Isolation and Characterization of Canine Adipose–Derived Mesenchymal Stem Cells

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ABSTRACT

This study is the first documentation of the isolation and extensive characterization of mesenchymal stem cells from canine adipose tissue. Methods previously used by our group to isolate and differentiate human adipose–derived mesenchymal stem cells (hAD-MSCs) have been modified and optimized for derivation of similar cells from canine adipose tissues. The canine adipose tissue–derived mesenchymal stem cells (cAD-MSCs) showed lower proliferation ability and were refractory to osteogenic and adipogenic differentiation under conditions employed to differentiate hAD-MSCs. The differentiation of cAD-MSCs into osteoblasts and adipocytes was effectively achieved under modified conditions, by using laminin-coated plates and peroxisome proliferative activated receptor, gamma (PPARγ) ligands, respectively. The formation of micromass was sufficient to induce chondrogenesis, unlike hAD-MSCs, which require transforming growth factor beta (TGF-β). These cells displayed anchorage-independent growth in soft agar, and their colony-forming efficiency in plastic was comparable with human counterparts. The cAD-MSCs expressed genes associated with pluripotency, while their differentiated progeny expressed appropriate lineage-specific genes. The optimization of growth and differentiation of cAD-MSCs should facilitate future stem cell–based reparative and regenerative studies in dogs. The dog is a promising biomedical model that is suitable for evaluation of novel therapies such as those employing stem cells in experimental and in spontaneous disease settings.

INTRODUCTION

Tissue engineering holds much promise for the regenerative treatment of various tissue disorders as well as for the delivery of therapeutic genes. One major area of great potential is the use of mesenchymal stem cells (MSCs) for the restoration of bone defects. By virtue of their cellular differentiation potential and trophic effects, MSCs are promising candidates for regenerative and reparative medicine. The MSCs exist in several connective tissue compartments, and have been isolated from various tissues. Adipose tissue is an attractive source of MSCs because of its abundance and ease of access with minimal donor site morbidity.

The current study was undertaken with the goal of isolation, expansion, and characterization of canine adipose tissue–derived mesenchymal stem cells (cAD-MSCs). Repair of major fractures, better outcomes of major reconstruction surgeries, and implants such as hip replacement are of equal concern in both human and veterinary medicine. The dog is a promising biomedical model for evaluation of novel therapies such as those employing stem cells in experimental and in spontaneous disease settings. Recent evidence on use of mesoangioblast stem cells for the treatment of muscular dystrophy in dogs demonstrated the potential of this model system.

MATERIALS AND METHODS

Isolation of cAD-MSCs

The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee. Methods
used to isolate MSCs from human adipose tissue were adapted to isolate MSCs from adult canine adipose tissue. The dogs ranged in age from 1 to 3 years, and were clinically healthy dogs undergoing experimental surgeries unrelated to this study.

Briefly, adipose tissue was collected from subcutaneous, omental, and inguinal fat depots of dogs, using standard surgical procedures. Each adipose tissue sample was weighed, and digested overnight at 37°C with collagenase type IA (Sigma, St. Louis, MO) (1 mg/mL) in D medium, which is a modified Eagle’s minimum essential medium (MEM) medium (5 mM medium/g of tissue) supplemented with N-acetyl-L-cysteine (NAC) (2 mM), L-ascorbic acid 2-phosphate (Asc 2P) (0.2 mM), penicillin, streptomycin, and amphotericin. Following centrifugation and washing of the pellet, cells were incubated (about 8 g of tissue/25 cm² flask) in D medium with 10% fetal bovine serum (FBS), NAC (2 mM), and Asc 2P (0.2 mM) in incubator supplied with humidified air and 5% CO₂. Unattached cells were removed the next day by washing with phosphate buffered saline.

Adherent cells were cultured in the K-NAC medium with 5% FBS. The K-NAC medium is a modified MCDB 153 medium (Keratinocyte-SFM, GIBCO–Invitrogen, Carlsbad, CA) supplemented with NAC (2 mM) and Asc 2P (0.2 mM). This low-calcium (0.09 mM) medium contains recombinant epidermal growth factor (rEGF, 5 ng/mL), bovine pituitary extract (BPE, 50 mg/mL), insulin (5 μg/mL), hydrocortisone (74 ng/mL), and 3,3',5-triiodo-DL-thyronine (T3) (6.7 ng/mL).

The medium was renewed every 3 days. The cells, grown to near confluence, were quantified, and subcultured or cryopreserved for further studies in K-NAC medium with 10% FBS and 10% dimethyl sulfoxide (DMSO).

**Differentiation of cAD-MSCs (osteogenesis, chondrogenesis, and adipogenesis)**

Cells derived from subcutaneous and omental fat and expanded in the K-NAC medium with 5% FBS were used for differentiation studies. The cells were treated by different induction cocktails in D medium with 10% FBS. All studies were carried out with the same number of controls.

**Osteogenesis.** Cells were plated at the seeding density of 1000 cells/cm² in six-well plates (regular or laminin-coated) and treated with dexamethasone (0.1 μM) or 1,25-dihydroxyvitamin D₃ (0.01 μM), Asc 2P (50 μM), and β-glycerophosphate disodium (10 mM) (DAG or VAG cocktail) in D medium containing 10% FBS for 6–8 weeks with medium change once in every 3 days. Alizarin red staining and von Kossa staining were carried out to detect calcified extracellular matrix deposits.

**Quantitative assay for calcium.** Calcium concentrations in treated and control plates were assayed with an inducibly coupled plasma-atomic emission spectrophotometer (Vista AX) (Varian, Victoria, Australia) using yttrium internal standard and cesium ionization suppressant at 370.602 nm.

**Chondrogenesis.** For chondrogenesis, micromass cultures of cells (1×10⁷ cells/10 μL) were incubated and formed in 24-well plates for 2.5 h and then treated by TGF-β1 (10 ng/mL), Asc 2P (50 μM), and insulin (6.25 μg/mL) (TAI cocktail) for 14 days, with medium change once in every 3 days. The micromasses were stained both grossly and histologically (5-micron-thick paraffin-embedded sections), with alcian blue for the presence of sulfated proteoglycan-rich matrix.

**Adipogenesis.** For adipogenesis, cells were plated at the seeding density of 10,000 cells/cm² in six-well plates, and treated with the conventional protocol of isobutylmethylxanthine (IBMX) (500 μM), dexamethasone (1 μM), indomethacin (100 μM), and insulin (10 μg/mL) (IDI cocktail) for 21 days with medium change once in every 3 days.

We optimized the adipogenic induction cocktail for cAD-MSCs. Cells were treated with rosiglitazone (5 μM), dexamethasone (1 μM), and insulin (5 μg/mL) (RDI cocktail) for 2 weeks with medium changed every 3 days. Induction efficacy of rabbit serum (5% or 10%) and high glucose (4.5 g/L) was also examined. Oil Red O staining was done to examine the lipid droplet formation.

**RT-PCR**

Total RNAs were extracted from cells using Versagene RNA Purification Kit (Gentra, Minneapolis, MN) and treated with DNase I to remove contaminating DNA. cDNAs were synthesized from 1 μg total RNA using random hexamer primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Primers derived from coding regions of respective genes in canine genome were used to amplify the target sites (Table 1). To ensure that primers would uniquely amplify the target transcripts, primers to some of the genes (OCT 4, BSP, and OSTEOCALCIN) were designed to flank an intron, allowing us to rule out genomic contamination by electrophoresis, stained with ethidium bromide, and visualized under UV light; digital images were captured with Alphalmager software. Respective tissue samples (bone, cartilage, and fat from dog) were used as positive controls to
validate the primers, and no template controls (water instead of cDNA) were used as negative controls.

For OCT4, NANOG, and SOX2 genes, PCR products were gel-purified using Qiaex II Gel Extraction Kit (Qiagen, Valencia, CA), sequenced with automated sequencer, and verified after sequence alignment with canine genome.

**Immunocytochemistry**

After osteogenic induction, cells were trypsinized and cytospin preparations were made. The cytospin slides were fixed in cold acetone (−20°C) for 3 min and washed twice with tris-buffered saline. Endogenous peroxidase was neutralized with 3% hydrogen peroxide for 6 min, and slides were incubated with 1:2 dilution of ready-to-use monoclonal mouse anti-human OSTEOCALCIN (BioGenex, San Ramon, CA) for 30 min, using an autostainer (BondMax) (Vision BioSystem, Norwell, MA). Polymer reagent (Vision BioSystem) was used for immunolabeling, and the immunoreaction was visualized with 3,3′-diaminobenzidine chromogen (Dako, Ft. Collins, CO) under a microscope followed by counterstaining with hematoxylin (SurgiPath, Richmond, IL).

### Table 1. Primers Used in RT-PCR

<table>
<thead>
<tr>
<th>Markers</th>
<th>Gene</th>
<th>Primer sequence (5'-3')</th>
<th>Amplicon Size</th>
<th>Ann. temp. (°C)</th>
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<td></td>
<td>OCT4</td>
<td>Forward GAGTGAGAGGCAACCTGGAG Reverse GTGAAGTGAGGGCTCCCAT A</td>
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<td>60</td>
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<td>Stemness</td>
<td>NANOG</td>
<td>Forward GAATAACCGAATTTGGAGCAG Reverse AGCGATTCCTCTCAAGTTG</td>
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<td>SOX2</td>
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<td></td>
<td>RUNX2</td>
<td>Forward GTCTCCTCCAGGAATGCTTC Reverse GGAAGTCGGGATGAGGAC</td>
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<td>60</td>
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<tr>
<td>Osteoblasts</td>
<td>COLIA1</td>
<td>Forward GTAGACACCCCTCTCAAGAC Reverse TCCACAGGAGAGGCTAGAC</td>
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<td>62</td>
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<td>OSTERIX</td>
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<td>60</td>
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<td>BSP</td>
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<td>60</td>
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<tr>
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<td>OSTEOCALCIN</td>
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<td>62</td>
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<td>COL2A</td>
<td>Forward GAAACTCTGCACCACTGGAATG Reverse GCTACAGGATCCTTGGA</td>
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<td>AGGREGAN</td>
<td>Forward ATCAAGCATGCTTACCAAGACA Reverse ATCAACCTCCAGGATGAC</td>
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<td>Chondrocytes</td>
<td>COMP</td>
<td>Forward TGTTGAGCAAGATGGATGGTG Reverse CACCCAGTGGGACTTCTCTG</td>
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<td>COL10A</td>
<td>Forward AGTAAAGAGGTAAGCGGATCTG Reverse TCTTGGGTCTATAAAGCTGGTTC</td>
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<td>SOX9</td>
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<td></td>
<td>PPARγ2</td>
<td>Forward ACACGTGGTGGGCTCCTTGAGT Reverse TGGCTCCCTAGGAAGTCACCAAAAGG</td>
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<td></td>
<td>CEBPα</td>
<td>Forward ATGCAAGAAGTGGGATGCAAGG Reverse GCGGCATTGCTGACTGGA</td>
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<td>Adipocytes</td>
<td>FABP4</td>
<td>Forward ATCAGTGGTCAAGGCGGATGTTG Reverse GACCTTCTCTGATCATCAGATGA</td>
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<td></td>
<td>LEPTIN</td>
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<td>60</td>
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<td>Housekeeping</td>
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<td>Forward TCTACATTGGGCACTGAGTGCAC Reverse TGAAGAGTTCAGGTTGCAACCA</td>
<td>136</td>
<td>60</td>
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*a Reaction contained 10% DMSO.*
After chondrogenic induction, the micromasses were fixed in 10% neutral-buffered formalin and embedded in paraffin block, and similar immunostaining protocol (as described in previous paragraph) was followed. 1:100 dilution of rabbit polyclonal anti-collagen 2 (DakoCytomation, Carpenteria, CA) was used as a source of primary antibody after validating the antibody with canine tissues, and a streptavidin–immunoperoxidase staining procedure (Dako) was used for immunolabeling.

Proliferation potential of cAD-MSCs

Cumulative population doubling level. For determination of the cumulative population doubling level (cpdl), 100,000 cells were plated in 75 cm² flask and grown in K-NAC medium containing 5% FBS until near confluence, to quantify the final cell yield and subcultured. The population doubling (pd) at each subculture was calculated by using the following equation: \( pd = \ln \left( \frac{N_f}{N_i} \right) / \ln 2 \), where \( N_i \) and \( N_f \) are initial and final cell numbers, respectively, and \( \ln \) is the natural log. The Pds of continuous subculture were added to obtain cpdl.

Colony formation efficiency in soft agar and on plastic. As described,7 50,000 cells in 3 mL of 0.33% agarose medium were plated on top of 3 mL prehardened 0.5% agarose medium in each triplicate 60 mm dishes with grids to aid colony counting. About 2.5 mL medium (K-NAC with 5% FBS) was then added and renewed every 3 days. The numbers of anchorage-independent colonies were scored after 3 weeks.

Colony-forming efficiency on plastic was assayed by plating 200 cells in each of triplicate 100 mm plates in K-NAC medium with 5% FBS as well as 10% FBS for 3 weeks. The colonies were then stained with 1% crystal violet, and scored.

RESULTS

Isolation of putative cAD-MSCs

cAD-MSCs were successfully isolated from subcutaneous and omental fat of three different dogs. As shown in Figure 1A, both serpiginous and fibroblast-like cells were observed in primary culture. These cells could maintain this phenotype and be expanded in culture (see confluent cells in Fig. 1B). Similar to human adipose–derived MSCs (hAD-MSCs), symmetric division of serpiginous (Fig. 1C, black arrow) and fibroblast-like cells (Fig. 1D, white arrow) as well as asymmetric division of serpiginous cells (Fig. 1D, black arrow) were evident at low density (Fig. 1D). These cells also displayed anchorage-independent growth in soft agar (Fig. 1E) at a frequency of 14.2 ± 3.7%. On a plastic surface, the colony-forming efficiency in K-NAC medium with 10% FBS was 17.75 ± 3.2%. It was considerably lower (4.67 ± 2.3%) with 5% FBS.

Some variability in proliferation rate was observed among initial cell cultures derived from different tissue sites, with cells derived from subcutaneous fat becoming confluent in 25 cm² flasks in 5–6 days as compared to those derived from omental fat, which took 11–12 days to reach confluency. This variation in proliferation capacity was also paralleled with cell yield studies in primary culture, where subcutaneous fat yielded the greatest number of cells/g of fat (Table 2). Although cells from inguinal fat were attached to the plastic surface, they showed little proliferation, and cell yield was not calculated for them. However, cells from

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of dogs</th>
<th>Cells/g of fat (mean ± SD)</th>
</tr>
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<tbody>
<tr>
<td>Subcutaneous</td>
<td>3</td>
<td>528,000 ± 236,000</td>
</tr>
<tr>
<td>Omental</td>
<td>3</td>
<td>182,000 ± 95,000</td>
</tr>
<tr>
<td>Inguinal</td>
<td>3</td>
<td>Much lower (not calculated)</td>
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</table>

FIG. 1. Phenotype of cAD-MSCs. Morphology of cAD-MSCs at low (A) and high (B) cell densities. The cultures contain serpiginous-shaped and cuboid or fibroblast-like cells. The serpiginous cells showed symmetrical (C) or asymmetrical (D) division. (E) Anchorage-independent colonies of cAD-MSCs developed in soft agar. Scale bar = 100 µm. (F) Cumulative pd of cAD-MSCs in K-NAC medium with 5% FBS or K-NAC:D (1:1) medium with 5% FBS. (G) mRNA expression of stemness markers OCT4, NANOG, and SOX2 in cAD-MSCs; β2 MICROGLOBULIN (BMG) is a housekeeping gene; M represents 100 bp DNA ladders with the lowest band representing 100 bps. Color images available online at www.liebertpub.com/ten.
subcutaneous and omental fats were apparently similar in terms of their efficiency to form colonies in soft agar and plastic (difference of less than 2 standard deviations from the above-mentioned mean values).

**Expression of stemness markers**

RT-PCR analysis revealed that cAD-MSCs express pluripotency-associated transcription factors, *OCT4*, *NANOG*, and *SOX2* (Fig. 1G).

**Life span of cAD-MSCs in different media**

The lifespan of cAD-MSCs derived from subcutaneous fat was 25 ± 1.2 cpdl in 83 days after eight passages, when cells were grown in the K-NAC medium with 5% FBS. The proliferation potential was significantly enhanced when these cells were grown in the 1:1 mixture of K-NAC and D medium supplemented with 5% FBS (cpdl = 40) in 66 days after nine passages at the cell seeding density of 400 cells/cm² (Fig. 1F). The proliferation potential of cAD-MSCs
isolated from omental fat was quite similar (difference of about 1.5 standard deviation from the above-mentioned mean value).

**Differentiation of cAD-MSCs**

The cAD-MSCs isolated from subcutaneous fat as well as those from omental fat were differentiated into three mesodermal lineages (osteogenic, chondrogenic, and adipogenic).

**Osteogenesis**

Upon induction with DAG cocktail, the cells became more cuboidal-like in phenotype, and continued to proliferate actively and formed cell aggregates that would roll in a sheet and easily detach (Fig. 2A–C). Deposition of calcified extracellular matrix was evident in treated cells that formed cell aggregates (Fig. 2D), but not in monolayer cells as revealed by alizarin red staining (Fig. 2E) as well as von Kossa (Fig. 2F). Cell phenotype did not change in untreated cells.

Laminin coating of plates not only prevented the clumping of cells during differentiation, but also induced extensive deposition of calcified extracellular matrix on monolayer cell culture following 6 weeks of treatment (Fig. 2G–I, displaying unstained, alizarin red, and von Kossa stained cells, respectively). This mineralization was more pronounced when 1,25-dihydroxyvitamin D₃ was used in the induction regimen (as shown in Fig. 2) instead of dexamethasone (not shown).

**RUNX2** expression was stronger in osteo-induced cells, although basal expression was noticed in noninduced cells as well (Fig. 2O). **COLIA1** was strongly expressed in both induced cells and control cAD-MSCs. On the other hand, expression of **OSTERIX, BSP, and OSTEOCALCIN** was seen only in induced cells, with no basal expression in control cells (Fig. 2O). After 20 days of osteogenic differentiation, the cells had clear diffuse cytoplasmic staining for OSTEOCALCIN, predominantly produced by osteoblasts (Fig. 2M, N).

Quantitative measurement of calcium showed that treated cells had about 16-fold higher level of calcium than in control cells (27.5 ± 8.6 μg per well [six-well plates] for treatment versus 1.7 ± 0.4 μg per well in control).

**Chondrogenesis**

Within a day after seeding the cAD-MSCs in micromass culture, three-dimensional aggregates were observed in both treated (Fig. 3A) and control wells (Fig. 3D). Both treated (Fig. 3B) and control micromasses (Fig. 3E) stained positive for alcian blue, indicating presence of sulfated proteoglycans. This was confirmed by staining paraffin-embedded sections corresponding to each group (Fig. 3C, treated; Fig. 3F, control). Putting the cells in micromasses (both treated and control) resulted in specific expression of **COL2A, AGGREGAN, COMP, COL10A, and SOX 9**, all of which were undetected in monolayer of cAD-MSCs expanded in K-NAC medium (Fig. 3I). Moreover, immunostaining of sections of both treated and control micromasses confirmed the expression of **COL2A** (Fig. 3G and H).

**Adipogenesis**

Following the induction regimen (IDII) that was effective for adipogenic differentiation of human MSCs, adipogenic differentiation of cAD-MSCs seemed to be limited (Fig. 4A, unstained; Fig. 4B, Oil Red O stained). The use of rabbit serum (5%) in combination with rosiglitazone (5 μM), dexamethasone (1 μM), insulin (5 μg/mL), and high glucose (4.5 g/L) considerably increased the adipogenic differentiation (in terms of both numbers and size of fat globules). Fat globules were noted within 4–5 days of treatment (Fig. 4C), which continued to increase in size, and stained positive with Oil Red O (Fig. 4D). In contrast, no differentiation was observed in the untreated group (Fig. 4E, unstained; Fig. 4F, Oil Red O stained). Expression of **PPARγ2, CEBPα, FABP4, and LPL** were specific to adipo-induced cells (Fig. 4G). Basal level of **LEPTIN** mRNA was observed in noninduced control cells, and expression level was increased following adipogenic induction (Fig. 4G).

Cells from both subcutaneous and omental fat from all the three donors were apparently equal in terms of their differentiation potential across all the three lineages tested. An estimation of randomly selected microscopic fields of treated plates showed that approximately 80% of the total cells underwent differentiation (judged by the pattern of staining).

**DISCUSSION**

This study has demonstrated for the first time the isolation of canine adult MSCs with extensive proliferation potential and multilineage differentiation ability from adipose tissue (cAD-MSCs). Although some studies have been done to demonstrate the in vivo potential of canine bone marrow–derived MSCs for tissue regeneration, immunogenecity, and gene delivery, most of them lacked in vitro characterization of the cells used for these purposes. A recent study has demonstrated the in vivo potential of BMP-2–modified cells isolated from canine adipose tissue, but those cells were not characterized for their proliferation potential, expression of stemness markers, and lineage-specific markers upon induction.

This study used the low-calcium medium supplemented with antioxidants for the expansion of MSCs, which had been shown to extend the life span of hAD-MSCs. Serpiginous-shaped cells, which divide symmetrically as well as asymmetrically, were interspersed in the primary culture and subsequent passages of cAD-MSCs. The
appearance of this stem cell morphology for cAD-MSCs paralleled hAD-MSCs\textsuperscript{7} and other stem cell types.\textsuperscript{14,15}

The proliferation potential of cAD-MSCs in K-NAC medium with 5% FBS (cpdl = 25 in 82 days) was higher than hAD-MSCs reported by Zuk \textit{et al.}\textsuperscript{16} (21 cpdl in 165 days after 13 passages), but lower than hAD-MSCs reported by Lin \textit{et al.}\textsuperscript{7} (35 cpdl in 62 days after seven passages) using the same medium. This may reflect the species difference in growth requirement or proliferation potential of AD-MSCs. However, this lower proliferation potential of cAD-MSCs could be greatly improved by using a medium with 1:1 mixture of K-NAC and D medium supplemented with 5% FBS (40 cpdl in 66 days after nine passages). Thus, these cells can be adequately expanded for a variety of therapeutic applications. Our study showed that canine adipose tissues from different sites showed considerable variation in terms of cell yield, with subcutaneous fat yielding the greatest number of MSCs/g of fat compared to omental and inguinal fat. Similar regional differences have been documented in various species.\textsuperscript{17,18}

\textit{OCT4}, \textit{NANOG}, and \textit{SOX2} are pluripotency markers, which are usually ascribed to embryonic stem cells. However, their expression has been documented in some of the somatic stem cells.\textsuperscript{19,20} cAD-MSCs expressed all of these three markers at mRNA level, suggesting the stemness of these cells isolated from canine fat. cAD-MSCs were able to undergo anchorage-independent growth, a property which has been documented for a number of human adult stem cells, including human mesenchymal\textsuperscript{7} and liver\textsuperscript{21} stem cells.

Following osteogenic induction of cAD-MSCs with conventional DAG cocktail, mineralization occurred only in discrete foci where cell aggregates formed. This pattern is different from hAD-MSCs,\textsuperscript{7,16} but is similar to observations in canine bone marrow–derived MSCs.\textsuperscript{22} Consistent with
the recently documented role of laminin in osteogenesis of human MSCs, inclusion of laminin in the induction regimen allowed differentiation and mineralization in the monolayer.

As had been demonstrated earlier, the use of 1,25-dihydroxyvitamin D$_3$ allowed more extensive deposition of calcified extracellular matrix after induction, compared to dexamethasone. Consistent with earlier studies in other systems, cAD-MSCs also expressed basal level of early stage transcription factor RUNX2 and extracellular matrix protein COLIA, which were upregulated following osteogenic induction. OSTEX, a late stage transcription factor, and two other osteoblast-specific markers, BSP and OSTEOCALCIN, were expressed only following osteogenic induction.

For hAD-MSCs, treatment with TGF-β1 is necessary for the formation of three-dimensional aggregates and chondrogenic differentiation. In contrast, cell–cell contact in micromass was sufficient for chondrogenic differentiation of cAD-MSCs. This finding is similar to that of bovine bone marrow–derived MSCS. Chondrogenic transcription factor, SOX9 and chondrocyte markers COL2A, AGGREGAN, COMP, and COL10A were expressed after micromass culture (both TGF-β1–induced and uninduced) of cAD-MSCs, whereas cells expanded in monolayer did not express any of these markers.

Canine MSCs were refractory to the commonly used induction condition for adipogenic differentiation of human MSCs, and needed significant optimization. Replacement of FBS with rabbit serum and inclusion of rosiglitazone and higher glucose concentration in the medium enhanced the adipogenic differentiation of canine MSCs. Adipogenic transcription factors PPARγ2 and CEBPα, and adipocyte markers FABP4 and LPL were specifically expressed following adipogenic induction, whereas LEPTIN was expressed at basal level, even in undifferentiated MSCs. In conclusion, this study has clearly demonstrated that canine adipose tissue is a promising source of MSCs. Immediate and clinically relevant uses of cAD-MSCs include repair of torn tendons, ligaments, and cartilage, as well as broken bones, which parallel needs in human medicine. Employment of stem cells both in experimental as well as spontaneously occurring disease states in the dog will provide rigorous model systems to assess the translation of stem cell–based therapy into clinics under both autologous and allogenic settings. Therefore, we propose that the methods demonstrated in this paper will allow pertinent issues of stem cell biology to be addressed in the canine model system and facilitate the realization of therapeutic potential of stem cells. However, there are species differences in the properties of adipose-derived MSCs. As with any study in a model organism, studies in dogs should be cautiously interpreted, even though they provide important insights into the biology and therapeutic potential of these cells. The exact nature or mechanism(s) underlying these differences should be explored in future studies.

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REFERENCES

12. Mosca, J.D., Hendricks, J.K., Buyaner, D., vis-Sproul, J., Chuang, L.C., Majumdar, M.K., Chopra, R., Barry, F., Murphy, M., Thiede, M.A., Junker, U., Rigg, R.J., Forestell, S.P.,


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