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Recloned dogs derived from adipose stem cells of a transgenic cloned beagle

Hyun Ju Oh[a], Jung Eun Park[a], Min Jung Kim[a], So Gun Hong[a], Jeong Chan Ra[b], Jung Youn Jo[b], Sung Keun Kang[b], Goo Jang[a], Byeong Chun Lee[a,*]

[a] *Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, Seoul, 151-742, Korea*
[b] *Central Research Center, RNL BIO, Seoul, 153-768, Korea*

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Abstract

A number of studies have postulated that efficiency in mammalian cloning is inversely correlated with donor cell differentiation status and may be increased by using undifferentiated cells as nuclear donors. Here, we attempted the recloning of dogs by nuclear transfer of canine adipose tissue-derived mesenchymal stem cells (cAd-MSCs) from a transgenic cloned beagle to determine if cAd-MSCs can be a suitable donor cell type. In order to isolate cAd-MSCs, adipose tissues were collected from a transgenic cloned beagle produced by somatic cell nuclear transfer (SCNT) of canine fetal fibroblasts modified genetically with a red fluorescent protein (RFP) gene. The cAd-MSCs expressed the RFP gene and cell-surface marker characteristics of MSCs including CD29, CD44 and thy1.1. Furthermore, cAd-MSCs underwent osteogenic, adipogenic, myogenic, neurogenic and chondrogenic differentiation when exposed to specific differentiation-inducing conditions. In order to investigate the developmental potential of cAd-MSCs, we carried out SCNT. Fused-couplets (82/109, 75.2%) were chemically activated and transferred into the uterine tube of five naturally estrus-synchronized surrogates. One of them (20%) maintained pregnancy and subsequently gave birth to two healthy cloned pups. The present study demonstrated for the first time the successful production of cloned beagles by nuclear transfer of cAd-MSCs. Another important outcome of the present study is the successful recloning of RFP-expressing transgenic cloned beagle pups by nuclear transfer of cells derived from a transgenic cloned beagle. In conclusion, the present study demonstrates that adipose stem cells can be a good nuclear donor source for dog cloning.

Keywords: Canine adipose-derived mesenchymal stem cells; Recloning; Somatic cell nuclear transfer; Red fluorescent protein

1. Introduction

Somatic cell nuclear transfer (SCNT) is a useful technology for basic research and production of transgenic animals for biomedical research. The SCNT approach permits the production of transgenic clones using targeted modification of the genome of the donor cells [1]. In spite of successful cloning of several mammalian species using adult somatic cells [2,3,4,5,6,7], adult fibroblasts are not incompetent for producing transgenic animals as compare to fetal fibroblasts because they have limited ability to proliferate in culture and are refractory to transfection and selection protocols designed to introduce transgenes [8,9].

As an alternative approach, fetal fibroblasts have been primarily chosen for producing SCNT-derived offspring because of their high developmental competence [9,10] and their potential for longer term survival

* Corresponding author. Tel.: +822-880-1269; fax: +822-873-1269.
E-mail address: bclee@snu.ac.kr (B.C. Lee).

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and genetic stability in culture, which are required for the duration of the transfection and screening processes [7,11]. For dog SCNT, fetal fibroblasts were cultured and wild-type and genetically modified beagles were successfully cloned using fetal fibroblasts [12,13]. In previous study, we produced a red fluorescent protein (RFP) expressed male cloned dogs using the male fetal fibroblasts with RFP gene [14]. Although they have the same genetic information, their coat color patterns were different from each other. For example, overall black and white pattern in the body of two puppies are not similar, and showed exclusively discrepancy. It was described as the phenotypic instability and we believe that the main reason for this instability could be inappropriate reprogramming of the donor nucleus [15,16].

The differentiation status of the nuclear donor cells has been assumed to contribute to the success of cloning since correct epigenetic reprogramming and the resulting changes in transcriptional control are the main processes involved in creating an embryo from a somatic nucleus [17]. In mice, there is evidence to suggest that a less-differentiated cell type can increase SCNT efficiencies compared to terminally differentiated cell types because the former are more easily reprogrammed [15,17,18,19,20]. When mouse embryonic stem (ES) cells from normally fertilized and SCNT embryos were used as nuclear donor cells, the number of viable offspring was increased compared to when mouse adult somatic cells were used [17,19,20,21]. Besides ES cells, several reports recently showed that adult stem cells from domestic animals used in SCNT are capable of producing viable offspring and can be genetically modified with positive selection [22,23]. They also are able to proliferate, self-renew and give rise to differentiated daughter cells.

Accordingly, the present study sought to use stem cells as nuclear donors in canine SCNT. Because there is no report of an ES cell line derived from dogs, we decided to employ adipose stem cells derived from inguinal region of a transgenic cloned dog. The aims of the present study were to examine if 1) canine adipose-derived mesenchymal stem cells (cAd-MSCs) can be a suitable donor cell type; 2) transgenic cloned dogs can be recloned using cAd-MSCs of transgenic dogs.

2. Materials and methods

2.1. Use and care of animals

In total, 17 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 the Institutional Animal Care and Use Committee (IACUC) of Seoul National University. In that regard, facilities for dog care and all procedures met or exceeded the standards established by the Committee for Accreditation of Laboratory Animal Care at Seoul National University.

2.2. Isolation and culture of canine adipose-derived mesenchymal stem cells

In order to isolate cAd-MSCs, adipose tissues were collected from inguinal region of a transgenic cloned beagle produced by SCNT of canine fetal fibroblasts modified genetically with RFP gene [24]. The adipose tissues were washed with phosphate-buffered saline (PBS) and then chopped with scissors in the dish. The chopped samples were digested with 1 mg/ml collagenase I (Gibco, Carlsbad, CA) under gentle agitation for 60 min at 37 °C, filtered through a 100 μm cell strainer and centrifuged at 1500 rpm for 5 min to obtain the cell fraction. The pellet was resuspended in 5% FBS-containing RKCM (RNL Bio media for MSC culture, RNL Bio Ltd, Seoul, Korea) and then centrifuged at 1,500 rpm for 5 min. After centrifugation, the floating non-stem cell adipocytes and supernant were removed and the cell pellet was collected. The cell fraction was cultured overnight at 37 °C, 5% CO_2, in RKCM medium. Next day, cell attachment was examined under a microscope, and non-adherent cells were removed by washing with PBS and the medium was changed to RKCM containing 5% FBS. The cell cultures were maintained over four to five days until confluence, and were cryopreserved at passage 0. The cells were cultured and expanded in 5% FBS-containing RKCM and used for characterization and the *in vitro* differentiation experiments at passage 2.

2.3. In vitro differentiation of canine adipose-derived mesenchymal stem cells

2.3.1. Osteogenic induction

At passage two, cAd-MSCs from transgenic cloned dogs were plated at 1 x 10^5 cells/ml in 5% FBS-containing RKCM. The culture medium was replaced with induction medium when cell confluency reached 50%. The osteoblast induction medium was NH Osteodiff medium (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were maintained in culture for 14 d, with 90% of the medium replaced every 3 d.

Staining of differentiated cells was done with Alizarin red S staining. The cells were fixed in 70% ethanol for 1 h, washed with PBS and stained with 40 mM Alizarin Red S solution (pH 4.2) for 1 h. After staining, cells were washed with PBS and then dried at room temperature. The cells were observed a microscope (Axiovert 300, Carl Zeiss, Germany) with a magnification of 100.

2.3.2. Adipogenic induction

At passage two, the cAd-MSCs were plated at 1 x 10^5 cells/ml in 5% FBS-containing RKCM. The medium was replaced with induction medium when cell confluency reached 50%. The adipocyte induction medium was NH Adipodiff medium (Miltenyi Biotec). The cells were maintained in culture for 21 d, with 90% of the medium replaced every 3 d. Differentiated adipocytes were stained with Oil red O. The cells were fixed in 10% formalin for 1 h, washed with PBS and stained with Oil red O solution (60% Oil Red O stock solution; 0.5% Oil Red O in isopropanol and 40% H_2O) for 1 h. The cells were then washed with PBS and observed with a microscope (Axiovert 300) with a magnification of 100.

2.3.3. Myogenic induction

At passage two, the cAd-MSCs were plated at 1 x 10^5 cells/ml in 5% FBS-containing RKCM. The medium was replaced with induction medium when cell confluency reached 50 % and maintained for 14 d. The induction medium was SKGM medium containing SKGM SingleOuots (LONZA, Walkersville, MD). After three washes in PBS, cells were fixed with 4% paraformaldehyde for 20 min and incubated in a blocking solution (10% goat and horse serum in PBS) for 2 h. The cells were then incubated with a primary antibody (a human anti-myosin, 1:500 dilution; Chemicon, Billerica, MA) at room temperature for 2 h or overnight at 4 °C. After three washes in PBS, cells were incubated with combinations of AlexaFluor 488-conjugated donkey anti-mouse secondary antibodies (1:1000 dilution; Chemicon), and then stained with DAPI for nucleic acid detection. The stained cells were observed with an inverted fluorescence microscope (Axiovert 300, Carl Zeiss, Germany) with a magnification of 100.

2.3.4. Neurogenic induction

At passage two, the cAd-MSCs were plated at 1 x 10^5 cells/ml in DMEM containing 10% FBS, 20 ng/ml EGF and 20 ng/ml FGF for 3 d and the medium was replaced with neuronal induction medium. The neuronal induction medium was DMEM containing 10% FBS, 2% dimethyl sulfoxide, 200 μM butylated hydroxyanisole, 1 μM hydrocortisone, 5 μg/ml insulin, 0.5 mM 3-Isobutyl-1-Metylxanthine and 1 mM adenosine 3'5- cyclic monophosphatd sodium salt monohydrate. Induction was terminated after 8 to 10 d of incubation at 37 °C. The cells were fixed with 4% paraformaldehyde for 20 min and incubated in a blocking solution (10% goat and horse serum in PBS) for 2 h. The cells were then incubated with either a human MAP2 antibody (1:200 dilution; Chemicon), a human NSE (neuron specific enolase) antibody (1:10 dilution; Abcam, Cambridge, MA), a human TUJ1 (beta III tubulin) antibody (1:1000 dilution; Abcam) or a human GFAP antibody (1:250 dilution; Chemicon) room temperature for 2 h or overnight at 4 °C. After three washes in PBS, cells were incubated with a combination of Alexa Fluor 488- or 555- conjugated donkey anti-mouse or anti- rabbit secondary antibodies (1:1000 dilution; Chemicon), and then stained with DAPI for nucleic acid detection. The stained cells were observed with an inverted fluorescence microscope (Axiovert 300) with a magnification of 100.

2.3.5. Chondrogenic induction

At passage two, the cAd-MSCs (2.5 x 10^5 cells) cultured in 5% FBS-containing RKCM were centrifuged at 500 g for 5 min and then resuspended in 0.5 ml of NH chondrogenic medium (Miltenyi Biotec) containing dexamethasone, ascorbate, insulin-transferrin-selenium, penicillin, sodium pyruvate, L-praline, L-glutamine and TGF-β. The cells were centrifuged again at 500 g for 5 min to form pellets. The pellets were maintained in culture using polypropylene tubes for 14 d, with 50% of the medium being replaced every three to four days. Differentiated cells were fixed in 10% formalin. After fixation, histological sections (5 μm) were deparafinized, hydrated in distilled water and stained with 1% Toluidine blue for 15 min. The sections were washed in distilled water, dehydrated through 95% and 100% alcohol, cleared in xylene for 3 min and mounted with a cover slip. The stained cells were observed with an inverted fluorescence microscope (Axiovert 300) with a magnification of 100.

2.4. Flow cytometry analysis

Trypsinized cAd-MSCs were suspended in PBS containing 5% bovine serum albumin (BSA) at a concentration of 2 x 10^5 cells/100 μl. The cells were stained with specific antibodies: CD29 (1:100, BD Biosciences, San Jose, CA), CD73 (1: 100, BD Biosciences), CD44 (1: 100, Serotec, Oxford, UK) or Thy1.1 antibody (1:100, Serotec) were FITC conjugated. CD31 (1:100, BD Biosciences), CD105 (1:100, BD Biosciences) or CD34 (1:100, Serotec) antibodies were phy-

coerythrin (PE)-conjugated. The antibodies used for characterization of cAd-MSC were selected and validated by the previous reports on characterization of human and canine MSCs [25,26]. CD34, CD44 and Thy1.1 are dog-specific antibodies and the other antibodies were derived from humans. The expression of the corresponding cell surface markers was assayed by FACS Calibur (BD Biosciences) using CELL Quest software.

2.5. Preparation of nuclear donor cells

The cAd-MSCs were further maintained in culture with 5% FBS-containing RKCM, passaged with 0.25% EDTA-trypsin (Gibco), cryopreserved with RKCM containing 10% DMSO (Sigma–Aldrich Corp., St Louis, MO) and stored in liquid nitrogen. The cAd-MSCs from passage numbers two to five were used as nuclear donor cells for SCNT. The cells were thawed, cultured prior to SCNT and then retrieved from the monolayer by trypsinization.

2.6. Somatic cell nuclear transfer, embryo transfer and pregnancy diagnosis

Collection of *in vivo* matured oocytes was performed about 72 h after ovulation as described in previous reports [4,27,28]. The oocytes were enucleated using micromanipulators (Nikon Narishige, Tokyo, Japan) under an inverted microscope equipped with epifluorescence. An Ad-MSC which was expressed RFP fluorescence under an inverted microscope equipped with RFP specific filter (510–560 nm, BA 590 nm) was introduced into the perivitelline space of an enucleated oocyte using previously reported procedures [4,29]. After microinjection, couplets were induced to fuse with two pulses of direct current of 72 V for 15 μs each using an Electro-Cell Fusion apparatus (NEPA GENE, Chiba, Japan). The fused couplets were activated by 4 min incubation with 10 μM calcium ionophore (Sigma–Aldrich Corp.) and reconstructed by 4 hrs incubation in 6-demethylaminopurine (Sigma–Aldrich Corp.). After reconstruction, cloned embryos were surgically transferred into the oviducts of naturally synchronous recipient dogs as described earlier [24,27,30]. Cloned embryos were placed in the ampullary portion of the oviduct using a 3.5 Fr Tom Cat Catheter (Sherwood, St Louis, MO). Pregnancy was detected between 25 and 30 d after embryo transfer with a SONOACE 9900 (Medison, Seoul, Korea) ultrasound scanner with 7.0 MHz linear-array probe. Pregnancy was monitored ultrasonographically every 2 wks after the initial confirmation and the number of fetal puppies was confirmed by radiography after Day 45.

2.7. Parental analysis for genotyping

Parentage analysis was performed on the nuclear donor fibroblasts, cloned dogs and surrogate recipients to confirm genetic identity. The following nine markers which proven for cloned dog genotyping in previously study [4,29] were selected for analysis: PEZ1, PEZ5, PEZ6, PEZ8, PEZ12, PEZ20, FH2010, FH2054 and FH2079. The isolated genomic DNA samples were dissolved in 50 μl TE and used for microsatellite assay with nine specific markers originally derived from dogs [4,29]. Length variations were assayed by PCR amplification with fluorescently labeled (FAM, HEX, and NED) locus-specific primers and PAGE on an automated DNA sequencer (ABI 373; Applied Biosystems, Foster City, CA). Proprietary software (GeneScan and Genotyper; Applied Biosystems) was used to estimate the PCR product size in nucleotides.

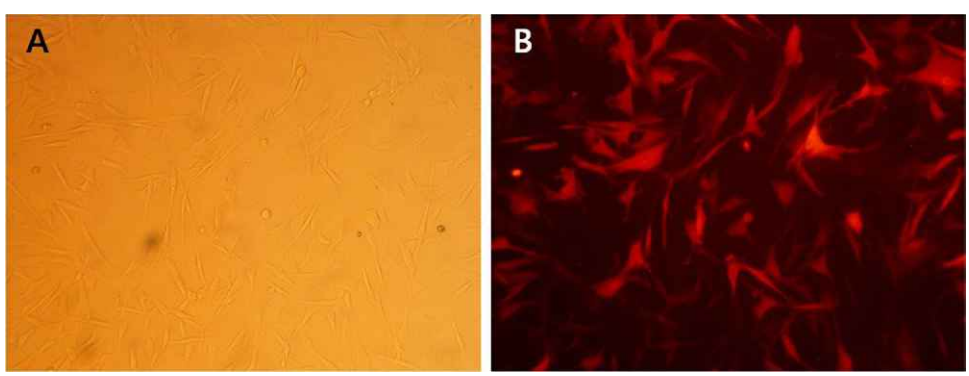
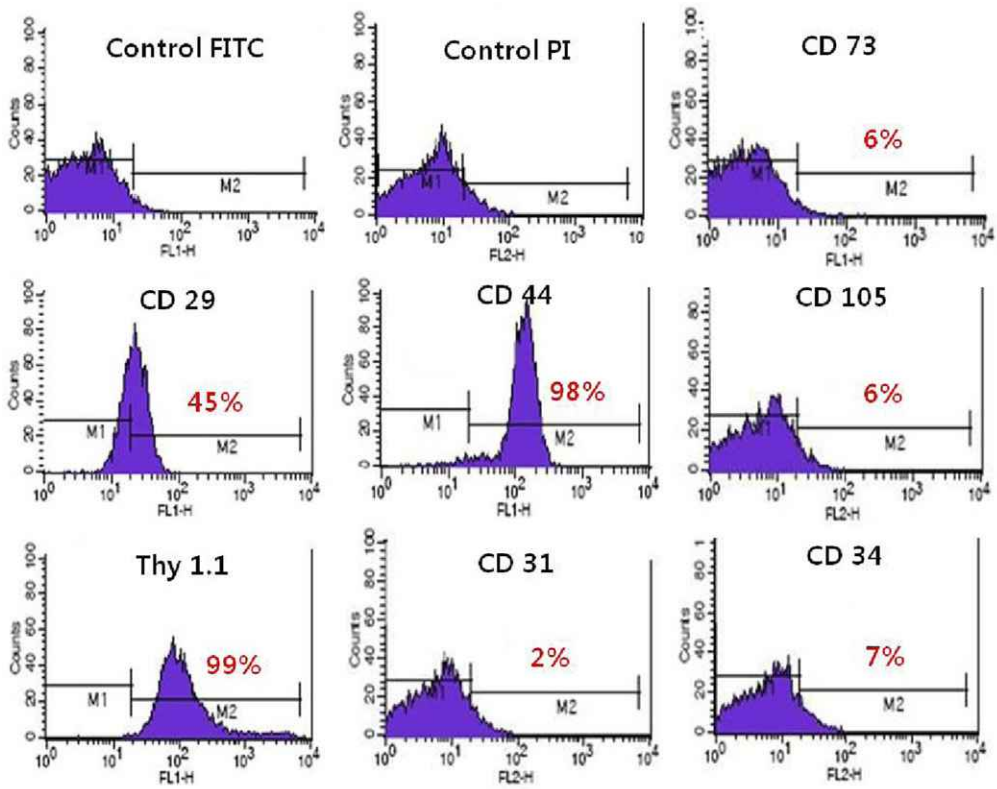


Fig. 1. Morphology of canine adipose mesenchymal stem cells derived from an RFP transgenic cloned beagle. (A) Visible light images. (B) Fluorescence images (x 200).



CD marker	Canine Ad-MSC	Human Ad-MSC [26]
CD29	+	+
CD44	+	+
CD71		+
Thy 1.1 (CD90)	+	+
Stro-1		+
CD11		-
CD14		-
CD31	-	-
CD34	-	-
CD45		-
CD73	-	+
CD105	-	+

Fig. 2. FACS analysis detecting CD29, CD44, Thy1.1, CD31, CD73, CD105 and CD34 antigen expression. The percentage of cells shows fluorescence intensity with specific antibody staining, as compared to nonspecific fluorescence (control). CD34, CD44 and Thy1.1 are dog-specific antibodies while CD31, CD34, CD44, CD73 and CD105 are human-specific antibodies.

3. Results

3.1. Characterization of adipose mesenchymal stem cells derived from RFP transgenic cloned dogs by FACS analysis

The cAd-MSCs had with a fibroblast-like morphology (Fig. 1A) and were attached to the plastic dish; RFP expression was confirmed by microscopic examination (Fig. 1B). The expression of mesenchymal stem cell markers in cAd-MSCs was determined by flow cytometry (Fig. 2). Cells were positive for CD29, CD44 and Thy 1.1, but negative for CD31, CD34, CD73 and CD105.

3.2. In vitro differentiation of adipose mesenchymal stem cells derived from RFP transgenic cloned dogs

Differentiation potentials of cAd-MSCs were investigated. The cAd-MSCs were differentiated into adipocytes, osteoblasts, myocytes, neural cells or chondrocytes. Expression of the RFP gene was confirmed by fluorescence microscopy (Fig. 3A2, 3B2 and 3C2; Fig. 4A2) (x 100). Osteogenic differentiation was confirmed by positive Alizarin Red S staining (Fig. 3A3 and 3A4). Mineralized deposits showed as a red color after staining, which reached nearly 100% cellular differentiation (Fig. 3A4). Myogenic differentiation was revealed morphologically with long, multinucleated cell-forming precursors of myotubes and confirmed by green fluorescence with the FITC-labelled human anti-myosin antibody (Fig. 3B1, 3B4). Chondrogenic differentiation was confirmed by toluidine blue O staining for proteoglycan, a chondrocyte marker. Lacunae formation with extracellular proteoglycan was observed (Fig. 3C3 and 3C4). Adipogenic differentiation was confirmed by positive Oil red O staining (Fig. 4A3, 4A4 and 4A5). Differentiated adipogenic cells accumulated lipid-rich vacuoles in the cytoplasm stained with Oil red O (Fig. 4A4 and 4A5), whereas the control group was not stained (Fig. 4A3). Neuro-

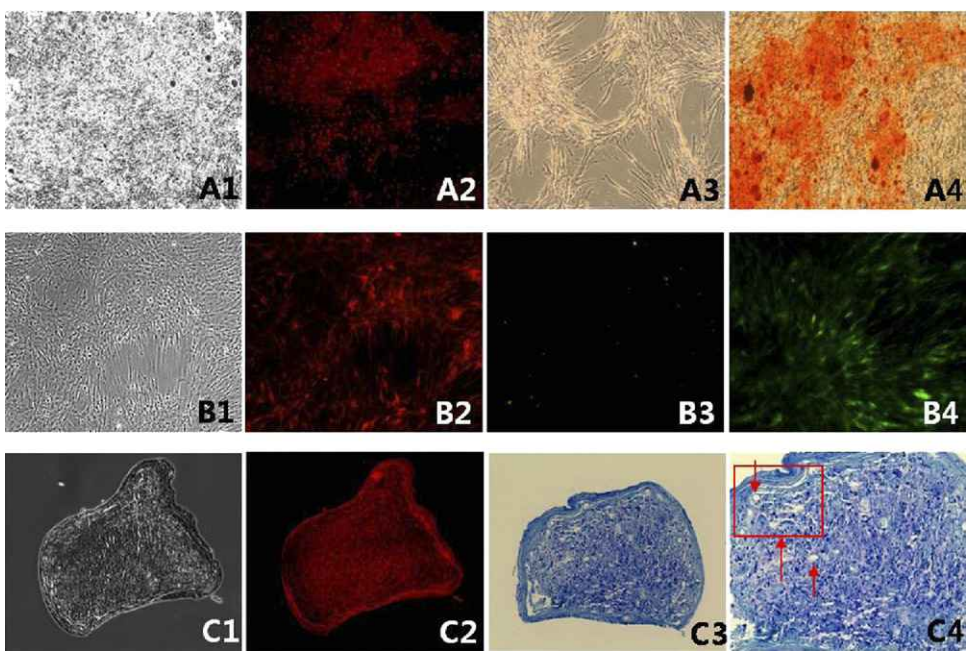


Fig. 3. *In vitro* osteogenic, myogenic and chondrogenic differentiation of RFP transgenic dog derived adipose mesenchymal stem cells by immunofluoresence at passage two. The cells transfected with RFP showed (A1) morphology of osteogenic differentiation and (A2) morphology of RFP labeling (x 100). (A3) osteogenic control cultured in normal adipose mesenchymal stem cell medium during 14 d showing negative Alizarin red s staining (x 100). (A4) osteogenic induction during 14 d culture showed morphological changes and mineralized deposits as indicated by positive Alizarin red S staining (x 100). (B1) cells showing morphology of myogenic differentiation and (B2) morphology of RFP labeling (x 200). (B3) the negative control of the myosin immunostaining (x 200). (B4) myogenic differentiation showing expression of myosin as positive immunostaining with myosin antibody (green color) (x 200). (C1) cells showing morphology of chondrogenic differentiation and (C2) morphology of RFP labeling (x 100). (C3) chondrogenic differentiation 21 d after induction showing lacunae with extracellular proteoglycan formation as evidenced by positive staining with toluidine blue O (x 100). (C4) lacunae as indicated by arrows (x 200). Every experiment was repeated three times.

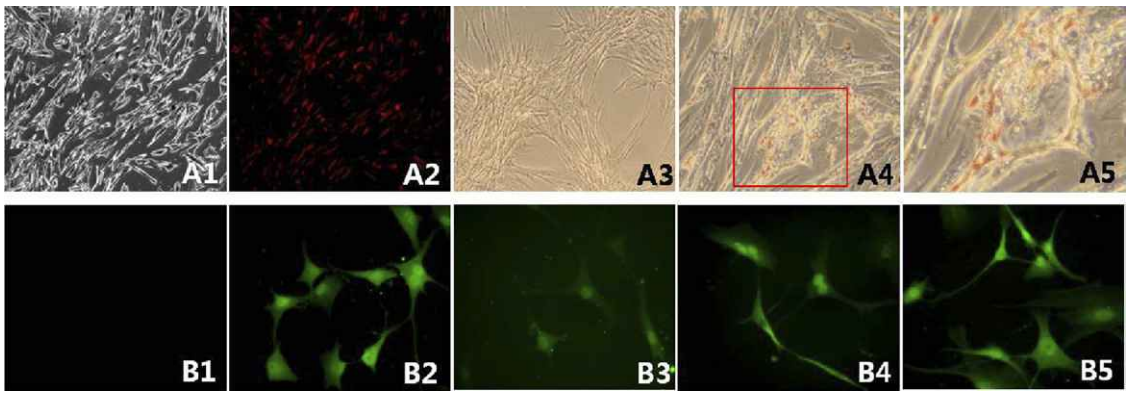


Fig. 4. *In vitro* adipogenic and neurogenic differentiation of transgenic dog derived adipose mesenchymal stem cells by immunofluoresence staining at passage two. (A1) morphology of adipogenic differentiation and (A2) morphology of RFP labeling (x 100) is shown. (A3) adipogenic control cultured in normal adipose mesenchymal stem cell medium during 21 d showed negative staining with Oil red O (x 100). (A4) adipogenic induction during 21 d showed morphological changes and accumulation of lipid-rich vacuoles in the cytoplasm as evidenced by positive Oil red O staining (x 100). (A5) lipid droplet deposition (vacuoles) demonstrating positive adipogenic induction. Morphology of neurogenic differentiation 10 d after induction showing large central bodies and neurites (x 400 of a quadrangle in A4). Neurogenic differentiation showing (B1) control, (B2) NSE antibody (green color), (B3) MAP-2 antibody (green color), (B4) TUJ1 antibody (green color) and (B5) GFAP antibody (green color) expression by positive immunostaining (x 400). Every experiment was repeated three times.

genic differentiation was confirmed by expression of neural cell markers (MAP-2, NSE or TUJ1 antibody; green) and astrocyte marker (GFAP; green) (Fig. 4B2, 4B3, 4B4 and 4B5).

3.3. Recloning of RFP transgenic pups using adipose mesenchymal stem cells

In total, 121 oocytes from 12 oocyte donor dogs were recovered and 109 of these oocytes were enucleated for cloning. The cAd-MSCs were injected into enucleated oocytes and fused by electric stimulation. The fused couplets (82/109, 75.2%; Table 1) were chemically activated and transferred into the uterine tubes of five naturally estrus-synchronized recipients. One of them (20%) maintained pregnancy until full-term and subsequently two healthy offspring were obtained by natural delivery on day 60 after embryo transfer (Table 2). The two cloned pups were genetically identical to the donor dog (Table 3). Also, their whole bodies were detected strong red fluorescence using a Leica inverted microscope equipped with a Texas red filter set (DsRed filter set; Biochemical Laboratory Services, Budapest, Hungary). The red fluorescence was produced by illumination with 540 ± 20 nm and detected by an emission filter with a maximal transmittance wavelength of 600 ± 25 nm (Fig. 5D). Interestingly, even under bright field illumination, the skin and claws of viable recloned pup appeared reddish and were readily distinguishable compared with a non-transgenic pup (Fig. 5B). Unfortunately, one pup died due to the surrogate mother's carelessness. Autopsy of the dead pup revealed no pathologic or anatomical abnormalities. Red fluorescence was seen in all of the tissues including

Table 1
Somatic cell nuclear transfer using canine adipose-mesenchymal stem cells

Replication	No. of oocyte donor dogs	Oocyte maturity	No. of flushed oocytes	No. of oocytes with transferred stem cells	No. of fused couplets
1	2	Mature	20	20	15
2	2	Aging	19	17	15
3	3	Mature	30	26	19
4	2	Mature	23	23	20
5	3	Aging/Mature	29	23	13
Total	12	—	121	109	82 (75.2%)[a]

[a] fused couplets/cell transferred oocytes (%).

Table 2
In vivo developmental ability of cloned embryos derived from canine adipose-mesenchymal stem cells

Recipient	No. of transferred embryos	Pregnancy	Size of litter (Birth weight, g)	Viability of offspring
A	12	−		
B	15	−		
C	24	−		
D	20	+	2 (260, 270)	50%
E	13	−		
Total	84	20%[a]	2.3%[b]	

[a] Pregnancies/recipients (%), [b] Size of litter/transferred embryos (%).

brain, heart, liver, kidney, lung, testis, muscle, intestine, thymus, spleen, adrenal gland, skin, bone and urinary bladder (data not shown).

4. Discussion

Adipose tissue is an attractive source for adult stem cells due to its abundance, plasticity in culture and relative simplicity of collection. Recent reports demonstrated that adipose stem cells have the potential to differentiate into other cell types, as well as having the potential for clinical applications [31,32]. Here, we demonstrated that 1) cAd-MSCs from cloned transgenic dogs have the capacity to differentiate into mesodermal and ectodermal lineages *in vitro*; 2) cAd-MSCs can be used to generate cloned pups by SCNT, i.e., production of stable, cloned RFP transgenic beagles.

The stem cell characteristics of cAd-MSCs established in this study were documented by two lines of evidence. The first line is the result of flow cytometry analysis using cell surface markers of mesenchymal stem cells. The cAd-MSC expressed mesenchymal stem cell markers including CD44 and Thy 1.1., but did not express hematopoietic or endothelial markers (CD31 and CD34). The flow cytometric measurements suggest that the primary cultures of cAD-MSCs may consist of heterogenous cell populations, e.g., 45% were CD29 positive, 6% were CD105 or CD73 positive and 7% were CD 34 positive. In humans, minimum criteria for characterization of Ad-MSC have already been established. The human Ad-MSC presented expression of markers CD105, CD73 and CD90, and exhibited no expression of the markers CD45, CD34, CD14, CD11b, CD79a or CD19 [33]. However, in the present study, the cAd-MSCs did not react with CD73 and CD105, which is reactive with human Ad-MSC. One major reason for the inconsistent pattern of the marker expression between human Ad-MSC and cAd-MSC could be due to the use of human CD73 and CD105 antibodies against cAd-MSCs. In line with our results, equine Ad-MSC did not obtain CD29 reactivity between human Ad-MSC and equine Ad-MSC [34]. Secondly, the most important criterion to qualify the cAd-MSCs as mesenchymal stem cells is their differentiation ability. The cAd-MSCs can serve as precursors to a broad spectrum of differentiated cell types. Neupane et al. [35] found that cAd-MSCs were able to differentiate along adipogenic and osteogenic pathways. We have assessed the multipotent characteristics of cAd-MSCs and showed that they successfully differentiated into osteogenic, adipogenic, myogenic and chondrogenic pathways in the mesodermal lineage and into neurogenic pathways in the ectodermal lineage, under specific culture conditions (Figs. 3 and 4).

It has been hypothesized that the genome of undifferentiated cells, such as stem cells, may be more easily reprogrammed by recipient oocytes during SCNT. The relationship between donor cell differentiation status and nuclear transfer success has been demonstrated in mice. Cloned mouse embryos derived from ES cells

Table 3
Microsatellite genotyping of recloned beagles

Marker	1st cloned dog	2nd cloned dog	Nuclear donor cell	Oocyte donor-1	Oocyte donor-2	Recipient
PEZ1	114	114	114	118/114	122/118	114
PEZ5	105/101	105/101	105/101	109/101	113/109	101
PEZ6	192/184	192/184	192/184	187	187/185	180
PEZ8	231	231	231	235/227	231	235/219
PEZ12	271/261	271/261	271/261	295/277	269	284
PEZ20	175	175	175	179/175	179	179/175
FH2010	231/227	231/227	231/227	239/231	235/227	235/231
FH2054	153/144	153/144	153/144	170/162	166/149	166/149
FH2079	273	273	273	277/273	290/269	273

Fig. 5. (A) The first dogs recloned by nuclear transfer of cAd-MSC derived from a transgenic cloned dog shown 2 d after birth. They are named Magic and Stem. (B) Recloned Magic which carries the RFP gene (left) and a non-transgenic puppy (control, right). Notice that the claws and pads of Magic are tinged with red even in bright field illumination. (C) Visible light image. (D) Fluorescence image.

showed significantly enhanced survival to term compared with those derived from somatic cell nuclei [18,20].

Therefore, we hypothesized that cAD-MSCs would be superior or at least equal in their suitability as nuclear donors than skin fibroblasts that are routinely used as donors. This possibility was tested in this study along with the purpose of recloning the RFP transgenic cloned dog. In this study, we provide evidence of cAD-MSCs' suitability as nuclear donors and the success of recloning the RFP transgenic cloned dog. Although we cannot exactly compare the efficiency between fibroblasts and Ad-MSCs, oocyte-donor cell couplets using cAd-MSCs fused at a rate of around 75%, which is similar to earlier reports from our group using adult or fetal fibroblasts [27,28,30,36]. The pregnancy rate, based on the number of pregnant recipients per total number of recipients, was similar to the pregnancy rate (15–30%) previously reported by our studies [27,28,30,36]. Here, we report the birth of the first recloned beagles from somatic stem cells and demonstrate the capacity of cAd-MSC for *in vivo* development. It is believed that the Ad-MSCs do not even compare favorably fibroblasts as nuclear donor and could be useful tool in dog SCNT. In agreement with our results, nuclear transfer using deer antler stem cells [37], porcine fetal somatic stem cells [23], hematopoietic stem cells [38], porcine skin-originated sphere stem cells [39] and porcine neural stem cells [40] have all successfully produced offspring.

Another important outcome of the present study is the successful recloning of an RFP-expressing transgenic cloned beagle by nuclear transfer of cells derived from this animal. The RFP cloned beagle produced in the previous study showed ubiquitous expression in its whole body [24]. The same was found in one of the two recloned beagles produced in this study, which strongly expressed RFP in the whole body and organs. Thus, recloning using cells derived from tissues of a transgenic animal can produce another transgenic clone carrying the foreign gene.

In conclusion, the present study demonstrates that cAd-MSCs exhibit multi-lineage differentiation potential and can be a good nuclear donor source for dog cloning. The present study demonstrated for the first time the successful production of cloned beagles by nuclear transfer of cAd-MSCs. Furthermore, we have demonstrated that recloning using cAd-MSCs is capable of producing multiple genetic modified clones, and that utilization of canine adipose derived-mesenchymal

stem cells may prove to be an excellent cell type for production of genetic disease model.

Acknowledgments

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