Canine Intra-Articular Multipotent Stromal Cells (MSC) From Adipose Tissue Have the Highest In Vitro Expansion Rates, Multipotentiality, and MSC Immunophenotypes

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Objective: To identify the optimum intra-articular multipotent stromal cell (MSC) tissue source in the canine stifle.

Study Design: Experimental.

Sample Population: Infrapatellar adipose tissue, synovium lining the joint capsule, and synovium surrounding the cranial cruciate ligament (CrCL) from normal stifles of 6 dogs.

Methods: Nucleated cell density for each tissue was determined, and cell doublings (CD) and doubling times (DT) were quantified for expansion rates. Adipogenic, osteogenic, and chondrogenic differentiation was confirmed with light microscopy. Fibroblastic, adipogenic, and osteogenic colony forming unit frequencies were determined for multipotentiality. Tissue-specific target gene expression was assessed, and percentages of CD29⁺, CD34⁺, CD44⁺, CD45⁺, and CD90⁺ cells quantified.

Results: Adipose tissue had the highest MSC density (ASC). The CD decreased with increasing passages for all cell types, and ASC values tended to be higher. Multipotentiality decreased with passage, but remained highest in ASC. Tissue-specific target gene expression was higher in induced versus noninduced cells, and ASCs had the highest upregulation across passages. Most cells were CD29⁺, CD44⁺, CD90⁺, and percentages decreased with passage. Within cell types, there were more CD29⁺ ASC in early passages and more CD44⁺ and CD90⁺ ASC in later passages.

Conclusions: ASC had the highest in vitro expansion rates, CFU frequencies, tissue-specific target gene expression, and percentages of MSC immunophenotypes.

Rupture of the cranial cruciate ligament (CrCL) is the leading orthopedic disorder of the canine stifle.¹,² Concomitant intra- and periarticular damage occurs with 70% of CrCL ruptures, and quality of life is reduced by the progressive pain, dysfunction, and osteoarthritis that results.³,⁴ Many tissues within the stifle have limited regenerative and reparative capacities, so surgical intervention is often necessary to restore joint function.⁵ Current surgical options do not necessarily restore damaged tissues or inhibit joint degeneration.⁶ The emerging field of tissue engineering with adult multipotent stromal cells (MSCs) has significant potential for generation of viable, functional, engineered stifle tissues to replace those lost to injury and degeneration.⁷

Ideal cell candidates for intra-articular tissue regeneration generally have high proliferative capacity, produce tissue-specific extracellular matrix and survive in the intra-articular environment.⁸ Orthotopic adult stromal cells have greater commitment to tissue-specific lineages than those from heterotopic sources.⁹ Intra-articular stromal cells have better expansion capacity and potential for stifle joint tissue regeneration than cells from other sources.¹⁰ Infrapatellar (IFP) adipose tissue and synovium are established intra-articular MSC sources, and CrCL cell isolates are reported to have higher expression of ligament specific phenotypic markers than peri-articular ligaments and tendons.¹¹,¹² For the potential benefits of orthotopic stromal cell tissue regeneration to be realized in clinical stifle therapies, it is critical to identify the optimum intra-articular source of adult MSCs. A direct comparison of cell isolate phenotypes and their in vitro expansion capacities and multipotentiality within individuals is key to achieving this goal.

Recently, canine IFP adipose-derived multipotent stromal cells (ASCs) were shown to have greater or equal expansion capacity to bone marrow MSCs and comparable multipotentiality after cryopreservation.¹³ Based on this information and the stated goal above, stromal cell tissue density, in vitro expansion rates, multipotentiality, and MSC cell immunophenotypes were quantified in 3 stifle tissues, adipose (ASC),
synovium lining the joint capsule (SSC), and synovium surrounding the CrCL (LSC), from all study subjects up to cell passage (P) 6 to test the hypothesis that values for the tested variables are higher for ASC.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were approved by the Institutional Animal Care and Use Committee.

Study Design

Synovium lining the joint capsule, IFP, and CrCL were collected from 1 normal stifle of 6 mixed breed female dogs (mean ± SEM weight, 23.1 ± 0.9 kg; mean age, 3.3 ± 0.4 years) undergoing stifle surgeries for reasons unrelated to the investigation. Stifles included in the study had no detectable radiographic changes or visible intra-articular abnormalities. The following assessments were performed on cells from all tissues in each dog: P0–6 cell doublings (CD) and doubling times (DT); P0, 3, and 6 CD29+/CD44+, CD90–, CD34, and CD45– cell percentages; P0, 3, and 6 fibroblastic, osteogenic, and adipogenic colony-forming unit frequencies; P0 chondrogenic pellet alcian blue (proteoglycan) staining; P0 and 3 stromal (undifferentiated) and differentiated (adipogenic, osteogenic, and chondrogenic) cell MSC and lineage-specific target gene mRNA levels. Cell immunophenotypes were also determined in the stromal vascular fraction (SVF) of pooled tissue harvests collected immediately postmortem from both normal stifles of 10 adult, female, mixed-breed dogs euthanatized for unrelated reasons. Pooling was necessary for adequate cell numbers, so only 1 SVF sample for each cell type was evaluated for nucleated cell density and with flow cytometry.

Cell Isolation and Expansion

Adipose tissue and joint capsular synovial cells were isolated according to a published protocol with minor modifications.13 Briefly, adipose tissue and synovium were minced into small pieces, and the CrCL surface was vigorously lavaged with PBS to detach the synovial membrane. Tissues were then digested with collagenase solution (DMEM-Ham’s F12, 0.1% type I collagenase [Worthington Biochemical Corporation, Lakewood, NJ], 1% bovine serum albumin). After 1 hour, the pellet resulting from centrifugation (260 ×g, 5 minutes) was resuspended in stromal medium (DMEM-Ham’s F12, 10% fetal bovine serum [FBS], 1% antibiotic/antimycotic solution) and filtered (100 µm, BD Falcon, Bedford, MA) after addition of an equal volume of red cell lysis buffer (0.16 mol/L NH₄Cl, 0.01 mol/L KHCO₃, 0.01% EDTA). The resulting SVF were seeded in 10 mm dishes (CellStar, Greiner, NC) at a density of 5 × 10⁵ cells/cm² in stromal medium and cultured under standard conditions (37°C, 5% CO₂). Medium was refreshed after 4 hours and then every 2–3 days. Subsequent passages were performed at 80% confluence with a cell seeding density of 5 × 10³ cells/cm². For purposes of this study, primary cell isolates were considered the SVF. The first cell passage of the primary cells was P0.

Cell Expansion (P0–6)

CD and cell doubling time (DT) were determined with duplicate cultures in 12-well plates at an initial seeding density of 5 × 10⁵ cells/cm².14 Cells were counted after 2, 4, and 6 days of culture. CD and DT were calculated with the formulae: $CD = \ln(N_F/N_i)/\ln(2)$ DT = $CT/CD$ (N_i: initial cell number; N_F: final cell number; CT: culture time).

Multipotentiality (P0, 3, 6)

Limiting-dilution assays to determine fibroblastic (CFU-F), adipogenic (CFU-Ad), and osteogenic (CFU-Ob) colony-forming unit frequencies.13 A total of 5 × 10⁵, 2.5 × 10⁵, 1.25 × 10⁴, 6.25 × 10³, 3.12 × 10², or 1.56 × 10² cells were placed in each well of one row (one concentration/row, 8 replicates/concentration) in a 96-well plate. Cells were cultured in stromal media for 7 days. Fibroelastic (CFU-F) colonies were then fixed with 1% paraformaldehyde and stained with 0.1% toluidine blue. For adipogenesis (CFU-Ad), cells were cultured in stromal medium for 7 days, followed by culture in adipogenic medium (DMEM-Ham’s F12, 3% FBS, 1% antibiotic/antimycotic solution, 33 µmol/L biotin, 17 µmol/L pantothenate, 1 µmol/L dexamethasone, 100 µmol/L indomethacin, 1 µmol/L insulin, 0.5 mmol/L isobutylmethylxanthine [IBMX], and 5 µmol/L rosiglitazone) for 21 days. Wells were then fixed as above and stained with oil red O. Similarly, osteogenesis wells were cultured in stromal medium for 7 days and then cultured in osteogenic medium (DMEM-Ham’s F12, 10% FBS, 1% antibiotic/antimycotic solution, 10 mmol/L β-glycerophosphate, 10 µmol/L dexamethasone, and 50 µg/mL sodium 2-phosphate ascorbate) for 21 days. Colonies were fixed in 70% ETOH and stained with 2% alizarin red. Wells were considered positive for MSC, adipogenesis, or osteogenesis when there were ≥10 toluidine blue-stained colonies, ≥10 oil red O-stained colonies, or ≥1 alizarin red-stained colonies, respectively. The ratio of negative to total wells in rows (1 for each concentration) containing negative wells was used to estimate the CFU for each concentration using the equation $F = e^{-x}$: ratio of negative to total wells within a row (concentration), e: natural logarithm constant 2.71, x: CFU number per well). Based on a Poisson distribution of a clonal cell lineage, the value of $F = 0.37$ occurs when the number of total cells plated in a well contains a single CFU.13,14 The mean value from each plate was used as the CFU for each animal/cell type/differentiation medium. For chondrogenesis, 2.5 × 10⁵ cells were centrifuged (200 ×g, 5 minutes) to form a pellet in a 1.5 mL microcentrifuge tube after 7 days of culture in stromal medium. The pellet was cultured in chondrogenic differentiation medium (DMEM-Ham’s F12, 3% FBS, 1% antibiotic/antimycotic solution,
50 μg/mL ascorbate phosphate, 100 mmol/L dexamethasone, 40 μg/mL proline, 2 mmol/L sodium pyruvate, 1% ITS, and 10 ng/mL TGF-β3) for 21 days with media changes every 2–3 days. Pellets were formalin-fixed, paraffin-embedded, sectioned (5 μm) and stained with alcoholic blue.

mRNA Levels

Total RNA was isolated from cells (RNAqueous Kit, Ambion, Austin, Tx), DNase-treated, and reverse-transcribed using oligo (dT) primers and Moloney murine leukemia virus reverse transcriptase. For chondrogenesis, pellet mRNA was extracted by homogenization in TRI Reagent (Sigma–Aldrich, St. Louis, MO). Target gene mRNA levels were quantified with qRT-PCR using SYBR Green technology (MJ Research Chromo4 Detector; Bio-Rad Laboratories, Hercules, CA). Canine-specific primers for MSC and adipogenic, osteogenic, and chondrogenic lineages were used (Table 1). Values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative gene expression was quantified according to 2^ΔΔCt.

Immunophenotype

Cell aliquots (10⁵) were resuspended in 200 mL PBS containing 5 μL antibody. Cells were incubated with antibodies specific for canine antigens or validated for canine cross reactivity against PE-CD34, FITC-CD44, PE-CD45, and PE-CD90 for 30 minutes at room temperature (Table 2). The cells were then washed with PBS and fixed with 4% paraformaldehyde. For CD29 staining, the cells were incubated with antibody for 30 minutes at room temperature. They were then incubated with a secondary antibody, FITC-conjugated goat anti-mouse IgG (Sigma–Aldrich) for 30 minutes, washed with PBS, and fixed as above. For negative autofluorescence control, cells were not incubated with antibodies. Cellular fluorescence was evaluated by flow cytometry using a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences, San Jose, CA). Cell percentages expressing each antigen were determined. Because of low SVF cell numbers, flow cytometry was performed on representative samples for each tissue type using pooled cells from unrelated dogs. Pooling was necessary for adequate cell numbers so only one sample was processed for each antibody.

Table 1  Primer Sequences

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<td>Leptin</td>
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<td>CCCCCTTGTTGGAGAGACA</td>
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<tr>
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<td>Col1</td>
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<td></td>
<td>Nanog</td>
<td>GAATAGCCGAATTGCAGCAG</td>
<td>AGCCAGTTCCCTCAGTGTTG</td>
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PPAR-γ, peroxisome proliferator-activated receptor gamma; Col1, collagen type I; OPG, osteoprotegerin; Col2, collagen type II; Sox2, sex determining region Y-box 2; Nanog, homeobox protein nanog.

Statistical Analysis

All results are presented as mean ± SEM. ANOVA models (GraphPad Prism, La Jolla, CA) were used to compare CD, DT, CFU frequency, RNA levels, and cell surface marker percentages among passages within cell types and among cell types within passages. Tukey’s post hoc tests were applied for multiple group comparisons (P < .05).

RESULTS

Cell Harvest

Using pooled samples, 3.4 × 10⁵ nucleated cells/g were isolated from IFP, 3.0 × 10⁵ cells/g from synovial membrane, and 1.1 × 10⁵ cells/g of CrCL (approximately 2.2 × 10⁵ nucleated cells/CrCL).

Cell Expansion

Day 2 and Day 4 cell counts were used as the initial number to calculate expansion rates on Days 4 and 6, respectively. Differences were not significant between days, so data were collapsed over this variable. The overall (P0–P6 combined) CD for ASC, SSC, and LSC were 1.7 ± 0.1, 1.5 ± 0.1, and 1.2 ± 0.1 CD/day, respectively (Table 3). The corresponding overall DT (P0–6) were 0.7 ± 0.1, 1.0 ± 0.2, and 1.3 ± 0.2 days/CD (Table 4). Within cell types, P6 ASC had significantly lower CD than P0, 1, 2, and 4, and significantly higher DT than P0–4. The P6 SSC had significantly lower CD and higher DT than all previous passages, and P6 LSC had significantly lower CD and higher DT than P0–4. There were no significant differences in CD among cell types for P0–3. For P4–6, LSC CD was significantly lower than ASC. The P5 SSC CD value was significantly higher than LSC. After P5, SSC, and LSC had significantly higher DT than ASC.

Multipotentiality

All cell types displayed characteristic adipogenic (Fig 1A–C), osteogenic (Fig 1D–F), and chondrogenic (Fig 1G–I) differentiation. The CFU frequency indicates the minimum


number of cells that includes one capable of forming a fibroblastic (CFU-F) colony or differentiating into osteogenic (CFU-Ob) or adipogenic (CFU-Ad) cell lineages. Results are presented as frequencies, so higher numbers indicate more cells required for a cell with the indicated differentiation capacity. Because of limited cell expansion past P4, P6 LSC were not included in CFU assays. Within cell types, the P6 ASC and SSC CFU-F were significantly higher than P0, and the LSC P3 CFU-Ad was significantly higher than P0, and the LSC P3 CFU-Ad was significantly higher than P0 (Table 5). Among cell types, P0 and P3 LSC had significantly higher CFU-F, CFU-Ad and CFU-Ob than ASC. The P3 SSC had significantly higher CFU-Ad than ASC, and P6 SSC had significantly higher CFU-Ad and CFU-Ob than P6 ASC.

**mRNA Levels**

Compared to SSC and LSC at the same passages, induced P0 and P3 ASC had significantly higher mRNA levels of peroxisome proliferator-activated receptor gamma (PPAR-γ) and leptin after adipogenic induction, osteoprotegerin (OPG) and collagen I (Col1) after osteogenic induction, and aggrecan (Fig 2A–F). After adipogenic and osteogenic induction, P0 and P3 ASC had significantly lower mRNA levels of homeobox protein nanog (Nanog) than SSC and LSC, and the same was true for sex determining region Y-box 2 (Sox2) expression after adipogenic induction. There were significantly lower mRNA levels of Sox2 in P3 ASC compared to LSC and SSC after osteogenic induction.

**Immunophenotype**

Most of P0 and P3 cells were CD29⁺, CD44⁺, CD90⁺, CD34⁺, and CD45⁺ (Fig 3), and the SVF of all cell types had lower percentages of CD44⁺ and CD90⁺ cells than P0 and 3 (Fig 4A–C). The SSC and LSC SVF had higher CD34⁺ percentages than the ASC SVF (Fig 4D). With increasing passages, the percentages of CD34⁺, CD29⁺, CD44⁺, and CD90⁺ cells tended to decrease in all cell types, while the percentages of CD45⁺ cells remained relatively constant (Fig 4E–H). Among cell types, the P0 ASC CD29⁺ percentage was significantly higher than the LSC (Fig 4A). The P3 ASC and SSC CD29⁺ percentages were significantly higher than LSC, and P6 ASC percentage significantly higher than SSC (Fig 4B). The CD44⁺ percentage was significantly higher for P3 SSC than ASC, though the P6 ASC percentage was significantly higher than P6 SSC. The P6 ASC CD90⁺ percentage was significantly higher than P6 SSC (Fig 4C). As above, because of limited cell expansion, flow cytometry was not performed on P6 LSC.

**DISCUSSION**

Stromal cell-mediated tissue regeneration is a promising approach for a wide range of medical applications. We have established the IFP as the optimum source of MSC in the canine stifle among those tissues evaluated. Consistent with the

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**Table 2** Antibodies for Flow Cytometry

<table>
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<th>Antibody</th>
<th>Label</th>
<th>Marker Expression</th>
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<th>Cat No.</th>
<th>Target Species</th>
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<td>β1 integrin</td>
<td>BD Biosciences</td>
<td>610468</td>
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<tr>
<td>CD34</td>
<td>PE</td>
<td>Hematopoietic progenitor (HSC)</td>
<td>BD Biosciences</td>
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<td>CD44</td>
<td>FITC</td>
<td>Hyaluronic acid receptor</td>
<td>eBiosciences</td>
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<td>CD45</td>
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<td>IgG</td>
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**Table 3** Canine ASC, SSC, and LSC Cell Doublings (CD/Day) for P0-6 (Mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>P0</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
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<tr>
<td>ASC</td>
<td>1.9 ± 0.2A,a</td>
<td>2.0 ± 0.2A,b</td>
<td>2.1 ± 0.2A,a</td>
<td>1.6 ± 0.2A,b,c</td>
<td>1.8 ± 0.2A,a</td>
<td>1.4 ± 0.6A,b,c</td>
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<td>1.9 ± 0.2A,a</td>
<td>1.8 ± 0.2A,a</td>
<td>1.7 ± 0.2A,a</td>
<td>1.7 ± 0.2A,a</td>
<td>1.5 ± 0.2A,a</td>
<td>1.3 ± 0.1A,a</td>
<td>0.5 ± 0.1B,b</td>
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<tr>
<td>LSC</td>
<td>1.5 ± 0.1A,a,b</td>
<td>1.8 ± 0.1A,a</td>
<td>1.5 ± 0.2A,a,b</td>
<td>1.4 ± 0.2A,a,b</td>
<td>1.1 ± 0.1B,b,c</td>
<td>0.8 ± 0.1B,c,d</td>
<td>0.4 ± 0.1B,d</td>
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Columns with different upper-case letters within passages and those with different lower-case letters within cell types are significantly different from each other (P < .05).

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**Table 4** Canine ASC, SSC, and LSC Doubling Times (Days/CD) for P0-6 (Mean ± SEM)

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<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
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<tbody>
<tr>
<td>ASC</td>
<td>0.6 ± 0.1A,a</td>
<td>0.6 ± 0.1A,a</td>
<td>0.5 ± 0.0A,a</td>
<td>0.8 ± 0.1A,a</td>
<td>0.6 ± 0.1A,a</td>
<td>0.8 ± 0.1A,a,b</td>
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<tr>
<td>SSC</td>
<td>0.6 ± 0.1A,a</td>
<td>0.7 ± 0.1A,a</td>
<td>0.7 ± 0.1A,a</td>
<td>0.7 ± 0.1A,a</td>
<td>0.8 ± 0.1A,a</td>
<td>0.9 ± 0.0A,b</td>
<td>3.0 ± 0.6B,b</td>
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<tr>
<td>LSC</td>
<td>0.7 ± 0.1A,a</td>
<td>0.6 ± 0.1A,a</td>
<td>0.8 ± 0.1A,a</td>
<td>0.8 ± 0.1A,a</td>
<td>1.0 ± 0.1A,a</td>
<td>1.6 ± 0.2B,b</td>
<td>3.7 ± 0.6B,c</td>
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Columns with different upper-case letters within passages and those with different lower-case letters within cell types are significantly different from each other (P < .05).
hypothesis, the MSC population, in vitro expansion rates, and multipotentiality were highest in ASCs compared to SSCs and LSCs within individuals up to P6 based on CD, CFU frequencies, MSC immunophenotypes, and target gene expression. As passages increased, CD, CFU frequencies, MSC immunophenotypes, as well as MSC, adipogenic and osteogenic lineage-specific gene expression decreased in all 3 cell types, suggesting some reduction in MSC numbers and multipotentiality. Notably, the ASCs retained these characteristics best through multiple cell passages. This information surrounding intra-articular MSCs may contribute to studies aimed at clinical translation of stromal cell technology.

Most stifle tissues are derived from interzonal mesenchyme, so MSCs derived from these tissues may share common features which are distinct from those residing elsewhere. Orthotopic cell sources have advantages for intra-articular tissue regeneration based on in vitro differentiation studies, possibly a result of exposure to the local joint environment during development potentiating cell differentiation into specialized joint tissues. Multipotent cell numbers, expansion capacity, and multipotentiality vary among the different tissues that contain MSCs within the femorotibial joint such as IFP, synovium, synovial fluid, and CrCL. We sought to identify and establish the best MSC

Table 5  Colony-Forming Unit Frequencies for Canine ASC, SSC, and LSC After Fibroblastic (CFU-F), Adipogenic (CFU-Ad), or Osteogenic (CFU-Ob) Induction (x 10^3).

<table>
<thead>
<tr>
<th></th>
<th>CFU-F</th>
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<th>CFU-Ad</th>
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<th>CFU-Ob</th>
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<td></td>
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<td>P6</td>
<td>P0</td>
<td>P3</td>
<td>P6</td>
<td>P0</td>
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<td>ASC</td>
<td>0.2 ± 0.1^A,a</td>
<td>0.6 ± 0.1^A,a</td>
<td>3.9 ± 0.8^B,b</td>
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<td>0.4 ± 0.1^A,a</td>
<td>6.6 ± 1.0^A,b</td>
<td>0.7 ± 0.2^A,a</td>
<td>1.3 ± 0.2^A,a</td>
<td>6.7 ± 1.0^A,b</td>
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<tr>
<td>SSC</td>
<td>0.3 ± 0.0^A,a</td>
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<td>1.5 ± 0.3^B,b</td>
<td>9.4 ± 1.6^B,c</td>
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<tr>
<td>LSC</td>
<td>1.3 ± 0.2^B,a</td>
<td>4.4 ± 0.9^B,b</td>
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Columns with different upper-case letters within passages and those with different lower-case letters within cell types are significantly different from each other (P < .05).
Figure 2  Fold change (mean ± SEM, n = 6, 3 replicates) of adipogenic (A,B), osteogenic (C,D), and chondrogenic (E,F) target genes in ASC, SSC, and LSC after the indicated inductions, normalized to GAPDH and relative to cells cultured in stromal medium. The fold change of MSC target genes (G,H) was evaluated for each cell type similarly after adipogenic and osteogenic induction. Columns with different letters within passages are significantly different from each other (P < .05).
Figure 3  Representative immunophenotyping of SVF, P0, P3, and P6 ASC, SSC, and LSC. Columns are different passages and rows different surface makers. The black graphs represent stained cells and the green graphs autofluorescence.
source for stifle-specific tissue regeneration by comparing some major intra-articular cell sources within individuals.

The ASC DT in this study was similar to previous reports for canine IFP and subcutaneous ASCs and human IFP ASCs.\textsuperscript{19,20} The DT for canine SSCs and LSCs in this study were significantly lower than those reported for human SSCs and LSCs.\textsuperscript{21} In addition to species differences, the human LSCs were harvested from the anterior cruciate ligament.
(ACL) core minus synovium, collagenase digestion was not performed and a different culture medium was used. Hence, differences in results between the studies are not unexpected. In our study, decreasing CD capacity with increasing passages in all cell types suggests that intra-articular MSCs from early passages may have the best expansion potential to meet therapeutic needs.

The ASC stromal, osteogenic, and adipogenic CFU frequencies in our study were higher than previously reported for dogs, and the LSC CFU-F frequencies were higher than that reported for human ACLs. Differences may be because of different cell sources and use of fresh versus revitalized cells. Indomethacin in the adipogenic medium in our study may have also contributed to higher CFU-Ad frequencies compared with the previous canine study. The higher adipogenic capacity of ASCs may also be because of a native preadipocyte population in P0. However, preadipocytes and multipotent cells are distinguished by the short preadipocyte lifespan in contrast to high expansion capacity and multipotentiality retained by MSCs through passages, so preadipocytes are unlikely to have affected later passages. Canine IFP ASCs have been reported to have similar osteogenic capacity to bone marrow-derived stromal cells (BMSCs), which supports the superior osteogenesis of the ASCs compared with SSCs and LSCs in our study. Information surrounding direct comparisons of the chondrogenic potential of stifle joint tissues is limited, but rat SSCs reportedly have better chondrogenic potential than MSCs from subcutaneous fat. Additionally, IFP ASCs had better chondrogenic potential than BMSCs and subcutaneous ASCs in elderly people with osteoarthritis. Though there was no difference in proteoglycan staining among intra-articular tissues evaluated in our study, higher mRNA levels of Col2 and aggrecan mRNA in ASC pellets may indicate higher chondrogenic potential.

The identity of the MSC populations within each tissue was confirmed, in part, by the presence of common MSC cell surface markers detected with flow cytometry. The mRNA levels of embryonic stromal cell markers previously used to identify canine MSCs in adult tissue, Nanog and Sox2, also supported the MSC identity of cell populations isolated from all 3 intra-articular tissues in our study. Cluster of differentiation (CD), also called cluster of determinants, are clusters of antigens that react with antibodies associated with various cell characteristics. They were originally used to identify different stages of lymphocyte maturation, but profiling of CD cell surface antigen expression with flow cytometry is now a standard mechanism by which MSC phenotypes are distinguished. The CD34+ cells within the SVF are consistent with human subcutaneous ASCs. Type B synoviocytes (fibroblast-like) are also CD34+. Because the LSCs were harvested from synovium around the ligament, it is reasonable that the SVF of LSCs as well as SSCs contained higher percentages of CD34+ cells. Plastic adherence appeared to effectively select against CD34+ cells with increasing passages.

The location, size, accessibility, and protective synovial capsule of the IFP make it a good potential MSC harvest option. It may be feasible to isolate cells for reapplication before closure during standard surgical procedures. Additionally, cells may be expanded in vitro and banked for later application. The number of autologous MSC harvested from the synovium or CrCL in a diseased or injured stifle may be limited because synovium undergoes significant pathogenic changes in the presence of inflammation, and the CrCL is often partially or completely ruptured in injured stifles. Despite this, it is feasible that synovium and CrCL may be potential MSC sources for tissue-specific engineering since they may contain less primitive cells than the IFP. Further research is necessary to identify and confirm this possibility.

The IFP is encapsulated and isolated from the local joint environment by synovium. Hence, MSCs niches within the IFP may therefore contain cells that receive less stimulation to induce differentiation and therefore remain more primitive compared to other intra-articular niches. Human IFP ASCs from osteoarthritic knees have comparable long-term cell expansion, multipotentiality, and MSC marker-expression as normal BMSCs. This is distinct from intra-articular SSCs, BMSCs, and cartilage-derived stromal cells, where cell morphology, proliferation, and multipotentiality are negatively affected by acute and chronic inflammation as well as the presence of OA. The results of our study suggest that the IFP is the optimum tissue source for adult MSC isolation in the canine stifle compared to joint capsular or ACL synovium. The orthotopic advantages as well as the relative isolation of MSCs within the IFP in the face of injury or disease further supports the advantages of this tissue source. Study findings are limited to normal tissues, and additional studies are necessary to confirm them in abnormal joints with distinctions between those affected by injury versus degenerative changes. Additionally, subcutaneous tissue may provide higher ASC yield from a greater volume of available tissue and reduce cell expansion necessary for therapeutic administration. In conclusion, this side by side comparison of canine stifle intra-articular tissue sources establishes the IFP as having the highest cell density and MSCs with the highest expansion capacity, multipotentiality, and MSC immunophenotype over multiple cell passages in vitro.

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