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Original Article

Bone morphogenetic protein-7 promoted the differentiation of bone marrow-derived mesenchymal stromal cells towards chondrocytes

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Abstract: Objectives: Bone marrow stromal cells (BMSCs) have been investigated for their use in the treatment of Osteoarthritis (OA) in animal models, but this approach is challenged by their poor ability to differentiate into chondrocytes, a major cell component in articular cartilage. Therefore, this study aimed to investigate how BMP-7 drives BMSC differentiation into chondrocytes. Methods: We isolated BMSCs and identified chondrocyte specific gene expression profiles after culture in permissive medium for 21 days, in order to improve the ability of BMSCs to differentiate. Results: We observed a significantly increased expression of bone morphogenetic protein-7 (BMP-7), aggrecan (ACAN), type II collagen (COL2A1), and SRY-related high-mobility-group box 9 (SOX-9), but the ALP, COLXA1, and runt-related transcription factor 2 (RUNX2) expressions were shown to have significantly decreased. In addition, several cytokines were upregulated, including transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-10. Due to the fact that the highest levels of upregulated BMP-7 were seen in the treated BMSCs, we induced its over-expression by plasmid mediated gene transfer. The results indicated that the over-expression of BMP-7 can cause the upregulation of ACAN, COL2A1, and SOX-9, but can also suppress that of ALP, COLXA1, and RUNX2 in a dose-dependent manner, demonstrating the pivotal role of BMP-7 in the regulation of downstream target gene expression. BMP-7 is critically involved in chondrogenesis. Further investigations also indicated that the knockdown of BMP-7 expression by shRNA suppressed ACAN, COL2A1, and SOX-9, but increased that of ALP, COLXA1, and RUNX2. Thus BMP-7 is a potent molecule in promoting BMSC differentiation towards the chondrocyte phenotype. Conclusion: The modulation of BMP-7 expression in BMSCs has the potential to increase BMSC chondrogenesis and BMP-7 can be used in the future as a molecular target in BMSC-based cell therapy for OA.

Keywords: Bone marrow stromal cells, bone morphogenetic protein-7, chondrocytes

Introduction

Osteoarthritis (OA) is characterized by the degeneration of articular chondrocytes. The subsequent inflammation contributes to disease pathogenesis and progression [1, 2]. Previous studies have indicated that levels of collagenase, proteoglycanase, matrix metalloproteinases, inducible nitric oxide synthase, nitric oxide, prostaglandin E2, interleukin (IL)-1β, IL-6, and IL-8 increase in the synovial fluid of the joints [3-6]. IL-1β expression of collagen type II, a cartilage specific proteoglycan from chondrocytes, is accompanied by the activation of MAP kinase and cartilage-specific transcription factors. These transcription factors include SRY-related high-mobility-group box 9 (SOX-9), a cartilage-specific transcription factor. However, caspase-3 is also upregulated in OA, suggesting that patients with OA have elevated levels of inflammation and cell apoptosis [7].

Anti-inflammatory reagents, anti-catabolic molecules, and rehabilitation can relieve pain and inflammation to a certain extent. For example, growth factors antagonize the catabolic actions of cytokines. Pretreatment of chondrocytes with insulin-like growth factor-1 and/or platelet derived growth factor-β may suppress IL-1β-

induced NF-κB activation [8]. Nevertheless, medicinal treatments are restricted to nonsteroidal anti-inflammatory drugs, which induce only temporary therapeutic effects and are accompanied by numerous side effects. The damaged tissue in OA patients cannot be effectively repaired. Stem cell-based therapies have emerged as a promising therapeutic approach in the treatment of this disease and their positive effects have been confirmed in animal models.

Mesenchymal stem cells (MSCs) are derived from synovial fluid in joints, adipose tissue, amniotic fluid, endometrium, the umbilical cord, and dental tissues [9-14]. MSCs express a number of markers, including cluster of differentiation (CD)44, CD90, CD105, and CD73. Following culture in permissive medium, these cells differentiate into different cell lineages, including bone, cartilage, and adipose tissues [15-17]. Currently, bone marrow-derived mesenchymal stem cells (BMSCs) have emerged as major cell sources for MSC-based therapy, due to their chondrogenesis properties and unlimited cell availability [15, 18].

Bone MSCs (BMSCs) are non-hematopoietic and multipotent stem cells, characterized by the expression of CD29, CD44, CD73, CD90, and CD105. However, human BMSCs do not express CD14, CD34, CD45 or human leucocyte antigen-antigen D. Previous studies have demonstrated that BMSCs have the capacity to differentiate into mesodermal lineages, including osteocytes, adipocytes, and chondrocytes [15, 19]. Not only do BMSCs possess the capacity to differentiate into chondrocytes, they also have immunomodulatory properties; they release numerous immune regulatory cytokines and mediators and are critical participants in immune suppression [20, 21]. They have thus been used to treat different autoimmune diseases, due to their immune regulatory property [22].

Bone morphogenetic protein (BMP-2/7) is a potent inducer of MSC differentiation. Human adipose tissue-derived MSCs express high levels of type II collagen, aggrecan, and SOX-9 mRNA after being treated with a library of Activin/BMP2 chimeric ligands. This indicates the acquisition of a chondrogenic phenotype in culture [23]. In addition, the over-expression of BMP-2 by recombinant adenovirus vector-mediated gene transfer has been demonstrated to significantly enhance the differentiation of rabbit synovial-derived MSCs into fibrocartilage cells [24]. Furthermore, the co-delivery of BMP-2/SOX-9 significantly increased the expression of type II collagen and aggrecan, and enhanced glycosaminoglycan (GAG) matrix formation [25]. The underlying mechanisms of BMP-2 in promoting chondrocyte differentiation are not well defined. It is possible that BMPs bind to cell surface receptors on MSCs and send signals to the nucleus to promote macromolecule synthesis and bone formation.

Previous studies have indicated that BMSCs stimulated with BMPs proliferate and differentiate into osteoblastic and chondrogenic lineages [26], BMP-7 promoted the differentiation of BMSCs into chondrocytes with increased type II collagen and GAG expression [19]. However, the mechanisms behind BMP-7-driven BMSC differentiation into chondrocytes remain unknown. To the best of our knowledge, the current study is the first to investigate the role of BMP-7 in promoting BMSC differentiation into chondrocytes and to determine the underlying molecular mechanisms. Thus, the current study provides a rationale for investigating chondrocyte lineage-targeted MSC differentiation through molecular intervention with BMP-7 in the treatment of patients with cartilage articular degeneration and injury.

Materials and methods

Isolation of BMSCs

All animal studies were approved by The Institute Research Medical Ethics Committee of Jinan University. BMSCs were generated from male Sprague-Dawley mice (aged 8-10 weeks; 30-40 g) provided by Laboratory Animal Facility of the Chinese Academy of Sciences (Shanghai, China). Briefly, bone marrow from the femur and tibia cavities was isolated from the anesthetized mice and was anticoagulated using heparin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), whereupon 1 ml PBS was added.

Culture of mouse mesenchymal stem cells and flow cytometry analysis

BMSCs were purified by Ficoll gradient (Fischer Scientific, Pittsburgh, USA) and plated in 35-mm dishes at a density of 4×10^5 cells/well. After 24 h, the cells were collected for identification of BMSCs markers. Antibodies (Abcam,

Table 1. Primer sequences for reverse transcription-quantitative polymerase chain reaction

Gene	Forward (5'-3')	Reverse (5'-3')
ACAN	CATGAGAGAGGCGAATGGAA	TGATCTCGTAGCGATCTTTCTTCT
COL2A1	TGGGTGTTCTATTTATTTATTGTCTTCCT	GCGTTGGACTCACACCAGTTAGT
SOX9	AGTACCCGCACCTGCACAAC	TACTTGTAGTCCGGGTGGTCTTTC
ALP	CCGATGGCACACCTGCTT	GGAGGCATACGCCATCACAT
COLXA1	CATGCCTGATGGCTTCATAAA	AAGCAGACACGGGCATACCT
RUNX2	GACGAGGCAAGAGTTTCACC	GGACCGTCCACTGTCACTTT
β-actin	GACAGGATGCAGAAGGAGATTACTG	CCACCGATCCACACAGAGTACTT

ACAN: aggrecan, COL2A1: type II collagen, SOX9: SRY-related high-mobility-group box 9, ALP: alkaline phosphatase, COLXA1: type I collagen, RUNX2: runt-related transcription factor 2.

Cambridge, MA, USA) against CD14 (ab1820-32), CD34 (ab81289), CD45 (ab10558), CD44 (ab157107), CD90 (ab225), and CD105 (ab-11414) were used for identifying cell purity and hematopoietic cell contamination. Cells were then washed twice with phosphate buffered saline (PBS) and stained samples were analyzed with a FACScan flow cytometer (Becton Dickson, Franklin Lakes, NJ, USA). Data were presented as percentage of positive stained cells.

Treatment of mouse mesenchymal stem cells

4×10^5 cultured BMSCs cells were seeded into a 6-well plate and were differentiated towards chondrocyte-like cells using a specific medium to promote spheroid formation. Spheroids were collected after 14, 28, and 46 days in chondrogenic medium and stained with hematoxylin, eosin, Safranin O, or Alcian blue to evaluate the extracellular matrix. Immunohistochemistry was performed to study collagen types I (COLI) and II (COLII), as well as aggrecan expression.

The BMSCs were then transfected with 1 nM and 5 nM BMP7 by Lipofectamine 200 (Invitrogen, Carlsbad, CA, USA) and maintained in culture for 21 days. Flesh medium was added every 3 days during cell culture. The same protocol was used for the transfection of 1 μg shRNA including shBMP7-1 and shBMP-7-2. The same amount of scramble shRNA was transfected as the negative control. All of the treated cells were collected for analysis 21 days after transfection.

Quantitative RT-PCR analysis

RNA was extracted from samples with Trizol reagent (Invitrogen) and cDNA was synthesized with cDNA synthesis kits (Invitrogen, Grand Island, NY). The primer sequences used to analyze the expression of murine gene transcripts are shown in **Table 1**. SYBR green PCR master mix (AB Applied Biosystems, Warrington, UK) was used for the real-time PCR reaction following the reaction conditions: 94°C 5 min, 50 cycles (94°C for 40 sec; 57°C for 30 sec, 72°C for 30 sec). Data were analyzed using 7500 systems DS software (AB applied Biosystems) and were normalized with internal control GAPDH and presented as ΔΔCt ± standard error.

Western blotting analysis

Cellular protein was extracted from the cultured cells using the RIPA lysis buffer (Promega, Madison, WI, USA) and the level of protein was measured with a BCA assay (Pierce). Western blot analysis was performed using primary antibodies against aggrecan (ACAN), type II collagen (COL2A1), SOX-9, ALP, COLXA1, and runt-related transcription factor 2 (RUNX2) (Cell Signaling, Beverly, MA, USA). The antibody against GAPDH (Sigma-Aldrich, St. Louis, MO, USA) was used as a loading control and the intensity was quantitatively analyzed with Image J software. The data are presented as the mean ratio of the densimetric intensity of the target protein to internal control GAPDH for each sample.

Alizarin red staining

After 21 days of osteogenic differentiation, the culture medium was removed, the cells were fixed with 4% paraformaldehyde for 30 minutes, and then were rinsed three times with PBS. The cells were then stained with 1% Alizarin red-Tris- HCl for 30 minutes (37°C). Finally, cells were observed under a light microscope (Olympus BX51, Japan).

Alkaline phosphatase (AKP) staining

After 21 days of osteogenic differentiation, the culture medium was removed and the cells were fixed with 4% paraformaldehyde for 30

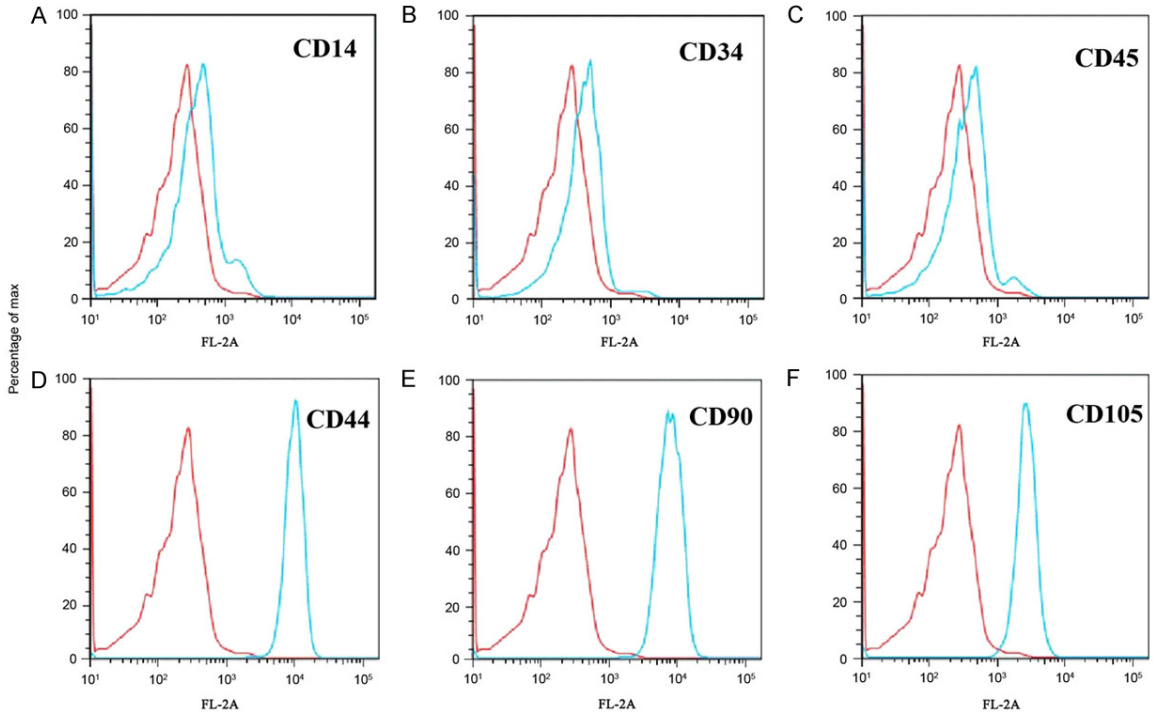

Figure 1. Identification of bone marrow-derived MSCs. The BMSCs were isolated from 8-10 week old mice. Following purification with Ficoll-Paque, cells were cultured for 24 h and then stained with antibodies against (A) CD14, (B) CD34, (C) CD45, (D) CD44, (E) CD90, and (F) CD105 (indicated by the blue line). The species matched isotype antibodies were used as negative controls (indicated by the red line). The cultured cells *in vitro* exhibited a fibroblast-like morphology in monolayer culture with the positive MSC specific markers CD44, CD90, and CD105, but the hematopoietic markers CD14, CD34, and CD45 were negative in the isolated MSC. The representative data from the MSCs of one mouse are presented. MSC: marrow stem cells, CD: cluster of differentiation.

minutes, before being incubated with incubation buffer (5 ml 3-β sodium glycerophosphate, 5 ml 2% barbital sodium, 10 ml distilled water, 10 ml 2% $CaCl_2$, and 2% $MgSO_4$) for 4 hours 37°C. Cells were then soaked in 2% cobalt nitrate for 5 min, and in 1% ammonium sulfide for 2 min. Finally, they were rinsed with water, dried, and examined with phase contract microscopy.

Statistical analysis

All data are expressed as means ± standard deviations. Statistical analysis was conducted using a one-way analysis of variance, followed by Tukey's post-hoc test. A p value <0.05 was considered to be a significant difference.

Results

Identification of bone marrow-derived mesenchymal stem cells

The cultured cells *in vitro* exhibited fibroblast-like morphology in a monolayer culture. Antibodies against CD14, CD34, CD45, CD44, CD90, and CD105 were used for immunostaining and species matched isotype antibodies were used as the negative controls. The expressions of the hematopoietic markers CD14, CD34, and CD45 were low, indicating a lower contamination of hematopoietic cells in the isolated BMSCs than in the negative controls (**Figure 1A-C**). However, flow cytometric analysis indicated that the MSCs expressed high levels of CD44, CD90, and CD105. The positive cells were >95%, indicating a high BMSC purity (**Figure 1D-F**).

MSC cells were differentiated into osteoblastic and chondrogenic lineages

Following 1 day of MSC cell culture, a chondrocyte permissive medium containing growth factors was added to cells and was maintained for 21 days. The levels of the osteoblastic and chondrogenic lineage markers were subsequently measured. BMSCs exhibited fibroblast-like morphology and RT-qPCR demonstrated

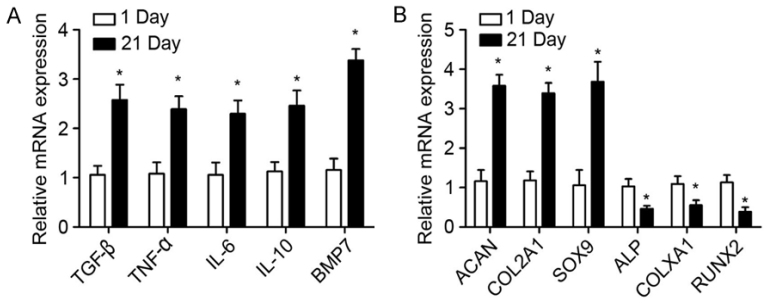

Figure 2. MSC cells differentiated into osteoblastic and chondrogenic lineages following 21 days of culture in chrondrocyte permissive medium. MSCs cultured in chondrocyte permissive medium for 21 days exhibited a fibroblast-like morphology. Levels of osteoblastic and chondrogenic lineage markers were analyzed using reverse transcription-quantitative polymerase chain reaction. Following 21 days culture, (A) TGF-β, TNF-α, IL-6, IL-10, BMP-7, and (B) ACAN, COL2A1, and SOX9 expressions were significantly increased but the expressions of ALP, COLXA1, and RUNX2 significantly decreased. Data are presented as the mean of DDCq normalized to the internal control GAPDH ± standard error of the mean. One of 3 independent experiments is presented, n=4, *P<0.05 vs. day 1 of initial cell culture. MSC: marrow stem cells, TGF-β: transforming growth factor-β, TNF-α: tumor necrosis factor-α, IL: interleukin, BMP: bone morphogenetic protein, ACAN: aggrecan, COL2A1: type II collagen, SOX9: SRY-related high-mobility-group box 9, ALP: alkaline phosphatase, COLXA1: type I collagen, RUNX2: runt-related transcription factor 2.

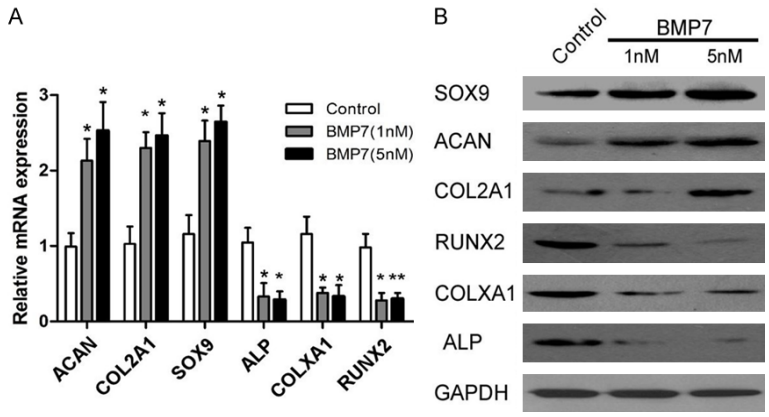

Figure 3. Overexpressing BMP-7 increased the differentiation of bone marrow stromal cells into chondrocytes *in vitro*. Bone marrow stromal cells were transfected with different concentrations (0, 1, and 5 nM) of plasmid encoding BMP-7, a secretory protein and maintained in permissive medium for 21 days. Cells transfected with empty plasmid were used as the negative control. (A) The expressions of chondrocyte specific protein markers (ACAN, COL2A1, and SOX9) and other relevant proteins (ALP, COLXA1, and RUNX2) were analyzed by reverse transcription-quantitative polymerase chain reaction. Data are presented as mean of DDCq ± standard error of the mean, n=3. Representative data from 3 independent experiments are presented. *P<0.05, **P<0.01 vs. day 1 negative control. (B) Target gene expression in the treated cells was confirmed by western blot analysis. An antibody against GAPDH was used as the internal loading control. Representative blot of 3 independent experiments is presented. BMP: bone morphogenetic protein, ACAN: aggrecan, COL2A1: type II collagen, SOX9: SRY-related high-mobility-group box 9, ALP: alkaline phosphatase, COLXA1: type I collagen, RUNX2: runt-related transcription factor 2.

that the expression of transforming growth factor (TGF)-β, tumor necrosis factor-α (TNF-α), IL-6, and IL-10 were significantly increased in BMSCs following 21 days of culture (P<0.05; **Figure 2A**). In addition, the expressions of chondrogenic lineage specific markers BMP-7, ACAN, COL2A1, and SOX-9 were significantly elevated (P<0.05), whereas the expressions of ALP, COLXA1, and RUNX2 decreased (P<0.05; **Figure 2**). These results indicate that MSC cells were differentiated into chondrocyte-like cells after 21 days of culture.

Overexpressing BMP-7 increased differentiation of MSCs into chondrocytes in vitro

A significant increase in the expression of BMP-7 protein was observed in the cultured cells, leading to the hypothesis that BMP-7 may facilitate the development of chondrocytes from BMSCs. In order to test this, cells were transfected with different concentrations of plasmid encoding BMP-7 one day after the initial cell culture. After 21 days of culture, following transfection with both 1 nM and 5 nM BMP-7, a significant increase in the expressions of the chondrocyte specific markers ACAN, COL2A1, and SOX-9 was observed, compared with their expressions in the controls transfected with empty plasmid (P<0.05; **Figure 3A**). Cells transfected with 5 nM BMP-7 encoding plasmid expressed the highest levels of these markers (**Figure 3A**). In contrast, the expressions of ALP, COLXA1, and RUNX2 were suppressed following BMP-7

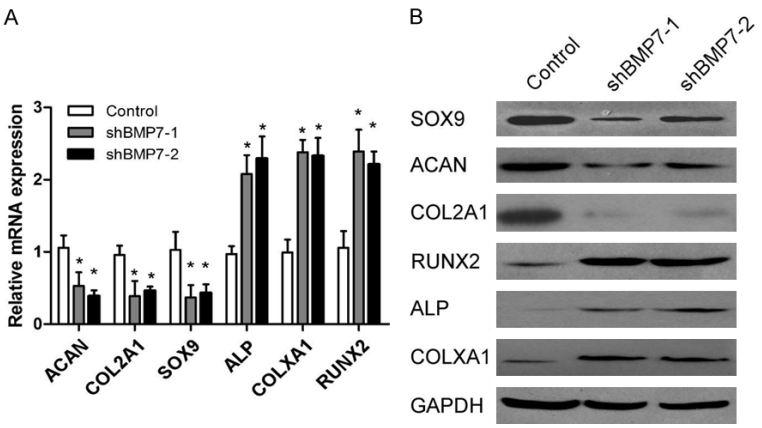

Figure 4. Knockdown of BMP-7 expression suppressed differentiation of bone marrow stromal cells into chondrocytes *in vitro*. Bone marrow stromal cells were transfected with two different silencing RNAs against BMP-7 in distinct coding regions. Cells transfected with the same amount of scramble shRNA were used as a negative control. (A) After 21 days of culture, the expressions of chondrocyte specific protein markers (ACAN, COL2A1, and SOX9) and other relevant proteins (ALP, COLXA1, and RUNX2) were analyzed by reverse transcription-quantitative polymerase chain reaction. Data are presented as mean of DDCq ± standard error of the mean, n=3. Representative data of 3 independent experiments are presented. *P<0.05, vs. day 1 negative control. (B) Target gene expression in the shRNA treated cells was confirmed by western blot analysis. An antibody against GAPDH was used as the internal loading control. Representative blot of 3 independent experiments is presented. BMP: bone morphogenetic protein, shRNA: short hairpin RNA, ACAN: aggrecan, COL2A1: type II collagen, SOX9: SRY-related high-mobility-group box 9, ALP: alkaline phosphatase, COLXA1: type I collagen, RUNX2: runt-related transcription factor 2.

overexpression (P<0.05; **Figure 3A**). Similar results were also seen when assessing protein levels using western blot analysis, with levels of the chondrocyte specific protein markers ACAN, COL2A1, and SOX-9 markedly increasing and those of ALP, COLXA1, and TUNX2 markedly decreasing following BMP-7 overexpression (**Figure 3B**). Therefore, the overexpression of BMP-7 facilitated BMSC chondrogenesis and may have potential implications in cartilage tissue engineering.

Knockdown of BMP-7 expression suppressed differentiation of MSCs toward chondrocytes in vitro

The current study demonstrates that BMP-7 is involved in the differentiation of BMSCs toward chondrocyte-like cells. Therefore, to confirm the role of BMP-7 in chondrocyte differentiation, shRNA covering two BMP-7 encoding sequences was synthesized. Scramble shRNA was used as a negative control. Following transfection into BMSCs and culture for 21 days, RT-qPCR demonstrated that the expressions of ACAN, COL2A1, and SOX-9 were significantly downregulated, (P<0.05; **Figure 4A**) compared with cells transfected with scramble shRNA. However, the expressions of ALP, COLXA1, and RUNX2 increased significantly in shBMP7-1 and shBMP7-2 transfected cells compared with cells transfected with scramble shRNA (P<0.05; **Figure 4A**). The western blot analysis also revealed similar results (**Figure 4B**). This indicates that BMP-7 pushes BMSCs towards chondrocyte-like differentiation *in vitro* and that the downregulation of BMP-7 may reverse these effects. The modulation and overexpression of BMP-7 may have beneficial effects in BMSC-based therapies for articular degeneration in patients with arthritis and articular injury.

BMP-7 expression modulated the differentiation of MSCs toward chondrocytes

Alizarin red staining was used to examine the mineralized nodules formed by BMSCs after 21 days of culture. AKP is a marker enzyme for mature osteoblasts. Light microcopy photographs (original magnification, 100X) and graphs showed the higher intensity of Alizarin Red S and ALP staining for BMSCs in cells transfected with 5 nM BMP-7 encoding plasmid, and the lower intensity in shBMP7-1 cells, which also indicated that BMP-7 expression modulated the differentiation of MSCs toward chondrocytes (**Figure 5**).

Discussion

Due to the feasibility and high capacity of BMSCs to differentiate into multiple lineages of cells, they have been recognized as a major stem cell source for tissue repair in many degenerative diseases, including OA, which is a common articular cartilage disease characterized by articular tissue degeneration, chondrocyte senescence, and apoptosis [2, 12]. Chronic inflammation serves an important role in the pathogenesis of OA and current therapeutics

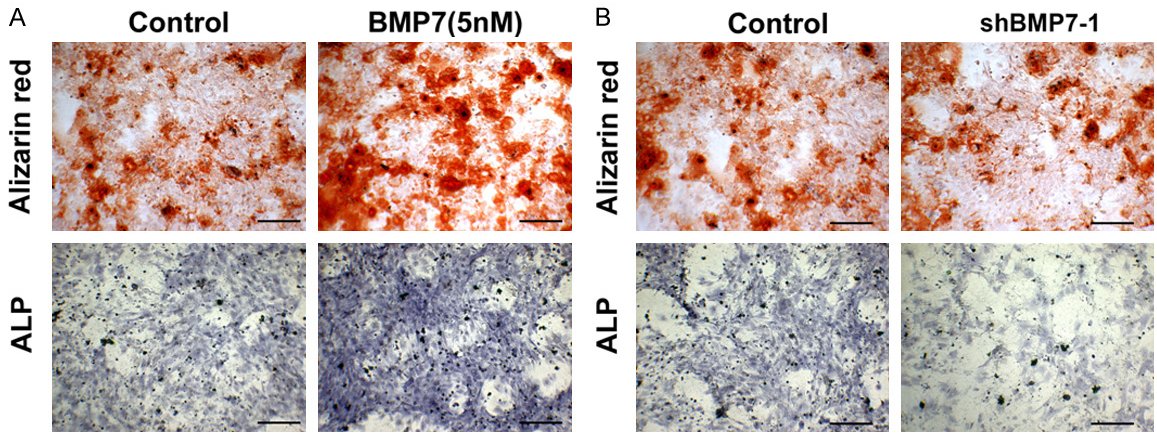

Figure 5. Alizarin red staining and Alkali phosphatase staining after 21 days of culture in chrondrocyte permissive medium. (A) Bone marrow stromal cells were transfected with 5 nM of plasmid encoding BMP-7. Cells transfected with empty plasmid were used as the negative control. (B) Bone marrow stromal cells were transfected with silencing RNA against BMP7-1. Cells transfected with the same amount of scramble shRNA were used as the negative control. ALP: alkaline phosphatase. Scale bar=50 μm, magnification: 100 ×.

using anti-inflammatory reagents may effectively relieve pain and reduce inflammation [7]. However, these therapeutic strategies are limited by the fact that anti-inflammatory medicines lack the ability to repair degenerated tissue. Therefore, cell-based replacement therapy has become essential in order to actually improve the functional recovery of tissue. Stem cells from adipose tissue, umbilical cords, and synovial fluid have been investigated in previous studies to facilitate repair in a number of degenerative diseases [14, 16, 17, 27]. However, these strategies are limited in scope, since they use stem cells from sources that cannot easily be replenished. Due to the sufficient sourcing of BMSCs from the marrow cavities of femurs and tibias in rodents, and to their self-renewing behavior, the capability of BMSCs in the present study to differentiate towards chondrocyte-like cells was investigated. The results revealed that isolated BMSCs expressed stromal cell markers, including CD44, CD90, and CD105, but lacked the hematopoietic cell markers CD14, CD34, and CD45. The purity of the isolated BMSCs was >95%. Further culture of BMSCs under permissive medium for 21 days led to the high expression of chondrocyte-like cell markers and a fibroblast-like morphology in a monolayer culture. BMP-7, ACAN, COL2A1, and SOX-9 are important chrondrocyte specific transcription factors and products, critically involved in cell differentiation, proliferation, and survival. SOX-9 has been reported to promote COL2A1 transcription and chondrocyte differentiation [28]. Following 21 days of culture, the expressions of these cell markers were significantly elevated ($P<0.05$). Our results indicate that these molecules might serve a critical role in chondrocyte differentiation. In contrast, we also observed the suppressed expression of ALP, COLXA1, and RUNX2 in the differentiated cells. A previous study demonstrated that ALP, COLXA1, and RUNX2 are suppressive regulators of chondrocytes and osteoblasts [29]. In osteoarthritic chondrocytes, RUNX2 is highly expressed but SOX-9 expression is reduced. Thus, the elevated expression of RUNX2 may suppress SOX-9 in normal chondrocytes [30]. Downregulation of these negative regulators is beneficial to the development of chrondrocytes and further investigations into their function in the present BMSC differentiation model by molecular intervention would be useful. In addition, the increased expressions of Th1 and Th2 cytokines, including TNF-α, TGF-β, IL-6, and IL-10 were observed in the differentiated cells. Their biological function in chrondrocyte differentiation is not currently well defined. According to previous studies, these cytokines promote endogenous progenitor cell differentiation into the target cells and are beneficial to tissue self-repair and regeneration during and following tissue inflammation and damage [31-33]. Hou, *et al.,* indicated that MiR-193b may inhibit early chondrogenesis by targeting TNF-α, TGF-β2, and TGF-β3 [34]. Increased levels of the cytokines TNF-α, TGF-β, IL-6, and IL-10, therefore,

have important implications in chondrocyte differentiation and regeneration.

BMP-7 is an important molecule that critically participates in chrondrocyte differentiation. In the present study, a significantly increased expression of BMP-7 was observed after 21 days of cell culture. According to previous studies, BMP-7 is an important chondrocyte specific marker. In order to investigate whether BMP-7 is involved in chrondrocyte differentiation after 21 days of culture, its overexpression was induced in BMSC cells, resulting in the enhanced expressions of ACAN, COL2A1, and SOX-9 , but the suppressed expressions of ALP, COLXA1, and RUNX2 in BMP-7 transfected cells compared with those in the empty vector transfected cells. These results were confirmed by RT-qPCR and western blot analysis, suggesting that BMP-7 signaling controls the downstream expression of protein expression and is critically involved in BMSC differentiation towards chrondrocyte-like cells. In order to further confirm the role of BMP-7 in chondrogenesis, BMP-7 expression was knocked down by transfecting cells with BMP-7 shRNA. The BMP-7 knockdown significantly suppressed the expressions of ACAN, COL2A1, and SOX-9, but enhanced the expressions of ALP, COLXA1, and RUNX2 compared with cells transfected with scramble shRNA.

In conclusion, BMP-7 is a potent inducer of chondrocyte-directed BMSC differentiation. Its overexpression increases the capability of BMSCs towards chondrocyte differentiation. The present study achieved novel insights into the role of BMP-7 in promoting chondrocyte-directed BMSC differentiation and provided a novel strategy for improving BMSC-based therapy to treat OA. Nevertheless, more direct and deeper data are needed in order to confirm the function of BMP-7 in the process of BMSC differentiation towards the chondrocyte phenotype.

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Disclosure of conflict of interest

None.

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References

[1] Conde J, Scotece M, Abella V, Lois A, Lopez V, Garcia-Caballero T, Pino J, Gomez-Reino JJ, Gomez R, Lago F and Gualillo O. Il-36alpha: a novel cytokine involved in the catabolic and inflammatory response in chondrocytes. Sci Rep 2015; 5: 16674.

[2] Sun Y, Zhou L, Lv D, Liu H, He T and Wang X. Poly(adp-ribose) polymerase 1 inhibition prevents interleukin-1beta-induced inflammation in human osteoarthritic chondrocytes. Acta Biochim Biophys Sin (Shanghai) 2015; 47: 422-430.

[3] Adams SB, Setton LA, Bell RD, Easley ME, Huebner JL, Stabler T, Kraus VB, Leimer EM, Olson SA and Nettles DL. Inflammatory cytokines and matrix metalloproteinases in the synovial fluid after intra-articular ankle fracture. Foot Ankle Int 2015; 36: 1264-1271.

[4] Tsuchida AI, Beekhuizen M, T HM, Radstake TR, Dhert WJ, Saris DB, van Osch GJ and Creemers LB. Cytokine profiles in the joint depend on pathology, but are different between synovial fluid, cartilage tissue and cultured chondrocytes. Arthritis Res Ther 2014; 16: 441.

[5] Valcamonica E, Chighizola CB, Comi D, De Lucia O, Pisoni L, Murgo A, Salvi V, Sozzani S and Meroni PL. Levels of chemerin and interleukin 8 in the synovial fluid of patients with inflammatory arthritides and osteoarthritis. Clin Exp Rheumatol 2014; 32: 243-250.

[6] Verbruggen G. Chondroprotective drugs in degenerative joint diseases. Rheumatology (Oxford) 2006; 45: 129-138.

[7] Csaki C, Mobasheri A and Shakibaei M. Synergistic chondroprotective effects of curcumin and resveratrol in human articular chondrocytes: inhibition of il-1beta-induced nf-kappab-mediated inflammation and apoptosis. Arthritis Res Ther 2009; 11: R165.

[8] Montaseri A, Busch F, Mobasheri A, Buhrmann C, Aldinger C, Rad JS and Shakibaei M. Igf-1 and pdgf-bb suppress il-1beta-induced cartilage degradation through down-regulation of nf-kappab signaling: involvement of src/pi-3k/akt pathway. PLoS One 2011; 6: e28663.

[9] Ahmed TA and Hincke MT. Mesenchymal stem cell-based tissue engineering strategies for repair of articular cartilage. Histol Histopathol 2014; 29: 669-689.

[10] Arufe MC, De la Fuente A, Fuentes-Boquete I, De Toro FJ and Blanco FJ. Differentiation of sy-

novial cd-105(+) human mesenchymal stem cells into chondrocyte-like cells through spheroid formation. J Cell Biochem 2009; 108: 145-155.
[11] Herlofsen SR, Hoiby T, Cacchiarelli D, Zhang X, Mikkelsen TS and Brinchmann JE. Brief report: importance of sox8 for in vitro chondrogenic differentiation of human mesenchymal stromal cells. Stem Cells 2014; 32: 1629-1635.
[12] Maumus M, Manferdini C, Toupet K, Peyrafitte JA, Ferreira R, Facchini A, Gabusi E, Bourin P, Jorgensen C, Lisignoli G and Noel D. Adipose mesenchymal stem cells protect chondrocytes from degeneration associated with osteoarthritis. Stem Cell Res 2013; 11: 834-844.
[13] Miyamoto C, Matsumoto T, Sakimura K and Shindo H. Osteogenic protein-1 with transforming growth factor-beta1: potent inducer of chondrogenesis of synovial mesenchymal stem cells in vitro. J Orthop Sci 2007; 12: 555-561.
[14] Wang ZH, Li XL, He XJ, Wu BJ, Xu M, Chang HM, Zhang XH, Xing Z, Jing XH, Kong DM, Kou XH and Yang YY. Delivery of the sox9 gene promotes chondrogenic differentiation of human umbilical cord blood-derived mesenchymal stem cells in an in vitro model. Braz J Med Biol Res 2014; 47: 279-286.
[15] Kang R, Zhou Y, Tan S, Zhou G, Aagaard L, Xie L, Bunger C, Bolund L and Luo Y. Mesenchymal stem cells derived from human induced pluripotent stem cells retain adequate osteogenicity and chondrogenicity but less adipogenicity. Stem Cell Res Ther 2015; 6: 144.
[16] Kim YS, Lee HJ, Yeo JE, Kim YI, Choi YJ and Koh YG. Isolation and characterization of human mesenchymal stem cells derived from synovial fluid in patients with osteochondral lesion of the talus. Am J Sports Med 2015; 43: 399-406.
[17] Manferdini C, Maumus M, Gabusi E, Piacentini A, Filardo G, Peyrafitte JA, Jorgensen C, Bourin P, Fleury-Cappellesso S, Facchini A, Noel D and Lisignoli G. Adipose-derived mesenchymal stem cells exert antiinflammatory effects on chondrocytes and synoviocytes from osteoarthritis patients through prostaglandin e2. Arthritis Rheum 2013; 65: 1271-1281.
[18] Yoo HJ, Yoon SS, Park SY, Lee EY, Lee EB, Kim JH and Song YW. Gene expression profile during chondrogenesis in human bone marrow derived mesenchymal stem cells using a cdna microarray. J Korean Med Sci 2011; 26: 851-858.
[19] Bai X, Li G, Zhao C, Duan H and Qu F. Bmp7 induces the differentiation of bone marrow-derived mesenchymal cells into chondrocytes. Med Biol Eng Comput 2011; 49: 687-692.
[20] Amarnath S, Foley JE, Farthing DE, Gress RE, Laurence A, Eckhaus MA, Metais JY, Rose JJ, Hakim FT, Felizardo TC, Cheng AV, Robey PG, Stroncek DE, Sabatino M, Battiwalla M, Ito S, Fowler DH and Barrett AJ. Bone marrow-derived mesenchymal stromal cells harness purinergenic signaling to tolerize human th1 cells in vivo. Stem Cells 2015; 33: 1200-1212.
[21] Bernardo ME and Fibbe WE. Mesenchymal stromal cells and hematopoietic stem cell transplantation. Immunol Lett 2015; 168: 215-21.
[22] Ullah I, Subbarao RB and Rho GJ. Human mesenchymal stem cells-current trends and future prospective. Biosci Rep 2015; 35.
[23] Peran M, Ruiz S, Kwiatkowski W, Marchal JA, Yang SL, Aranega A, Choe S and Izpisua BJ. Activin/bmp2 chimeric ligands direct adipose-derived stem cells to chondrogenic differentiation. Stem Cell Res 2013; 10: 464-476.
[24] Fu P, Zhang L, Wu H, Cong R, Chen S, Ding Z and Hu K. [in vitro differentiation of synovial-derived mesenchymal stem cells infected by adenovirus vector mediated by bone morphogenetic protein 2/7 genes into fibrocartilage cells in rabbits]. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 2013; 27: 345-352.
[25] Cha BH, Kim JH, Kang SW, Do HJ, Jang JW, Choi YR, Park H, Kim BS and Lee SH. Cartilage tissue formation from dedifferentiated chondrocytes by codelivery of bmp-2 and sox-9 genes encoding bicistronic vector. Cell Transplant 2013; 22: 1519-1528.
[26] Dorman LJ, Tucci M and Benghuzzi H. In vitro effects of bmp-2, bmp-7, and bmp-13 on proliferation and differentation of mouse mesenchymal stem cells. Biomed Sci Instrum 2012; 48: 81-87.
[27] Zheng P, Ju L, Jiang B, Chen L, Dong Z, Jiang L, Wang R and Lou Y. Chondrogenic differentiation of human umbilical cord bloodderived mesenchymal stem cells by coculture with rabbit chondrocytes. Mol Med Rep 2013; 8: 1169-1182.
[28] Maneix L, Servent A, Poree B, Ollitrault D, Branly T, Bigot N, Boujrad N, Flouriot G, Demoor M, Boumediene K, Moslemi S and Galera P. Up-regulation of type ii collagen gene by 17beta-estradiol in articular chondrocytes involves sp1/3, sox-9, and estrogen receptor alpha. J Mol Med (Berl) 2014; 92: 1179-200.
[29] Musumeci G, Mobasheri A, Trovato FM, Szychlinska MA, Graziano AC, Lo FD, Avola R, Mangano S, Giuffrida R and Cardile V. Biosynthesis of collagen i, ii, runx2 and lubricin at different time points of chondrogenic differentiation in a 3d in vitro model of human mesenchymal stem cells derived from adipose tissue. Acta Histochem 2014; 116: 1407-1417.
[30] Orfanidou T, Iliopoulos D, Malizos KN and Tsezou A. Involvement of sox-9 and fgf-23 in runx-2 regulation in osteoarthritic chondrocytes. J Cell Mol Med 2009; 13: 3186-3194.

[31] Albiero ML, Amorim BR, Martins L, Casati MZ, Sallum EA, Nociti FJ and Silverio KG. Exposure of periodontal ligament progenitor cells to lipopolysaccharide from escherichia coli changes osteoblast differentiation pattern. J Appl Oral Sci 2015; 23: 145-152.

[32] Cao W, Cao K, Cao J, Wang Y and Shi Y. Mesenchymal stem cells and adaptive immune responses. Immunol Lett 2015; 168: 147-53.

[33] Carreira BP, Santos DF, Santos AI, Carvalho CM and Araujo IM. Nitric oxide regulates neurogenesis in the hippocampus following seizures. Oxid Med Cell Longev 2015; 2015: 451512.

[34] Hou C, Yang Z, Kang Y, Zhang Z, Fu M, He A, Zhang Z and Liao W. Mir-193b regulates early chondrogenesis by inhibiting the tgf-beta2 signaling pathway. Febs Lett 2015; 589: 1040-1047.