Comparison of cell proliferation and epigenetic modification of gene expression patterns in canine foetal fibroblasts and adipose tissue-derived mesenchymal stem cells

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Abstract

Objectives: This study compared rate of cell proliferation, viability, cell size, expression patterns of genes related to pluripotency and epigenetic modification between canine foetal fibroblasts (cFF) and canine adipose tissue-derived mesenchymal stem cells (cAd-MSc).

Materials and methods: Proliferation pattern, cell viability as well as cell size at each passage of cFF and cAd-MSc were measured when cultures reached confluence. In addition, real-time PCR was performed to investigate expression of *Dnmt1*, *HDAC1*, *OCT4*, *SOX2*, *BAX*, *BCL2* genes with reference to β -actin gene expression as an endogenous control in both cell lines.

Results: cFF and cAd-MSc differed in number of generations, but not in doubling times, at all passages. Mean cell size of cAd-MSc was significantly smaller than that of cFF. Cell viability was significantly lower in cFFs and apoptotic level was significantly lower in cAd-MSc compared to passage-matched cFF. In the expression of genes related to pluripotency and epigenetic modification, level of *HDAC1* in cAd-MSc was significantly higher than in cFF, but expression of *Dnmt1* did not differ between the two groups. *OCT4* and *SOX2* were significantly more highly expressed in cAd-MSc compared to cFF.

Conclusions: cAd-MSc have higher stem-cell potential than cFF in terms of proliferation patterns, epigenetic modification and pluripotency, thus cAd-

MSc could be more appropriate than cFF as donors of nuclei in somatic cell nuclear transfer for transgenesis.

Introduction

Dogs have high potential as animal models for human genetic diseases as they not only exhibit similarities in physiology but also share a large number of disease types with humans (<http://omia.angis.org.au>, accessed Feb 2012) (1,2). In addition, it has become possible to generate transgenic model dogs that exhibit specific human disease traits (2). For this purpose, somatic cell nuclear transfer (SCNT) has emerged as the most suitable technique, as alternatives such as pronucleus injection or germline-transmissible embryonic stem cells are not available, unlike in other species (3,4).

Foetal fibroblasts are preferred as donors of nuclei for SCNT used in producing transgenic dogs, as they have excellent proliferative ability and are capable of being genetically modified, with the successful end product of production of live offspring (3,5–7). Using virus-driven, gene-inserted foetal fibroblasts as donor cells, cloned dogs were produced that continuously expressed a red fluorescent protein gene, and conditionally expressed a green fluorescent protein (3,4). However, the donor cells become senescent and unusable because stable transgene-expression, homologous recombination or multiple transfections require a long time for *in vitro* culture (6,8). In addition, using dog foetal fibroblasts raises unresolved issues that cause considerable emotional reaction and heated ethical debate, mainly because of the need to destroy fetuses.

As an alternative to foetal cells, recent reports indicate that there are mesenchymal stem-cell (MSC) lines that can be maintained sufficiently long for homologous recombination events to take place (9,10).

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MSC can proliferate for many passages in culture and show constant population expansion; furthermore, MSC have the ability to give rise to several differentiated cell types (11,12). In this study, we chose MSC derived from adipose tissue. Adipose tissue presents a number of advantages with respect to other investigated stem-cell sources as, they are easily obtained from lipoaspiration or minimally invasive surgery, and can be readily expanded in number to generate hundreds of millions of cells from a small quantity of fat (13). Because of these properties, canine adipose-derived stem cells (cAd-MSC) have been shown to have considerable therapeutic potential for use in models of several disease processes including treatment of human malignancies. In addition, a recent report has demonstrated that cAd-MSC can generate cloned pups when used as donor cells in SCNT (14).

This study is comprised of a basic experiment to establish criteria for selecting suitable nuclear donor cells for generating transgenic cloned dogs, by comparison analysis of various aspects of Ad-MSC and foetal fibroblasts. The aims of the study were to compare canine foetal fibroblast (cFF) and cAd-MSC for: (i) proliferation rates, viability and cell size; (ii) expression patterns of *HDAC1* and *Dnmt1* genes associated with epigenetic modification, and of *OCT4* and *SOX2* associated with pluripotency and early embryonic development.

Materials and methods

Isolation and culture of foetal fibroblasts

Two fetuses were obtained from pregnant beagles at the 28th day of gestation and transported to the laboratory immersed in phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA, USA) containing antibiotics. The fetuses were washed three times in PBS, then heads and other organs were removed and the remains were minced using a surgical blade. Minced embryonic tissues were suspended in PBS and centrifuged at 300 *g* for 3 min. Pelleted tissues were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% (v/v) foetal bovine serum (FBS; Invitrogen), 1 mM glutamine (Invitrogen), 25 mM NaHCO₃ and 1% (v/v) minimal essential medium (MEM) and 1% (v/v) nonessential amino acid solution (Invitrogen), at 38.0 °C, in a humidified atmosphere of 5% CO₂ and 95% air. After 7–10 days incubation, a fibroblast monolayer was established from the tissue explants. Cells were maintained in culture, passaged and cryopreserved in 10% dimethyl sulfoxide (DMSO), then stored in liquid nitrogen. In this study,

we used two such established foetal cell lines for all experiments.

Isolation and culture of adult adipose-derived mesenchymal stem cells

Adipose tissues were collected from the inguinal region of two dogs, a Beagle and a Doberman. From these, two cAd-MSC lines were established as previously described (14). Briefly, adipose tissues were digested with 1 mg/ml collagenase I (Invitrogen) for 60 min at 37 °C. Digested tissues were filtered through a 100-µm cell strainer and centrifuged at 300 *g* for 5 min to obtain a cell pellet, which then was resuspended in RKCM-P (RNL Bio media for MSC culture; RNL Bio Ltd, Seoul, Korea) containing 5% FBS and centrifuged at 300 *g* for 5 min. After centrifugation, supernatant was discarded and the pellet was collected. Cells were cultured overnight at 37 °C under 5% CO₂ in air, in RKCM-P. Cell adhesion was examined under a microscope 24 h later and cell layers were washed in PBS; culture medium was changed using fresh RKCM-P. Cells were maintained in culture for 4–5 days until 90% confluence was achieved (passage 0), then cryopreserved in 10% DMSO and stored in liquid nitrogen. Cells were cultured and expanded in RKCM-P and used for characterization. Two cAd-MSC cell lines were used for all experiments.

Flow cytometric analysis

Trypsinized cAd-MSCs (2×10^5 cells) were suspended in 100 µl of PBS containing 5% FBS. They were then stained with FITC-conjugated CD29, CD73, CD105, CD31, CD34 and CD45 (1:10; BD Pharmingen, San Diego, CA, USA) antibodies and FITC-conjugated CD44 and CD90 (Serotec, Oxford, UK) antibodies. Immunophenotype of cAd-MSCs was analysed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using CELL Quest software.

Measurement of cell population doubling time, cell viability and size

Cryopreserved cFF and cAd-MSC were thawed and subcultured at 1×10^5 cells per 35-mm tissue culture dish (Falcon, Oxnard, CA, USA). Growth rate of the cell population was calculated using the equation below to determine doubling time. Number of cells counted at each passage when populations were confluent, was inserted into the equation. Furthermore, cell viability and cell size at each passage were measured using Countess (Invitrogen) according to the manufacturer's instructions.

$$\text{Doubling time} = \frac{\text{Total time elapsed}}{\text{Number of generation}}$$

$$N = N_0 \times 2^x$$

[N = final cell number, N_0 = initial cell number (1×10^5), x = generation number of exponential growth, total time elapsed = time until confluence]

Total RNA extraction

Cell pellets from passages 3 to 7 of cFF and cAd-MSC were used. Total RNAs were extracted from the pellet of each group using Easy-spin^{TM} (DNA-free) Total RNA Extraction Kit (iNtRON Biotechnology Inc., Kyunggi, Korea) according to the manufacturer's instructions, with slight modifications where needed. Total RNAs were eluted from all samples following the manufacturer's protocol (iNtRON Biotechnology Inc.). Eluted total RNAs were calculated by spectrophotometry, equalized for concentration across all samples and immediately stored at -80°C until used for reverse transcription (RT) and real time polymerase chain reaction (PCR).

Reverse transcription

Total RNA was subjected to RT for detecting expression of *HDAC1*, *Dnmt1*, *OCT4*, *SOX2*, *BAX* and *BCL-2*, which are related to epigenetic modification or pluripotency, apoptosis and expression of β -actin as control. cDNA was synthesized from 1 μg of total RNA using a SuperScript^{TM} III First-Strand cDNA Synthesis Kit (Invitrogen Life Technologies, Barcelona, Spain)

following the manufacturer's instructions with slight modifications where required. Briefly, total RNAs were taken (5–8 μl) in 0.5 ml PCR tubes, and then 1 μl of 50 μM oligo(dT)20 and 1 μl of 10 mM dNTP mix were added. DEPC-treated water was added to tubes to make up to 10 μl mixtures, which were incubated at 65°C for 5 min, then placed on ice for 2 min. In each tube, 2 μl RT buffer (10 $\times$), 4 μl 25 mM MgCl_2 , 2 μl 0.1 M DTT, 1 μl RNaseOut^{TM} (40 U/ μl) and 1 μl SuperScript^{TM} III RT (200 U/ μl) were added and mixed gently. Mixtures were centrifuged briefly and incubated for 50 min at 50°C . Reactions were terminated at 85°C for 5 min and chilled on ice, then collected by brief centrifugation, and 1 μl RNase H (2 U/ μl) was added to each tube and incubated for 20 min at 37°C . Synthesized cDNAs were stored at -20°C until used for real-time PCR. All products except total RNA were supplied with kits.

Real-time PCR

Real-time PCR was performed according to the instructions of the supplier (Takara Bio Inc., Shiga, Japan) with slight modification. Primer sequences used for real-time PCR are shown in Table 1. In brief, all primers were standardized using a standard curve. A PCR plate (MicroAmp optical 96-well reaction plate, Singapore) was made by adding 2 μl cDNA, 1 μl (10 pM/ μl) forward primer, 1 μl (10 pM/ μl) reverse primer, 8 μl SYBR Premix Ex Tag (Takara Bio Inc.), 0.4 μl ROX Reference Dye (Takara Bio Inc.) and 9.6 μl nuclease-free water (Ambion Inc., Austin, TX, USA). For each sample, four replications were made per plate. Wells were capped using optical 8-cap Strip (Applied Biosystems, Carlsbad, CA, USA). Plates were then vortexed and centrifuged briefly in a plate spinner. Real-time PCR was

Table 1. Primer sequences used for quantitative PCR.

Gene	Primer sequences (5'→3')	GeneBank no.	Product size (bp)
Beta-actin	F-GCTACGTCGCCCTGGACTTC R-GCCCGTCGGGTAGTTCGTAG	NM_001003349	86
HDAC1	F-GCTGCACCATGCAAAGAAGT R-TCGCCGTGGTGAATATCAAT	XM_859623	129
DNMT1	F-CCCAGACCGCTTCTACTTCC R-ACTTGGCTCGCATGTTTGAG	XM_533919	148
OCT4	F-CGAGTGAGAGGCAACCTGGAGA R-CCACACTCGGACCACATCCTTC	XM_538830	114
SOX2	F-CAGACCTACATGAACGGCTCGC R-CCTGGAGTGGGAGGAGGAGGTA	XM_545216	147
BAX	F-ACTTTGCCAGCAAAGTGGTG R-AGGAAGTCCAGTGTCCAGCC	NM_001003011	88
BCL2	F-TGAGTACCTGAACCGGCATC R-GTCAAACAGAGGCTGCATGG	NM_001002949.1	100

performed using 7300 Real Time PCR System (Applied Biosystems) according to the manufacturer's instructions.

Statistical analysis

Rates of cell proliferative and levels of gene expression from real-time PCR were analysed by paired Student's *t*-test (GraphPad Prism version 5; Graphpad Incorporation, San Diego, CA, USA). All data were derived from experiments repeated at least five times.

Results

Comparison of cell proliferation patterns between canine foetal fibroblasts and adipose-derived mesenchymal stem cells

cFF and cAd-MSC had similar spindle-shaped morphology in culture (Fig. 1). cAd-MSC were determined by flow cytometry to be positive for CD29, CD44, CD73, CD90 and CD105, but negative for CD31, CD34 and CD45 (data not shown). To investigate cell proliferation, cells were cultured until they reached confluence and subcultures were stopped when number of generation fell to zero. Growth curves for both cell lines were typical 'S-shaped' curves (Fig. 2), representing normal cell growth pattern. Number of generation and doubling time were measured at each passage. There were significant differences between the two groups in number of generations, but not in doubling time, at all passages (Fig. 3a, b). In the 3–6th passages, cAd-MSC had higher proliferation rates than cFF. However, doubling time of cAd-MSC increased more rapidly from the 7th passage compared to cFF. Cell size and viability of both cell types were measured at each passage and averaged over all passages from 3rd to 9th. Mean size of cAd-MSC was significantly smaller than that of cFF (Fig. 3c, $P < 0.0001$). Cell viability was significantly higher and apoptotic level was significantly lower in cAd-MSC than cFF (Fig. 4a,b; $P < 0.001$).

Expression levels of genes related to epigenetic modification and pluripotency between foetal fibroblasts and adipose-derived mesenchymal stem cells

Relative expression of genes related to epigenetic modification and pluripotency was analysed in cells derived from cFFs and cAd-MSCs. As shown in Fig. 5a,b, *Dnmt1* expression was similar in cFF and cAd-MSC, while *HDAC1* had significantly higher expression in cAd-MSC than in cFF ($P < 0.05$). As shown in Fig. 5c, d, *OCT4* and *SOX2* expression in cAd-MSC was significantly higher than in cFF ($P < 0.05$).

Discussion

Ad-MSC have applications in various research areas, such as cell therapy and tissue engineering, especially in bone reconstruction (15), and in animal cloning (14). Use of these cells has clear advantages such as easy access to subcutaneous adipose tissue, simple isolation procedures, abundant quantities and less ethical concerns (16,17). In SCNT research, however, there have been only few reports using Ad-MSC, compared to FF, which are frequently used for SCNT. In this study, we performed comparative analyses of cell proliferation and gene expression between cFF and cAd-MSC.

First, cFF and cAd-MSC were assessed during continuous-passage culture for their proliferation patterns and viability. As shown in Figs 1 and 2, cAd-MSC had fibroblast-like morphology and sigmoidal growth curves in culture. In line with our results, the same features have been reported in Ad-MSC of other species such as horse and human (17–19). The proportion of live cells was significantly higher and early apoptotic level significantly lower in cAd-MSC than in cFF (Fig. 4a,b). Interestingly, doubling time of cAd-MSC increased more rapidly from the 7th passage compared to cFF (Fig. 3a), although cAd-MSC proliferated more actively than cFF, as shown by cumulative population doublings (Fig. 3b). Increased doubling time of late passages was similar to MSC derived from other tissues such as amniotic

Figure 1. Photographs of cell growth in fourth passage. Morphology of (a) canine foetal fibroblasts (cFF) and (b) adipose tissue-derived mesenchymal stem cells (cAd-MSC) (magnification 100 $\times$).

Figure 2. Growth curve of cFF and cAd-MSC. The sigmoidal curve (lag phase, log phase, plateau) growth pattern observed in cFF and cAd-MSC at the 4th passage. Equal numbers of cells (1×10^5) were seeded in triplicate and aliquots were counted daily during a period of 8 days. (a) Growth curve of cFF by two foetal cell lines. (b) Growth curve of cAd-MSC by two cAd-MSC lines.

Figure 3. Cellular proliferation pattern and cell size. (a) Doubling time of cFF and cAd-MSC; (b) The generation number of cFF and cAd-MSC; (c) Cell size of cFF and cAd-MSC. Different superscripts (a, b) represent significant differences between groups ($P < 0.05$). Each experiment was performed at least five times using cFF (pooled data from two foetal cell lines) and cAd-MSC (pooled data from two cAd-MSC lines) during the 3–9th passages. Data show mean $\pm$ SEM of the two cell lines in each group.

Figure 4. Cellular viability of cFF and cAd-MSC. (a) Average viability of cFF and cAd-MSC; (b) Expression profiles of BAX/Bcl2 in cFF and cAd-MSC. Different superscripts (a, b) represent significant differences between groups ($P < 0.05$). Each experiment was performed at least five times using cFF (pooled data from two fetal cell lines) and cAd-MSC (pooled data from two cAd-MSC lines) during the 3–9th passages. Data show mean $\pm$ SEM of the two cell lines from each group.

fluid, amnion and umbilical cord of dogs (20). It may be that self-renewal capacity of cAd-MSC is greater than that of cFF in early passages, but decreases with increasing mRNA expression level of pluripotency passage number. It is recommended that transgenic cell lines be established until the 4–5th passage through selec-

tion, when cAd-MSCs are used as donor cells in SCNT. Generally, cell size increased when subjected to continuous subculture for transfection (21). The finding that the size of cAd-MSC was maintained irrespective of passage number might indicate cAd-MSC to be appropriate donor cells.

Figure 5. Expression level of genes related to epigenetic modification and pluripotency. Expression profiles of (a) DNMT1, (b) HDAC1 related to epigenetic modification; and (c) OCT4, (d) SOX2 related to pluripotency in cFF, cAd-MSC. Different superscripts (a, b) represent significant differences between groups ($P < 0.05$). Each experiment was performed at least five times. Data show mean $\pm$ SEM of the two cell lines from each group.

Secondly, we investigated expression of epigenetic- and pluripotency-related genes, in cFF and cAd-MSC. Epigenetic changes in DNA methylation and chromatin histone modifications are known to regulate gene expression (22–24). HDAC1 is an enzyme associated with histone de- or hypo-acetylation. Acetylation of histones by histone acetyltransferases stimulates transcription, whereas deacetylation of histones by HDACs is correlated with transcriptional repression and nucleosomal structure stabilization (25). DNA methylation is maintained by methyltransferase *Dnmt1* (26) that controls self-renewal and differentiation of stem cells, and is critical for progenitor cell maintenance and self-renewal in mammalian somatic tissues (27,28). In the present study, HDAC1 expression in cAd-MSC was significantly higher than in cFF, whereas there was no significant difference in *Dnmt1* between cAd-MSC and cFF. HDAC1/2 multiprotein corepressor complex is essential for pluripotency maintenance in embryonic stem (ES) cells, and loss of HDAC1 leads to differentiation of ES cells (29). In addition, HDAC1 is essential for unlimited cell proliferation in mouse ES cells (30). It may be that proliferative capacity and undifferentiated status of cAd-MSC are superior to those of cFF. Thus, cAd-MSC are recommended as donor cells for SCNT as they do not become altered during long-term culture for selection after transfection.

With this in mind, we next observed that pluripotent genes in cAd-MSC had significantly higher expression patterns compared to cFF. OCT4 and SOX2 are related to pluripotency and are well-known transcription factors that reprogramme differentiated somatic cells into ES cell-like pluripotent stem cells

(31). Although OCT4 has been considered to be expressed only in ES cells, recent research has reported that OCT4 is expressed in bone marrow MSC, in equine cord blood cells and in adult human fibroblasts (27,28,32). Similarly, cAd-MSC showed significantly higher expression levels of OCT4 and SOX2 compared to cFF. It is believed that high expression levels of pluripotency regulators like OCT4 and SOX2 may be essential for 'stemness' maintenance of cAd-MSC. Furthermore, high expression levels of Oct4 and Sox2, which are required for embryo cleavage stages and blastocyst differentiation (33,34), should induce positive effects on reprogramming and development of cloned embryos produced by SCNT, using cAd-MSC donor cells.

In conclusion, our results demonstrated that cAd-MSC have more stem cell potential than cFF in terms of their proliferation patterns, epigenetic modification and pluripotential ability. We recommend that cAd-MSC are more appropriate than cFF as nucleus donors in SCNT used for transgenesis. Further studies are warranted on efficiency of establishing a stable transgenic cell line using cAd-MSC and production of transgenic cloned dogs using transgenic cAd-MSC.

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