Original article

Phenotypic characteristics of feline adipose-derived stem cells affected by cell passage number

S. Panasophonkul[1], P. Samart[1], K. Kongon[1], A. Sathanawongs[2]

[1] Department of Companion Animal and Wildlife Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand 50100
[2] Department of Veterinary Biosciences and Veterinary Public Health, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand 50100

Abstract

In this study we evaluated the influence of passage number on the phenotypic characteristics of feline adipose-derived stem cells (ASCs) in order to develop a broader understanding of their dynamics. Feline ASCs were individually isolated from five domestic cats and subjected to proliferative culture at passage (P) 2, 6 and 10. The cells at each sub-passage were examined in regard to their phenotypic properties associated with multipotent mesenchymal stem cells (MSCs), such as morphology, proliferation kinetics, self-renewal, and expression of MSCs-specific surface markers. The differentiation capacity into adipocytes and osteoblasts was also identified. Feline ASCs appeared with a fibroblast-like morphology with minimal alteration through P10. The rate of cell proliferation gradually decreased, while cell doubling time gradually increased with each passage. A significant decrease in CFU-F efficiency was observed with increasing cell passage number. The ASC population uniformly expressed their characteristic markers CD44 and CD90, but did not express the hematopoietic marker CD45. However, MSC markers gradually decreased in the later passage stages. Feline ASCs were capable of undergoing both adipogenesis and osteogenesis at P2. These findings suggested that the phenotypic characteristics of feline ASCs could be affected by long-term passages, which is potentially very important in regard to their therapeutic application.

Key words: adipose-derived stem cell, feline, phenotypic characteristic, cell passage number

Introduction

Mesenchymal stem cells (MSCs) have widely been used in clinical applications for treating degenerative diseases in both human and animal patients (Fortier and Travis 2011, Stoltz et al. 2011), as they have the unique property of self-renewal and are capable of differentiating into various types of cells or tissues (Keating 2012). Mesenchymal stem cells isolated from adipose tissue (ASCs) now are receiving more attention in pre-clinical and clinical studies of veterinary medicine, since these cells are more easily isolated,

with a higher yield from donor animals (Bassi et al. 2012, Cawthorn et al. 2012). Moreover, ASCs apparently have no significant differences in relation to their morphology and immunosuppressive properties when compared to MSCs derived from other sources (Carrade and Borjesson 2013).

Although ASCs have been isolated and characterized in several species (Zuk et al. 2001, Wan et al. 2007, De Mattos et al. 2009, Guercio et al. 2013) very few studies have investigated feline ASCs. Webb et al. (Webb et al. 2012) demonstrated that feline MSCs isolated from adipose tissue had a proliferation rate higher than those isolated from bone marrow, and expressed a high level of CD44, CD90, and CD105, but did not express CD4 and MHC class II. These findings were confirmed by a later study of Kono et al. (2014), showing that ASCs isolated from domestic cats were positive for CD44, CD90, CD105, and alpha smooth muscle actin, and negative for CD14, CD34, and CD45. Moreover, the application of ASCs in therapeutic purposes has recently been reported in spontaneously occurring diseases in feline patients. Quimby et al. (2013) demonstrated the succession of autologous transplantation using feline ASCs into the renal cortex of cats with chronic kidney disease. Another study using autologous ASCs resulted in modest improvement of inflammation and promotion of tissue regeneration in feline patients suffering from chronic gingivostomatitis (Arzi et al. 2016).

These clinical applications require many cells that can be cultured *in vitro* while maintaining their stem cell capacity during serial passages. However, previous studies have suggested that long-term passage could affect the potential of ASCs. Requicha et al. (2012) demonstrated that canine ASCs showed a continuous decrease of MSC marker expressionfrom passage (P) P1 to P4, and also reduced osteogenic differentiation capacity. Similarly, in equine ASCs, a negative CD13 and positive CD44 and CD90 were expressed differently after the first three passages (Carvalho et al. 2009). Moreover, human ASCs exhibited a dramatic increase of doubling time from 45 hours to 90 hours after P6 (Wall et al. 2007). On the other hand, the variations in the pattern of ASCs proliferation were observed in equine ASCs until P4, and then the propagation of cells ceased after P8 (Braun et al. 2010). Although these studies have been carried out in diverse species, data concerning the long-term effects of cell culture have not been reported in felines.

In the present study we therefore aimed to isolate feline ASCs and evaluate the influence of serial passage on their phenotypic characteristics, including morphology, growth kinetics, colony-forming efficiency, and the expression pattern of MSCs (CD44, CD90) and hematopoeitic-specific surface markers (CD45). Furthermore, the lineage differentiation capacity into adipocytes and osteoblasts of feline ASCs was also analyzed. This should be valuable information in regard to responsive treatment in feline animal models.

Materials and Methods

This study was conducted according to the experimental practices and standards approved by the Ethical Committee for Animal Use, Faculty of Veterinary Medicine, Chiang Mai University, Thailand (Accession No. S10/ 2557). Subcutaneous adipose tissue samples were collected from 5 healthy cats (aged 1-3 years, 2-5 kg) under the informed consent of the owners. All chemicals were purchased from Sigma-Aldrich, St Louis, USA, unless otherwise specified.

Harvesting of subcutaneous adipose tissue

Cats were generally anesthetized intramuscularly with 0.5 mg/kg xylazine HCl (Troy Laboratories, Australia) and 7 mg/kg zoletil (Virbac Laboratories, France). Anesthesia was then maintained intravenously with the same dose of zoletil. A small surgical incision was made in the flank and abdominal region, and approximately 2 g of inguinal subcutaneous adipose tissue was harvested. The specimens were placed in sterile 100 ml bottles with sterile phosphate buffered saline (PBS) containing 1% antibiotics/ antimycotics (AA) (Invitrogen, Carlsbad, CA, USA) (washing solution), and then transported to the laboratory within 2 hours at room temperature. During surgery, the animals were monitored by anesthesia protocols until recovery.

Isolation of adipose-derived stem cells

The ASCs were isolated by mechanical and enzymatic methods as described previously (Kono et al. 2014) with minor modifications. Briefly, after weighing the harvested adipose tissue, it was minced and digested with 10 ml of pre-warmed PBS containing 0.075% collagenase type I (Worthington, Lakewood, NJ, USA), and then incubated in a water bath at 37ºC for 60 min. The tubes were vigorously shaken every 5 min to ensure uniform digestion. Upon digestion, collagenase was inactivated by adding an equal volume of low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% AA,

followed by centrifugation at 1200 rpm for 5 min to remove the supernatant. The cell pellet was re-suspended in the same volume of erythrocytes lysis buffer (160 mM NH_4Cl in PBS), incubated at room temperature for 10 min, and then centrifuged. The supernatant was discarded and the cell pellet was re-suspended in DMEM containing 0.375% Herpes and 1% AA. The cell suspension was filtered through a sterile cell strainer 70-µm nylon mesh and centrifuged for 5 min. The obtained cells were then re-suspended in fresh culture medium [DMEM supplemented with 15% FBS, 1% AA, 0.5% non-essential amino acid (NEAA) and 0.5% L-glutamine (Invitrogen)]. The cells were seeded in 75 cm^2 cell culture flasks at 1 x 10^4 cells/ cm^2 and cultured at 37ºC under 5% CO_2 with medium changed every 3 days. At 80-90% confluence, cells were trypsinized and then sub-cultured until P10 or cryopreserved for further processing.

The cells were thawed in a 37ºC water bath until the last ice crystals disappeared; the cells were then rinsed with 9 ml of stop solution, centrifuged at 1200 rpm for 5 min and the supernatant carefully removed. The cells were re-suspended in culture medium and cultured at a concentration of 1 x 10^4 cells/ cm^2 under the same conditions.

Phenotypic characteristics of feline ASCs

All phenotypic characteristics of feline ASCs, including morphology, growth kinetics, population doubling time (PDT), colony-forming unit fibroblast (CFU-F) assay and immunophenotypic properties, were examined at passages 2, 6, and 10.

Cell morphology

Initially, the cells were plated onto a 35 mm culture plate at a density of 3,000 cells/ cm^2. The cells were fixed with 4% paraformaldehyde (PF) and then stained with Giemsa solution for 20 min. Excess stain was washed with distilled water and the plates were dried in air. Images of staining were examined and captured under a phase-contrast microscope with a digital sight camera (Nikon Eclipse TE 2000-U, Tokyo, Japan) for their morphology.

Growth kinetics and population doubling time

Growth rate and population doubling time (PDT) of feline ASCs were examined at different passages. The cells were seeded at a density of 3,000 cells/ cm^2 into 12-well plates and cultured for 10 days. The cells were trypsinized and counted at days 3, 5, 7 and 10 using a hemocytometer with trypan blue viability staining. The growth curves were plotted and the population doubling times (PDT) were calculated at day 10 as follows: PDT=CT/CD; CD = ln(Nf/Ni) × ln2, where CD is the cell number, CT is the cell culture time, Nf is the initial cell number at day 0 and Ni is the final cell number at day 10 (Vidal et al. 2006).

Colony-forming unit fibroblast assay

The cells from the same samples were cultured in 6-well plates at a density of 200 cells/ well in culture medium. After plating, the cells were incubated at 37ºC and 5% CO_2 for 14 days and the medium was changed every 3 days. After 14 days of culture, colonies were fixed with 4% PF in PBS for 20 min. The fixed cells were washed three times with PBS for 5 min each, exposed to 0.5% crystal violet in methanol for 5 min, and washed twice in distilled water. ASCs colonies were then counted using a stereo microscope (ATL10, American Scientific, USA). Cell clusters consisting of more than 20 nucleated cells were considered as a colony. Data were reported as CFU-F efficiency (%), calculated as the number of colonies/number of seeded cells x 100.

Flow cytometry analysis

Feline ASCs were immunologically examined by flow cytometry to analyse the expression of specific stem cell surface markers. To perform the flow cytometry, the cells from P2, P6 and P10 were trypsinized with 0.25% trypsin plus 0.04% EDTA (Invitrogen) and blocked with PBS containing 0.2% bovine serum albumin (BSA). These cell aliquots (1 x 10^5 cells/ sample) were then incubated with primary antibodies in the dark for 30 min on ice. The following monoclonal antibodies were used: fluorescent isothiocyanate (FITC)-conjugated anti-human CD44 (1:200, clone: IM7, eBioscience, USA), allophycocyanin (APC)-conjugated anti-human CD90 (1:40, clone: 5E10, eBioscience, USA) and phycoerythrin (PE)-conjugated CD45 (1:100, clone: RA3-6B2, Biolegend, USA), in staining solution (PBS containing 0.2% BSA and 0.1% sodium azide (NaN_3). After incubation, the cells were washed twice with staining solution for 5 min each, and the cells were then fixed in 0.5 ml of cold 2% paraformaldehyde in PBS and stored at 4ºC prior to analysis. The cells were analyzed using a CyAn ADP flow cytometer (Beckman Coulter, California, USA) using the
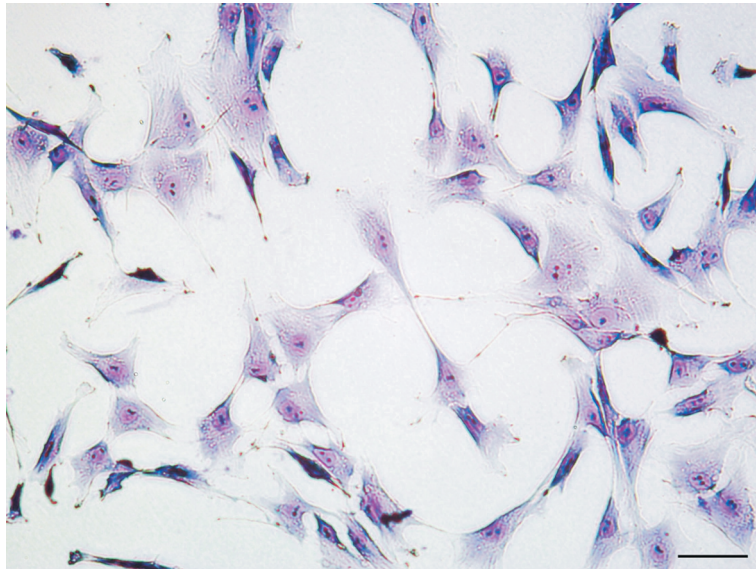
Fig. 1. Representative images showing morphology of feline ASCs obtained from subcutaneous fat tissue of domestic cats at passage 2. The cells were stained with Giemsa and observed under phase contrast. These cells exhibited a spindle-shaped fibroblastic morphology with a flat polygonal shape. Scale bars = 50 μm. ASCs, adipose-derived stem cells.

FlowJo Vx 10.0 software package to examine the expression of specific MSCs surface markers. A minimum of 10,000 events were counted for each analysis.

ASC Differentiation assays

In this study, the differentiation potentials into adipocytes and osteoblasts of feline ASCs at the 2nd passage were also identified in order to confirm their MSC properties. Adipogenic and osteogenic differentiation were induced as previously described (Kyllönen et al. 2013, Wang et al. 2015) with minor modification. Briefly, before induction, the cells from P2 were seeded in twelve-well plates at 4 x 10^4 cells/ cm^2 and allowed to reach confluence (90%-100%) for 3-7 days in basal medium (10% FBS in mixer of 25% low-glucose DMEM and 75% high-glucose DMEM supplemented with 1% NEAA, 1% L-glutamine and 1% AA). For adipogenic differentiation, confluent cells were cultured in adipogenic medium consisting of basal medium supplemented with 1 μM dexamethasone, 0.5 mM 3-isobutyl-L-methylxanthine (IBMX), 200 μM indomethacin and 2% insulin-transferrin-selenium-X for 3 days; the cells were then cultured in maintenance medium consisting of basal medium supplemented with 1% insulin-transferrin-selenium-X for 24 hours. This sequence was repeated 3 times. The cells in the control group were cultured in basal medium without any inducers. To evaluate intracellular lipid accumulation, then the cells were fixed and stained with 0.5% (v/v) oil red O in isopropanol for 30 min and were examined under

a phase-contrast microscope. For osteogenic differentiation, confluent cells were cultured in osteogenic medium consisting of basal medium supplemented with 10 nM dexamethasone, 10 mM β-glycerophosphate and 250 μM L-ascorbic acid, and the medium was changed every 3 days. The cells in the control group were cultured in basal medium without inducers. After 3 weeks of differentiation, the cells were fixed and stained with 1% alizarin red S for 30 min at room temperature to detect calcium node formation and then were examined under a phase-contrast microscope.

Statistical analysis

The results are shown as means ± standard error of mean (SE). Statistical analyses were performed with SPSS version 21.0 (IBM, Armonk, NY, USA). The results of growth rates, PDT, CFU-F, and the expression of MSC surface markers were compared using One-way analysis of variance (ANOVA), with Tukey post-hoc test to analyse the specific sample pairs for significant differences. Differences were considered significant at $p<0.05$. Technical triplicates of each sample were used in all the assays.

Results

Isolation, Culture and Morphology of ASCs

Under our culture conditions, adipose-derived stem cells (ASCs) were successfully isolated from
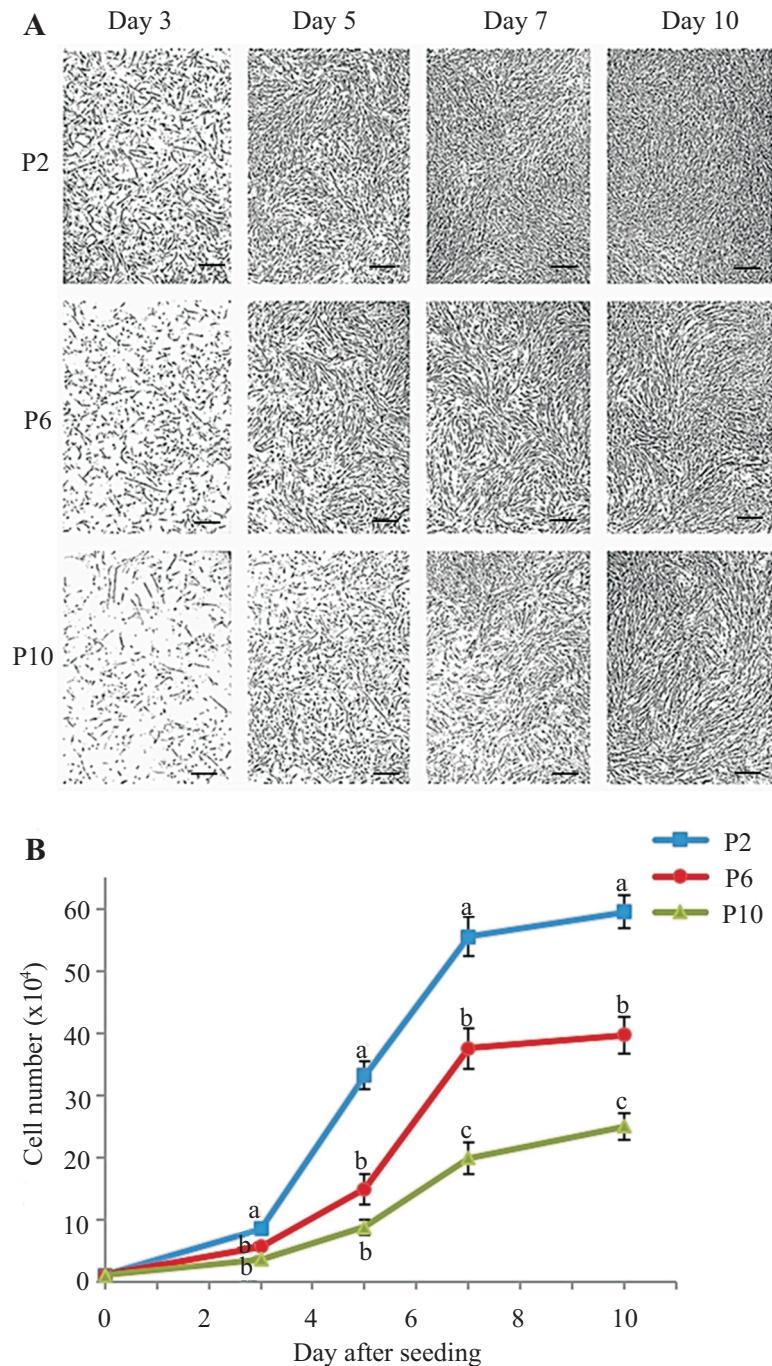
Fig. 2. Growth kinetics of feline ASCs at passages 2, 6 and 10. (A) Representative images showing the density of feline ASCs at different periods of culture time. (B) Growth curves of feline ASCs. Cell population growth rates increased after 3 days of culture, and then slowed after day 7. Results are expressed as mean ± SE. Superscripts a, b, c show significant differences (p<0.01) Scale bars = 100 μm.

subcutaneous adipose tissue of all domestic cats. The yield of stromal vascular fraction cells after isolation with enzymatic digestion was $1.5\pm0.02 \times 10^5$ cells/ g. These obtained stromal vascular fraction cells completely adhered to the plastic culture flask, and revealed an elongated to a spindle or shuttle shape with a large nucleus in the cytoplasm by day 3 of the cell

culture (Fig. 1). Afterwards, the cells reached 80 to 90% confluence with fibroblast-like morphology, forming a highly homogenous monolayer of the culture by day 8~10 of the culture. After primary culture, feline ASCs reached confluence in 6 to7 days until P5, and then the culture period gradually increased. Cell morphology (size and shape) of all feline ASCs persis-
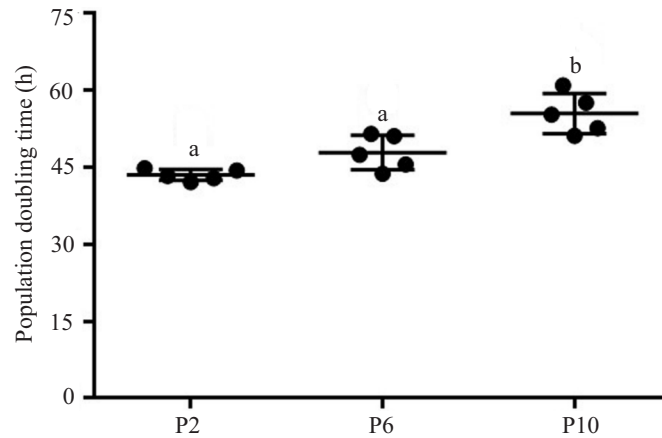
Fig. 3. Population doubling times of feline ASCs at passages 2, 6, and 10. Significant differences were identified until passage 10. Results are expressed as mean ± SE. Superscripts a, b show significant differences (p<0.01).
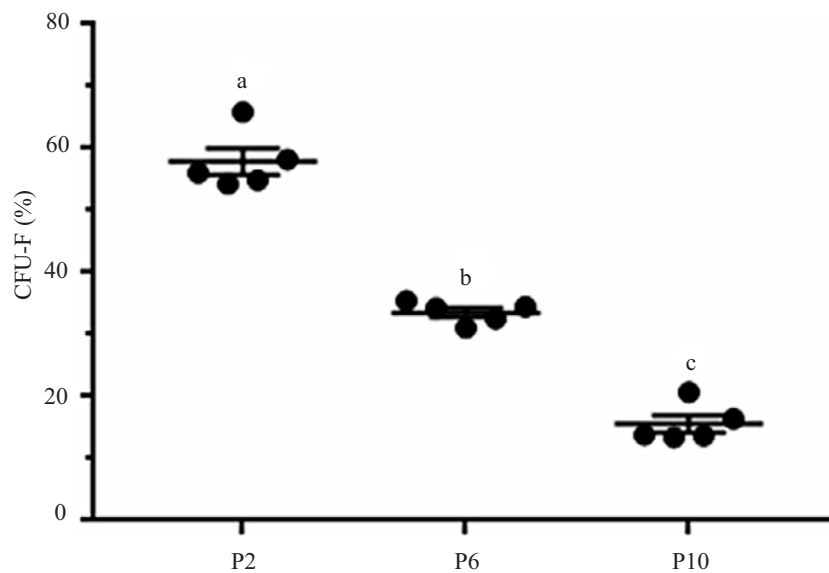

Fig. 4. Cloning efficiency of feline ASCs at passages 2, 6, and 10. The colony-forming rates were significantly decreased when the cells were cultured along all studied passages. Results are expressed as mean ± SE. Superscripts a, b, c reflect significant differences (p<0.001).

ted with only minimal alterations and showed a high degree of cell viability (more than 80%, data not shown) up to P10.

Growth kinetics and population doubling time (PDT)

Growth kinetic studies were conducted at the selected passages (P2, P6 and P10) as representative of early, middle and late periods, respectively (Fig. 2A). During the culture, feline ASCs displayed a growth pattern as a multi-logarithmic phase until P10 (Fig. 2B). The ASCs began growing more rapidly after 3 days of culture, and then their population growth started to slow down after day 7 of culture. Feline ASCs had sufficient proliferation rates at each pas-

sage, particularly at P2 and P6. By comparing the growth curves, the proliferation rate of feline ASCs was significantly decreased from P2 to P10 (p<0.01). The doubling time of feline ASCs cultured at the same time point was determined after 10 days of culture. Figure 3 shows the time required for population doubling, which significantly increased when the passage number exceeded P2 (43.5±0.5 hours) to P10 (55.5±1.7 hours) and P6 (47.8±1.5 hours) to P10 (p<0.01).

Colony forming unit-fibroblast (CFU-F) assay

The presence of colony forming units was observed at P2, P6 and P10 under a stereo microscope at day 14 (Fig. 4A). Feline ASCs revealed a significant
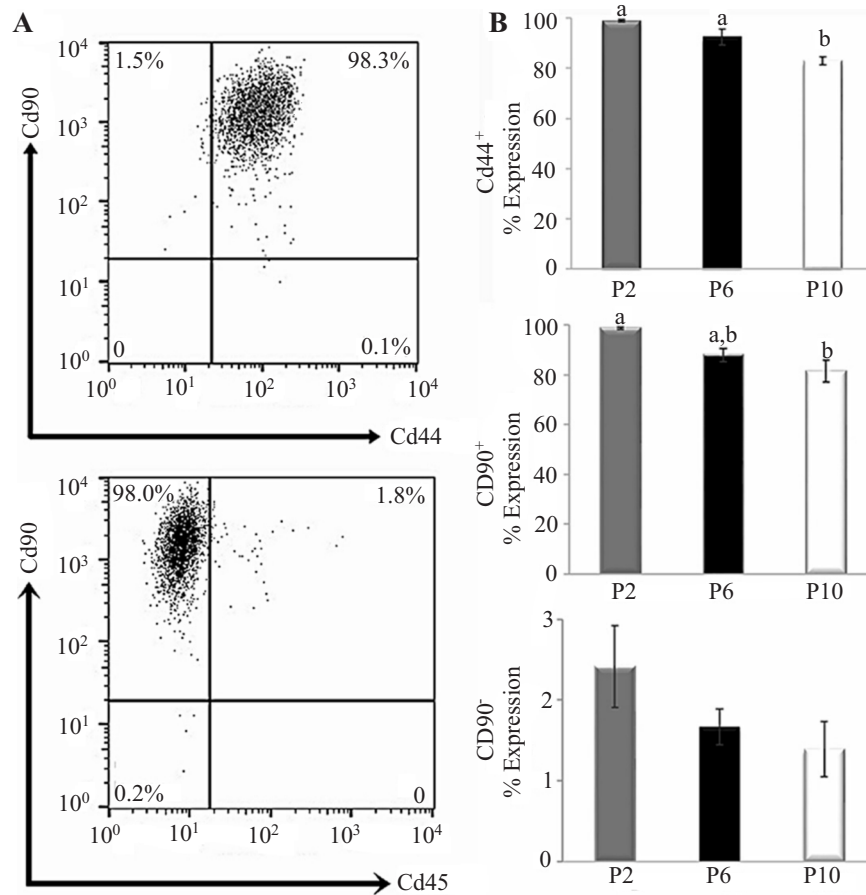
Fig. 5. Representative flow cytometry analyses of feline ASCs. (A) Dot plots represent flow cytometer analysis of stained cells with CD44, CD90, and CD45 at P2. Feline ASCs were negative for hematopoietic markers CD45 but were strongly positive for CD44 and CD90. (B) The percentages of expression levels of CD44, CD90, and CD 45 among P2, P6, and P10 are shown. A significant decrease was observed when the cells were cultured through passage 10. Results are expressed as mean ± SE. Superscripts a, b indicate significant differences (p<0.05).
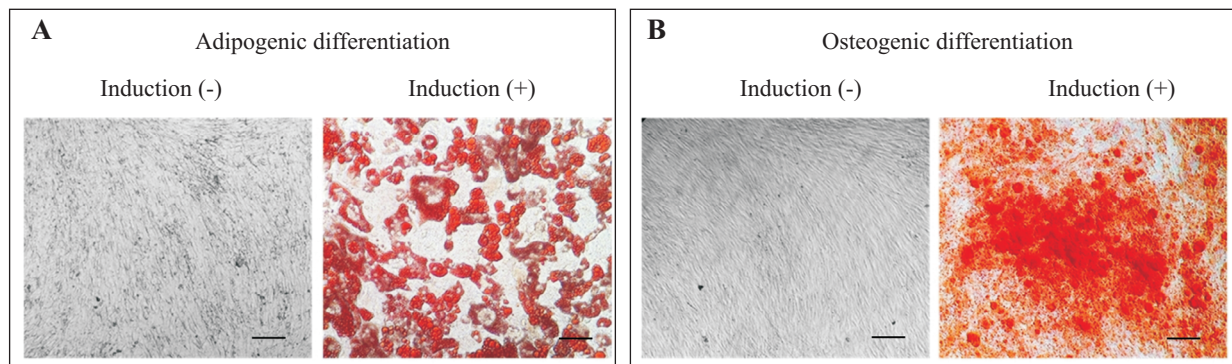

Fig. 6. The differentiation potential of feline ASCs at passage 2. Oil Red O staining for the adipogenic differentiation (A) and Alizarin Red S staining for osteogenic differentiation (B) were performed after feline ASCs were cultured in either basal medium (Induction –) or differentiation medium (Induction +) for 12 and 21 days, respectively. Scale bars = 100 μm.

decrease of CFU-F efficiency when the cells were cultured along all studied passages, from P2 (57.9±2.1%) to P6 (34.3.4±0.8%) and P10 (15.6±1.4%) (p<0.001; Fig. 4B).

Immunophenotyping of ASCs by flow cytometry analysis

The ASCs surface markers, including CD44, CD45 and CD90, were examined by flow cytometry

(Fig. 5A). The feline ASCs were uniformly positive for the MSCs markers CD44 and CD90, and were negative for the hematopoietic markers CD45 at all passages. Although each feline ASC cell line was strongly positive for CD44 and CD90 (more than 80%) through passage 10, a significant decrease in the expression levels of both CD markers was observed during sequential sub-passages (Fig. 5B). The down-regulation of CD44 was found to be statistically significant from P2 (98.9±0.4%) to P10 (82.9±1.6%) (p0.001) and P6 (92.4±3.1%) to P10 (p<0.05) while CD90 was only statistically significant from P2 (98.6±0.5%) to P10 (81.4±4.3%) (p<0.005).

ASCs differentiation assays

We demonstrated that the cells at P2 were able to differentiate into mesodermal lineages include adipocytes and osteoblasts, based on histological examination. After 12 days of adipogenic differentiation, Spindle-shaped ASCs changed to oval-shaped cells contained a number of intracellular lipid droplets which stained positive for oil red O, while after 21 days of osteogenic differentiation, the cells became aggregate and created mineralized nodules that stained positive for alizarin red S. Negative staining was observed under control conditions of both inductions. (Fig. 6).

Discussion

Stem cells harvested from adipose tissue have been applied clinically due to the ease of isolation and relative abundance of stem cells in adipose tissue. Similarly to other species, feline adipose-derived stem cells (ASCs) have therapeutic potential for tissue regeneration and repair, but only if the cells can be cultured and are capable of sequential long-term passage. To address these concerns, the present study was conducted to investigate the effects of sequential passage on the phenotypic properties of the cells, including growth kinetics, population doubling time (PDT), colony forming unit-fibroblast (CFU-F), and expression of MSC-specific surface markers. The differentiation potential of feline ASCs was further assessed. Cells isolated from adipose tissues of all domestic cats displayed a spindle-shaped morphology as described by Webb et al. (2012) and Kono et al. (2014), relevant to the criteria for stem cells as determined by The International Society for Cell Therapy (Dominici et al. 2006). During culture, we also found that these cells formed nodular aggregates followed by detachment when approaching 100% confluence

which is similar to a current report concerning feline ASCs (Kono et al. 2014). In contrast, this phenomenon has not been observed in ASCs harvested from humans, rabbits, rats, and mice (Zuk et al. 2001, 2002, Arrigoni et al. 2009), suggesting that species-specific properties influence the cell adhesion.

The growth kinetics of feline ASCs from P2 to P10 were examined. The proliferation rate of feline ASCs decreased gradually with passage number from P2 to P10. Similar patterns have been observed in guinea pig ASCs from P2 to P8 (Aliborzi et al. 2016) as well as canine ASCs from P1 to P6 (Lee et al. 2014), where the proliferation rate and the number of cultured cells were decreased after P5. Feline ASCs displayed significantly longer doubling times with increasing passage number, particularly at P10 (55.5 hours). Recently, similar results were reported in canine ASCs with increasing doubling times of 1.73 days at P4 to 4.99 days at P6 (Lee et al. 2014). Moreover, Wall et al. (2007) described PDTs of 45 h up to P5 in human ASCs and then a dramatic increase to 90 h for the cells at P6 and later. Interestingly, the PDTs in our study at P6 and P10 were less (47.8 h and 55.5 h) compared to those in the latter reports. It is possible that species-specific differences exist, as well as differences in culture conditions (e.g., supplementing with L-glutamine and NEAA as in our study) that could affect the proliferation ability of the cells (Schwarz et al. 2012, Roxburgh et al. 2016). The similarity of CFU-F efficiency was also indicated in feline ASCs with a dramatic reduction of colony formation with increasing passage number. Our results are similar to those reported in cattle, where CFU-F efficiency was significantly higher in P3 than P7, and also higher in P7 than P11 (Lu et al. 2014). These findings indicated that the self-renewal capacity of ASCs was gradually reduced with increasing culture time.

To further characterize these cells, cell surface markers were examined using flow cytometry. Feline ASCs displayed typical features of MSCs, bearing CD44 and CD90 and lacking the hematopoietic marker CD45, in agreement with the other reports on felines (Webb et al. 2012, Kono et al. 2014), guinea pigs (Aliborzi et al. 2016), humans (Russo et al. 2014), and equines (Alipour et al. 2015). While there was no significant difference in the expression of CD44 and CD90 from P2 (98.9% and 98.6%) to P6 (92.4% and 87.8%), fewer P10 cells expressed CD44 and CD90 (82.9% and 81.4%), respectively. Similarly, Requicha et al. (2012) reported that the expression of MSCs markers (CD90, CD73, and CD105) decreased with increasing number of sub-passages in canine ASCs. Noticeably, the declines in CD expression in this study seem to be related to the results of PDT and CFU efficiency, which was significantly increased after P6,

suggesting that the decreasing expression of one or several MSCs markers after continuous sub-passages may compromise the proliferation capacity as well as the self-renewal of feline ASCs, thus limiting their *in vitro* expansion (Requicha et al. 2012).

Cell differentiation capacity has been considered as an important characteristic of MSCs, and is represented by distinct changes in cell appearance, including size and shape, membrane potential and metabolic activity (De Schauwer et al. 2011). Adipogenic and osteogenic differentiation were thus identified by staining with Oil Red O and Alizarin Red S, respectively at P2. All feline ASCs differentiated into adipogenic and ostogenic lineages, similar to previous reports from other species (Arrigoni et al. 2009, Vieira et al. 2010, Kono et al. 2014, Lee et al. 2014, Lu et al. 2014).

Our present study clearly demonstrated that feline ASCs could be isolated from inguinal subcutaneous fat tissue of domestic cats, and that the cells exhibit the phenotypic properties of MSCs, able to differentiate into adipogenic and osteogenic lineages, making them promising candidates for clinical regenerative approaches. Nevertheless, the obtained results revealed that these feline ASCs exhibit a progressive decrease in proliferation and self-renewal capabilities, as well as the expression of MSC markers under long-term passages. These aspects thereby should be taken into consideration in future applications. Our data will be a useful tool for further research in feline species not only in the domestic cat, but also in wild cats and their relatives.

Acknowledgements

References

Aliborzi G, Vahdati A, Mehrabani D, Hosseini SE, Tamadon A (**2016**) Isolation, characterization and growth kinetic comparison of bone marrow and adipose tissue mesenchymal stem cells of guinea pig. Int J Stem Cells 9: 115-123.

Alipour F, Parham A, Kazemi MH, Dehghani H (**2015**) Equine adipose-derived mesenchymal stem cells: phenotype and growth characteristics gene expression profile and differentiation potentials. Cell J 16: 456-465.

Arrigoni E, Lopa S, de Girolamo L, Stanco D, Brini AT (**2009**) Isolation, characterization and osteogenic differentiation of adipose-derived stem cells: from small to large animal models. Cell Tissue Res 338: 401-411.

Arzi B, Mills-Ko E, Verstraete FJ, Kol A, Walker NJ, Badgley MR, Fazel N, Murphy WJ, Vapniarsky N, Borjesson DL (**2016**) Therapeutic Efficacy of Fresh, Autologous Mesenchymal Stem Cells for Severe Refractory Gingivostomatitis in Cats. Stem Cells Transl Med 5: 75-86.

Bassi G, Pacelli L, Carusone R, Zanoncello J, Krampera M (**2012**) Adipose-derived stromal cells (ASCs). Transfus Apher Sci 47: 193-198.

Braun J, Hack A, Weis-Klemm M, Conrad S, Treml S, Kohler K, Walliser U, Skutella T, Aicher WK (**2010**) Evaluation of the osteogenic and chondrogenic differentiation capacities of equine adipose tissue-derived mesenchymal stem cells. Am J Vet Res 71: 1228-1236.

Carrade DD, Borjesson DL (**2013**) Immunomodulation by mesenchymal stem cells in veterinary species. Comp Med 63: 207-217.

Cawthorn WP, Scheller EL, MacDougald OA (**2012**) Adipose tissue stem cells: the great WAT hope. Trends Endocrinol Metab 23: 270-277.

De Mattos Carvalho A, Alves AL, Golim MA, Moroz A, Hussni CA, de Oliveira PG, Deffune E (**2009**) Isolation and immunophenotypic characterization of mesenchymal stem cells derived from equine species adipose tissue. Vet Immunol Immunopathol 132: 303-306.

De Schauwer C, Meyer E, Van de Walle GR, Van Soom A (**2011**) Markers of stemness in equine mesenchymal stem cells: a plea for uniformity. Theriogenology 75: 1431-1443.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (**2006**) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315-317.

Fortier LA, Travis AJ (**2011**) Stem cells in veterinary medicine. Stem Cell Res Ther 2: 9.

Guercio A, Di Bella S, Casella S, Di Marco P, Russo C, Piccione G (**2013**) Canine mesenchymal stem cells (MSCs): characterization in relation to donor age and adipose tissue-harvesting site. Cell Biol Int 37: 789-798.

Keating A (**2012**) Mesenchymal stromal cells: new directions. Cell Stem Cell 10: 709-716.

Kono S, Kazama T, Kano K, Harada K, Uechi M, Matsumoto T (**2014**) Phenotypic and functional properties of feline dedifferentiated fat cells and adipose-derived stem cells. Vet J 199: 88-96.

Kyllonen L, Haimi S, Mannerstrom B, Huhtala H, Rajala KM, Skottman H, Sandor GK, Miettinen S (**2013**) Effects of different serum conditions on osteogenic differentiation of human adipose stem cells *in vitro*. Stem Cell Res Ther 4: 17.

Lee KS, Kang HW, Lee HT, Kim HJ, Kim CL, Song JY, Lee KW, Cha SH (**2014**) Sequential sub-passage decreases the differentiation potential of canine adipose-derived mesenchymal stem cells. Res Vet Sci 96: 267-275.

Lu T, Xiong H, Wang K, Wang S, Ma Y, Guan W (**2014**) Isolation and Characterization of Adipose-derived Mesenchymal Stem Cells (ADSCs) from Cattle. Appl Biochem Biotechnol 174: 719-728.

Quimby JM, Webb TL, Habenicht LM, Dow SW (**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.

Requicha JF, Viegas CA, Albuquerque CM, Azevedo JM, Reis RL, Gomes ME (**2012**) Effect of anatomical origin and cell passage number on the stemness and osteogenic differentiation potential of canine adipose-derived stem cells. Stem Cell Rev 8: 1211-1222.

Roxburgh J, Metcalfe AD, Martin YH (**2016**) The effect of medium selection on adipose-derived stem cell expansion and differentiation: implications for application in regenerative medicine. Cytotechnology 68: 957-967.

Russo V, Yu C, Belliveau P, Hamilton A, Flynin LE (**2014**) Comparison of human adipose-derived stem cells isolated from subcutaneous, omental, and intrathoracic adipose tissue depots for regenerative applications. Stem Cells Transl Med 3: 206-217.

Schwarz C, Leicht U, Rothe C, Drosse I, Luibl V, Rocken M, Schieker M (**2012**) Effects of different media on proliferation and differentiation capacity of canine, equine and porcine adipose derived stem cells. Res Vet Sci 93: 457-462.

Stoltz JF, Decot V, Huseltein C, He X, Zhang L, Magdalou J, Li YP, Menu P, Li N, Wang YY, de Isla N, Bensoussan D (**2011**) Introduction to regenerative medicine and tissue engineering. Biomed Mater Eng 22: 3-16.

Vidal MA, Kilroy GE, Johnson JR, Lopez MJ, Moore RM, Gimble JM (**2006**) Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. Vet Surg 35: 601-610.

Vieira NM, Brandalise V, Zucconi E, Secco M, Strauss BE, Zatz M (**2010**) Isolation, characterization, and differentiation potential of canine adipose-derived stem cells. Cell Transplant 19: 279-289.

Wall ME, Bernacki SH, Loboa EG (**2007**) Effects of serial passaging on the adipogenic and osteogenic differentiation potential of adipose-derived human mesenchymal stem cells. Tissue Eng 13: 1291-1298.

Wan DC, Siedhoff MT, Kwan MD, Nacamuli RP, Wu BM, Longaker MT (**2007**) Refining retinoic acid stimulation for osteogenic differentiation of murine adipose-derived adult stromal cells. Tissue Eng 13: 1623-1631.

Wang X, Liu C, Li S, Xu Y, Chen P, Liu Y, Ding Q, Wahapu W, Hong B, Yang M (**2015**) Effects of continuous passage on immunomodulatory properties of human adipose-derived stem cells. Cell Tissue Bank 16: 143-150.

Webb TL, Quimby JM, Dow SW (**2012**) In vitro comparison of feline bone marrow-derived and adipose tissue-derived mesenchymal stem cells. J Feline Med Surg 14: 165-168.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (**2001**) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7: 211-228.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (**2002**) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13: 4279-4295.