b) 7-week-old second cloned dog derived from cells of Baekdu, an elite rescue dog.

Table 2 Analysis of genomic DNA testing with canine-specific microsatellite markers in donor cells, cloned dogs and surrogate females

Name	Donor cell		Clone 1		Recipient 1		Clone 2		Recipient 2	
PEZ1	123	119	123	119	123	119	123	119	115	115
FHC2054	180	176	180	176	155	155	180	176	151	151
FHC2010	236	224	236	224	232	228	236	224	224	224
PEZ5	103	103	103	103	111	111	103	103	111	111
PEZ20	180	176	180	176	196	188	180	176	177	173
PEZ12	268	268	268	268	283	283	268	268	276	264
PEZ3	128	128	128	128	124	124	128	128	131	124
PEZ6	188	176	188	176	183	183	188	176	184	184
PEZ8	226	226	226	226	238	233	226	226	233	229
FHC2079	274	270	274	270	274	270	274	270	278	274

Table 3 Sequence alignments within 661 bases of the hypervariable region of mtDNA

Sample ID	Nucleotide positions*															
	15435	15518	15526	15595	15612	15632	15639	15643	15652	15800	15814	15815	15912	15955	16003	16083
Reference (U96639 v.2)	G	A	C	C	T	C	T	A	G	T	C	T	C	C	A	A
Donor cell	G	A	C	C	T	C	T	A	G	T	C	T	C	C	A	A
Clone 1	A	C	T	T	C	T	G	G	A	C	T	C	T	T	G	G
Oocyte donor 1	A	C	T	T	C	T	G	G	A	C	T	C	T	T	G	G
Clone 2	G	A	C	C	T	C	T	A	G	T	C	T	C	C	A	A
Oocyte donor 2-1	A	C	T	T	C	T	G	G	A	C	T	C	T	T	G	G
Oocyte donor 2-2	G	A	C	C	T	C	T	A	G	T	C	T	C	C	A	A

*The nucleotide positions were numbered from those of GenBank accession no U96639 v.2, and 661 bases (from 15431 to 16091) were examined.

subtest. Scores of social dominance and sound sensitivity were different between the two cloned dogs ($P < 0.001$), but there was no difference in the other tests (Fig. 2). From the interpretation of all 10 subtests, the two cloned puppies were classified as being the same type 3 (dogs of this type accept humans and leaders easily).

DISCUSSION

Cloning or SCNT is one of the assisted reproductive technologies (ARTs) currently used in animal reproduction. Although SCNT is inefficient compared with other ARTs such as *in vitro* fertilization or artificial insemination (Oback & Wells 2007), SCNT and banking of somatic cells for SCNT are still utilized to multiply elite animals with desired phenotypic traits and to produce genetically modified animals (Brophy *et al.* 2003; Faber *et al.* 2004; Yonai *et al.* 2005; Hoshino *et al.* 2009).

For animal cloning, embryo activation is a key step in the development of cloned embryos (Alberio *et al.* 2001). Correct activation is essential to support normal development of cloned embryos. Generally, the combination of ionomycin or ionophore and 6-DMAP is a routine tool for the activation of cloned embryos. Ionomycin or ionophore are widely used to increase intracellular Ca^{2+} levels in activated oocytes (Morgan & Jacob 1994). 6-DMAP, a protein serine/threonine kinase inhibitor (Neant & Guerrier 1988), contributes to DNA synthesis (Ledda *et al.* 1996) and accelerates pronucleus formation in activated, reconstructed embryos. Many studies have been reported for establishing a species-specific activation protocol because optimal treatment time and concentration of 6-DMAP is critical for this step (Choi *et al.* 2004; Lan *et al.* 2005). However, during 6-DMAP treatment, little attention has been paid to effects of culture medium on embryo development. Therefore, the present study investigated the effects of two different culture media used for 6-DMAP treatment after calcium ionophore treatment of fused couplets on SCNT efficiency. With respect to full-term development of cloned embryos, there was no difference between oocytes activated in SOF-DMAP and PZM-DMAP. This result shows that the type of medium used in 6-DMAP culture has no effect on dog cloning efficiency.

Next, we investigated whether the resulting cloned dogs have potential as candidates for rescue dogs. Our study compared the behavioral patterns of the two cloned dogs using PAT, which can predict the performance of a puppy and its expected temperament they will have in adulthood. Also, specific tests predictive of their future employment as adult service dogs have been developed (Slabbert & Odendaal 1999). The necessity of applying the PAT at an early age has been emphasized because early prediction of a dog's adult behavior could save costs of training and reduce time wasted to train doubtful puppies. The present study was carried out using the Vollhard puppy behavioral test with modification

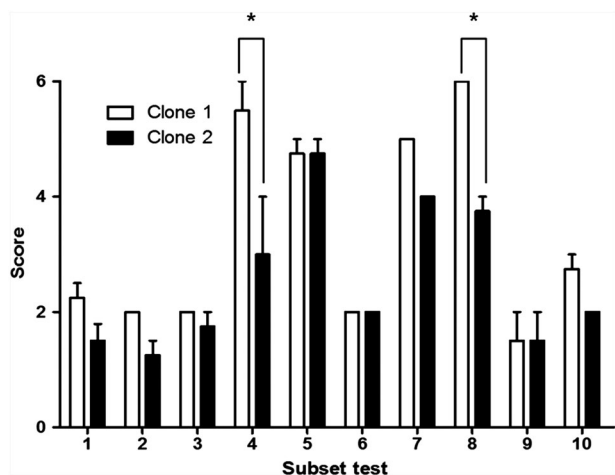


Figure 2 Average scores of two cloned puppies for each subtest (Puppy Aptitude Test). The subtests in numerical order are: 1) social attraction, 2) following, 3) restraint, 4) social dominance, 5) elevation dominance, 6) retrieving, 7) touch sensitivity, 8) sound sensitivity, 9) sight sensitivity and 10) stability. *The value is significant when $P < 0.001$.

(The American Kennel Club AkC 1985; Burghardt 2003). Although the two cloned dogs did not show a perfectly identical score throughout the 10 subtests, overall they showed a similar tendency. Interpretation of the PAT scores indicated that the two cloned dogs mostly belong to type 3, that is, the dog accepts human leaders easily, adapts well to new situations and is generally good with children and elderly people, as well as making a good obedience prospect, and it usually has a common sense approach to life (The American Kennel Club AkC 1985). Thus, the two cloned dogs were evaluated as possessing the appropriate temperament for rescue training.

Also, we performed PAT test using 7-week-old shepherds (control) born by natural mating. Six controls were littermates, and grew up in the same environment. The result was interesting as follows: two belonged to type 2 and two belonged to type 3. The other two controls showed a result that is type 4 (data not shown). In other words, the control group derived from natural mating showed various types in PAT test, not the same as the cloned animals.

In conclusion, the present study demonstrates that dogs cloned using donor cells derived from one elite rescue dog have similar behavioral characteristics. The numbers of elite working dogs in diverse fields can be increased by using the SCNT technique with donor cells derived from a small piece of tissue from elite working dogs; this tissue fragment can be collected once only, cultured, passaged and frozen for many future cloning applications. Further monitoring of the similarities of the performance of cloned dogs in lifesaving and security activities is required and is ongoing.

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