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RESEARCH ARTICLE

Effect of Optimized Treatment of Donor Cells on the Efficiency of Production of SCNT-Cloned Mastiffs

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ABSTRACT

Somatic cell nuclear transfer (SCNT) is an alternative potential tool for the conservation of endangered. In this study, somatic cells were collected from a purebred 9-month-old male mastiff and an 11-month-old female mastiff. Oocytes that had been matured *in vivo* were retrieved from outbred dogs by laparoscopy. We used cycling cells as donor cells for SCNT. A total of 289 oocytes were reconstructed with each male or female somatic cell and then fused/activated simultaneously by electrical stimulation. Finally, 224 embryos were transferred to 16 recipients that had been synchronized naturally. The efficiency of delivery of cloned dogs (7.1%) was threefold higher than in previous reports. Moreover, one surrogate delivered four identical cloned female Tibetan Mastiff puppies; another three surrogates each delivered triplets. Microsatellite analysis demonstrated the genotypic identity of the cloned puppies. Thus, our study has demonstrated techniques that improve significantly the overall efficiency of SCNT in the canine species.

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INTRODUCTION

We previously reported successful production of cloned canids, but the efficiency was extremely low (Lee *et al*., 2005). It is particularly difficult to analyse the *in vitro* developmental viability of cloned canine embryos because no *in vitro* culture (IVC) system has been established for them. Although various efforts have been made to establish the technology, the efficiency of the procedure has not improved greatly (Hossein *et al*., 2008; Kim *et al*., 2009). Many factors can affect the efficiency of somatic cell nuclear transfer (SCNT), some of which have been reported to be successful in the development of canine SCNT embryos. These factors include the collection of oocytes *in vivo* as recipient cytoplasm (Hossein *et al*., 2008), preparation of somatic cells for donor nuclei, the SCNT procedure used (Kim *et al*., 2009), fusion/activation (Jang *et al*., 2008; Kim *et al*., 2009), and embryo transfer (ET) (Fayrer-Hosken, 2007). If these factors were optimized, dogs could be cloned by SCNT with high efficiency.

Polo-like kinases (Plks) are one of the genes involved in cell cycle regulation (Tsvetkov, 2004). Kues *et al*. (2000) reported that expression of Plks was a suitable marker for determining cell cycle stage in porcine fetal fibroblasts and should lead to the identification of optimal cell culture conditions for donor cells in porcine SCNT. However, expression of Plks in canine fibroblasts had not been reported previously. When non–green fluorescent protein (GFP) transfected cells are used as donor nuclei in SCNT, there is no way to confirm their cell cycle stage or viability (Hyun *et al*., 2003), yet SCNT efficiency depends on the donor cell cycle stage (Wilmut *et al*., 1997). However, in studies of SCNT cloned dogs, donor nuclei, which are considered to be the most important factor, have been neglected.

Considering the virtually unlimited value of cloned canids in critical biotechnology applications, including gene conservation of endangered canids, we analysed the effect of cell-cycle synchronisation methods, including the use of cycling canine adult skin fibroblasts (CASFs), on the cell-cycle stage and viability of donor nuclei. We then

investigated proliferation and *Plk*-1/-4 gene expression in CASFs to identify the optimal state of SCNT donor cells. Additionally, we report here an advanced fusion/ activation method and describe the high efficiency in production of cloned dogs using SCNT of optimised cells into enucleated oocytes.

MATERIALS AND METHODS

Chemicals: All chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO), unless otherwise stated.

Estimation of time of ovulation: The time of ovulation was estimated from the serum progesterone concentration, as reported by Hase *et al*. (2000). The day on which the progesterone concentration initially reached 4.0 ng/ml or more was regarded as the day of ovulation. On vaginal cytology during ovulation, superficial epithelial cells usually represented greater than or equal to 90% of the epithelial cells (Evans and Savage, 1970).

Collection of oocytes, preparation of donor cells and SCNT: All surgeries were performed under general anaesthesia using the procedure published by Kim *et al*. (2009). Donor nuclei were obtained from canine fibroblasts using skin biopsies taken from a purebred 9-month-old male mastiff and an 11-month-old female mastiff. Prior to SCNT, cells were thawed and cultured for 3–4 days until confluent. Individual cells were retrieved from monolayers by trypsinization for 30 s. The SCNT procedure was modified from that reported by Kim *et al*. (2009).

Determination of cell cycle and cell death by FACS: The cellular DNA content was determined by staining cells with propidium iodide and measurement of fluorescence in a Becton Dickinson FACScan (FACSCalibur, BD; San Jose, CA). Canine fibroblasts were harvested by trypsinization and fixed in cooled 70% methanol (-20°C). The cells were then incubated for 2 h in a solution containing 1 mg/ml RNase and 20 µg/ml propidium iodide. Subsequently, the cells were analysed in a Becton Dickinson FACScan (FACSCalibur, BD; San Jose, CA). The proportions of cells in the G0/G1, S, and G2/M phases and those undergoing apoptosis were estimated using the Modfit cell cycle analysis program (CellQuest, BD).

Cell proliferation assays: The CASFs from early and late passages were cultured in 96-well microtitre plates in DMEM supplemented with 10% FBS. The cell proliferation assay was performed using an EZ-CyTokox kit (Daeillab, EZ-CyTox, Korea) according to the manufacturer's manual. Briefly, 10 µL of kit reagent (10% of culture medium) was added to each well after 24–96 h of incubation. The cells were incubated for 2 h and then shaken thoroughly for 1 min on a shaker. The absorbance of the treated and untreated samples was measured using a microtitre plate reader at 450 nm, according to the filters available for the microplate reader (Molecular Devices, SpectraMax M2/M2e).

Real-time quantitative PCR analysis: To analyse the relative abundance of mRNA of the *Plk*-1/4 genes, CASFs were harvested on day 4 after thawing. Messenger RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For reverse transcription, 1.5 µg total RNA per 40 µL reaction mixture was converted to complementary cDNA using an M-MLV reverse transcriptase kit (Invitrogen Corporation, Carlsbad, CA). Real-time PCR amplification was conducted using the ABI7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). A Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used to provide real-time PCR quantification of the desired PCR products. The real-time PCR reaction mixture consisted of 50 ng of cDNA and 1 µl each of forward and reverse primers (see Table 3) in a total volume of 20 µl. Three replications were performed, and the mRNA level of each sample was normalized to GAPDH mRNA levels. The relative levels of mRNA were determined by the ratio of 2-^ΔΔ where Ct = threshold cycle for target amplification, ΔCt = Cttarget gene (*Plk*-1 or *Plk*-4) - ΔCtinternal reference (GAPDH), and ΔΔCt = ΔCt sample - ΔCt calibrator.

Embryo transfer: The cloned embryos were transferred surgically into the oviducts of naturally synchronized females after simultaneous fusion and activation. The surgeries were performed using the same procedure published by Kim *et al*. (2009).

Statistical analysis: Differences among treatments were analysed with SPSS using one-way analysis of variance (ANOVA). Data are expressed as means ± SD. Comparisons of mean values among treatments were performed using Tukey's least significant difference (LSD) test. Descriptive statistics are used to present the data. Data were analysed using the unpaired *t*-test with Welch's correction. In each case, a P value of <0.05 was considered significant.

RESULTS

Cell cycle analysis of CASFs: To reduce the detrimental effects of synchronization treatments, contact inhibition and serum starvation, we analysed the cell cycle stages in cycling cells by FACS (Table 1). The G0/G1 rates of cycling at day 1 (p2d1, 61.1%), day 2 (p2d2, 69.5%) and day 3 (p2d3, 76.1%) were significantly lower than for synchronized cells ($P<0.05$). No significant differences in the G0/G1 stage of cycling cells at day 4 (p2d4, 88.1%), contact inhibition (85.7%) and serum starvation (93.4%) were observed. The proportion of cells in the S phase under conditions of serum starvation (1.6%) was significantly lower than other conditions ($P<0.05$). This indicates that cells cycling at day 4 are able to induce a natural synchronization of the cell cycle without artificial treatments.

Analysis of apoptosis and proliferation at different passages: Given the result of experiment 1, cells cycling at day 4 were used subsequently as the optimal condition for natural synchronization. To evaluate the effect of cell passage number on cell proliferation and the rate of apoptosis, we used passages 2, 4, 7, 10 and 11 of cycling cells (Fig. 1; Table 2). As shown in Fig. 1, the rate of

proliferation of cells at passage 11 (O.D. 1.22±0.15) was significantly lower than that of cells at passage 2 (O.D. 1.67±0.04) (P<0.05), but it was not different from those at passage 4 (O.D. 1.56±0.08), passage 7 (O.D. 1.50±0.05) or passage 10 (O.D. 1.51±0.10). Thus, passage 11 was excluded from the analysis of apoptosis. In the analysis of cell cycle phase and apoptosis at different passages (Table 2), we observed that, after passage 7, there was a tendency to decrease the proportion of cells in the G0/G1 phase. The G0/G1 rate of cells at passage 10 (p10d4, 72.6%) was significantly lower than at passages 2 and 4 (P<0.05), but it was not different from passage 7 (Table 2). The rate of apoptosis in cells at passage 10 (16.0%) was higher than at the other passages.

Table 1: Relative mean (±SD) percentages of canine fibroblast cells in various cell cycle phases and subject to different cell treatments

Culture treatment		Cell cycle phase (gated, %)		
		G0/G1	S	G2/M
Cycling	p2d1	61.1±1.4[a]	28.9±0.4[a]	10.0±1.0[a]
	p2d2	69.5±1.0[b]	22.5±0.6[b]	8.0±0.4[ab]
	p2d3	76.1±0.7[c]	14.6±0.2[c]	9.3±0.3[ab]
	p2d4	88.1±1.3[d]	4.9±0.9[d]	7.1±1.7[ab]
Contact inhibition	p2d5	85.7±0.7[d]	4.8±0.4[d]	9.4±0.4[ab]
Serum starvation	p2d5	93.4±0.9[d]	1.6±0.1[e]	5.0±0.9[b]

[a-d] Within the same column, values with different superscripts differed significantly (P<0.05).

Table 2: Relative mean (±SD) percentages of canine fibroblast cells in different cell cycle phases at different passages

Passages	Cell cycle phase (gated, %)			Dead cell (sub G, %)
	G0 / G1	S	G2 / M	
p2d4	91.4±0.6[a]	7.8±0.6	0.8±0.3[a]	4.1±1.7[a]
p4d4	91.5±0.4[a]	5.7±0.4	2.8±0.7[ab]	2.3±0.3[a]
p7d4	87.4±0.7[ab]	6.0±0.2	6.6±0.6[ab]	7.3±0.4[a]
p10d4	72.6±8.0[b]	17.8±11.1	9.6±3.1[b]	16.0±2.0[ab]

[a-b] Within the same column, values with different superscripts differed significantly (P<0.05).

Expression of *Plk* genes in CASFs: We performed semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and real-time PCR (Table 3) to monitor cell cycle synchronization at the molecular level by measuring the expression of cell cycle-regulated *Plk* genes at passages 2, 4, 7 and 10. Samples of adult skin fibroblasts cycling at day 4 were tested by RT-PCR amplification. Semi-quantitative multiplex RT-PCR, using the housekeeping GAPDH gene as an internal standard, showed that all passages expressed the *Plk*-1 genes (Fig. 2A). Using the *Plk*-1 expression level of passage 2 cells as a standard (control, 1.00 arbitrary unit), the expression level of *Plk*-1 mRNA in passage 4 cells (0.70 arbitrary units) was found to be significantly higher than that in passage 7 (0.57 arbitrary units) or passage 10 (0.32 arbitrary units) (P<0.05). The level of expression of *Plk*-4 mRNA in passage 4 (0.3 arbitrary units) was also significantly higher than in passages 7 and 10 (Fig. 2B). In passages 7 and 10, only faint bands (measuring 0.10 and 0.05 arbitrary units) of *Plk*-4 mRNA were detected. Thus, the semi-quantitative multiplex RT-PCR assay of *Plk*-1/-4 was found to be a reliable tool for monitoring the cell viability of canine fibroblasts.

Production of cloned Tibetan Mastiffs: To assess the effect of the optimized treatment of donor cells on the efficiency of cloning dogs using SCNT, we transferred 224 cloned embryos into the oviducts of 16 surrogate dams by surgical embryo transfer (ET). Fifty-four cloned embryos were transferred into four surrogates as a control group. In control experiments, the pregnancy rate (25%) and production efficiency of dog cloning (1.8%) were similar to the results of the previous report (pregnancy rate, 22.2%; production efficiency, 2.0%). Under the conditions of the donor cells described above, donor cells of passages 4 and 5 cultured at day 4 were used for SCNT. The delivery efficiency of cloned dogs (7.1%) was more than three fold higher than when the previous methods

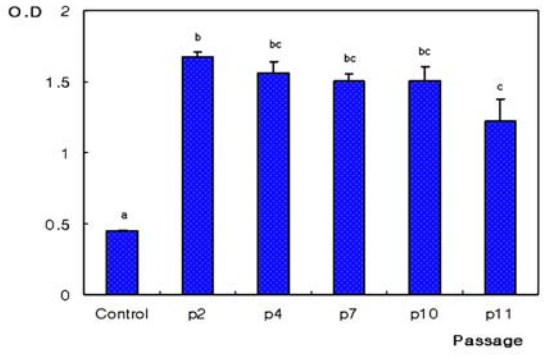

Fig. 1: Proliferation assay of different passages in adult skin fibroblast cells of Tibetan Mastiffs. Cells from early (2, p2) to late (11, p11) passages were plated on to 96-well plates (0.1–1 × 10^4cells/well). Ez-cytox reduction was monitored by optical density (O.D.) at 450 nm. Control represents the blank (P<0.05). Ten replicates were used.

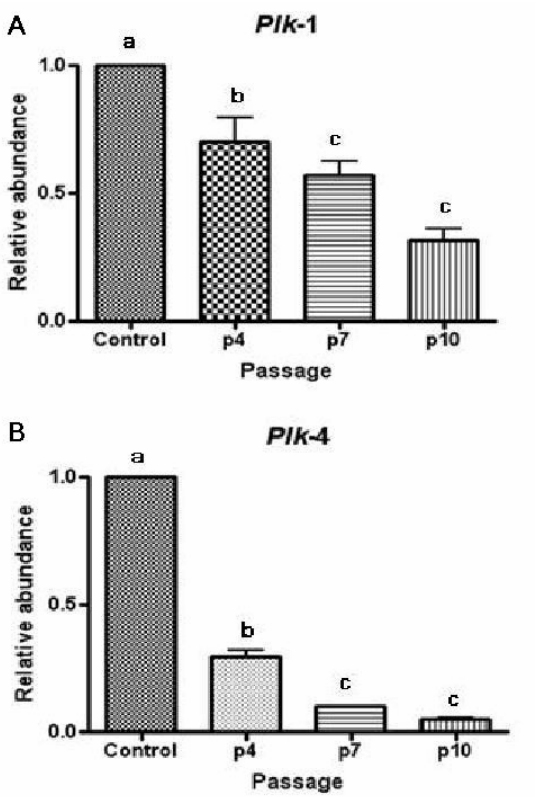

Fig. 2: Real-time quantitative PCR of *Plk*-1 and *Plk*-4 gene transcripts. The relative abundance of mRNA transcripts of the *Plk*-1 (A) and *Plk*-4 (B) genes was measured at different passages (2, 4, 7 and 10) of adult skin fibroblast cells. Three replications were performed three times and the mRNA level of each sample was normalized to that of GAPDH mRNA.

Table 3: Primers and sizes of amplification products

Gene	GenBank accession no.	Sequence	Product size	Melting temperature (°C)
GAPDH	NM_001003142	L : acgggaaacttgtcatcaacgg R : agtgagccccagccttctcca	150 bp	60°C
Plk-1	XM_547435	L : cccagaacaagatgtgtgcggt R : tgagcagcagcaacgtagacaaga	290 bp	60°C
Plk-4	XM_533295	L : caatggaacgatgtcactcagca R : gaggtgtctggtctggaaatgga	247 bp	60°C

Table 4: Effect of optimised treatment of Tibetan Mastiff skin fibroblast cells on production efficiency of SCNT-cloned puppies

Preparation type of donor cell for SCNT	No. of oocytes		No. of recipients			No. of puppies born (%[b])
	All used	Fused (%)	Received embryos	Pregnant at day 30 (%[a])	Carried full term (%[a])	
Control	76	54 (71.0)	4	1 (25.0)	1 (25.0)	1 (1.8)[c]
Optimised treatment	289	224 (77.5)	16	6 (37.5)	6 (37.5)	16 (7.1)[d]

[a]Percentage was calculated from the number of recipients that received embryos; [b]Percentage was calculated from the number of embryos transferred; [c, d] Within the same column, values with different superscripts differed significantly (P<0.05).

were employed (Table 4). Furthermore, the cloned puppies exhibited a strong phenotypic resemblance to each other as well as to their respective somatic cell donors.

DISCUSSION

Khammanit *et al*. (2008) reported the donor cells were synchronized at the G0/G1 phase by serum starvation or contact inhibition on cell cycle synchronization in canine fibroblasts. In agreement with that report, we also demonstrated that treatment using serum starvation/contact inhibition induced synchronization at G0/G1 (Table 1). However, while artificial synchronization using serum starvation/contact inhibition is used widely to synchronize donor cells, it often increases the proportion of apoptotic cells (Kues *et al*., 2000) and causes high embryonic losses after SCNT (Lawrence *et al*., 2005). For this reason, we tried to use a natural condition of cycling cells to use instead of the G0/G1 phase distribution used in artificial synchronization; we found that cells cycling at day 4 were in a state that resembled synchronization, but without the detrimental effects. Thus, cell cycle synchronization of donor cells by artificial synchronization is not required for canine SCNT.

The number of donor cell passages is also an important factor in SCNT. Kubota *et al*. (2000) reported that up to 15 passages of long-term cultured skin fibroblasts did not decrease cloning competence in bovine SCNT. Thus, we examined whether their strategy could be applied to canine SCNT using assays of the rates of proliferation and apoptosis (Fig. 1 and Table 2). In our canine fibroblast cells, however, cells in passage 11 showed a decreased rate of proliferation. In addition, the rate of apoptosis in cells at passage 10 was significantly higher than in cells at other passages, and cells from passage 7 had a decreased G0/G1 phase. In agreement with our results, Li *et al*. (2003) demonstrated that the rate of nuclear remodelling decreased significantly when a large number of passages were used in equine SCNT, and Mammone *et al*. (2006) reported that apoptotic cell death increased with senescence in normal human skin fibroblast cells. This phenomenon suggests that long-term culture may induce decreased viability and alter genetic or epigenetic regulation in the donor cells. For this reason, we analyzed the expression of cell cycle-regulated Plk genes (Fig. 2). Because the *Plk*-1 gene is primarily responsible for mitotic functions, and also because its protein levels are high in the S and G2/M phases and low after mitosis (Tsvetkov, 2004), we measured the level of expression of *Plk*-1 mRNA at different passages (p4, p7 and p10), using passage 2 as a standard. We therefore analysed centriole biogenesis induced by *Plk*-4 in mammalian cells at different passages of donor cells. Cells prior to passage 7 had levels of *Plk*-1/-4 mRNAs comparable to normal healthy cells (Fig. 2). This indicates that selection for high expression of *Plk*-1/-4 in donor cell candidates may compensate for the deficiency in mitotic modulators in the enucleated ooplasm during SCNT.

Using optimized donor cells, we then prepared Tibetan Mastiff cells cultured at day 4 of passages 4 and 5 and attempted the simultaneous fusion/activation method for SCNT. We reported previously that delayed activation with 6-DMAP was better than protocols based on electrical activation for the production of SCNT cloned puppies (Kim *et al*., 2009; Hossein *et al*., 2009a; Hossein *et al*., 2009b). However, in the present study we found that the simultaneous electrical fusion/activation protocol was simple and efficient and that no further chemical stimulation was required for postactivation (Table 4); Hyun *et al*. (2003) also reported the successful cloning of piglets by SCNT using electrical stimulation alone.

The Tibetan Mastiff is the oldest breed of dog in the world, and it is at the edge of extinction (Li *et al*., 2008). Li *et al*. (2008) believe that protection of and research on the Tibetan Mastiff is extremely urgent, yet few studies have been carried out, particularly at the molecular level. Despite the fact that most Tibetan Mastiff puppies are born between December and January (because their oestrous period usually takes place during late autumn), we have demonstrated that this optimized SCNT protocol could overcome the limitation of the seasonal reproductive characteristics of Tibetan Mastiffs (this study was carried out in April) by using oocytes and surrogates from other breeds. Therefore, our results suggest that SCNT represents a means of increasing the number of individuals within a population, and that it provides an efficient strategy for the preservation of genetic material, especially in the conservation of extinction-endangered species that reproduce poorly in captivity. Both male and female animals could be cloned and reintroduced into their natural environments. To date, we have also produced SCNT cloned dogs from breeds of all sizes, from small (beagles, Hossein *et al*., 2009b), to medium (Golden Retriever, Kim *et al*., 2009; Missy, Hossein *et al*., 2009a) and large (Tibetan Mastiff). The SCNT procedure

can thus be utilized to generate cloned dogs regardless of breed.

Conclusion: The present study has demonstrated that: 1) cell-cycle synchronization of donor cells by serum starvation/contact inhibition is not required, 2) *Plk*-1/-4 mRNA can be used to select the donor cells, and 3) electrical stimulation alone is sufficient to activate SCNT embryos for the production of cloned dogs by SCNT. As a result, we were able to use somatic cells collected from both female and male Tibetan Mastiffs to produce 10 female and 6 male mastiffs.

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