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Original Article

Transforming growth factor-β1 and bone morphogenetic protein-2 can induce bone mesenchymal stem cells to differentiate into cartilage cells

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Abstract: Objective: To explore whether transforming growth factor β1 (TGF-β1) and bone morphogenetic proteins-2 (BMP-2) are able to induce bone mesenchymal stem cells (BMSCs) to differentiate into cartilage cells. Methods: The BMSCs from 10 healthy SD rats were obtained and then induced by TGF-β1 and BMP-2. Meanwhile, the normal cartilage cells served as the positive control and the pure BMSCs (without any added induction factors) as the negative control. Then, all the cells including the control and the experimental groups were cultured for 14 d, during which glycosaminoglycan was detected by toluidine blue staining method at 7th and 14th d, respectively. Besides, the morphology of the BMSCs and their surface antibody were also determined by using a microscope and a flow cytometry, respectively. Further, type II collagen and its mRNA expression were also detected by means of western blot and reverse transcription polymerase chain reaction (RT-PCR), respectively. Results: Microscope observation revealed that the BMSCs showed a spindled-fibroblast morphology. There was no expression of cluster of differentiation 11b/c (CD11b/c) and CD45 observed in the BMSCs, whereas the expression of CD29 and CD90 could be observed. The analysis result of toluidine blue indicated the acidic glycosaminoglycan components could be detected in the induced BMSCs. After inducing the BMSCs for 7 d, the expression of type II collagen was significant, which was in line with the positive control group. However, the negative control did not show any expression of type II collagen ($P<0.05$). After 14 d of induction, RT-PCR analysis results also indicated the occurrence of the amplified product of type II collagen in the experimental group, and the expression was significantly higher than the negative control ($P<0.05$). Conclusion: TGF-β1 and BMP-2 can induce the differentiation of the BMSCs into cartilage cells, thus showing a great potential in cartilage tissue engineering.

Keywords: Transforming growth factor-β1, bone morphogenetic protein-2, bone marrow mesenchymal stem cells, chondrocyte

Introduction

Stem cells are commonly defined as the cells with potent self-renew ability and differentiation potential. It can differentiate into various mature cells with special functions [1]. Thus, it can be used to provide regenerative medical materials [2, 3]. Also, due to its characteristics of multiple differentiation and self-generation, stem cells are clinically widely used to amend damaged tissues and organs, which is favorable to organ transplantation. Besides, mesenchymal stem cells are also proven to possess immune-regulatory properties [4, 5]. When tumors are formed, bone mesenchymal stem cells (BMSCs) usually attach to the peripheral tumor tissues, and thus BMSCs are regarded as one of the important micro environmental factors. Generally, BMSCs can regulate the tumor metastasis through various pathways [6, 7]. The in vitro directed differentiation of BMSCs can be affected by many factors, among which the cell growth factors and local micro environments are believed to be significant ones [8]. Transforming growth factor β1 (TGF-β1) belongs to polypeptide family and can act as an important growth factor which participate in various biological activities, including promoting cell proliferation and regulating cell differentiation [3, 4]. Bone morphogenetic proteins-2 (BMP-2)

Table 1. RT-PCR sequence specific primers

Genes	Primer sequence	PCR product length
Type II collagen	F-5'-GCG AGT CTT GCG TCT ACC C-3'	385 bp
	R: 5'-GTC TTG CCC CAC TTA CCG-3'	
β-actin	F: 5'-GCG AGT CTT GCG TCT ACC C-3'	607 bp
	R: 5'-ACA GAG TAC TTG CGC TCA GGA G-3'	

Note: RT-PCR, reverse transcription polymerase chain reaction.

is considered one of the tissue growth factors in articular cartilage tissue engineering which can regulate the cell cycle of cartilage cells and can also affect the differentiation of stem cells. In addition, BMP-2 can promote mesochondrium synthesis and secretion [5]. In this study, the BMSCs of 10 healthy SD rats were obtained, and then the cells were induced by adding TGF-β1 and BMP-2. The detailed report is attached below.

Materials and methods

Experimental rats

Ten SPF Wister rats aged 3-8 weeks and weighed 112-120 g were provided by Shanghai Laboratory Animal Co. Ltd. The sex factor of the rats were not considered in this work. Then, their BMSCs were obtained for further use. In the process, the operation was strictly according to the *Guidelines of Treating Experimental Animals of China, released in 2006* [3].

Reagents and instruments

Insulin, DMEM/F12 culture media, 0.25% pancreatin-0.02% EDTA were purchased from Gibco Company (U.S.A). Besides, TGF-β1 and BMP-2 were provided by Peprotech Company (U.S.A). Rabbit Anti-mouse PE cluster of differentiation 90 (CD90) and CD11b/c, CD4 were purchased from Biolegend Company (Germany) and Invitrogen Company (U.S.A), respectively. Rabbit Anti-mouse PE CD29 was provided by eBioscience Company (Germany).

The instruments included: an 5% CO_2 incubator at 37°C (Forma Company, U.S.A), a high speed refrigerated centrifuge (Biofuge, Germany), an inverted phase contrast microscope (Olympus, Japan), a superclean bench (Medical Equipment Factory of Shanghai Medical Instruments Co., Ltd, China), a thermal cycler (Biometra, U.S.A) and a ultraviolet spectrophotometer (DU-600, Beckman Coulter, U.S.A) as well as a Flow cytometer (FACSCalibur, Becton Dickinson, Germany).

Methods

The separation and culture of BMSCs: After anaesthetizing the rats with ketamine, the femurs and tibias of the rats were obtained, and then the marrows were washed out using the sterilized phosphate buffer saline. After fully oscillation, the lymphocyte separation liquid was added into the marrow solution at a ratio of 2:1 and then was fully mixed. Afterwards, the mixture solution was centrifuged at 1,006.2 g for 30 min, after which the individual cells in the interface of the liquids were extracted and transferred to DMEM culture media. Then, the cells were subjected to primary culture in an incubator until achieving 80% of cell fusion. Subsequently, the cells were subjected to subculture using 0.25% trypsin. During the primary culture, the culture media was renewed every three days.

The separation and culture of the cartilage cells: After anaesthetizing the rats, their cartilage tissues were obtained and cut into the dices with a volume of roughly 1 mm^3. Then, 0.25% trypsin was used to digest the dices for 30 min. After centrifuging the matrix at 1,500 rmp for 10 min, the supernatant fraction was removed, and subsequently 0.2% of NB4 collagenase was added. The matrix was incubated in a thermostatic oscillator at 37°C for 2 h, after which the matrix was centrifuged at 1,500 rmp for 10 min. The cartilage cells were then inoculated into a culture dish with a diameter of 100 mm at a density of about $2.5*10^6$. Subsequently, 8 mL cell culture liquid was added into the dish, which was then subjected to conventional incubation.

The induction experiment: The BMSCs with the added TGF-β1 (10 μg/L) and BMP-2 (100 μg/L) was taken as the experimental group. The BMSCs without any added TGF-β1 and BMP-2 was taken as the negative control. Meanwhile, the cartilage cells served as the positive control. They were induced for 7 d and 14 d, respectively.

The detection of CD genotype on the surface of the BMSCs: The BMSCs was digested by adding 0.25% pancreatin and 0.02% EDTA for 5 min, and then the process was terminated by adding complete medium. Subsequently, the cell concentration was adjusted to approxi-

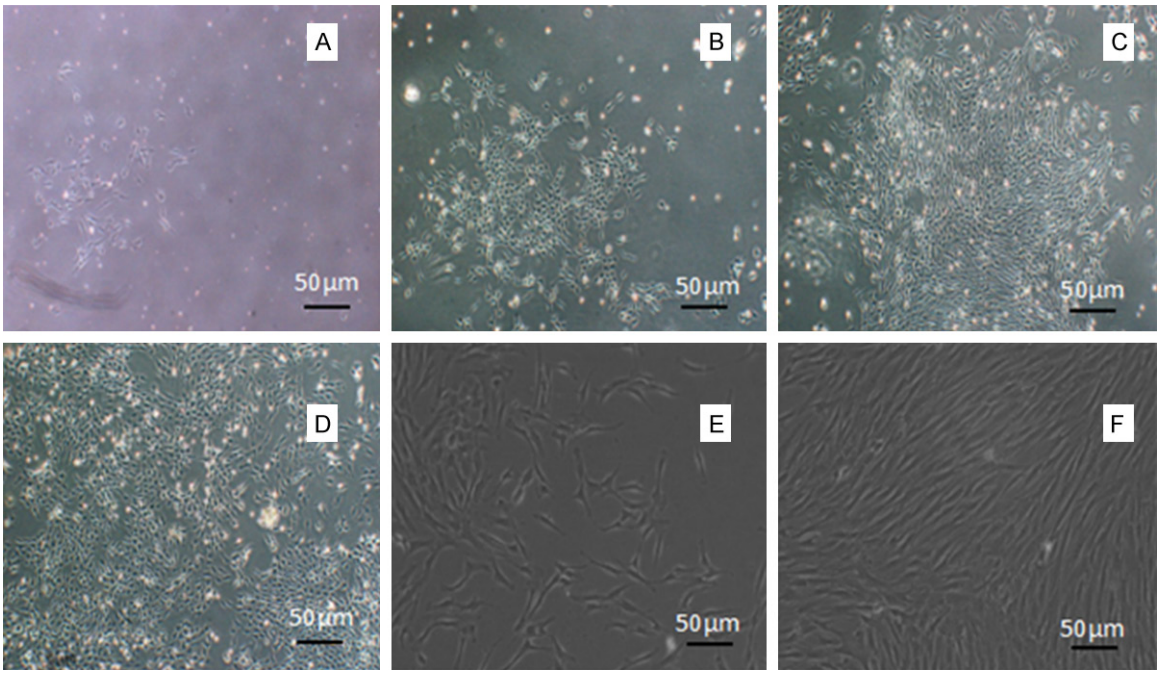

Figure 1. The morphology of the BMSCs. (A) The morphology of the BMSCs subjected to primary culture (after 3 d); (B) The morphology of the BMSCs after 5 d; (C) The morphology of the BMSCs after 7 d; (D-F) The morphology of the P1 generation of BMSCs. The magnification of (A-D) is 40×, and the magnification of (E, F) is 100×. BMSCs, bone mesenchymal stem cells.

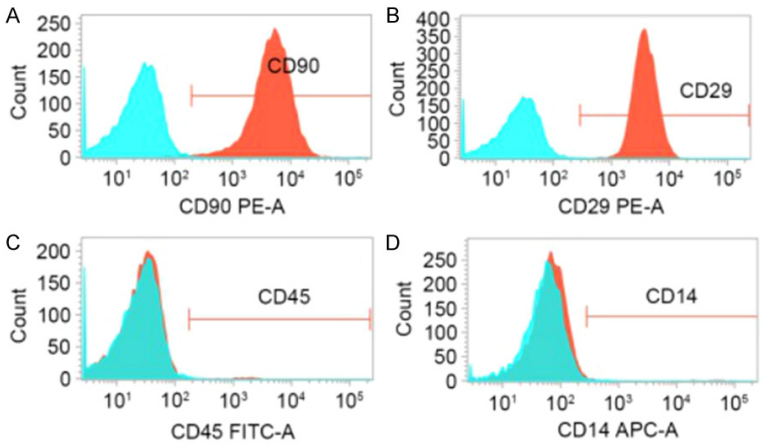

Figure 2. The CD phenotype on the surface of the BMSCs. A: CD11; B: CD45; C: CD29; and D: CD90. BMSCs, *bone mesenchymal stem cells.* CD, *cluster of differentiation.*

mately 1.0*10^7 per mL. Then, 1.5 μL PE-labeled CD11b/c, CD45, CD29, CD90 was added to 100 μL cell suspensions, respectively, and the suspension was incubated under a light-proof environment at room temperature for 30 min. After that, PBS solution was used to wash the suspension at least twice. Prior to conducting the flow cytometry analysis, the cells should be re-suspended using 0.3 mL PBS solution.

The identification of the differentiation of BMSCs into cartilage cells: The BMSCs was inoculated into a 12-well culture plate at a density of 5*10^3 per cm^2 for subculture. When the BMSCs reached 60% fusion, the chondrogenic differentiation liquid was added. The induction process lasted for two weeks, during which the chondrogenic differentiation liquid was renewed twice a week. After the culture liquid was removed and the cells were washed with PBS solution, 4% paraformaldehyde was added and the matrix was hold for 15 min so as to fix the cells. Subsequently, 1% Borax - toluidine blue solution was added to stain the cells for 20 min, after which the cells were washed with PBS solution at least twice. Then, the cells were subjected to microscope observation.

The detection of type II collagen by using Western blot: After inducing the BMSCs for 7 d, PBS solution was used to wash the cells, includ-

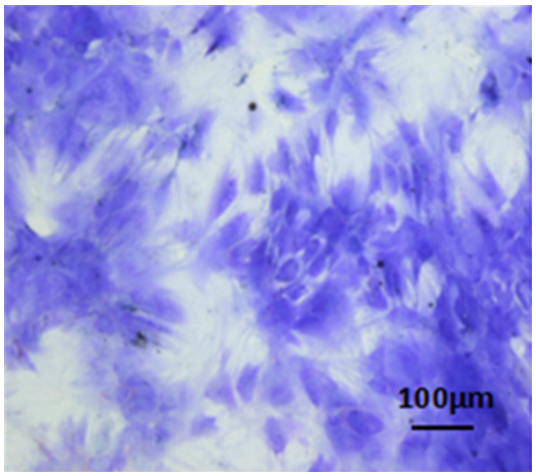

Figure 3. The histochemical detection result of the BMSCs after toluidine blue staining. The blue color represents the matrix compositions of the cartilage cells. BMSCs, bone mesenchymal stem cells.

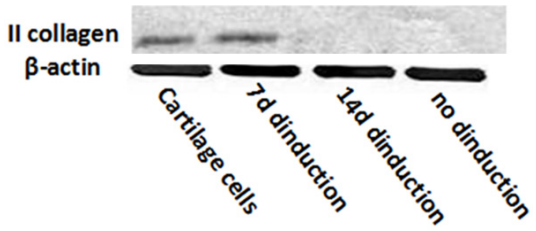

Figure 4. The Western blot detection of type II collagen before and after the induction of the BMSCs by TGF-β1 and BMP-2. BMSCs, bone mesenchymal stem cells; TGF-β1, transforming growth factor β1; BMP-2 bone morphogenetic proteins-2.

ing the experimental group, the positive control group and the negative control group. Then, 2 mL RIPA lysis buffer was added into the cells. After homogenization, ice bath (30 min), and centrifugation (1,000 r/min for 3 min), the supernatant was collected and then subjected to ultrasonic treatment so as to fully broke nucleus. After centrifuging the mixture at 12,000 rmp at 4°C for 5 min, 10 μL supernatant was collected for detection of protein concentration. Briefly, after the separating gel and spacer gel were prepared, the supernatant was subjected to electrophoresis (the voltage was 120 V) and transmembrane. The excessive part of the membrane was cut off and the direction was also labeled. After adding the blocking liquid and holding the membrane for 1 h, the rabbit anti human type II collagen antibody (1:1,000) and HRP labeled secondary antibody (1:3,000) were added to conduct Western Blot detection. A Bio-Rad Chemi Doc MP was used for imaging analysis and Image Lab Software (Version 2.0.1, Bio-Rad) was used to detect the optical density.

The detection of mRNA of type II collagen by using reverse transcription polymerase chain reaction (RT-PCR): After inducing the BMSCs for 21 d, 0.5 mL Trizol was added to the cells, which was then repeatedly crashed so as to separate nucleic acid. Subsequently, chloroform and isopropanol were in sequence added into the solution, after which the solution was centrifuged at 12,000 g at 4°C for 10 min. After removing the supernatant, 1 mL 75% ethanol which contained 0.1% DEPC was added to the precipitate to wash the RNA. After that, the solution was centrifuged at 8,000 g at 4°C for 5 min, after which the supernatant was removed and the precipitate was collected and subjected to natural air drying. Subsequently, after 200 μL DEPC solution was added into the dried RNA precipitate, an ultraviolet spectrophotometer was used to detect RNA concentrations. The first strand cDNA was synthesized by using a Kit, and the synthesized product was then subjected to RT-PCR amplification. Then, 2% agarose gel electrophoresis was conducted and the result was analyzed by the gel imaging analysis system. The result was expressed as the ratio of the integral absorbance of type II collagen to the integral absorbance of the reference gene β-actin. The detailed information is exhibited in **Table 1**.

Statistical analysis

All the data were analyzed using SPSS 23.0 software. The expression results of type II collagen and its mRNA expression levels were presented as the mean ± standard deviation. The difference between groups was analyzed with independent t-test. $P<0.05$ indicates a statistically significant difference between groups.

Results

The morphology of BMSCs

As shown in **Figure 1**, after the marrow suspensions were subjected to adherent culture for 24 h, it can be observed that part cells showed obvious growth. After 3 d, there was spindle or fusiformis shaped BMSCs observed (**Figure 1A**). Besides, a small amount of BMSCs showed

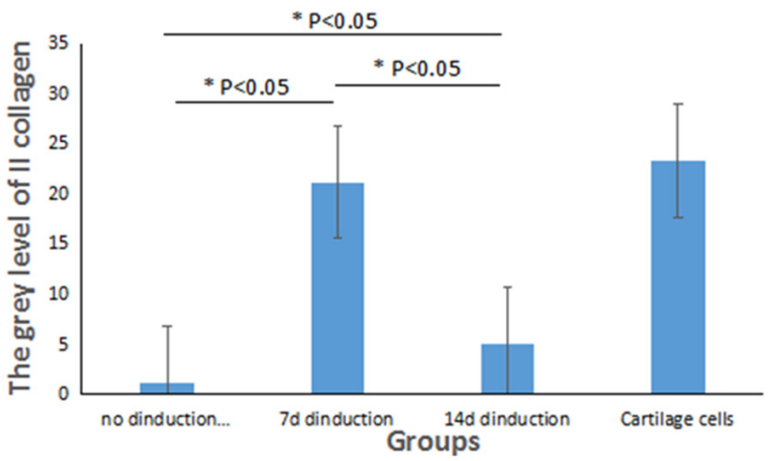

Figure 5. The gray level analysis of type II collagen staining. *P<0.05 means a significant difference between groups.

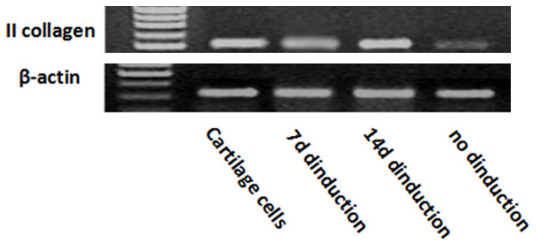

Figure 6. The RT-PCR result of the genes related to type II collagen before and after the induction of the BMSCs. BMSCs, bone mesenchymal stem cells; RT-PCR, reverse transcription polymerase chain reaction.

polygon or irregular shapes, with the significantly observed nucleus and a high nuclear-cytoplasmic ratio. Meanwhile, some small cellular clusters were observed to begin to form. With the increased culture time, the BMSCs became bigger (**Figure 1B**, **1C**), showing the feature of typical fusion growth. When cultured for 7-10 d, the fiber-shaped BMSCs gradually fused and formed sheet-like morphology, indicating a flourishing growth. Besides, the BMSCs subjected to subculture showed uniform fusiformis morphology, with a relatively dense distribution (**Figure 1D-F**), suggesting the cells had good viability and were suitable for the further functional study.

CD genotype on the surface of the BMSCs

The detection results of flow cytometry are exhibited in **Figure 2**. The results suggested there was no observed expression of CD11b/c and CD45, but the expression of CD29 and CD90 was observed. The result was in line with the phenotypic traits of CD of the BMSCs.

The identification of histochemistry and cytochemistry

As shown in **Figure 3**, the BMSCs induced by TGF-β1+ BMP-2 showed rounder cell body, less cytoplasm and smaller nucleus. After toluidine blue staining, there was significant acidic glycosaminoglycan compositions (blue color in **Figure 3**), indicating that the cultured BMSCs can be induced to differentiate into cartilage cells.

The expression of type II collagen

After inducing the BMSCs by TGF-β1 and BMP-2 for 7 d, the western blot was performed and the results are shown in **Figures 4** and **5**. The results suggested that the induced BMSCs can express type II collagen, and the expressed results were in line with the normal cartilage cells. In comparison, the negative group showed no expression of type II collagen, and the difference between the induced group (the positive control) and the non-induced group (the negative control) was statistically significant (t=24.17, t=5.58 and P<0.05).

The expression of genes of type II collagen

After inducing the BMSCs for 14 d, we performed the RT-PCR analysis and the result is shown in **Figures 6** and **7**. The results indicated that the amplified product of the genes of type II collagen was detected, which was agreement with the normal cartilage cells. However, there was no amplified product observed in the non-induced group (the negative control). The difference between the induced group and non-induced group was statistically significant (t= 19.86, t=20.03, P<0.05).

Discussion

Stem cells refer to the cells that have the ability of multiple directional differentiation and self-proliferation. Usually, stem cells are inactive, whereas when the organs are damaged, stem cells can differentiate into the related cells to amend the damaged ones [9]. At present,

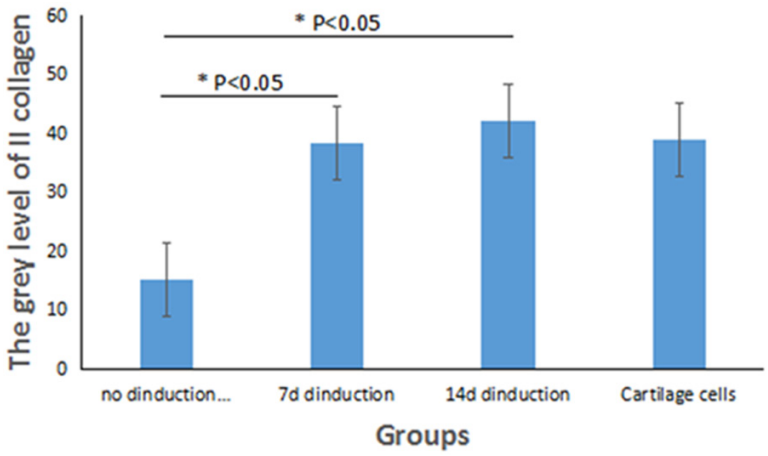

Figure 7. The gray level analysis of type II collagen staining. *P<0.05 means a significant difference between groups.

BMSCs is still the focus of the investigation of adult stem cells. In this study, we found that P2-P3 of BMSCs showed relatively flourishing growth and high proliferation rate. Even when the cells were subcultured at a ratio of 1:10-15 for a week, the fusion of the BMSCs could be up to 90%.

The BMSCs in human have the following features: (1) the good adhesion under the standard culture conditions, (2) being able to express CD105, CD73 and CD90, but did not express CD34, CD45, CD11a and HLA-DR, (3) can differentiate into bone cells, cartilage cells and fat cells [10-13]. In this study, the analysis results of flow cytometry revealed that the BMSCs expressed CD29 and CD90 but did not express CD11b/c and CD45, confirming the obtained cells was BMSCs.

TGF-β1 is a polypeptide and usually regarded as an important growth factor [14]. TGF-β1 participates in various biological activities, not only promoting cell proliferation but also regulating cell differentiation, including the differentiation of cartilage cells [15]. The surface of BMSCs can excrete and express the receptors of TGF-β1, thus enabling TGF-β1 to induce BMSCs to differentiate into cartilage cells [16]. Besides, TGF-β1 can also synthesize type II collagen and proteoglycan, thereby maintaining the stable phenotype of chondrocytes [17]. Nixon et al., reported that TGF-β1 could promote the differentiation of BMSCs into cartilage cells [18].

BMP-2 is a commonly used growth factor in bioengineering and is also related to the cell cycle of cartilage cells. Besides, it plays an important role in the differentiation of stem cells. In brief, BMP-2 can regulate the formation of cartilage matrix, especially playing a crucial role in the generation of glycosaminoglycan. In addition, BMP-2 can also promote the expression of Sox9 and Noggin. For example, there are some reports revealing that a potent regulatory mechanism of the differentiation of cartilage cells can be achieved by using Sox9 to control Noggin [19, 20].

The major compositions of cartilage include collagenous fibers and proteoglycan. Cartilage cells can excrete collagen protein and acid mucopolysaccharide. Collagen protein and acid mucopolysaccharide are main composition of type II collagen, and thus they are usually regarded as the marker of cartilage cells. In this study, after inducing BMSCs by TGF-β1 and BMP-2 for 14 d, we observed that the morphology of BMSCs changed from its original fusiform shape to polygon. Additionally, the positive result of toluidine blue staining suggested that the differentiated BMSCs can excrete glycosaminoglycan, and the western blot and RT-PCR analysis results also revealed the induced BMSCs could express type II collagen. Hence, these results may suggest that TGF-β1 and BMP-2 can induce the differentiation of BMSCs into cartilage cells.

Overall, although TGF-β1 and BMP-2 can induce BMSCs to differentiate into cartilage cells, the mechanism needs to be further investigated because the directional differentiation of BMSCs is a rather complex process.

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Disclosure of conflict of interest

None.

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