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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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 Lymphoprep™ protocol (1.077 ± 0.001 g/ml) (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/ml of penicillin and 0.1 mg/ml of streptomycin and incubated at 37°C in 5% CO₂ 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 × 10⁴ cells/cm² per subculture. All subcultures were allowed to reach confluence of 80–90% before passaging. At each passage (P0–P3), 1 × 10⁶ 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 ng 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 aggregan, dedifferentiation gene, type I collagen, 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.
RESULTS

Morphology and proliferation characterization of cBMSC

On average, P0 cultures reached 80–90% confluence within 11 ± 2 days (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 proliferated in circular patches (Fig. 1C). Subsequent passages (P1–P3) established colony forming unit-fibroblasts (CFU-F) and revealed reduced CFU-F formation from P1−P3. Although cBMSC demonstrated a chondrogenic phenotype, they concomitantly expressed some cartilage-specific 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. Interestingly, Sox-9 mRNA bands expression especially for aggrecan type II collagen and type X collagen were markedly upregulated from P1–P3 compared to P0 but there was no significant difference (P>0.05) between the passages (Fig. 3). Sox-9 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.

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 markers, when compared to P0.

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 only significantly upregulated from P1–P3 compared to P0 but there was no significant difference 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.

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), and 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).

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| 5ˈ-CTGACCGGAAGCCTCAGCTGG-3ˈ | 129 | NM_001003142.1 |
| Sox-9        | 773–792| 5ˈ-CGATGCTGCTTCCACTACCT-3ˈ | 179 | NM_001002978.1 |
| Type I Collagen | 565–583| 5ˈ-GGCGAGGAGGCCACGGA-3ˈ | 164 | NM_001003187.1 |
| Type II Collagen | 724–743| 5ˈ-GGCGGCTGCACTCGTGT-3ˈ | 215 | NM_001006951.1 |
| Type X Collagen | 150–169| 5ˈ-GTGGATACCGGACTTGTG-3ˈ | 117 | XM_003639401.1 |
| Aggrecan     | 294–313| 5ˈ-GGGATACCCATGTCACGCTC-3ˈ | 111 | NM_001113455.1 |
| HIF-2α       | 4127–4146| 5ˈ-CACTGCCAACGGCTGCACT-3ˈ | 72 | XM_531807.3 |

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

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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 collagen (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.
differentiation within 8 (± 2) days of culture as verified by the expression of cartilage-specific gene markers, characteristic similarly described elsewhere [2, 5, 7, 13]. In the present study, BMSC demonstrated independent chondrogenic differentiation. cBMSC progressed from being elongated spindle-shaped, to small bipolar cells then to large polygonal fibroblastic-like cells, a phenotype confirmed that the cells had MSC character by their ability to adhere to plastic culture plate under standard culture conditions [5]. We determined the chondrogenic potential and phenotype retention decreases significantly in passage-dependent pattern marked by decreased expression of 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.

**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 described 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 showed remarkable influence in an autocrine and / or paracrine manner [2, 3]. To the contrary, another study using bovine calves BMSC culture expanded up to P2 undergo independent chondrogenesis but the chondrogenic potential and phenotype retention decreases significantly in in vivo conditions as verified by the expression of cartilage-specific gene markers, Sox-9, type II collagen and aggrecan [20]. The 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 an autocrine and / or paracrine manner [2, 3].

| 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 |
| (n=12) | Pearson Correlation | 1 | −0.802b | 0.566 | −0.789b | 0.599a | −0.754b | 0.157 |
| Sig. (2-tailed) | 0.002 | 0.055 | 0.002 | 0.04 | 0.005 | 0.626 |
| Sox-9 | Pearson Correlation | −0.802b | 1 | −0.769b | 0.659a | 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 | −0.769b | 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 | −0.789b | 0.659a | −0.213 | 1 | 0.192 | 0.433 | −0.524 |
| (n=12) | Sig. (2-tailed) | 0.02 | 0.02 | 0.507 | 0.55 | 0.16 | 0.08 |
| Type X collagen | Pearson Correlation | −0.599a | 0.32 | −0.324 | 0.192 | 1 | 0.684a | 0.166 |
| (n=12) | Sig. (2-tailed) | 0.04 | 0.31 | 0.304 | 0.55 | 0.014 | 0.607 |
| Aggrecan | Pearson Correlation | −0.754b | 0.399 | −0.401 | 0.433 | 0.684a | 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).
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 expression 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 type I collagen expression with a significant negative correlation between passaging and type II collagen expression indicating 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 cartilage proteoglycan (C2G2) sites of type X collagen. 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 passaging and a positive significant correlation with aggrecan gene expression. This aberration expression 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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