



Phenotypic characterization of equine synovial fluid-derived chondroprogenitor cells

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Abstract

Background: Progenitor cells exist in most tissues and body fluids. Synovial fluid chondroprogenitor cells have been described in several species; however, the specific phenotypic characteristics of these cells have not been defined. This study addressed the impacts of joint location and donor age variation on synovial fluid chondroprogenitor numbers, and determined whether synovial fluid chondroprogenitors express hypertrophic characteristics or a non-endochondral phenotype in chondrogenic culture.

Methods: Synovial fluid was aspirated from the joints of healthy adult horses, and cells in the aspirates were expanded through two monolayer passages. The number of colony-forming units (CFU) in each aspirate was assessed after 14 days. Population doublings (PD) and PD times were calculated during the subsequent two passages. Passage 3 cells were used to determine osteogenic and adipogenic capacities, or were transferred to pellet cultures in chondrogenic medium containing TGF- β 1 or BMP-2. Bone marrow-derived MSCs and primary articular chondrocytes were cultured under similar conditions to provide reference values for chondrogenesis. Chondrogenesis was assessed by measuring collagen type II protein and sulfated glycosaminoglycan secretion, expression of chondrogenic mRNAs, and induction of ALP activity.

Results: CFU counts varied considerably, but the majority of aspirates contained <50 CFU/ml. PDs were consistently between 2.0 and 3.0 during both passages. PD times varied from 0.2-0.4 days. Proliferation was not significantly influenced by anatomical location or donor age. Equine synovial fluid progenitors expressed osteogenic, adipogenic and chondrogenic phenotypes under appropriate conditions. Under chondrogenic conditions, synovial fluid cells increased collagen and aggrecan mRNA expression to levels comparable to bone marrow MSCs, although collagen type II protein secretion was less than half that of articular chondrocytes. In contrast to bone marrow MSCs, synovial fluid chondroprogenitors did not express collagen type X or increase ALP activity in response to TGF- β 1 or BMP-2. Consistent with these observations, Runx2 and Mef2C expression in synovial fluid chondroprogenitors was 20-40 fold lower than in bone marrow MSCs.

Conclusions: Synovial fluid chondroprogenitors are capable of robust non-hypertrophic chondrogenesis, whether stimulated by TGF- β 1 or by BMP-2. These results indicate that synovial fluid cells are phenotypically suitable for articular cartilage repair/regeneration, although strategies to accelerate cell proliferation and synthesize a functional cartilage matrix *in vivo* will be required for clinically feasible cell-based therapies.

Keywords: Chondroprogenitor, synovial fluid, chondrogenesis, MSC

Introduction

Mesenchymal stem cells (MSCs) are characterized on the

basis of their ability to form colonies from single 'founder' cells, maintain extensive proliferative capacity over multiple

passages and differentiate along several mesenchymal lineages. As a consequence, MSCs hold considerable promise for tissue engineering and regenerative medicine applications to repair musculoskeletal tissues; particularly tissues such as articular cartilage [1,2] that have limited intrinsic reparative capacity. Much of the initial MSC research was focused on bone marrow MSC (BM-MS) populations; however, it is now well established that MSCs are present in most tissues and body fluids. Despite similarities in isolation protocols, MSCs derived from different tissues demonstrate significant variation in their ability to adopt specific phenotypes, synthesize functional extracellular matrices [3-5] and modulate immunological and inflammatory pathways during tissue repair [6,7].

The presence of MSCs in synovial fluids was first reported in 2004 [8], and subsequent studies have corroborated this finding in several model species, including the horse [9-12]. The proliferative and differentiation capacities of synovial fluid-derived progenitor cells have varied between studies [9-13], but in all cases, these cells were capable of chondrogenesis. However, the specific chondrogenic lineage of synovial fluid-derived chondroprogenitors (SF-CP) has not been defined. The majority of MSCs express a hypertrophic phenotype under chondrogenic conditions [14-16], with endochondral characteristics that are less than optimal for articular cartilage repair. Given that cell populations within the synovial cavity share developmental and spatial proximity to articular chondrocytes, SF-CPs could be expected to express a phenotype more consistent with articular chondrocytes than the hypertrophic phenotype adopted by other MSC populations.

This study was designed to determine whether SF-CP numbers vary with anatomical location or with age, to assess the chondrogenic capacity of equine SF-CPs and determine whether SF-CPs express a hypertrophic or non-hypertrophic phenotype. SF-CPs were isolated from three clinically relevant joint locations from horses of differing ages. After monolayer expansion, SF-CPs were stimulated to undergo chondrogenesis in standard chondrogenic medium supplemented with transforming growth factor beta 1 (TGF- β 1), and in medium containing bone morphogenetic protein 2 (BMP-2). BMP-2 stimulates hypertrophic differentiation in competent chondrogenic populations [17,18]. The chondrogenic responses of SF-CPs were compared with the activities of primary articular chondrocytes and bone marrow-derived MSCs (BM-MS) cultured under similar conditions. The study was designed to address the hypothesis that SF-CPs express a non-hypertrophic chondrogenic phenotype.

Materials and methods

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Illinois. Unless otherwise stated, chemical reagents were purchased from Sigma (Sigma-Aldrich Chemical Co, St Louis, MO), and cell culture media and reagents were purchased from Fisher Scientific (Fisher Scientific, Pittsburgh, PA).

Isolation and expansion of synovial fluid-derived cells

Synovial fluid was aspirated from diarthrodial joints of healthy, adult horses that were clinically and radiographically free of joint disease, immediately prior to arthroscopic surgery or immediately after euthanasia for the reasons other than musculoskeletal disease. 1 ml of synovial fluid was diluted in 10 ml of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 100 units of sodium penicillin/ml, 100 μ g of streptomycin sulfate/ml (basal medium) and seeded into 100 mm culture plates. The primary cultures were monitored daily for cell attachment and formation of colonies. After 14 days, distinct colonies, defined as focal clusters of at least 25 cells, were counted across the entire surface of the culture dishes, under low power microscopy.

At 80-90% confluence, the primary synovial fluid cultures were lifted with 0.05% Trypsin/EDTA and the cell suspensions were assessed for cell number and viability by trypan blue exclusion. The cells were re-seeded at 1×10^4 cells/cm² in basal medium and expanded through two passages. The population doublings (PD) of each SF-CP passage were calculated as: Log_2 (final cell number/initial cell number). PD times (PDT) during each passage were calculated by dividing the PD value by the time, in days, of each passage. PD and PDT outcomes were stratified according to donor age. Horses less than 2 years of age represented untrained/minimally exercised donors. Horses between 2 and 4 years of age were in active work, while the majority of horses greater than 4 years of age were comparatively sedentary.

Articular chondrocyte isolation and culture

Fully differentiated chondrocytes were isolated from articular cartilage from four healthy adult horses and cultured as pellets, as previously described [19]. Cartilage was diced and digested with 0.2% collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ) overnight. Isolated chondrocytes were pelleted by centrifugation (2.5×10^5 cells per pellet) at 390 rcf in 1.5 ml microcentrifuge tubes. The pellets were maintained in chondrogenic medium (DMEM, supplemented with insulin/transferrin/selenous acid (ITS), 37.5 μ g/ml ascorbic acid, 100 μ g/ml sodium pyruvate, 100 units of sodium penicillin/ml, 100 μ g of streptomycin sulfate/ml) for three days, and then transferred to low-attachment culture plates (Corning Incorporated, Corning, NY) for the remainder of the 20-day experiments. These pellets were assessed using the chondrogenesis protocols detailed below, to provide comparative "primary chondrocyte" benchmarks for the synovial fluid-derived cell activities.

Bone marrow-derived mesenchymal stem cell isolation and culture

Bone marrow aspirates were collected from the tuber coxae of four healthy adult horses, as previously described [20], using a Jamshidi biopsy needle. 10-15 ml of marrow aspirate was

collected into 30 ml syringes containing 1,000 IU of heparin. The aspirates were diluted with 15 ml of PBS and centrifuged at 300xg for 15 minutes. The supernatant was removed and the pellets were re-suspended in PBS and centrifuged as before. The pelleted cells were re-suspended in 15 ml of DMEM supplemented with 10% FBS, 300 µg of L-glutamine/ml, 100 units of sodium penicillin/ml, 100 µg of streptomycin sulfate/ml, and 1mM of sodium pyruvate/ml and seeded in 75-cm² flasks. The flasks were left undisturbed at 37°C in a 5% CO₂ atmosphere with 90% humidity for five days to allow cells to attach to the culture dishes. After the initial five days, the medium was replaced every 2-3 days with 12 ml of fresh medium until the primary bone marrow aspirates approached confluency. BM-MSCs were expanded through two passages as for the synovial fluid-derived cells.

Tri-lineage differentiation and analyses

Osteogenesis

After two monolayer passages, SF-CPs were seeded in 12-well plates, at 1x10⁴ cells per cm², cultured in osteogenic medium (basal medium supplemented with 100 nM dexamethasone, 50 µg ascorbic acid/ml, and 10 mM glycerol-2-phosphate). Osteogenic differentiation was assessed after 14 days. Alizarin Red stain retention was used to detect mineralized matrix formation. Cells were fixed with 10% formalin for 30 minutes and then washed three times with distilled water. 1 ml of fresh 2% Alizarin Red solution (pH 4.1) was added to each well at room temperature for 20 minutes. The stain was removed and the monolayers were washed with distilled water until the rinsed solution was clear. Mineral deposits within the cell layers were stained bright red. Representative images of stained monolayers were acquired using a Leica DMIL microscope and DFC320 digital camera (Leica Microsystems, Leica Application Suite -LAS- version 2.6.R1, Wetzlar, Germany).

Osteogenic monolayers were also stained for alkaline phosphatase (ALP) activity. Cell layers were fixed with 10% formaldehyde for 1 minute and were washed three times with distilled water. An alkaline dye (Procedure No. 86 AP, leukocyte) was added to each well at room temperature for 15 minutes. The cell layers were washed three times with distilled water, then neutral red solution was added for 5 minutes to counterstain the monolayers. Cells clusters exhibiting ALP activity were stained blue. Representative images of stained monolayers were obtained, as for the Alizarin red assays.

Adipogenesis

Following monolayer expansion, synovial fluid-derived cells were seeded in 12-well plates at 5x10³ cells per cm² and cultured in adipogenic medium (basal medium supplemented with 10 µg insulin/ml, 10⁻⁶ M dexamethasone, 100 µM indomethacin, and 0.5 mM isobutylmethylxanthine) for 14 days. At day 14, adipogenesis was assessed by Oil Red O staining to detect intra-cellular accumulation of lipoproteins. Cells were fixed with 10% formalin for 30 minutes, and were washed

three times with PBS. Oil Red O solution (0.36% in 60% isopropanol) was added to each well at room temperature for 50 minutes. Oil Red O solution was removed and the monolayers were washed three times with distilled water. Hematoxylin solution was added for 15 minutes, and the unbound stain was removed. Representative images of stained monolayers were obtained, as above.

Chondrogenesis

After the second passage of monolayer expansion, synovial fluid-derived cells were trypsinized and the cells were counted and re-suspended in chondrogenic medium supplemented with 10 ng TGF-β1/ml (R&D Systems, Minneapolis, MN), then pelleted (2.5x10⁵ cells per pellet) by centrifugation at 390 rfu in 1.5 ml microcentrifuge tubes. After 3-5 days, pellets were gently aspirated from the microcentrifuge tubes and transferred in groups to six-well ultra-low attachment culture plates (Corning) and were maintained in chondrogenic medium for 20 days. In parallel experiments, 100 ng BMP-2/ml (R&D Systems) was substituted for TGF-β1 in the chondrogenic medium to provide a putative stimulus for hypertrophic differentiation. On days 10 and 20, pellets were aspirated from the culture medium, snap-frozen in liquid nitrogen and stored at -20°C for biochemical assessments of chondrogenesis, or at -80°C for RNA isolation. Chondrogenesis was assessed by measuring the expression of chondrocyte-specific genes, collagen type II protein, sulfated glycosaminoglycan (sGAG) secretion, and ALP activity, as described below.

RNA isolation, reverse transcription and quantitative PCR (qPCR)

Snap-frozen pellets were homogenized in the phenol-based dissociation agent, TRIzol[®] (Invitrogen Corporation, Carlsbad, CA). RNA was isolated according to the manufacturer's recommended 'high salt' precipitation protocol, and purified using RNeasy 'RNA Clean Up' protocol (QIAGEN Inc, Valencia, CA) that included DNase treatment to remove any genomic contamination. Complementary DNA was generated from 1 µg of total RNA using a commercial reverse transcription kit (Superscript TM First-Strand Synthesis System[®] for RT-PCR, Invitrogen), following the manufacturer's recommended protocol.

Collagen type II, aggrecan, collagen type X, Sox9, Mef2C and Runx2 mRNAs were measured by fluorescent qPCR, as previously described [21]. The primers used for qPCR analyses are listed in Table 1. Quantitative PCR was performed using 5 µL of diluted cDNA template (1:10 dilution) combined with 20 µL of a mixture composed of 12.5 µL 1 x SYBR Green Mastermix (Bio-Rad Laboratories, Hercules, CA), 1 µL each of the 10 µM forward and reverse primer stocks and 5.5 µL DNase/RNase-free water in a 96-well microplate. Each sample was run in duplicate. The reactions were performed in an iCycler iQ (Bio-Rad Laboratories) using the following conditions: initial denaturation for 3 minutes at 95°C, 40 cycles of denaturation

Table 1. Primers used in the qPCR reactions.

Gene (amplicon size)	Primers	Annealing temperature
EF1- α (328 bp)	S 5' CCCGGACACAGAGACTTCAT A 5'AGCATGTTGTCACCATTC	62.1°C
Collagen type II (223 bp)	S 5' AGCAGGAATTTGGTGTGGAC A 5' TCTGCCAGTTCAGGTCTCT	62.1°C
Collagen type X (244 bp)	S 5' TGCCAACCAGGGTGTAACAG A 5' ACATTACTGGGGTGCCGTTTC	62.1°C
ALP (260 bp)	S 5' CCACGTCTTCACATTTGGTG A 5' AGACTGCGCCTGGTAGTT	54.2°C
Aggrecan (202 bp)	S 5' GACGCCGAGAGCAGGTGT A 5' AAGAAGTTGTCGGGCTGGTT	62.1°C
Mef2c (155 bp)	S 5' CCCAACTTTGAGTGCCAGT A 5' ATGTGAGGTCTCCACCCATC	55.3°C
Sox9 (304 bp)	S 5' GAACGCACATCAAGACGGAG A 5' CTGGTGGTCTGTGTAGTCGT	56.2°C
Runx2 (115 bp)	S 5' CAGACCAGCAGCACTCCATA A 5' GAGCGTCAACACCATTC	56.8°C

at 95°C for 10 seconds, annealing temperature (Table 1) for 30 seconds and polymerase extension at 72°C for 20 seconds. The presence of a single amplicon was monitored by melting curve analyses. Sterile water was used as a 'no template' negative control for each of the PCR reactions to monitor the possibility of contamination.

The qPCR data were normalized to expression of the reference gene, elongation factor-1 alpha (EF1- α) that remains stably expressed during chondrogenesis and *in vitro* culture of chondrocytes [18-22]. The level of expression for each target gene was calculated as $2^{\Delta Ct}$ and the comparative ΔCt method was used to determine relative gene expression levels [23].

Collagen type II protein secretion

A collagen type II ELISA kit (Chondrex Inc, Redmond, WA) was used to measure collagen type II protein within pellets, as previously described [22]. Three pellets from each group were dissolved in 0.05M acetic acid (pH 2.8-3.0 with formic acid) and were digested in pepsin solution at 4°C overnight. The following day, 10% of 10xTBS was added and the pH was adjusted to 8.0 with 1N sodium hydroxide. The intra- and inter-crosslinkages within collagen molecules were digested with pancreatic elastase (0.1mg/ml in 1xTBS, pH7.8-8.0) on a rotating platform at 4°C overnight. The capture antibody solution was added (100 μ l/well) and incubated at 4°C overnight. The following morning, the wells were washed 6 times in wash buffer. The samples (100 μ l) and type II collagen standard were added to the plate wells and incubated at room temperature for 2 hours. The plate was then washed with wash buffer 6 times. The detection antibody solution (100 μ l) was added to each well and incubated at room temperature for 2 hours, following by rinsing with wash buffer 6 times. Streptavidin peroxidase solution (100 μ l) was then added and incubated

at room temperature for 1 hour. After washing, 100 μ l of OPD-Urea H₂O₂ solution was immediately added to each well. After 30 minutes, 50 μ l of stop solution (2N sulfuric acid) was added and the Optical Density (OD) values were measured spectrometrically at 405nm wavelength using a FLUOstar Optima Microplate Reader (BMG LABTECH, Durham, NC).

Sulfated glycosaminoglycan measurement

The dimethyl methylene blue dye-binding (DMMB) assay was used to measure the secretion and accumulation of sGAGs, using three pellets from each sample [20-22]. Pellets were digested in 250 μ l of papain digestion buffer at 65°C overnight. 50 μ l of lysate and 200 μ l of DMMB reagent were added to 96-well plates and Optical Density (OD) values was measured at 530 nm (FLUOstar Optima Microplate Reader, BMG Lab Technologies).

Alkaline phosphatase activity

ALP activity was measured using an assay that monitored conversion of p-nitrophenol phosphate to p-nitrophenol, using three pellets from each experimental group [21]. Pellets were homogenized in 2% Triton-X100 on ice, and then were centrifuged at 2x10³ rpm for 15 minutes at 4°C to remove insoluble debris. ALP reagent (100 μ l; Sigma 104 phosphatase substrate) and 100 μ l of lysate were added to 96-well plate wells. After 10 minutes incubation, the p-nitrophenol in each well was measured spectrometrically at 405nm wavelength (FLUOstar OPTIMA, BMG Lab Technologies). 2% Triton-X 100 alone was used as the negative control and murine growth plate cartilage lysate was used as the positive control.

Pellet DNA content

Pico green fluorescence assay (Invitrogen) was used to measure

pellet DNA content. Duplicate 100 μ l papain digest aliquots were diluted 1:5 in 1xTE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) and were transferred to black 96-well microplates, along with calf thymus DNA standards. Pico Green reagent (0.5 μ l diluted in 100 μ l of 1xTE buffer) and added to each sample and standard. The microplate was placed in the dark to prevent reagent photo-degradation. After a 5 minute incubation, sample fluorescence was measured at 485 nm wavelength (FLUOstar OPTIMA, BMG Lab Technologies).

Histologic examination

Representative pellets from each chondrogenesis experiment were fixed in 4% paraformaldehyde for 24 hours, then positioned in cassettes using HistoGel (Richard-Allan Scientific, Radnor, PA), transferred to PBS solution and stored at 4°C. The pellets were dehydrated in alcohol, embedded in paraffin, sectioned at 8 μ m and stained with toluidine blue. Cross-sectional images of the pellets were acquired using the Nanozoomer 2.0 HT Digital Pathology System machine (Hamamatsu Photonics K.K., Hamamatsu, Japan) at 20x objective.

Statistical analyses

The CFU and proliferation outcomes were derived from 30 synovial fluid isolates, and assessed by one way ANOVA. The quantitative assays detailed above were routinely run using three replicates from each cell isolate. These replicate results were averaged to generate a single data point. Chondrogenesis experiments driven by TGF- β 1 and by BMP-2 were repeated five times, using SF-CPs from independent donors. Quantitative indices of phenotype or biosynthesis were assessed by one-way ANOVA. Dunnett's post hoc test was applied, using 'time zero (T⁰)' data sets as reference controls, to determine whether SF-CP responses to chondrogenic stimulation were statistically significant. Statistical analyses were performed by Prism 5.0. Data were presented as the mean \pm standard error of the mean (SEM). A p value < 0.05 was considered to be statistically significant.

Results

Monolayer expansion

There was considerable variation in the CFU counts in synovial fluid samples, ranging from 10 to 150 colonies per ml of fluid; however, there were less than 50 CFUs/mL in the majority of samples (Figure 1). There were no statistical differences between the CFU counts from synovial fluid aspirated from equine carpal, metatarsophalangeal (fetlock) or tibiotarsal (hock) joints, although, overall, carpal aspirates had lower CFUs than fetlock and hock aspirates. Primary isolates required 4-6 weeks to approach confluence, whereas subsequent passages reliably achieved confluence within 6-10 days. The PD of P1 and P2 cultures ranged between 2.0 and 3.0 (Figure 2A), corresponding to a confluent cell concentration of approximately 5-8 \times 10⁴ cells/cm². The PDTs varied between 0.2 and 0.35 days (Figure 1). PD and PDT values were not significantly

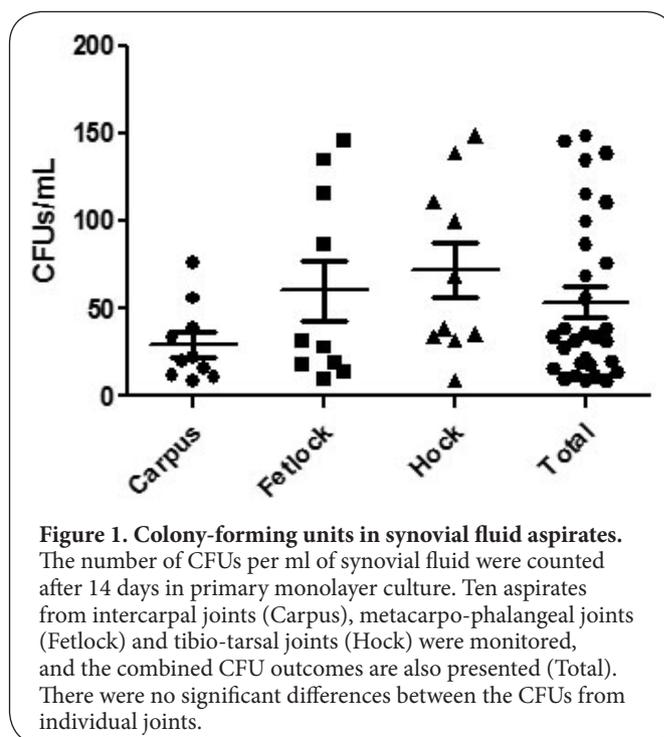


Figure 1. Colony-forming units in synovial fluid aspirates. The number of CFUs per ml of synovial fluid were counted after 14 days in primary monolayer culture. Ten aspirates from intercarpal joints (Carpus), metacarpo-phalangeal joints (Fetlock) and tibio-tarsal joints (Hock) were monitored, and the combined CFU outcomes are also presented (Total). There were no significant differences between the CFUs from individual joints.

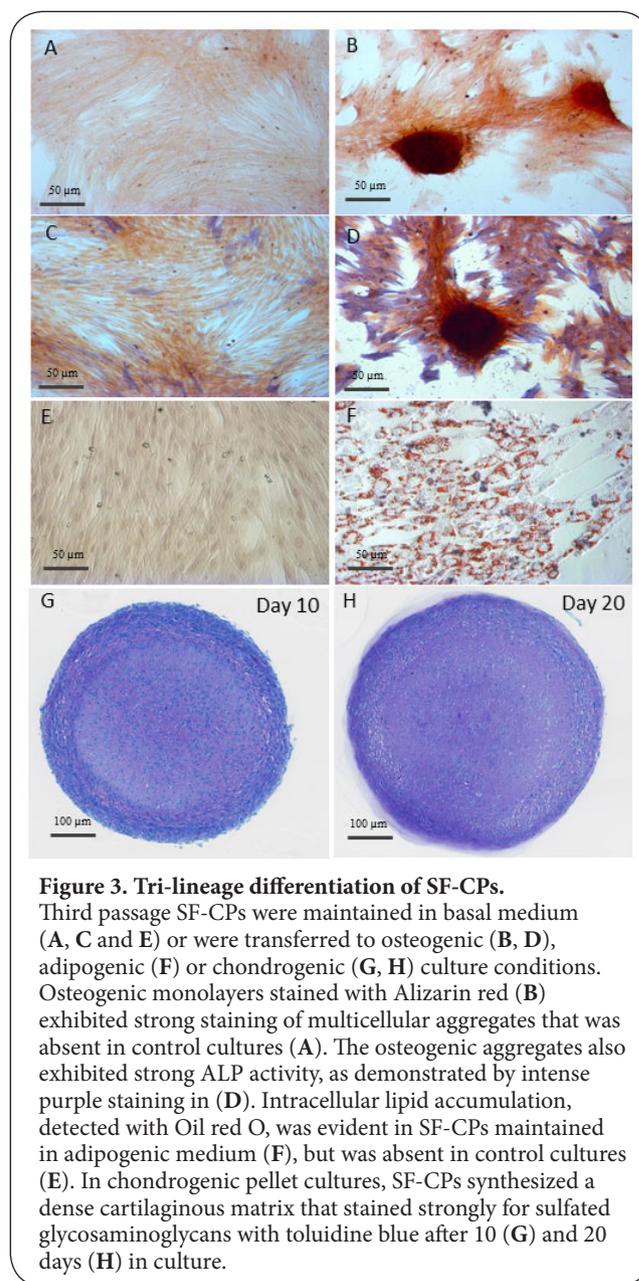
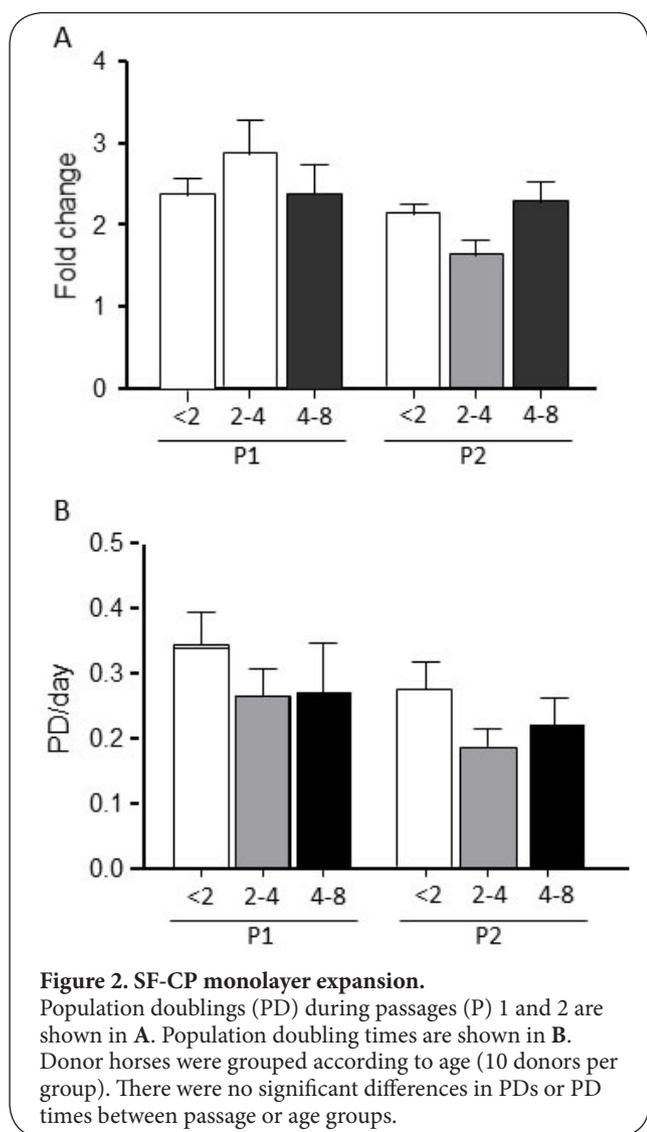
influenced by the age of the donor.

Osteogenic and adipogenic differentiation

Synovial fluid-derived cells cultured in osteogenic medium for 14 days formed characteristic multicellular nodules that stained intensely with Alizarin Red (Figure 2B) and ALP (Figure 3D), compared with cells maintained in basal medium (Figures 3A and 3C, respectively). SF-CPs cultured in adipogenic medium for 14 days developed intracellular lipid droplets, identified by Oil Red O accumulation, whereas cells cultured without adipogenic medium showed no evidence of intracellular lipid formation (Figures 3E and 3F).

Synovial fluid-chondrogenitor chondrogenesis

SF-CPs cultured in chondrogenic medium supplemented with TGF- β 1 synthesized a dense cartilaginous matrix that stained strongly with toluidine blue (Figures 3G and 3H). Serial DNA measurements indicated that pellet cell numbers remained stable during the culture interval, so matrix component outcomes are presented in terms of 'per pellet' values. Collagen type II mRNA levels were significantly up-regulated at both day 10 and day 20 (Figure 4A). At day 20, collagen type II expression matched that of chondrogenic BM-MSCs and was approximately 50% of collagen type II expression in primary articular chondrocytes. Collagen type II protein secretion also increased significantly (Figure 4B), with the majority of protein secretion occurring in the first 10 days of pellet culture. SF-CP collagen secretion was equivalent to chondrogenic BM-MSC pellets, but both progenitor cell groups deposited only around



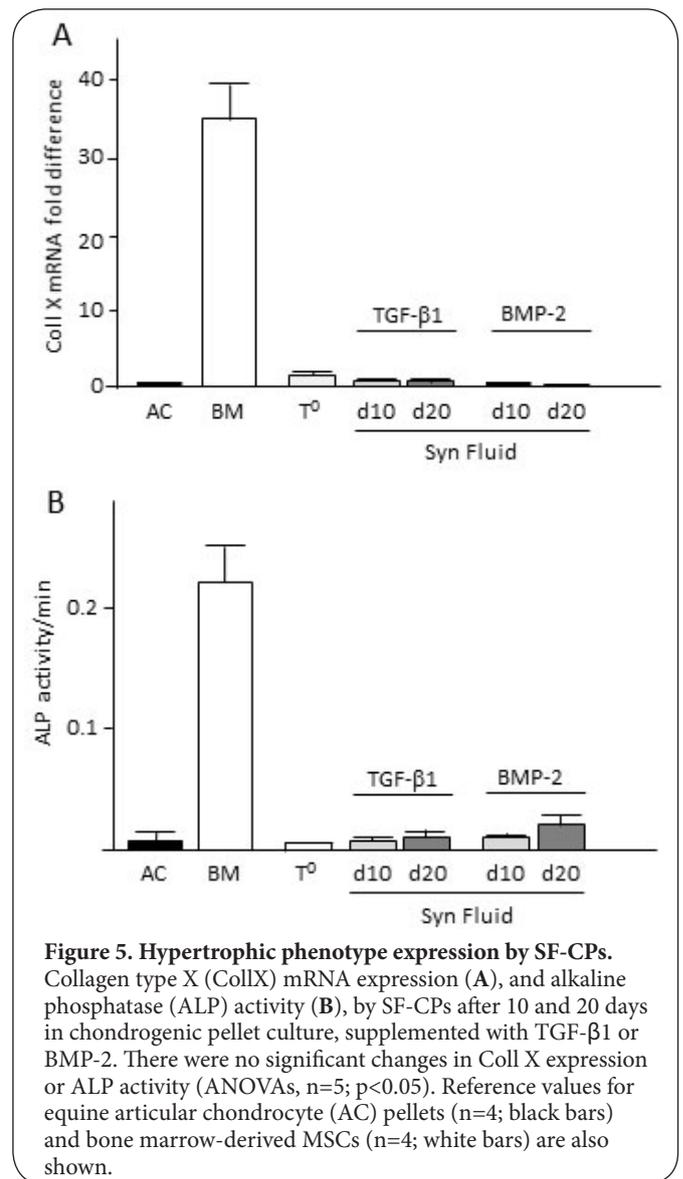
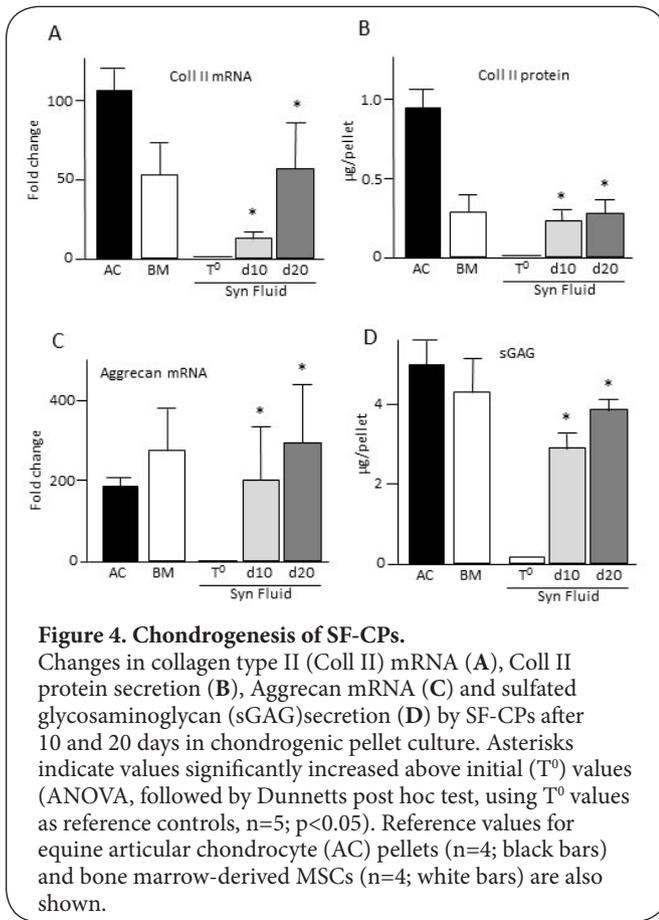
30% of the collagen secreted by articular chondrocytes. Aggrecan mRNA expression was also significantly increased (over 200-fold) in SF-CPs cultured under chondrogenic conditions (Figure 4C), to levels comparable with BM-MSCs and articular chondrocytes. SF-CPs deposited 3-4 µg sGAGs in each pellet; values comparable to sGAG contents of BM-MSC and chondrocyte pellets (Figure 4D).

Assessment of hypertrophic differentiation

The capacity of SF-CPs to express a hypertrophic phenotype was assessed in standard chondrogenic medium, stimulated by TGF-β1, and in medium substituted with BMP-2. BMP-2 stimulated SF-CP collagen type II protein (BMP-2: 0.21 +/- 0.04 µg/pellet vs. TGF-β1: 0.23 +/- 0.08 µg/pellet) and sGAG secretion (BMP-2: 3.59 +/- 1.38 µg/pellet vs. TGF-β1: 3.88 +/- 0.67 µg/pellet) similarly to TGF-β1. Of particular importance to the objective of this study, collagen type X mRNA levels were

not increased during chondrogenesis driven by either factor (Figure 5A), and remained at levels comparable to expression by articular chondrocytes. In comparison, collagen type X expression by BM-MSCs was 30-40 fold above that of SF-CPs. Similarly, there was no significant increase in ALP activity in SF-CP pellets (Figure 5B). In contrast, ALP activity was 10-20 fold higher in BM-MSC pellets. Collectively, these results indicate that chondrogenic SF-CPs do not express phenotypic markers characteristic of the transient, hypertrophic phenotype of chondrocytes engaged in endochondral ossification.

To address this issue further, we measured the expression of Sox9, Runx2 and Mef2C mRNAs; transcription factors required for chondrogenesis and hypertrophic differentiation [24,25].



Sox9 mRNA expression was comparable in SF-CPs and BM-MSCs (Figure 6A); however, expression of the hypertrophy-linked transcription factors Runx2 and Mef2C was over 20 and 40 times higher, respectively, in BM-MSCs than in SF-CPs (Figures 6B and 6C).

Discussion

This study was conducted to determine the isolation and proliferation parameters of SF-CPs in equine synovial fluid, and to determine whether these cells express a hypertrophic or non-hypertrophic phenotype under chondrogenic conditions. Cells capable of establishing colonies and proliferating extensively through multiple passages were present in all the synovial fluid aspirates used in this study. The CFU concentrations in synovial fluids were not significantly influenced by the anatomical source of the aspirates. Surprisingly, donor age had no significant effect on initial CFU concentrations or cell proliferation. The cell populations expanded in monolayer culture expressed osteogenic, adipogenic and chondrogenic phenotypes under standard *in vitro* culture conditions, consistent with findings in similar studies [4,5,10,12], and reflecting the multi-lineage potential requirement for MSCs.

SF-CPs synthesized a cartilaginous matrix quantitatively comparable to that of BM-MSCs and fully differentiated

articular chondrocytes maintained under similar culture conditions. SF-CPs did not express biomarkers characteristic of the hypertrophic phenotype (collagen type X mRNA and ALP induction), clearly supporting the hypothesis of the study. This was the case under standard chondrogenesis conditions driven by TGF-β1, and also when the hypertrophy-inducing growth factor, BMP-2, was used to drive differentiation. That Runx2 and Mef2c expression was not induced in SF-CPs, even though collagen type II and sGAG secretion were comparable to BM-MSC synthesis, strongly suggests that SF-CPs are 'hard-wired' for a non-hypertrophic phenotype, rather being less sensitive to chondrogenic stimuli than other MSC populations. Collectively, these results suggest that SF-CPs are phenotypically appropriate for articular cartilage repair applications, although this will need to be rigorously tested *in vivo*. Similar

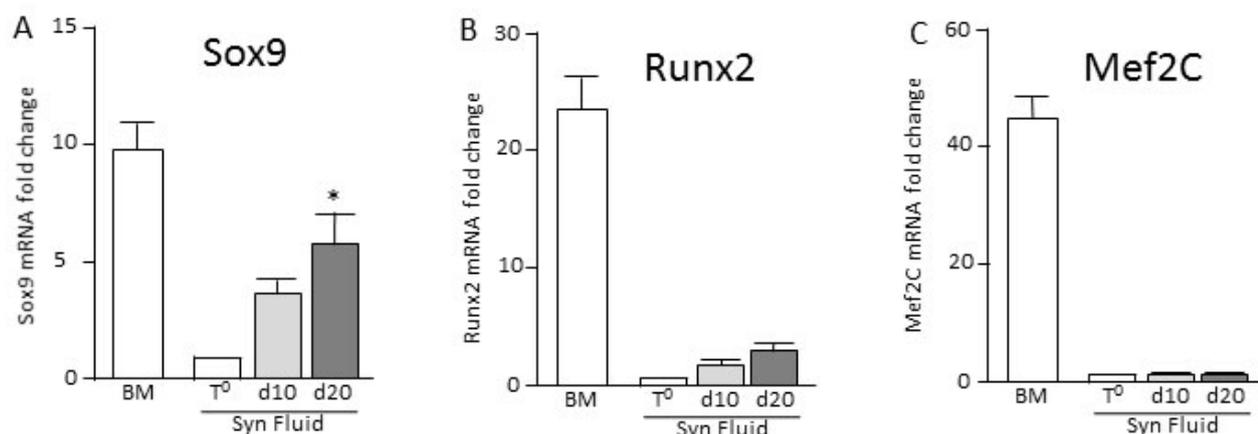


Figure 6. Chondrogenic transcription factor expression by SF-CPs.

Sox 9 mRNA expression (A), Runx2 mRNA expression (B) and Mef2C mRNA expression (C) by SF-CPs after 10 and 20 days in chondrogenic pellet culture, supplemented with TGF- β 1. Sox9 mRNA levels increased over time, reaching significance at day 20, as indicated by the asterisk (ANOVA, followed by Dunnett's post hoc test, using T⁰ values as reference controls, n=5; p<0.05). There were no significant changes in Runx2 or Mef2C mRNA levels. Reference values for bone marrow-derived MSCs (n=4; white bars) are also shown.

chondroprogenitor populations have not performed well in *in vivo* chondrogenesis [26,27] and cartilage defect models [28,29] and equine SF-CP collagen synthesis was substantially less than that of articular chondrocytes in the current study. Effective strategies to improve synthesis and maintenance of a functional cartilage matrix will be required to capitalize on the chondrogenic capacities of SF-CPs and other progenitor populations for clinical applications.

Accepting the phenotypic suitability of SF-CPs for articular cartilage applications, the initial *in vitro* expansion of primary synovial fluid cells from aspirates required several weeks; an unfeasible time frame for most clinical applications. The time required to generate clinically useful cell numbers could be reduced by collecting larger volumes of synovial fluid and/or by aspirating fluid from several joints, given that arthrocentesis is a minimally invasive procedure, compared to bone marrow or adipose tissue harvesting. *In vitro* expansion of SF-CPs can also be substantially accelerated by fibroblast growth factor-2, reducing expansion times by more than 50%, without compromising subsequent chondrogenesis [21], as with bone marrow MSCs [30]. Alternatively, endogenous SF-CPs could be directly recruited to sites of cartilage damage to improve healing, by modulating chemokine concentrations at sites of injury [2,31,32] and utilizing the intrinsic chondrogenic properties of synovial fluid [33].

The non-hypertrophic phenotype of SF-CPs provides some indication of their source. Progenitor cell populations have been identified in several intra- and peri-articular tissues; synovial membrane [34], subchondral bone marrow space [35], intra-articular adipose tissue [36], and articular cartilage surface [37]. Adipose-derived MSCs [14] and synovial membrane-derived progenitors [15] express a hypertrophic

phenotype under chondrogenic conditions. BM-MSCs also express hypertrophic characteristics during chondrogenic differentiation [16] and, regardless, the joints sampled in these experiments were clinically normal and did not have sufficient cartilage loss to provide direct communication with the subchondral marrow compartment. In contrast, progenitors from the articular cartilage surface express a non-hypertrophic chondrogenic phenotype [37], consistent with the outcomes of our experiments. In healthy joints, it is highly plausible that the low numbers of SF-CPs are shed directly from the cartilage surfaces. The source(s) and associated phenotypic profiles of intra-synovial chondroprogenitors in pathological joints are likely to be very different. Consistently SF-CP numbers are increased in osteoarthritic and traumatized joints, and comparative transcriptional and synovial chemokine analyses indicate that the synovial membrane is the most likely source of SF-CPs in these disease states [8,10,38,39]. In severely traumatized and arthritic joints, stem cells from the subchondral marrow cavity could also contribute to the intra-synovial SF-CP population [35]. The phenotypic spectrum of SF-CPs in pathological joints warrants further investigation, to determine whether they can be utilized to improve clinical resolution of intra-articular disease.

Conclusions

Synovial fluid chondroprogenitors are capable of robust non-hypertrophic chondrogenesis, whether stimulated by TGF- β 1 or by BMP-2. These results indicate that synovial fluid cells are phenotypically suitable for articular cartilage repair/regeneration, although strategies to accelerate cell proliferation and stimulate functional cartilage matrix will be required to develop clinically feasible cell-based therapies.

The non-hypertrophic phenotype of SF-CPs suggest that, under normal conditions, they originate from the progenitor population on the articular cartilage surface. In pathological contexts, it is likely that SF-CPs are derived from several intra- and peri-articular sources.

List of abbreviations

ALP: Alkaline phosphatase
BM-MSC: Bone marrow-derived mesenchymal stem cell
BMP-2: Bone morphogenetic protein 2
CFU: Colony-forming unit
DMMB: Dimethyl methylene blue
DNA: Deoxyribonucleic acid
EDTA: Ethylenediaminetetraacetic acid
EF1- α : Elongation factor -1 alpha
ELISA: Enzyme-linked immunosorbent assay
FBS: Fetal bovine serum
ITS: Insulin/transferrin/selenous acid
MSC: Mesenchymal stem cell
mRNA: Messenger ribonucleic acid
OD: Optical density
P: Passage
PBS: Phosphate-buffered saline
PD: Population doubling
PDT: Population doubling time
qPCR: Quantitative polymerase chain reaction
RCF: Relative centrifugal force
SEM: Standard error of the mean
SF-CP: Synovial fluid-chondroprogenitor
sGAG: Sulfated glycosaminoglycan
TGF- β 1: Transforming growth factor beta 1

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	YC	MB	HP	MS
Research concept and design	✓	✓	--	✓
Collection and/or assembly of data	✓	✓	✓	✓
Data analysis and interpretation	✓	--	✓	✓
Writing the article	✓	--	--	✓
Critical revision of the article	✓	--	--	✓
Final approval of article	--	--	--	✓
Statistical analysis	✓	--	--	✓

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References

1. Koga H, Engebretsen L, Brinchmann JE, Muneta T and Sekiya I. **Mesenchymal stem cell-based therapy for cartilage repair: a review.** *Knee Surg Sports Traumatol Arthrosc.* 2009; **17**:1289-97. | [Article](#) | [PubMed](#)
2. Richter W. **Mesenchymal stem cells and cartilage in situ regeneration.** *J Intern Med.* 2009; **266**:390-405. | [Article](#) | [PubMed](#)
3. Danisovic L, Varga I, Polak S, Ulicna M, Hlavackova L, Bohmer D and Vojtassak J. **Comparison of in vitro chondrogenic potential of human mesenchymal stem cells derived from bone marrow and adipose tissue.** *Gen Physiol Biophys.* 2009; **28**:56-62. | [PubMed](#)
4. Vidal MA, Robinson SO, Lopez MJ, Paulsen DB, Borkhsenius O, Johnson JR, Moore RM and Gimble JM. **Comparison of chondrogenic potential in equine mesenchymal stromal cells derived from adipose tissue and bone marrow.** *Vet Surg.* 2008; **37**:713-24. | [Article](#) | [PubMed Abstract](#) | [PubMed FullText](#)
5. Yoshimura H, Muneta T, Nimura A, Yokoyama A, Koga H and Sekiya I. **Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle.** *Cell Tissue Res.* 2007; **327**:449-62. | [Article](#) | [PubMed](#)
6. Bochev I, Elmadjian G, Kyurkchiev D, Tzvetanov L, Altankova I, Tivchev P and Kyurkchiev S. **Mesenchymal stem cells from human bone marrow or adipose tissue differently modulate mitogen-stimulated B-cell immunoglobulin production in vitro.** *Cell Biol Int.* 2008; **32**:384-93. | [Article](#) | [PubMed](#)
7. Lee WJ, Hah YS, Ock SA, Lee JH, Jeon RH, Park JS, Lee SI, Rho NY, Rho GJ and Lee SL. **Cell source-dependent in vivo immunosuppressive properties of mesenchymal stem cells derived from the bone marrow and synovial fluid of minipigs.** *Exp Cell Res.* 2015; **333**:273-88. | [Article](#) | [PubMed](#)
8. Jones EA, English A, Henshaw K, Kinsey SE, Markham AF, Emery P and McGonagle D. **Enumeration and phenotypic characterization of synovial fluid multipotential mesenchymal progenitor cells in inflammatory and degenerative arthritis.** *Arthritis Rheum.* 2004; **50**:817-27. | [Article](#) | [PubMed](#)
9. Jones EA, Crawford A, English A, Henshaw K, Mundy J, Corscadden D, Chapman T, Emery P, Hatton P and McGonagle D. **Synovial fluid mesenchymal stem cells in health and early osteoarthritis: detection and functional evaluation at the single-cell level.** *Arthritis Rheum.* 2008; **58**:1731-40. | [Article](#) | [PubMed](#)
10. Sekiya I, Ojima M, Suzuki S, Yamaga M, Horie M, Koga H, Tsuji K, Miyaguchi K, Ogishima S, Tanaka H and Muneta T. **Human mesenchymal stem cells in synovial fluid increase in the knee with degenerated cartilage and osteoarthritis.** *J Orthop Res.* 2012; **30**:943-9. | [Article](#) | [PubMed](#)
11. Ando W, Kutcher JJ, Krawetz R, Sen A, Nakamura N, Frank CB and Hart DA. **Clonal analysis of synovial fluid stem cells to characterize and identify stable mesenchymal stromal cell/mesenchymal progenitor cell phenotypes in a porcine model: a cell source with enhanced commitment to the chondrogenic lineage.** *Cytotherapy.* 2014; **16**:776-88. | [Article](#) | [PubMed](#)
12. Murata D, Miyakoshi D, Hatazoe T, Miura N, Tokunaga S, Fujiki M, Nakayama K and Misumi K. **Multipotency of equine mesenchymal stem cells derived from synovial fluid.** *Vet J.* 2014; **202**:53-61. | [Article](#) | [PubMed](#)
13. Alegre-Aguaron E, Desportes P, Garcia-Alvarez F, Castiella T, Larrad L and Martinez-Lorenzo MJ. **Differences in surface marker expression and chondrogenic potential among various tissue-derived mesenchymal cells from elderly patients with osteoarthritis.** *Cells Tissues Organs.* 2012; **196**:231-40. | [Article](#) | [PubMed](#)
14. Estes BT, Wu AW and Guilak F. **Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6.** *Arthritis Rheum.* 2006; **54**:1222-32. | [Article](#) | [PubMed](#)
15. Shirasawa S, Sekiya I, Sakaguchi Y, Yagishita K, Ichinose S and Muneta T. **In vitro chondrogenesis of human synovium-derived mesenchymal stem cells: optimal condition and comparison with bone marrow-derived cells.** *J Cell Biochem.* 2006; **97**:84-97. | [Article](#) | [PubMed](#)
16. Sheehy EJ, Buckley CT and Kelly DJ. **Oxygen tension regulates the osteogenic, chondrogenic and endochondral phenotype of bone marrow derived mesenchymal stem cells.** *Biochem Biophys Res Commun.* 2012; **417**:305-10. | [Article](#) | [PubMed](#)

17. Kugimiya F, Kawaguchi H, Kamekura S, Chikuda H, Ohba S, Yano F, Ogata N, Katagiri T, Harada Y, Azuma Y, Nakamura K and Chung UI. **Involvement of endogenous bone morphogenetic protein (BMP) 2 and BMP6 in bone formation.** *J Biol Chem.* 2005; **280**:35704-12. | [Article](#) | [PubMed](#)
18. Stewart MC, Kadlcek RM, Robbins PD, MacLeod JN and Ballock RT. **Expression and activity of the CDK inhibitor p57Kip2 in chondrocytes undergoing hypertrophic differentiation.** *J Bone Miner Res.* 2004; **19**:123-32. | [Article](#) | [PubMed](#)
19. Stewart MC, Saunders KM, Burton-Wurster N and Macleod JN. **Phenotypic stability of articular chondrocytes in vitro: the effects of culture models, bone morphogenetic protein 2, and serum supplementation.** *J Bone Miner Res.* 2000; **15**:166-74. | [Article](#) | [PubMed](#)
20. Stewart AA, Byron CR, Pondenis HC and Stewart MC. **Effect of dexamethasone supplementation on chondrogenesis of equine mesenchymal stem cells.** *Am J Vet Res.* 2008; **69**:1013-21. | [Article](#) | [PubMed](#)
21. Bianchessi M, Chen Y, Durgam S, Pondenis H and Stewart M. **Effect of Fibroblast Growth Factor 2 on Equine Synovial Fluid Chondroprogenitor Expansion and Chondrogenesis.** *Stem Cells Int.* 2016; **2016**:9364974. | [Article](#) | [PubMed Abstract](#) | [PubMed FullText](#)
22. Oshin AO, Caporali E, Byron CR, Stewart AA and Stewart MC. **Phenotypic maintenance of articular chondrocytes in vitro requires BMP activity.** *Vet Comp Orthop Traumatol.* 2007; **20**:185-91. | [Article](#) | [PubMed](#)
23. Livak KJ and Schmittgen TD. **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.** *Methods.* 2001; **25**:402-8. | [Article](#) | [PubMed](#)
24. Kim IS, Otto F, Zabel B and Mundlos S. **Regulation of chondrocyte differentiation by Cbfa1.** *Mech Dev.* 1999; **80**:159-70. | [Article](#) | [PubMed](#)
25. Arnold MA, Kim Y, Czubyrt MP, Phan D, McAnally J, Qi X, Shelton JM, Richardson JA, Bassel-Duby R and Olson EN. **MEF2C transcription factor controls chondrocyte hypertrophy and bone development.** *Dev Cell.* 2007; **12**:377-89. | [Article](#) | [PubMed](#)
26. De Bari C, Dell'Accio F and Luyten FP. **Failure of in vitro-differentiated mesenchymal stem cells from the synovial membrane to form ectopic stable cartilage in vivo.** *Arthritis Rheum.* 2004; **50**:142-50. | [Article](#) | [PubMed](#)
27. Marcus M, De Bari C, Dell'Accio F, Charles W and Archer CW. **Articular chondroprogenitor cells maintain chondrogenic potential but fail to form a functional matrix when implanted into muscles of SCID mice.** *Cartilage.* 2014; **5**:231-40. | [Article](#)
28. Wilke MM, Nydam DV and Nixon AJ. **Enhanced early chondrogenesis in articular defects following arthroscopic mesenchymal stem cell implantation in an equine model.** *J Orthop Res.* 2007; **25**:913-25. | [Article](#) | [PubMed](#)
29. Frisbie DD, McCarthy HE, Archer CW, Barrett MF and McIlwraith CW. **Evaluation of articular cartilage progenitor cells for the repair of articular defects in an equine model.** *J Bone Joint Surg Am.* 2015; **97**:484-93. | [Article](#) | [PubMed](#)
30. Stewart A, Byron C, Pondenis H and Stewart M. **The effect of fibroblastic growth factor-2 on equine monolayer stem cell expansion and subsequent chondrogenesis.** *Am J Vet Res.* 2007; **68**:941-945.
31. Zhang S, Muneta T, Morito T, Mochizuki T and Sekiya I. **Autologous synovial fluid enhances migration of mesenchymal stem cells from synovium of osteoarthritis patients in tissue culture system.** *J Orthop Res.* 2008; **26**:1413-8. | [Article](#) | [PubMed](#)
32. Gorter R, Kruegel J and Miosge N. **New insights into cartilage repair - the role of migratory progenitor cells in osteoarthritis.** *Matrix Biol.* 2012; **31**:206-13. | [Article](#) | [PubMed](#)
33. Hegewald AA, Ringe J, Bartel J, Kruger I, Notter M, Barnewitz D, Kaps C and Sittinger M. **Hyaluronic acid and autologous synovial fluid induce chondrogenic differentiation of equine mesenchymal stem cells: a preliminary study.** *Tissue Cell.* 2004; **36**:431-8. | [Article](#) | [PubMed](#)
34. De Bari C, Dell'Accio F, Tylzanowski P and Luyten FP. **Multipotent mesenchymal stem cells from adult human synovial membrane.** *Arthritis Rheum.* 2001; **44**:1928-42. | [Article](#) | [PubMed](#)
35. Koelling S, Kruegel J, Irmer M, Path JR, Sadowski B, Miro X and Miosge N. **Migratory chondrogenic progenitor cells from repair tissue during the later stages of human osteoarthritis.** *Cell Stem Cell.* 2009; **4**:324-35. | [Article](#) | [PubMed](#)
36. Wickham MQ, Erickson GR, Gimble JM, Vail TP and Guilak F. **Multipotent stromal cells derived from the infrapatellar fat pad of the knee.** *Clin Orthop Rel Res.* 2008; **412**:196-212.
37. McCarthy HE, Bara JJ, Brakspear K, Singhrao SK and Archer CW. **The comparison of equine articular cartilage progenitor cells and bone marrow-derived stromal cells as potential cell sources for cartilage repair in the horse.** *Vet J.* 2012; **192**:345-51. | [Article](#) | [PubMed](#)
38. Lee DH, Sonn CH, Han SB, Oh Y, Lee KM and Lee SH. **Synovial fluid CD34(-) CD44(+) CD90(+) mesenchymal stem cell levels are associated with the severity of primary knee osteoarthritis.** *Osteoarthritis Cartilage.* 2012; **20**:106-9. | [Article](#) | [PubMed](#)
39. Morito T, Muneta T, Hara K, Ju YJ, Mochizuki T, Makino H, Umezawa A and Sekiya I. **Synovial fluid-derived mesenchymal stem cells increase after intra-articular ligament injury in humans.** *Rheumatology (Oxford).* 2008; **47**:1137-43. | [Article](#) | [PubMed](#)

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