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Citation	Journal of veterinary medical science, 80(11), 1681-1687 https://doi.org/10.1292/jvms.18-0202
Issue Date	2018-11
Doc URL	https://hdl.handle.net/2115/73200
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	Journal of veterinary medical science 80 (11) 1681-1687.pdf



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The Journal of Veterinary Medical Science

FULL PAPER

Surgery

Independent chondrogenic potential of canine bone marrow-derived mesenchymal stem cells in monolayer expansion cultures decreases in a passage-dependent pattern

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J. Vet. Med. Sci.
80(11): 1681–1687, 2018
doi: 10.1292/jvms.18-0202

Received: 10 April 2018
Accepted: 27 August 2018
Published online in J-STAGE: 11 September 2018

ABSTRACT. Although chondroinductive growth factors are considered necessary for chondrogenesis of bone marrow-derived mesenchymal stem cells (BMSC), independent and spontaneous chondrogenesis has been previously demonstrated in adult horses, bovine calves and adult human BMSC. Surprisingly, adult canine BMSC under similar culture conditions previously failed to demonstrate chondrogenesis. The present study evaluated independent chondrogenic potential of BMSC sourced from three young dogs in the absence of known chondroinductive factors. BMSC were culture expanded in 10% DMEM up to third passage (P3). At each passage, the phenotype of BMSC was evaluated by RT-PCR gel electrophoresis and qPCR. BMSC exhibited a chondrogenic phenotype in the absence of dexamethasone and TGF-β1 as verified by the expression of *Sox-9*, *type II collagen* and *aggrecan*. *Sox-9* was significantly downregulated ($P<0.05$) from P1–P3 compared to P0 while *type II* and *X collagen*, and *aggrecan* were significantly downregulated at P3 compared to P0. There was a significant ($P<0.01$) negative correlation between passaging and *Sox-9*, *type II collagen* and *aggrecan* gene expression. These results indicate that independent chondrogenic potential and phenotype retention of BMSC decreases in a passage-dependent pattern. Therefore, caution should be exercised for future experiments evaluating the chondrogenic potential of BMSC after extensive expansion cultures in 10% DMEM.

KEY WORDS: bone marrow-derived mesenchymal stem cell, chondrogenesis, dog, monolayer culture, phenotype

Currently, the clinical use of mesenchymal stem cells (MSC) in veterinary medicine is in its early stages for the treatment of tendon, ligament, or osteoarthritic (OA) cartilage lesions in horses or dogs [8]. Canine bone marrow-derived mesenchymal stem cells (cBMSC) offer a significant promise as a multipotent source for cell-based therapies and could form the basis for the differentiation and cultivation of cartilage tissue grafts to repair and regenerate OA joint [5]. Bone marrow-derived mesenchymal stem cells (BMSC) have potential for extensive monolayer expansion *in vitro* and undergo chondrogenesis when cultured with growth medium supplemented with different growth factors such as transforming growth factor (TGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and bone morphogenesis proteins (BMP) [2, 5, 16, 18, 19, 23, 25, 27, 32]. Although these growth factors are considered necessary, spontaneous independent chondrogenic differentiation of BMSC derived from adult horses [7], bovine calves [2, 3] and human [10] has been demonstrated in the absence of these factors. BMSC derived from adult horses were previously shown to exhibit a chondrogenic phenotype in monolayer culture system [7] whereas those derived from bovine calves [2, 3] and human [10] used the micromass/pellet culture system which is well-known to promote differentiation of MSC towards the chondrocytic lineage by promoting strong cell to cell interaction because of contact mediated signalling, formation of junctional complexes and increased potential for exchange of homocrine factors. Surprisingly, cBMSC from adult dogs failed to demonstrate chondrogenic phenotype in chondrogenic induction medium without dexamethasone and TGF or when cells were cultured in standard medium containing 10% fetal calf serum (FCS) in micromass/pellet culture or high density culture [5]. However, it is not yet clear whether the failure of adult cBMSC to undergo independent chondrogenic differentiation despite using micromass culture is an indication of reduced chondrogenic potential related to extensive monolayer expansion cultures or is

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specific to adult canine BMSC.

Therefore, the present study was undertaken to clarify the findings of the previous study and evaluate the independent chondrogenic potential of BMSC sourced from young dogs in monolayer expansion cultures in the absence of known chondroinductive factors.

MATERIALS AND METHODS

Collection site and dog preparation

The study was performed using cBMSC aseptically collected by the proximal femur approach from femoral bone marrow of three experimental Beagle dogs (mean age: 11.5 months; range: 11–12 months). The use of experimental dogs was in accordance with Hokkaido University Institutional Animal Care and Use Committee guidelines (approval number: 12-0059). Briefly, dogs were put under general anesthesia (GA) using propofol (Intervet, Tokyo, Japan) at 6 mg/kg intravenously for induction and maintained on isoflurane (Intervet) and oxygen. For pain management, each dog was administered meloxicam (Boehringer-Ingelheim Animal Health, Tokyo, Japan) at 0.2 mg/kg subcutaneously. The limb from where the bone marrow aspirate was collected was aseptically prepared by clipping the hair around the proposed site of collection, scrubbed with povidone iodine and then 70% ethanol applied to further disinfect the site.

cBMSC isolation and culture

A modification of the protocol previously described for isolation and culture of MSC was used [28]. Briefly, mononuclear cells (MC) were collected by gradient centrifugation using a modification of Lymhoprep™ protocol (1.077 ± 0.001 g/m*l*) (Axis Shield POC, Oslo, Norway). After centrifugation, MC were collected from the sample/medium interface and plated in polystyrene culture plates containing Dulbecco's Modified Eagle Medium (DMEM) (GIBCO BRL, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Nichirei Biosciences Inc., Tokyo, Japan, Batch #: 83300104), 100 IU/m*l* of penicillin and 0.1 mg/m*l* of streptomycin and incubated at 37°C in 5% CO_2 in a humidified chamber without disturbing the plates for 4 days. Thereafter, non-adherent cells were removed, adherent cells gently washed with 1 x PBS and fresh medium added. Medium was changed every 48 hr until the cells reached about 80–90% confluence. At confluence, primary (P0) monolayer colony forming unit cells were gently washed twice with 1 x PBS and detached using pre-warmed TrypLE™ Select CTS™ (GIBCO). Cells were passaged up to third passage (P3) in 100 mm culture plates at seeding density of 1×10^4 cells/cm^2 per subculture. All subcultures were allowed to reach confluence of 80–90% before passaging. At each passage (P0–P3), 1×10^6 cells were collected and total RNA extracted using TRIZol® Kit (Invitrogen, Life Technologies, Carlsbad, CA, U.S.A.), according to the manufacturer's instructions to evaluate expression of chondrogenic phenotype genes. Cell viability and total cell density was determined using 0.5% Trypan blue stain and manual haemocytometer cell counting method. The cells morphology and proliferation characterization was determined using a light microscope.

Chondrogenic phenotype analysis

Total RNA was quantified by spectrophotometry at 260 nm. Total of 500 *n*g RNA was reverse transcribed into cDNA using M-MLV RT kit (Invitrogen) and amplified by PCR using TaKaRa Ex taq (TaKaRa Bio, Tokyo, Japan) according to manufacturer's recommended protocol. This technique was employed to detect the expression of chondrogenic specific-genes, *Sox-9*, *type II collagen* and *aggrecan*, dedifferentiation gene, *type I collage*n, chondrocyte hypertrophy gene, *type X collagen* and *hypoxia inducible factor-2alpha* (*HIF-2α*) in monolayer cultured cBMSC. The PCR conditions were an initial denaturation of 94°C for 1 min followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec and then a finishing step of 72°C for 1 min. All PCR reactions were validated by the presence of a single band on gel electrophoresis. In addition, quantitative real-time PCR (qPCR) was performed with KAPA SYBR® FAST qPCR kit (KAPA biosystems, Woburn, MA, U.S.A.) by the two step method to determine the fold-changes in the target genes between the passages. cDNA template with the amount of 2 *μl* was added to each 16 *μl* of kapa mix and 2 *μl* specific primers premixture. qPCR conditions were an initial denaturation of 95°C for 20 sec followed by 40 cycles of 95°C for 3 sec and 60°C for 20 sec then a pre-melt condition of 60°C for 90 sec followed by a final melt step. All qPCR reactions were validated by the presence of a single peak in the melt curve analysis. The standard curve method was used to determine the fold-changes in mRNA expression between the passages with P0 as the calibrator. All target genes expression were normalized against the reference gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). The sequences, product size and accession codes for each of primers used in the experiments are indicated in Table 1. Primer sequences for all genes were designed using data published on the National Center for Biotechnology Information (NCBI) website using NCBI's standard and pairwise BLAST programs.

Data analysis

Quantitative data was analyzed using SPSS Version 16.0 (SPSS Inc., Chicago, IL, U.S.A.). Analysis of variance (ANOVA) was used to compare relative gene expression between passages. Where significant difference was observed, *post hoc* multiple comparisons were performed with Bonferroni. Bivariate correlation analysis was performed to model the relationship between passaging and relative gene expression. Significant difference was defined as $P<0.05$.

Table 1. Sequence of primers used for RT-PCR to evaluate chondrogenic phenotype gene expression by cBMSC in monolayer expansion cultures

Name of gene	Domain	Primer[a)]	Fragment[b)] (bp)	Accession
GAPDH	664–683	5'-CTGAACGGGAAGCTCACTGG-3'	129	NM_001003142.1
	773–792	5'-CGATGCCTGCTTCACTACCT-3'		
Sox-9	565–583	5'-GCCGAGGAGGCCACCGAACA-3'	179	NM_001002978.1
	724–743	5'-CCCGGCTGCACGTCGGTTTT-3'		
Type I Collagen	150–169	5'-GTGGATACGCGGACTTTGTT-3'	164	NM_001003187.1
	294–313	5'-GGGATACCATCGTCACCATC-3'		
Type II Collagen	4127–4146	5'-CACTGCCAACGTCCAGATGA-3'	215	NM_001006951.1
	4322–4341	5'-GTTTCGTGCAGCCATCCTTC-3'		
Type X Collagen	1782–1801	5'-TTTCTCCTACCACGTGCATG-3'	117	XM_003639401.1
	1879–1898	5'-GAAGCCTGATCCAGGTAGCC-3'		
Aggrecan	6569–6588	5'-ACTTCCGCTGGTCAGATGGA-3'	111	NM_001113455.1
	6660–6679	5'-TCTCGTGCCAGATCATCACC-3'		
HIF-2α	1248–1267	5'-TGCAAAGCACGGGGGCTACG-3'	72	XM_531807.3
	1300–1319	5'-GGCTGCAGGTTGCGAGGGTT-3'		

a) Primers for forward & reverse sense are presented in a 5' to 3' orientation, b) The expected fragment size, *GAPDH*= glyceraldehyde-3-phosphate dehydrogenase, *HIF-2α*=Hypoxia inducible factor-2alpha.

RESULTS

Morphology and proliferation characterization of cBMSC

On average, P0 cultures reached 80–90% confluence within 11 ± 2 day (mean ± SD). Subsequent passages (P1–P3) from all three (3) dogs were able to reach confluence within 8 ± 2 days of passaging, indicating a high proliferation rate. Cell viability as determined using Trypan blue exclusion stain indicated viability of >95% at all passages. The morphological events of the cBMSC as observed using a light microscope showed the appearance of adherent elongated large, spindyloid fibroblastic-like cells on the surface of the culture plates with many non-adherent cells on day 4 of P0 culture (Fig. 1B). Following removal of non-adherent cells and medium change, there was a rapid morphological change of cells to small rounder to bipolar spindle-shaped cells that established colony forming unit-fibroblasts (CFU-F) and proliferated in circular patches (Fig. 1C). Subsequent passages (P1–P3) were characterized by bipolar to polygonal spindle-shaped cells (Fig. 1D–F) with reduced CFU-F ability especially from P2–P3 when compared to P0.

Chondrogenic phenotype evaluation

cBMSC demonstrated a chondrogenic phenotype at all passages (P0−P3) as verified by the expression of cartilage-specific gene markers, *Sox-9, type II collagen* and *aggrecan* (Fig. 2). Notably, there was a passage-dependent decrease in cells retention of a chondrogenic phenotype as evidenced by decreased *Sox-9*, *type II collagen* and *aggrecan* mRNA bands expression especially for *Sox-9* which was barely detectable in some passages albeit cells continuously expressing distinct type II collagen and aggrecan genes (Fig. 2). The observed passage-dependent decrease in chondrogenic potential of cBMSC appeared to be related to the reduced CFU-F formation from P1−P3. Although cBMSC demonstrated a chondrogenic phenotype, they concomitantly expressed *type I* and *X collagen* genes, with the former being predominantly expressed at all passages compared to other genes while the latter demonstrated a passage-dependent downregulation (Fig. 2). *HIF-2α* gene was expressed at all passages and appeared to be upregulated with subsequent passaging (Fig. 2).

Quantitative real-time PCR (qPCR) was performed to verify and quantify the findings of RT-PCR gel electrophoresis. *Sox-9*, *type II collagen* and *aggrecan* were all downregulated in a passage-dependent pattern (Fig. 3). However, compared to P0, only *Sox-9* was significantly downregulated ($P<0.05$) at all passages (P1−P3) while *type II collagen* was only significantly downregulated at P3 compared to P0 and aggrecan was significantly downregulated at P2−P3 compared to P0. *Type I collagen* expression was markedly upregulated from P1−P3 compared to P0 but there was no significant difference ($P>0.05$) between the passages (Fig. 3). Interestingly, *type X collagen* expression followed a similar pattern to cartilage-specific genes and was significantly downregulated ($P<0.05$) at P3 compared to P0. There was no significant difference in *HIF-2α* gene expression between the passages although it showed a passage-dependent increase from P1−P3 (Fig. 3).

Bivariate correlation analysis demonstrated a significant negative correlation between passaging and *Sox-9* (Pearson Correlation= −0.802, $P=0.002$), passaging and *type II collagen* (Pearson Correlation= −0.789, $P=0.002$), passaging and *type X collagen* (Pearson Correlation= −0.599, $P=0.04$) and, passaging and *aggrecan* (Pearson Correlation= −0.754, $P=0.005$). *Type I collagen* (Pearson Correlation=0.566, $P=0.055$) and *HIF-2α* (Pearson Correlation=0.157, $P=0.626$) showed a non-significant positive correlation with passaging (Table 2).

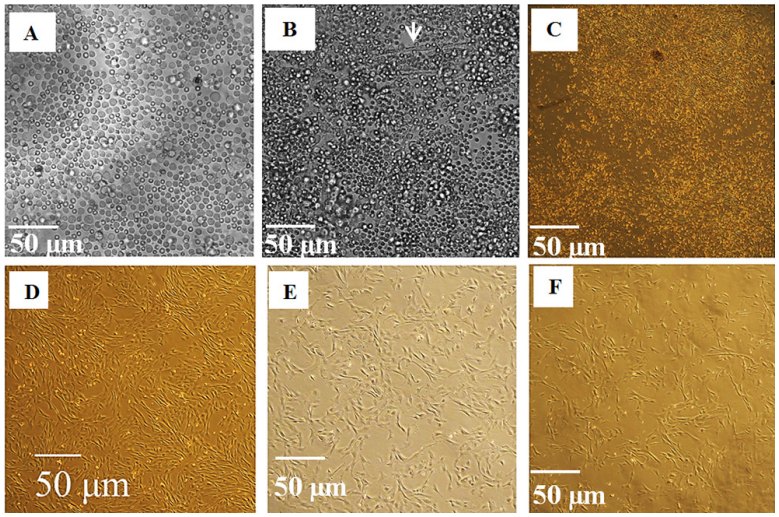


Fig. 1. Photomicrographs of monolayer cultured canine bone marrow-derived mesenchymal stem cells (cBMSC) maintained in 10% FBS DMEM. (A) Day 1, primary culture (P0) showing non-adherent mononuclear cells immediately following seeding, (B) Day 4, P0 culture showing appearance of adherent elongated spindle-shaped fibroblastic-like cells just before removing non-adherent cells, (C) Day 10, P0 culture showing circular patches of small bipolar colony forming unit-fibroblasts (CFU-F), (D) Day 4, first passage (P1) culture showing bipolar to polygonal shaped cells, (E) Day 5, second passage (P2) and (f) Day 6, third passage (P3) showing larger polygonal shaped cells with reduced CFU-F formation. Scale Bars: 50 μm.

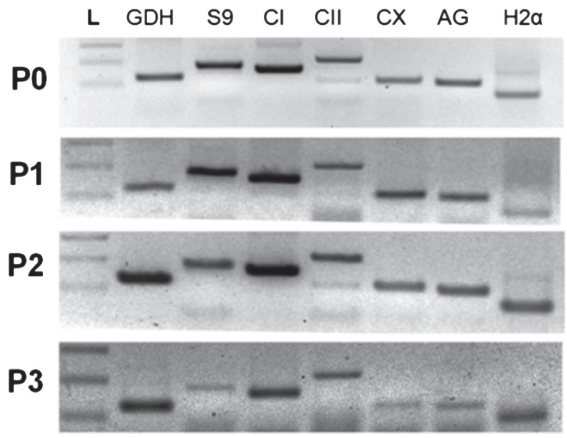


Fig. 2. Expression of chondrogenic specific-genes, *Sox-9* (S9), *type II collagen* (CII) and *aggrecan* (AG), dedifferentiation gene, *type I collage*n (CI), chondrocyte hypertrophy gene, *type X collagen* (CX) and *hypoxia inducible factor-2alpha* (*HIF-2α*) as determined by RT-PCR gel electrophoresis in monolayer cultured canine bone marrow-derived mesenchymal stem cells (cBMSC). RNA was collected from monolayer expansion culture of cBMSC at every passage from P0 to P3. Results represent one (1) experiment of three (3) independent experiments for each passage evaluated.

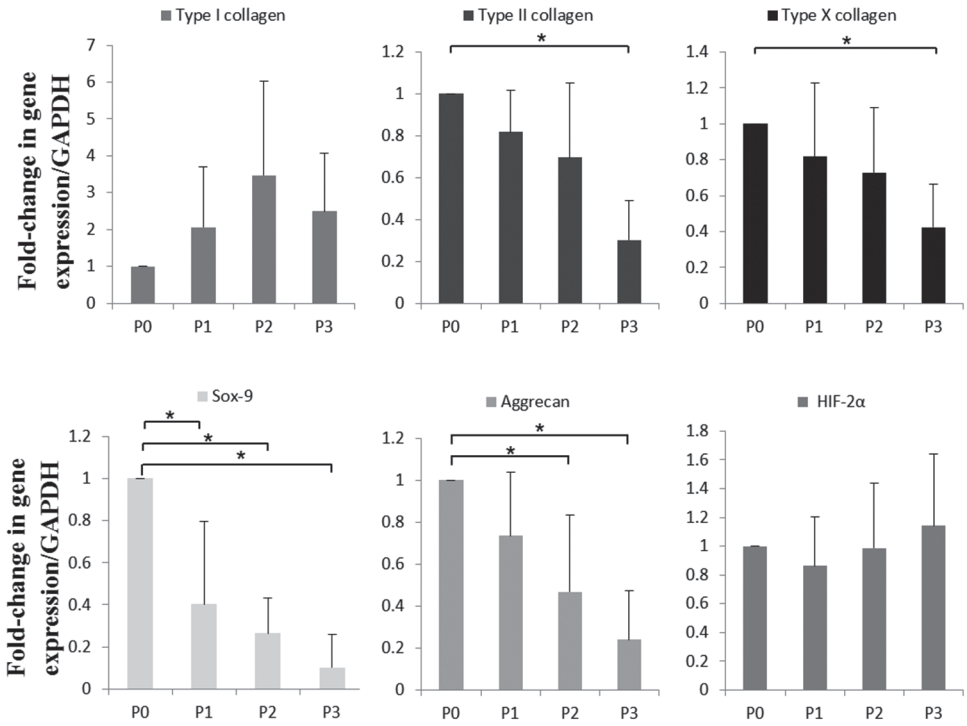


Fig. 3. Bar graphs showing the fold-changes in gene expression between passages of monolayer expansion cultures of canine bone marrow-derived mesenchymal stem cells (cBMSC). Gene expression for *Sox-9*, *type I* (*COLI*), *II* (*COLII*) and *X* (*COLX*) *collagen*, *aggrecan* (*AGG*), and *hypoxia inducible factor-2alpha* (*HIF-2α*) were compared between passages with the primary culture (P0) as the calibrator to which fold-changes between the passages were determined. Gene expressions were normalized with the reference gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). All fold-changes in gene expression are expressed as mean ± 95% confidence interval (CI) of three (3) independent experiments. Significant difference was defined as *P<0.05.

Table 2. Bivariate correlation analysis between passaging and gene expression of cBMSC in monolayer expansion cultures

		Passage	*Sox-9*	*Type I collagen*	*Type II collagen*	*Type X collagen*	*Aggrecan*	*HIF-2α*
Passage	Pearson Correlation	1	−0.802[b)]	0.566	−0.789[b)]	−0.599[a)]	−0.754[b)]	0.157
(n=12)	Sig. (2-tailed)		0.002	0.055	0.002	0.04	0.005	0.626
Sox-9	Pearson Correlation	−0.802[b)]	1	−0.769[b)]	0.659[a)]	0.32	0.399	−0.341
(n=12)	Sig. (2-tailed)	0.002		0.003	0.02	0.31	0.199	0.278
Type I collagen	Pearson Correlation	0.566	−0.769[b)]	1	−0.213	−0.324	−0.401	−0.179
(n=12)	Sig. (2-tailed)	0.055	0.003		0.507	0.304	0.197	0.577
Type II collagen	Pearson Correlation	−0.789[b)]	0.659[a)]	−0.213	1	0.192	0.433	−0.524
(n=12)	Sig. (2-tailed)	0.002	0.02	0.507		0.55	0.16	0.08
Type X collagen	Pearson Correlation	−0.599[a)]	0.32	−0.324	0.192	1	0.684[a)]	0.166
(n=12)	Sig. (2-tailed)	0.04	0.31	0.304	0.55		0.014	0.607
Aggrecan	Pearson Correlation	−0.754[b)]	0.399	−0.401	0.433	0.684[a)]	1	0.361
(n=12)	Sig. (2-tailed)	0.005	0.199	0.197	0.16	0.014		0.249
HIF-2α	Pearson Correlation	0.157	−0.341	−0.179	-0.524	0.166	0.361	1
(n=12)	Sig. (2-tailed)	0.626	0.278	0.577	0.08	0.607	0.249	

a) Correlation is significant at the 0.05 level (2-tailed). b) Correlation is significant at the 0.01 level (2-tailed).

DISCUSSION

The results of the study confirms that cBMSC undergo independent chondrogenesis in monolayer culture in the absence of dexamethasone and TGF-β1 previously shown to be necessary for chondrogenic differentiation of adult canine BMSC [5]. We confirmed that the cells had MSC character by their ability to adhere to plastic culture plate under standard culture conditions and differentiate into chondrocytic lineage *in vitro* as per previously defined criterion [6]. In addition, the cells demonstrated multilineage differentiation ability into chondrogenic, adipogenic and osteogenic lineage (S1 Fig.) as verified by Alcian blue, Oil red O, and Alizarin red S positive stain, respectively based on previously described protocol [5]. Morphologically, from P0−P3, cBMSC progressed from being elongated spindle-shaped, to small bipolar cells then to large polygonal fibroblastic-like cells, a characteristic similarly described elsewhere [2, 5, 7, 13]. In the present study, BMSC demonstrated independent chondrogenic differentiation within 8 (± 2) days of culture as verified by the expression of cartilage-specific gene markers, *Sox-9*, *type II collagen* and *aggrecan*. The independent chondrogenic potential and retention of chondrogenic phenotype of cBMSC significantly decreased in a passage-dependent pattern marked by decreased expression of *Sox-9*, *type II collagen* and *aggrecan* with highly expressed *type I collagen*. The observed concomitant expression of *type I* and *X collagen* is an indication of loss of spatiotemporal chondrogenic differentiation signals in BMSC leading to formation of a fibrocartilage-like phenotype.

Chondrogenesis *in vivo* is characterized by condensation of mesenchyme cells that enlarges through appositional growth by recruiting the cells from the outer layer, which differentiate into chondroblasts and begins secreting the molecules that form the extracellular matrix [4, 16, 29]. However, in the present study, condensing of cells using pellet/micromass culture was not used instead we evaluated the potential of cBMSC to undergo independent chondrogenesis in monolayer culture system. Independent chondrogenic differentiation of adult horses BMSC has been previously reported in monolayer culture with medium supplemented with 10% FBS and was verified by a chondrocytic phenotype shift in expression from type I to *type II collagen*, and an increase in quantity and molecular size of proteoglycans synthesized over time [7]. Surprisingly, adult dogs cBMSC showed no expression of *type II collagen* and cartilage-specific proteoglycan (CSPG) in high density monolayer culture in chondrogenic induction medium without TGF-β1 or in cell culture medium containing 10% FCS [5]. To the contrary, the present study demonstrates that cBMSC undergo independent chondrogenic differentiation but the chondrogenic potential and phenotype retention decreases significantly in a passage-dependent pattern as shown by decreased expression of *Sox-9*, *type II collagen* and *aggrecan* with increased expression of *type I collagen*. Our laboratory previously showed that bovine calves BMSC culture expanded up to P2 undergo independent chondrogenesis in pellet culture and during the process known chondrogenic growth factors TGF-β1 and β2, BMP-6, and FGF-2 showed remarkable influence in a autocrine and / or paracrine manner [2, 3]. To the contrary, another study using bovine calves BMSC expanded up to P4 prior to differentiation failed to exhibit a chondrogenic phenotype [20]. However, similar to the findings of the present study, the chondrogenic potential of human MSC has also been shown to be vulnerable to monolayer expansion cultures. Human adult BMSC expanded in standard growth medium (DMEM-LG + 10% FBS) supplemented with FGF-2 prior to differentiation in aggregate cultures in chondrogenic medium supplemented with dexamethasone and TGF-β1 exhibited chondrogenic differentiation at all passages tested compared to cells that were expanded in only standard growth medium prior to differentiation in aggregate cultures that differentiated along the chondrogenic lineage after P1 but exhibited only marginal differentiation after P4 and failed to form cartilage after P7 [27]. Taken together, these findings suggest that caution must be taken when evaluating the *in vitro* chondrogenic potential of MSC intended for cartilage tissue regeneration because their chondrogenic potential reduces significantly with extensive expansion cultures in standard growth medium.

Sox-9 is a known positive regulator of articular cartilage differentiation, chondrocyte proliferation, and transition to a non-mitotic

hypertrophic state and is necessary for chondrogenic differentiation both before and after mesenchymal condensations [12, 17, 26]. In this study, *Sox-9* mRNA was highly expressed in P0 cBMSC and this was paralleled with increased expressional levels of *type II collagen*, *aggrecan* and *type X collagen*. Albeit *Sox-9* is essential for the induction and maintenance of chondrocyte phenotype differentiation, other factors may be involved in regulating the promoter activity of *type II collagen* and *aggrecan* as we observed distinct *type II collagen* and *aggrecan* gene expression in some culture passages were *Sox-9* was significantly downregulated and this is in agreement with observations made elsewhere [30].

The expression of *type I collagen* is normally considered as a premature cartilage marker during cartilage differentiation *in vivo* which at a later stage of chondrification is then replaced by *type II collagen* as the differentiation of MSC proceeds [14]. *Type I collagen* is also associated with *in vitro* dedifferentiation of monolayer cultured articular chondrocytes [9, 11, 22]. We observed an increase in t*ype I collagen* expression with a significant negative correlation between passaging and *type II collagen* expression an indication of a major shift to a fibroblastic-like phenotype. This phenotype shift is similar to findings by other studies that have evaluated chondrogenic differentiation of MSC [2, 10, 14, 23] which makes monolayer culture system for chondrogenic differentiation undesirable for cartilage tissue engineering.

Similar to our findings, the expression of *type X collagen* during *in vitro* chondrogenic differentiation of MSC has been previously reported by others [1, 10, 23, 29, 31]. Interestingly, *type X collagen* showed a significant negative correlation with passaging and a positive significant correlation with aggrecan gene expression. This aberrant expression of *type X collagen* during early stages of BMSC chondrogenic differentiation has previously raised questions on its use as a marker for chondrogenesis and chondrocyte hypertrophy of BMSC differentiation [23]. Hypertrophic chondrocytes secrete *type X collagen* which establishes the framework for subsequent calcification and endochondral ossification [24, 29]. In articular cartilage chondrocytes, the expression of *type X collagen* indicates the terminal stage of chondrocyte differentiation and is characterized by increase in cell volume, extracellular matrix remodelling and expression of hypertrophy related factors including runt-related transcription factor (Runx2), alkaline phosphatase (ALP) and Indian Hedgehog (Ihh) [21, 29]. While *Sox-9* is a suggested negative regulator of *type X collagen* with its over-expression reported to significantly downregulate expression of *type X collagen* [17], we observed a non-significant positive correlation between *Sox-9* and *type X collagen* expression. In immature chondrocytes, there is restricted and reciprocal expression of the *collagen X* gene in hypertrophic chondrocytes and *Sox-9* which epitomise the precise spatiotemporal control of gene expression *in vivo* as chondrocytes progress through phases of differentiation [17]. However, during *in vitro* chondrogenesis as observed in this study and other studies, the critical spatiotemporal cues observed *in vivo* are not present and the majority of the MSC population continues to express both *type II* and *X collagen* concomitantly [29]. This *in vitro* aberration in MSC has been partly attributed to altered DNA methylation status at 2 cytosine-guanine dinucleotides (CpG) sites of *type X collagen*. Methylation-based *type X collagen* gene silencing is established in cartilage tissue and human articular chondrocytes but altered in MSC at 2 CpG sites and their demethylation during *in vitro* chondrogenesis may facilitate induction of *type X collagen* [33].

Lastly, we evaluated the expression of *HIF-2α* (also designated endothelial PAS domain protein-1 or *EPAS1*), a homolog of *HIF-1α* and member of the basic helix-loop-helix/PAS transcription factor family. *HIF-2α* is regulated via oxygen-dependent degradation and is involved in control of the hypoxic response via activation of target genes, in a manner similar to that of *HIF-1α*. *HIF-2α* mRNA was expressed in all culture passages with no significant difference between the passages or significant correlation with passaging. Increased expression of *HIF-2α* mRNA has been reported to improve chondrogenic differentiation of human BMSC and stem cells derived from the infrapatellar fat pad under hypoxic conditions [1, 15] and therefore our results may reflect its important role during and after chondrogenic differentiation.

The present study is limited by the lack of protein expression analysis to clarify whether the observed phenotype based on mRNA expression correlates with the active protein synthesis by these BMSC. Nonetheless, whether the observed mRNA transcription of the selected genes evaluated does not result in translation of the active proteins, the results clearly demonstrate the passage-dependent epigenetic changes that occur in cBMSC in monolayer expansion cultures.

In conclusion, the study demonstrates independent chondrogenic differentiation of cBMSC in monolayer culture in the absence of dexamethasone and TGF-β1 previously shown to be necessary. Our results show that independent chondrogenic potential and chondrogenic phenotype retention of cBMSC in monolayer expansion cultures in standard growth medium significantly decreases in a passage-dependent pattern. Therefore, caution should be exercised when evaluating the chondrogenic potential of BMSC intended for cartilage tissue engineering especially after extensive expansion cultures in standard growth medium.

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