



Altered properties of feline adipose-derived mesenchymal stem cells during continuous *in vitro* cultivation

Bo-Yeon LEE^{1) #}, Qiang LI^{1) #}, Woo-Jin SONG^{1) #}, Hyung-Kyu CHAE¹⁾,
Kyeong KWEON¹⁾, Jin-Ok AHN¹⁾ and Hwa-Young YOUN^{1) *}

¹⁾Department of Veterinary Internal Medicine, College of Veterinary Medicine, Seoul National University, Seoul 08826, Republic of Korea

ABSTRACT. Cytotherapy with mesenchymal stem cells (MSCs) has been studied in many species, and often requires *in vitro* cell expansion to obtain therapeutic doses of stem cells. Because the characteristics of MSCs, such as self-renewal and multi-lineage differentiation, can be altered by long-term culture, it is important to maintain stemness during cultivation. This study assessed the changes in the characteristics of feline adipose tissue-derived (fAT)-MSCs during *in vitro* passaging. Stem cells isolated from the adipose tissue of donor cats were cultured for seven sub-passages. Proliferation capacity was analyzed by calculating the cell doubling time and by colorimetric assay. Expression of stem cell-specific markers was evaluated by quantitative reverse transcription (qRT)-PCR and immunophenotyping. Expression of adipogenic and osteogenic differentiation markers was also measured by qRT-PCR. Histochemical staining and measurement of β -galactosidase activity were conducted to detect cellular senescence. The cell proliferation rate decreased significantly at passage 5 (P5). Gene expression levels of pluripotency markers (*Sox2*, *Nanog* and *Klf4*) and stem cell surface markers (*CD9*, *CD44*, *CD90* and *CD105*) decreased during continuous culture; in most assays, statistically significant changes were observed at P5. The ability of cells to undergo adipogenic or osteogenic differentiation was inversely proportional to the number of passages. The proportion of senescent cells increased with the number of passages. These results suggest that repeated passages alter the proliferation and multipotency of fAT-MSCs. In clinical trials, early-passage cells should be used to achieve the maximum therapeutic effect.

KEY WORDS: cat, differentiation, mesenchymal stem cell, passage, proliferation

J. Vet. Med. Sci.

80(6): 930–938, 2018

doi: 10.1292/jvms.17-0563

Received: 19 October 2017

Accepted: 5 April 2018

Published online in J-STAGE:

18 April 2018

Mesenchymal stem cells (MSCs) are undifferentiated cells that can be extracted from bone marrow [1], adipose tissue [21], umbilical cord blood [42], muscle [37], and dental pulp [24]. MSCs are characterized by their self-renewal, multipotency, and ability to differentiate into diverse tissues such as connective tissue, muscle, and blood vessels [5]. Because of these characteristics, MSCs play an important role in restoring tissue function when an organ or tissue is damaged. Adipose-derived stem cells have been particularly widely studied because they can be easily extracted and isolated in large quantities [6].

The therapeutic applications of stem cells, which take advantage of these characteristics, have been studied in various species, including humans, dogs, and cats [16, 20]. In cats, Quimby *et al.* [26] and Parys *et al.* [23] independently confirmed the safety of intravenous and intraperitoneal injection of feline adipose-derived stem cells. Case reports have described the therapeutic application of feline adipose-derived stem cells for the treatment of various diseases, including chronic kidney disease [25], asthma [33], gingivitis [2], and enteropathic diseases [36].

In general, the number of cells that must be injected for MSCs to elicit treatment effects is 10^5 – 10^8 cells/kg [39], and thus an *in vitro* expansion process is required to obtain sufficient numbers of stem cells. In both humans and dogs, sequential *in vitro* passaging of adipose-derived stem cells leads to cell aging, a decrease in the rate of proliferation, and changes in gene expression patterns and differentiation potency [7, 17]. Because such changes in cell characteristics are directly related to therapeutic applications and the efficacy of stem cell treatments, it is important to maintain a balance between cell expansion and stemness. For feline cells, few studies have investigated changes in stem cell characteristics with sequential passaging. This study aimed to assess changes in the proliferation capacity, differentiation potency, and molecular expression patterns of feline adipose tissue-derived (fAT)-MSCs during long-term culture.

*Correspondence to: Youn, H.-Y.: hyyoun@snu.ac.kr

#These authors contributed equally to this work.

©2018 The Japanese Society of Veterinary Science



This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)

MATERIALS AND METHODS

Isolation, expansion and storage of FAT-MSCs

Adipose tissue was obtained from three healthy adult female domestic short-haired cats during ovariohysterectomy at the Seoul National University (SNU) Veterinary Medicine Teaching Hospital. Their owners provided informed written consent for research use. The blood analysis and imaging findings of the donor cats were normal. In addition, the cats were free of infection by feline leukemia virus and feline immunodeficiency virus. The procedure was approved by the Institutional Animal Care and Use Committee of SNU and the protocol was performed in accordance with approved guidelines. Tissue samples were washed four times in Dulbecco's phosphate buffered saline (DPBS; PAN-Biotech, Aidenbach, Germany) containing 1% penicillin-streptomycin (PS; PAN-Biotech), finely minced in a petri dish with sterile scissors, and digested with 0.1% collagenase I (Gibco/Life Technologies, Carlsbad, CA, U.S.A.) solution for 60 min at 37°C. After digestion, three volumes of high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS; PAN-Biotech) was added to neutralize the sample. The adipose tissue mixture was then centrifuged at $1,200 \times g$ for 5 min. The supernatant was removed, and the pellet containing cells was resuspended in 5 ml high-glucose DMEM. The cell suspension was passed through a 70- μm cell strainer to remove undigested debris and then centrifuged at $1,200 \times g$ for 5 min. Erythrocytes in the pellet were eliminated by adding 2 ml of red blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO, U.S.A.), and the cell solution was incubated for 10 min at 37°C. The sample was washed in five volumes of DPBS and centrifuged again at $1,200 \times g$ for 5 min. After removing the supernatant by suction, cells were resuspended in high-glucose DMEM and seeded onto a 100-mm ϕ cell culture dish at a density of 3,000/cm². Cells were incubated at 37°C and 5% CO₂ in high-glucose DMEM containing 20% FBS and 1% PS. During cell expansion, the culture media was changed every 2–3 days. For all passages from P0 to P7, cultured cells were seeded at a density of 10,000/cm² in 100-mm ϕ cell culture dishes for subculture at 70–80% confluency using 1 ml of 0.25% trypsin-EDTA (PAN-Biotech).

To preserve cells from each passage, 1×10^6 cells were stocked in cryopreservation medium composed of 80% FBS, 10% DMEM and 10% dimethyl sulfoxide (Daejung Chemicals & Metals, Siheung, Korea) and stored in liquid nitrogen as described previously [39].

Flow cytometry

Flow cytometry was used to evaluate the expression of cluster of differentiation (CD) MSC markers. Cryopreserved cells at P1 were thawed and cultured in culture medium in a 100-mm ϕ culture dish. Cultured cells were detached from the plate with 0.25% trypsin-EDTA when confluency reached 80%. The obtained cells were washed with DPBS and divided into three conical tubes, each containing 1×10^6 cells. Cells were suspended in 30 μl DPBS and 3 μl monoclonal antibodies against the following proteins: CD9, CD44 (GeneTex, Irvine, CA, U.S.A.), CD34-phycoerythrin (PE) and CD45-fluorescein isothiocyanate (FITC; eBiosciences, San Diego, CA, U.S.A.). For CD9 and CD44, indirect immunofluorescence was performed with goat anti-mouse IgG-FITC and goat anti-rat IgG-PE (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for each marker. Non-stained cells were used as controls for autofluorescence. Cell fluorescence was analyzed with a flow cytometer (FACS Aria II; BD Biosciences, Franklin Lakes, NJ, U.S.A.). A minimum of 10,000 events were counted for each sample, and all data were analyzed using FlowJo7.6.5 (Tree Star, Inc., Ashland, OR, U.S.A.).

Tri-lineage differentiation

For adipogenic and osteogenic differentiation, P1 cells at 80% confluency were cultured in a 24-well plate in appropriately conditioned differentiation medium. The adipogenic differentiation medium was composed of high-glucose DMEM containing 10% FBS, 5% insulin–transferrin–selenium-X (Invitrogen, Carlsbad, CA, U.S.A.), 1 μM dexamethasone (Sigma-Aldrich), and 0.5 mM 3-isobutyl-L-methylxanthine (IBMX, Sigma-Aldrich) according to Kono *et al.* [14]. The StemPro Osteogenesis Differentiation kit (Gibco/Life Technologies) was used to evaluate osteogenic differentiation according to the manufacturer's instructions. For chondrogenic differentiation, 10 μl of highly concentrated cell suspension ($5 \times 10^6/\text{ml}$) was first seeded in the middle of the plate and incubated with culture medium for two days. Cells were then washed twice with PBS and cultured with the StemPro Chondrogenesis Differentiation kit (Gibco/Life Technologies). Cells in adipogenic and osteogenic differentiation media were each cultured for one week, and cells in chondrogenic differentiation medium were cultured for two weeks. After differentiation, all cells were fixed with 4% paraformaldehyde. Cells were then stained with oil red O, 1% alizarin red, or Alcian blue (all Sigma-Aldrich) to confirm adipocyte, osteoblast, and chondrocyte differentiation, respectively. Before their use in downstream experiments, cells were characterized by immunophenotyping and multi-lineage differentiation.

Growth kinetics

During sequential passages, cell doubling times (DTs) were calculated in triplicate cultures. The numbers of cells and culture times (CTs) were used to calculate DTs based on the following formula:

$$\text{DT} = \text{CT} \times \frac{\ln 2}{\ln \left(\frac{\text{N1}}{\text{N0}} \right)}$$

where N0 represents the initial cell number and N1 represents the cells number at the time of harvest.

Table 1. qRT-PCR primer sequences

Gene	Forward	Reverse
<i>GAPDH</i>	ACGATGACATCAAGAAGGTG	CATACCAGGAAATGAGCTTG
<i>Oct4</i>	GGAGTCCCAGGACATCAAAG	GCCTGCACAAGTGTCTCTGC
<i>Sox2</i>	GAGTGGAAACTTTTGTCTGGAGAC	GGCAGTGTGACTTATCCTTCTTCA
<i>Nanog</i>	TTTGCTGTAACGTATCTGGG	CCAGGCTTCTATTCTATCACCAG
<i>Klf4</i>	TTACCACTGTGACTGGGATG	CTTCATGTGTAAGGCGAGGT
<i>CD9</i>	CCAAGTGTATCAAATACCTGCTCTT	ATAAACTCCTGTGTAGAAGCTGGAA
<i>CD44</i>	TGGGTTGTTTGGCATCCAGTGC	CGTTTTCTTCAGTTGGTTCCCAGCC
<i>CD90</i>	TGAGAAGAAGAAGCACGTGA	ACGTGGAGTTCACATGTGTA
<i>CD105</i>	TATGCGTCTGAACATCGTCA	GTGTGCGAGTAGATGTACCA
<i>PPAR-γ</i>	GGGAGTTTCTAAAGAGCCTGAG	GTGCCTCAATGGGCTTCACATTGAGC
<i>COL1α1</i>	ATCACCTACCACTGCAAGAACAG	GTGGTTTTGTATTTCGATCACTGTC

Colorimetric cell proliferation assay

An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide; Sigma-Aldrich] assay was used to evaluate the proliferation ability of MSCs at each passage. MSCs at P1, P3, P5 and P7 were seeded at 5×10^3 cells per well in a 96-well microtiter plate and cultured in five replicates at 37°C and 5% CO₂ for two days. At 24 and 48 hr after cultivation, the culture medium was removed and 10 μl of MTT solution in 5 mg/ml PBS was added. The plate was then placed in the dark at 37°C. After 4 hr, the supernatant was aspirated and 100 μl dimethyl sulfoxide was added to each well as a detergent. The plate was incubated in the dark at 37°C for 10 min. The absorbance of each well at 570 nm was measured using a microplate reader.

RNA extraction and gene expression analysis

Total RNA from MSCs at P1, P3, P5 and P7 was extracted using the Easy-BLUE Total RNA Extraction kit (Intron Biotechnology, Sungnam, Korea) according to the manufacturer's instructions. Briefly, cells from each passage were transferred to a tube containing 1 ml of Easy-BLUE, and 200 μl chloroform was added to extract the homogenate. Total RNA was precipitated with isopropanol, and the resulting pellet was washed with ethanol. After air-drying, the RNA pellet was resuspended in 30 μl of distilled water. The concentration and purity of RNA samples were measured with a spectrophotometer (Implen, Munich, Germany) at 260 and 280 nm. Samples with absorbance ratios (260/280) higher than 1.8 were used for subsequent analysis. First-strand cDNA was synthesized with LaboPass M-MuLV Reverse Transcriptase (Cosmo Genetech, Seoul, Korea) using the primers listed in Table 1.

Quantitative real-time (qRT)-PCR analysis was performed in duplicate in 96-well plates with StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) using SYBR Green dye (AMPIGENE qPCR Green Mix Hi-ROX; Enzo Life Sciences, Farmingdale, NY, U.S.A.). The following amplification program was used: polymerase activation for 2 min at 95°C, followed by 40 cycles of denaturation for 5 sec at 95°C and annealing and extension for 25 sec at 60°C. Melting curve analysis was carried out from 65°C to 97°C to evaluate the specificity of PCR products. Feline glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a reference to compare gene expression, and the Ct value of each gene was normalized to that of *GAPDH*.

Senescent cell identification

Senescent cells were identified using the Cellular Senescence Assay kit (CELL BIOLABS, San Diego, CA, U.S.A.), which detects senescence-associated β-galactosidase (SA-β-gal) activity at pH 6.0. The number of senescence positive and negative cells was calculated under a light microscope.

Statistical analysis

All data were analyzed using GraphPad Prism v.6.01 software (GraphPad Inc., La Jolla, CA, U.S.A.). All data are displayed as the means ± standard deviation. In comparisons of multiple sample groups, *P*-values <0.05 were considered statistically significant.

RESULTS

Identification of fAT-MSCs

Cells obtained from feline adipose tissue at P1 were characterized by morphology, immunophenotyping, and tri-lineage differentiation. Three days after seeding, spindle-shaped cells were adhered to plastic culture plates (Fig. 1A). Cells were positive for expression of CD9 and CD44 and negative for CD34 and CD45 (Fig. 1B). fAT-MSCs differentiated into adipogenic, osteogenic, and chondrogenic cells (Fig. 1C).

Cell proliferation assay

Overall, proliferation rates decreased with an increasing number of passages. While there was no significant difference in proliferation rates from P1 to P4, marked suppression of the expansion rate was observed at P5 (*P*<0.001). After P5, cells exhibited

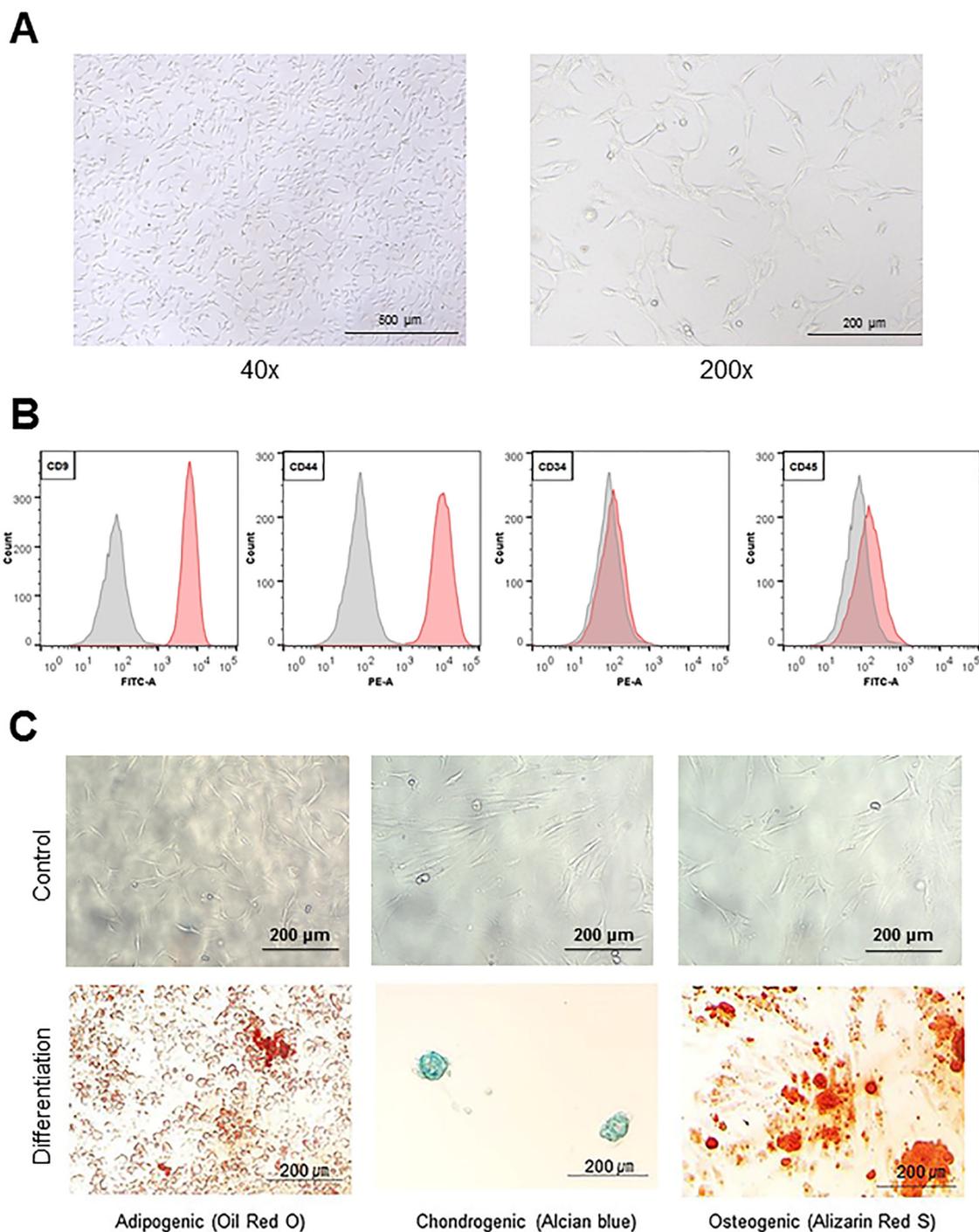


Fig. 1. Characterization of fAT-MSCs. P1 cells were used for identification. (A) Typical fibroblast-like spindle-shaped cells adhered to a plastic cell culture dish are shown. Original magnification: 40 × (left) and 200 × (right). (B) Immunophenotypic analysis was conducted using flow cytometry for positive markers CD9 and CD44 and negative markers CD34 and CD45. (C) Adipogenic (oil red O stain), osteogenic (alizarin red S stain), and chondrogenic (Alcian blue stain) differentiation abilities of fAT-MSCs (from left to right). Original magnification: 200 ×. Respective negative control cells are shown.

a persistent increase in the DT, which became significant at P7 (Fig. 2A). Furthermore, cells exhibited a reduced ability to absorb MTT over time, as shown in Fig. 2B, with significantly reduced absorbance from P5 ($P < 0.001$).

Altered expression of MSC markers with subsequent passages

The mRNA expression levels of pluripotency genes and stem cell surface markers were assessed by qRT-PCR. Expression of

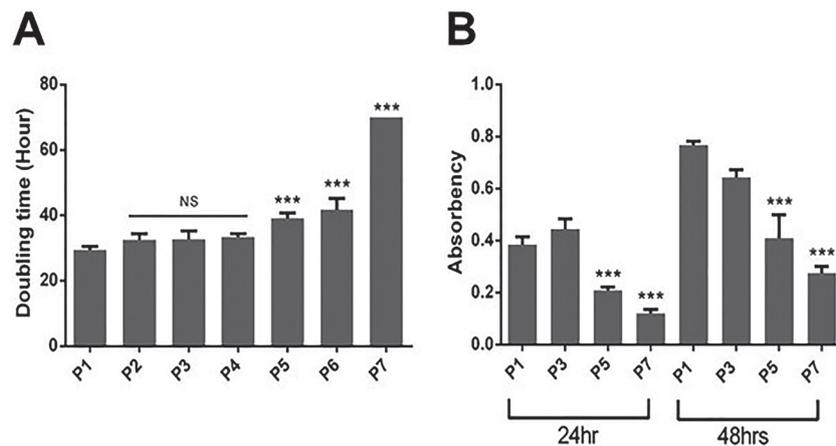


Fig. 2. Decreased proliferation ability during passaging. (A) Cell doubling time during continuous subcultures. (B) Results of MTT assay at 24 and 48 hr after seeding. *** $P < 0.001$ vs. P1 cells.

pluripotency genes including *Sox2*, *Klf4*, and *Nanog* decreased with subsequent passages (Fig. 3A). *Oct4* was not expressed in cells at any passage (data not shown). Expression levels of stem cell surface markers, including *CD9*, *CD44*, *CD90* and *CD105*, were also significantly reduced during subculture. A particularly notable decrease was observed in the expression of *CD9* and *CD44* from P3 ($P < 0.001$ for both, Fig. 3B).

Protein expression of stem cell markers was measured by flow cytometry. After all passages, most fAT-MSCs expressed CD9 and CD44. Other markers, including CD34 and CD45, were not expressed or were rarely expressed. The expression of CD9 and CD44 proteins was reduced following P3, which is consistent with the qRT-PCR results (Fig. 3C).

Effect of continuous culture on the differentiation capability of fAT-MSCs

The expression of adipogenesis and osteogenesis differentiation marker genes was analyzed by qRT-PCR. Compared to P1, expression of the adipogenic marker peroxisome proliferator-activated receptor gamma (*PPAR γ*) was significantly reduced at P5 (Fig. 4A). Similarly, expression of the osteogenic marker collagen 1 α 1 (*COL1 α 1*) was significantly reduced at P5 (Fig. 4B).

Elevated cell senescence with increasing passages

With an increasing number of passages, the cells became larger and their shapes became heterogeneous. Senescent cells were not observed at P1. At P3, approximately 0.7% of cells exhibited SA- β -gal activity. At P5 and P7, 2.8 and 5% of cells, respectively, were stained blue (Fig. 5A–D). Expression of SA- β -gal activity increased significantly beginning at P5 ($P < 0.001$; Fig. 5E).

DISCUSSION

Recently, the clinical applications of MSCs have been widely studied in many species and for many diseases [8]. After separation from the source, cells must be cultured *in vitro* to obtain a therapeutic dose of stem cells. Numerous studies have shown that the characteristics of MSCs can become altered during prolonged culturing [9, 10, 27]. According to a study by Lee *et al.* [18] in dogs, passages beyond P6 led to a significant increase in proliferation time and decrease in the differentiation index of cells. However, no similar studies have been conducted for cats. In the present study, fAT-MSCs from healthy cats were used to assess changes in proliferation and stem cell differentiation potency for each sequential passage. We also confirmed a correlation between such changes and cell aging.

To achieve effective cytotераpy treatments using MSCs, stem cells that have been injected into the body must appropriately replace damaged organ tissue and produce sufficient anti-inflammatory effects through the paracrine system [28]. The proliferative ability and multipotency of stem cells must also be maintained to achieve proper cell proliferation and differentiation into target tissues. A decrease in proliferation rate with sequential passages has been reported in cats and various other species [27, 35, 38, 39]. In the present study, the proliferation rate decreased successively from P1 to P7, which became statistically significant beginning at P5. Based on these results, significant differences in stem cell characteristics are expected to occur after P5, and thus subsequent experiments examined only P1, P3, P5 and P7.

We next examined mRNA expression levels for various factors that determine stem cell characteristics, such as pluripotency markers (*Oct4*, *Sox2*, *Nanog* and *Klf4*) and stem cell surface markers (*CD9*, *CD44*, *CD90* and *CD105*). Interestingly, among these pluripotency markers, *Oct4* expression was not observed regardless of the number of passages, while expression of the remaining factors decreased with an increasing number of passages. When the mRNA levels of all detected factors were compared with those at P1, significant decreases were detected beginning at P5. In the case of stem cell surface markers, a significant decrease in *CD9* expression was observed beginning at P3, while significant reductions in *CD44*, *CD90* and *CD105* expression were observed

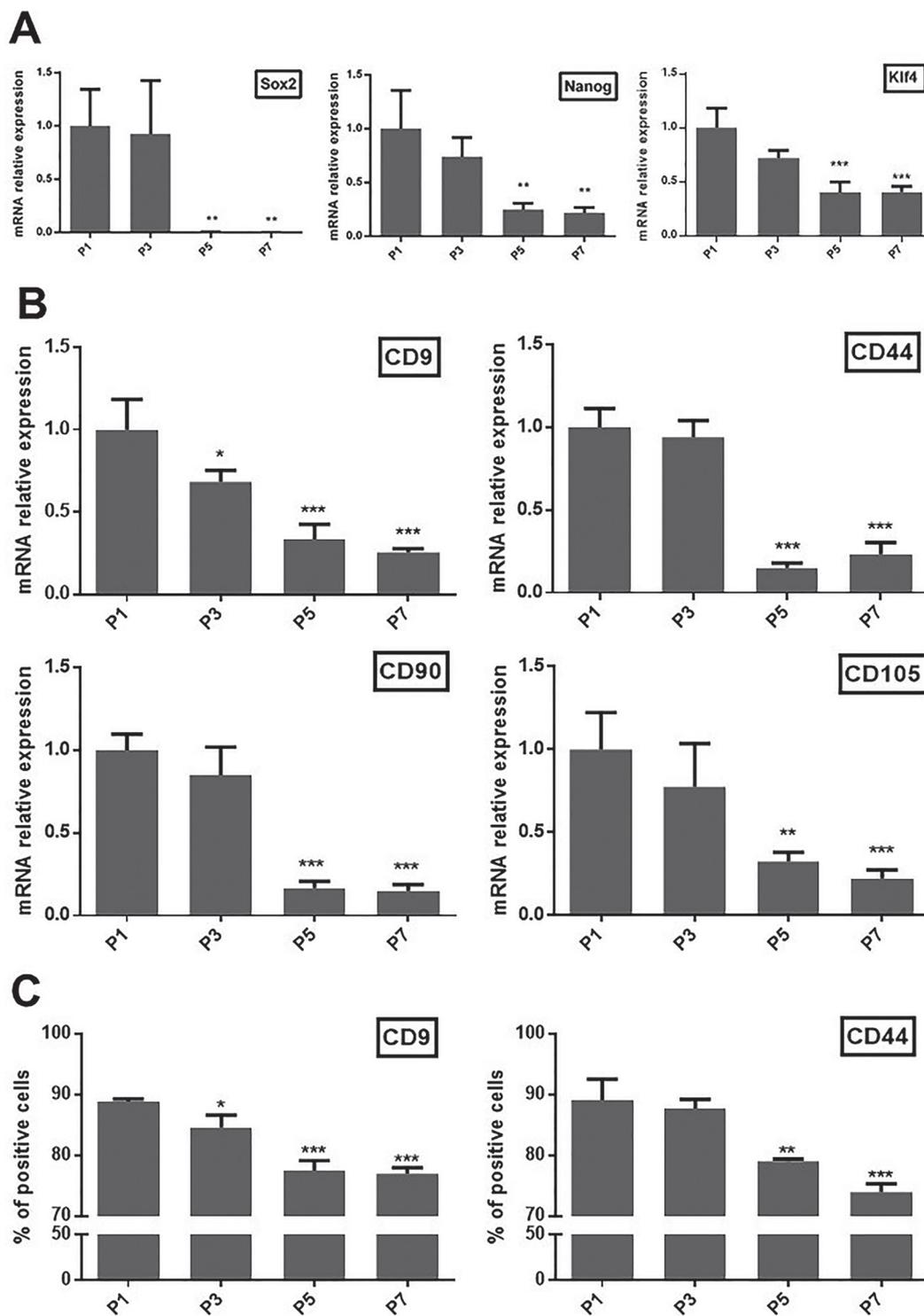


Fig. 3. Expression of pluripotency markers and cell surface markers at P1, P3, P5 and P7 (A) Relative mRNA expression levels of pluripotency markers *Sox2*, *Nanog* and *Klf4*. (B) Relative mRNA expression levels of stem cell surface marker genes *CD9*, *CD44*, *CD90* and *CD105*. (C) Cell surface protein expression analyzed by flow cytometry. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. P1 cells.

at P5. In the subsequent flow cytometry experiment, corresponding decreases in protein expression were confirmed. Based on a previous study, the pluripotency marker genes tested in the present study are involved in self-renewal as well as the maintenance of cell pluripotency in various species [19, 29, 41]. Classically, it is thought that leukemia inhibitory factor and the activation of Stat3 play key roles in maintaining stem cell pluripotency [31]. A recent study, however, revealed that mouse embryonic stem cells overexpressing Nanog maintain their pluripotency without leukemia inhibitory factor activity, indicating the existence of an

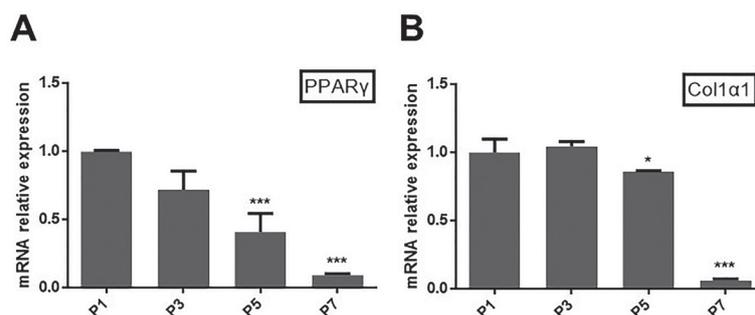


Fig. 4. mRNA expression levels of (A) adipogenic (*PPAR γ*) and (B) osteogenic (*COL1 α 1*) differentiation markers with sequential passages were analyzed. * $P < 0.05$, *** $P < 0.001$ vs. P1 cells.

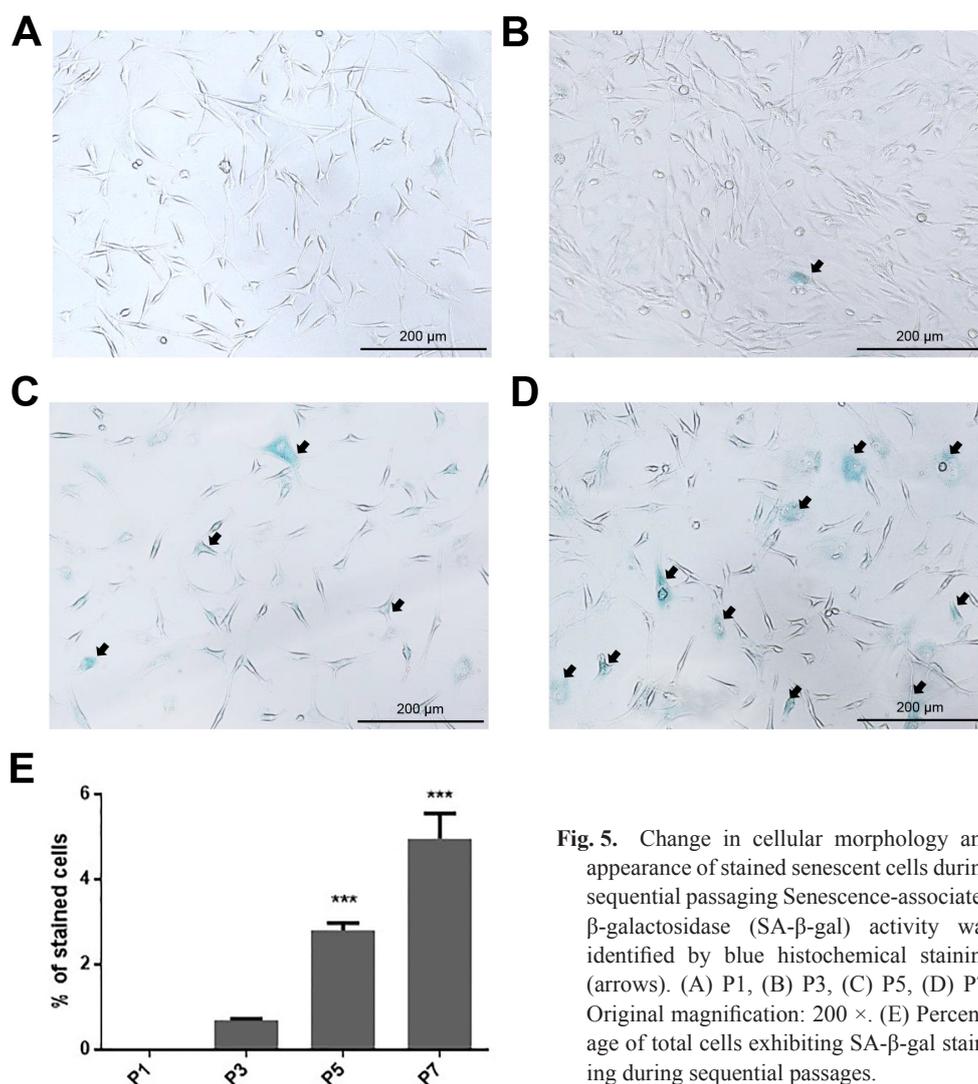


Fig. 5. Change in cellular morphology and appearance of stained senescent cells during sequential passaging. Senescence-associated β -galactosidase (SA- β -gal) activity was identified by blue histochemical staining (arrows). (A) P1, (B) P3, (C) P5, (D) P7. Original magnification: 200 \times . (E) Percentage of total cells exhibiting SA- β -gal staining during sequential passages.

independent pathway for pluripotency maintenance [22, 30]. In addition, *Klf4*, which acts upstream of *Nanog* and is the direct target of *Stat3*, is known to be an important factor in pluripotency preservation [40]. Based on these studies, *Nanog* and *Klf4* may act as key regulatory factors in the maintenance of pluripotency and self-renewal, and reductions in the expression levels of these proteins are thought to lead to reduced stemness. As the cell surface markers *CD9*, *CD44*, *CD90* and *CD105* are closely related to stem cell proliferation [12], migration [43], and differentiation [3, 13], decreases in their expression levels with sequential passages is expected to greatly influence the efficacy of stem cell therapies.

To confirm the results described above, the mRNA expression levels of adipogenic and osteogenic differentiation markers were

evaluated to determine changes in actual differentiation potency with sequential passages. We observed significant decreases in both *PPAR γ* (adipogenic differentiation marker) and *COL1a1* (osteogenic differentiation marker) after P5. These results are consistent with those of previous studies assessing the differentiation ability of bone-marrow-derived stem cells from humans and dogs at each passage [11, 18, 32]. In contrast, a study of bone-marrow-derived stem cells from pigs showed that adipogenic differentiation increased with sequential passages [34]. It appears that changes in differentiation potency with sequential passages differ among species and according to external conditions, such as the composition of the culture medium.

Finally, when evaluating senescence-associated enzyme activity during cell aging, stained cells first appeared at P3, and the percentage of stained cells in a specific field of view increased significantly beginning at P5. Additionally, cell morphologies gradually became diversified with increasing passages, accompanied by an increase in cell size. In typical cells, cell aging is accompanied by changes in gene expression patterns [4]. Such changes can either prevent cells from responding to mitogenic signals or affect reproduction or differentiation by altering their metabolic status. Therefore, it is thought that increasing numbers of senescent stem cells, which accumulate with sequential passages, may cause a reduction in treatment effects during *in vivo* applications. Additionally, although it has not been confirmed in feline stem cells, it has been reported that human senescent fibroblasts can cause malignant transformation of cells through interactions with adjacent cells [15]. Therefore, whether aging cells cause diverse changes to adjacent cells through this mechanism requires further analysis.

There is a limitation in this study. We obtained fAT-MSCs from three cats. Although they showed similar abilities of proliferation and differentiation depending passages, larger populations should be evaluated.

In the present study, we confirmed the changes in the characteristics of feline stem cells that occurred with long-term *in vitro* culture. We observed a continuous decrease in the self-renewal ability and pluripotency of these cells over subsequent passages during *in vitro* culture, with significant differences in the expression of most of the cell surface markers, which commenced at P5. Although the effects of applying aged stem cells in cats are unknown, we recommend the use of early-passage cells, particularly those before P5, for efficacious therapeutic application of stem cells in cats.

CONFLICT OF INTEREST. There are no conflicts of interest to declare.

ACKNOWLEDGMENT. This work was supported by the Research Institute for Veterinary Science and BK21 PLUS Program for Creative Veterinary Science Research.

REFERENCES

1. Al-Nbaheen, M., Vishnubalaji, R., Ali, D., Bouslimi, A., Al-Jassir, F., Megges, M., Prigione, A., Adjaye, J., Kassem, M. and Aldahmash, A. 2013. Human stromal (mesenchymal) stem cells from bone marrow, adipose tissue and skin exhibit differences in molecular phenotype and differentiation potential. *Stem Cell Rev.* **9**: 32–43. [Medline] [CrossRef]
2. Arzi, B., Mills-Ko, E., Verstraete, F. J., Kol, A., Walker, N. J., Badgley, M. R., Fazel, N., Murphy, W. J., Vapniarsky, N. and Borjesson, D. L. 2016. Therapeutic efficacy of fresh, autologous mesenchymal stem cells for severe refractory gingivostomatitis in cats. *Stem Cells Transl. Med.* **5**: 75–86. [Medline] [CrossRef]
3. Aslan, H., Zilberman, Y., Kandel, L., Liebergall, M., Oskouian, R. J., Gazit, D. and Gazit, Z. 2006. Osteogenic differentiation of noncultured immunoisolated bone marrow-derived CD105+ cells. *Stem Cells* **24**: 1728–1737. [Medline] [CrossRef]
4. Campisi, J. and d'Adda di Fagagna, F. 2007. Cellular senescence: when bad things happen to good cells. *Nat. Rev. Mol. Cell Biol.* **8**: 729–740. [Medline] [CrossRef]
5. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D. and Horwitz, E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**: 315–317. [Medline] [CrossRef]
6. Gimble, J. and Guilak, F. 2003. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* **5**: 362–369. [Medline] [CrossRef]
7. Gruber, H. E., Somayaji, S., Riley, F., Hoelscher, G. L., Norton, H. J., Ingram, J. and Hanley, E. N. Jr. 2012. Human adipose-derived mesenchymal stem cells: serial passaging, doubling time and cell senescence. *Biotech. Histochem.* **87**: 303–311. [Medline] [CrossRef]
8. Hoffman, A. M. and Dow, S. W. 2016. Concise review: Stem cell trials using companion animal disease models. *Stem Cells* **34**: 1709–1729. [Medline] [CrossRef]
9. Izadpanah, R., Kaushal, D., Kriedt, C., Tsien, F., Patel, B., Dufour, J. and Bunnell, B. A. 2008. Long-term *in vitro* expansion alters the biology of adult mesenchymal stem cells. *Cancer Res.* **68**: 4229–4238. [Medline] [CrossRef]
10. Katsara, O., Mahaira, L. G., Iliopoulou, E. G., Moustaki, A., Antsaklis, A., Loutradis, D., Stefanidis, K., Baxevas, C. N., Papamichail, M. and Perez, S. A. 2011. Effects of donor age, gender, and *in vitro* cellular aging on the phenotypic, functional, and molecular characteristics of mouse bone marrow-derived mesenchymal stem cells. *Stem Cells Dev.* **20**: 1549–1561. [Medline] [CrossRef]
11. Kim, J., Kang, J. W., Park, J. H., Choi, Y., Choi, K. S., Park, K. D., Baek, D. H., Seong, S. K., Min, H. K. and Kim, H. S. 2009. Biological characterization of long-term cultured human mesenchymal stem cells. *Arch. Pharm. Res.* **32**: 117–126. [Medline] [CrossRef]
12. Kim, Y. J., Yu, J. M., Joo, H. J., Kim, H. K., Cho, H. H., Bae, Y. C. and Jung, J. S. 2007. Role of CD9 in proliferation and proangiogenic action of human adipose-derived mesenchymal stem cells. *Pflugers Arch.* **455**: 283–296. [Medline] [CrossRef]
13. Knudson, W. and Loeser, R. F. 2002. CD44 and integrin matrix receptors participate in cartilage homeostasis. *Cell. Mol. Life Sci.* **59**: 36–44. [Medline] [CrossRef]
14. Kono, S., Kazama, T., Kano, K., Harada, K., Uechi, M. and Matsumoto, T. 2014. Phenotypic and functional properties of feline dedifferentiated fat cells and adipose-derived stem cells. *Vet. J.* **199**: 88–96. [Medline] [CrossRef]
15. Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y. and Campisi, J. 2001. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc. Natl. Acad. Sci. U.S.A.* **98**: 12072–12077. [Medline] [CrossRef]

16. Le Blanc, K., Frassoni, F., Ball, L., Locatelli, F., Roelofs, H., Lewis, I., Lanino, E., Sundberg, B., Bernardo, M. E., Remberger, M., Dini, G., Egeler, R. M., Bacigalupo, A., Fibbe, W., Ringdén O., Developmental Committee of the European Group for Blood and Marrow Transplantation 2008. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* **371**: 1579–1586. [[Medline](#)] [[CrossRef](#)]
17. Lee, K. S., Cha, S. H., Kang, H. W., Song, J. Y., Lee, K. W., Ko, K. B. and Lee, H. T. 2013. Effects of serial passage on the characteristics and chondrogenic differentiation of canine umbilical cord matrix derived mesenchymal stem cells. *Asian-Australas. J. Anim. Sci.* **26**: 588–595. [[Medline](#)] [[CrossRef](#)]
18. Lee, K. S., Kang, H. W., Lee, H. T., Kim, H. J., Kim, C. L., Song, J. Y., Lee, K. W. and Cha, S. H. 2014. Sequential sub-passage decreases the differentiation potential of canine adipose-derived mesenchymal stem cells. *Res. Vet. Sci.* **96**: 267–275. [[Medline](#)] [[CrossRef](#)]
19. Loh, Y. H., Wu, Q., Chew, J. L., Vega, V. B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., Wong, K. Y., Sung, K. W., Lee, C. W., Zhao, X. D., Chiu, K. P., Lipovich, L., Kuznetsov, V. A., Robson, P., Stanton, L. W., Wei, C. L., Ruan, Y., Lim, B. and Ng, H. H. 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat. Genet.* **38**: 431–440. [[Medline](#)] [[CrossRef](#)]
20. Marx, C., Silveira, M. D. and Beyer Nardi, N. 2015. Adipose-derived stem cells in veterinary medicine: characterization and therapeutic applications. *Stem Cells Dev.* **24**: 803–813. [[Medline](#)] [[CrossRef](#)]
21. Oedayringsingh-Varma, M. J., van Ham, S. M., Knippenberg, M., Helder, M. N., Klein-Nulend, J., Schouten, T. E., Ritt, M. J. and van Milligen, F. J. 2006. Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure. *Cytotherapy* **8**: 166–177. [[Medline](#)] [[CrossRef](#)]
22. Pan, G. and Thomson, J. A. 2007. Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Res.* **17**: 42–49. [[Medline](#)] [[CrossRef](#)]
23. Parys, M., Nelson, N., Koehl, K., Miller, R., Kaneene, J. B., Kruger, J. M. and Yuzbasiyan-Gurkan, V. 2016. Safety of intraperitoneal injection of adipose tissue-derived autologous mesenchymal stem cells in cats. *J. Vet. Intern. Med.* **30**: 157–163. [[Medline](#)] [[CrossRef](#)]
24. Perry, B. C., Zhou, D., Wu, X., Yang, F. C., Byers, M. A., Chu, T. M. G., Hockema, J. J., Woods, E. J. and Goebel, W. S. 2008. Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use. *Tissue Eng. Part C Methods* **14**: 149–156. [[Medline](#)] [[CrossRef](#)]
25. Quimby, J. M., Webb, T. L., Gibbons, D. S. and Dow, S. W. 2011. Evaluation of intrarenal mesenchymal stem cell injection for treatment of chronic kidney disease in cats: a pilot study. *J. Feline Med. Surg.* **13**: 418–426. [[Medline](#)] [[CrossRef](#)]
26. Quimby, J. M., Webb, T. L., Habenicht, L. M. and Dow, S. W. 2013. Safety and efficacy of intravenous infusion of allogeneic cryopreserved mesenchymal stem cells for treatment of chronic kidney disease in cats: results of three sequential pilot studies. *Stem Cell Res. Ther.* **4**: 48. [[Medline](#)] [[CrossRef](#)]
27. Roobrouck, V. D., Ulloa-Montoya, F. and Verfaillie, C. M. 2008. Self-renewal and differentiation capacity of young and aged stem cells. *Exp. Cell Res.* **314**: 1937–1944. [[Medline](#)] [[CrossRef](#)]
28. Sharma, R. R., Pollock, K., Hubel, A. and McKenna, D. 2014. Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices. *Transfusion* **54**: 1418–1437. [[Medline](#)] [[CrossRef](#)]
29. Shi, Y. and Ai, W. 2013. Function of KLF4 in stem cell biology. pp. 317–343. In: *Pluripotent Stem Cells* (Bhartiya, D. and Lenka, N. eds), InTech, Winchester.
30. Silva, J., Nichols, J., Theunissen, T. W., Guo, G., van Oosten, A. L., Barrandon, O., Wray, J., Yamanaka, S., Chambers, I. and Smith, A. 2009. Nanog is the gateway to the pluripotent ground state. *Cell* **138**: 722–737. [[Medline](#)] [[CrossRef](#)]
31. Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M. and Rogers, D. 1988. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**: 688–690. [[Medline](#)] [[CrossRef](#)]
32. Stenderup, K., Justesen, J., Clausen, C. and Kassem, M. 2003. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone* **33**: 919–926. [[Medline](#)] [[CrossRef](#)]
33. Trzil, J. E., Masseur, I., Webb, T. L., Chang, C. H., Dodam, J. R., Cohn, L. A., Liu, H., Quimby, J. M., Dow, S. W. and Reiner, C. R. 2014. Long-term evaluation of mesenchymal stem cell therapy in a feline model of chronic allergic asthma. *Clin. Exp. Allergy* **44**: 1546–1557. [[Medline](#)] [[CrossRef](#)]
34. Vacanti, V., Kong, E., Suzuki, G., Sato, K., Canty, J. M. and Lee, T. 2005. Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture. *J. Cell. Physiol.* **205**: 194–201. [[Medline](#)] [[CrossRef](#)]
35. Vidal, M. A., Walker, N. J., Napoli, E. and Borjesson, D. L. 2012. Evaluation of senescence in mesenchymal stem cells isolated from equine bone marrow, adipose tissue, and umbilical cord tissue. *Stem Cells Dev.* **21**: 273–283. [[Medline](#)] [[CrossRef](#)]
36. Webb, T. L. and Webb, C. B. 2015. Stem cell therapy in cats with chronic enteropathy: a proof-of-concept study. *J. Feline Med. Surg.* **17**: 901–908. [[Medline](#)] [[CrossRef](#)]
37. Young, H. E., Steele, T. A., Bray, R. A., Hudson, J., Floyd, J. A., Hawkins, K., Thomas, K., Austin, T., Edwards, C., Cuzzourt, J., Duenzl, M., Lucas, P. A. and Black, A. C. Jr. 2001. Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. *Anat. Rec.* **264**: 51–62. [[Medline](#)] [[CrossRef](#)]
38. Zaim, M., Karaman, S., Cetin, G. and Isik, S. 2012. Donor age and long-term culture affect differentiation and proliferation of human bone marrow mesenchymal stem cells. *Ann. Hematol.* **91**: 1175–1186. [[Medline](#)] [[CrossRef](#)]
39. Zhang, N., Dietrich, M. A. and Lopez, M. J. 2014. Therapeutic doses of multipotent stromal cells from minimal adipose tissue. *Stem Cell Rev.* **10**: 600–611. [[Medline](#)] [[CrossRef](#)]
40. Zhang, P., Andrianakos, R., Yang, Y., Liu, C. and Lu, W. 2010. Kruppel-like factor 4 (Klf4) prevents embryonic stem (ES) cell differentiation by regulating Nanog gene expression. *J. Biol. Chem.* **285**: 9180–9189. [[Medline](#)] [[CrossRef](#)]
41. Zhang, S. and Cui, W. 2014. Sox2, a key factor in the regulation of pluripotency and neural differentiation. *World J. Stem Cells* **6**: 305–311. [[Medline](#)] [[CrossRef](#)]
42. Zhang, X., Hirai, M., Cantero, S., Ciubotariu, R., Dobrila, L., Hirsh, A., Igura, K., Satoh, H., Yokomi, I., Nishimura, T., Yamaguchi, S., Yoshimura, K., Rubinstein, P. and Takahashi, T. A. 2011. Isolation and characterization of mesenchymal stem cells from human umbilical cord blood: reevaluation of critical factors for successful isolation and high ability to proliferate and differentiate to chondrocytes as compared to mesenchymal stem cells from bone marrow and adipose tissue. *J. Cell. Biochem.* **112**: 1206–1218. [[Medline](#)] [[CrossRef](#)]
43. Zhu, H., Mitsuhashi, N., Klein, A., Barsky, L. W., Weinberg, K., Barr, M. L., Demetriou, A. and Wu, G. D. 2006. The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix. *Stem Cells* **24**: 928–935. [[Medline](#)] [[CrossRef](#)]