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Overexpression of Bone Morphogenetic Protein-1 Promotes Osteogenesis of Bone Marrow Mesenchymal Stem Cells *In Vitro*

Authors' Contribution:

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Data Collection B

Statistical Analysis C

Data Interpretation D

Manuscript Preparation E

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Background: Osteogenesis of bone marrow mesenchymal stem cells (BMSCs) is an important research topic in the application of bone tissue engineering. Bone morphogenetic protein-1 (BMP-1) is important in bone formation and stability, but its effects on the osteogenesis of BMSCs are unclear. This study aimed to investigate the association of BMP-1 with the osteogenic capacity of BMSCs.

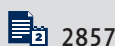
Material/Methods: Primary rabbit BMSCs were cultured and divided into a BMP-1-overexpressing group, a Green Fluorescent Protein-expressing (GFP) group, and a Control group. The transfection efficiency of BMP-1 was tested by Western blotting. Cell viabilities, alkaline phosphatase (ALP) activities, Ca²⁺ concentrations, and gross examinations of BMSC sheets were examined at different times. The osteogenic marker collagen I was assessed by immunohistochemical analysis.

Results: The cell viability, ALP activity, and Ca²⁺ content of the BMP1-overexpressed group were significantly enhanced compared with the GFP group and Control group. Immunohistochemistry staining results showed that BMP-1 promoted the expression of type I collagen in BMSCs sheets.

Conclusions: Our results suggest that the overexpression of BMP-1 can promote the osteogenesis of BMSCs and provides an improved method of cell-based tissue engineering.

MeSH Keywords: **Alkaline Phosphatase • Bone Morphogenetic Protein 1 • Immunohistochemistry**

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Background

With the deepening of stem cell and regenerative medicine research, cell-based tissue engineering bone research and development have become the fastest growing and most active field [1]. Bone marrow mesenchymal stem cells (BMSCs) are considered to be clinically valuable tissue-engineered seeding cells due to their multi-directional differentiation potential [2]. Their effects on osteogenesis and bone repair have been extensively studied [3,4]. However, researchers found that BMSCs are only partially transformed into osteoblasts after transplantation, so the osteogenic capacity is weak [5]. Therefore, enhancing the osteogenic differentiation of BMSCs to improve their osteogenic efficiency is an important research focus.

Bone morphogenetic proteins (BMPs), as osteogenic factors, have been verified to play important roles in bone remodeling and regeneration [6]. More than 20 kinds of BMPs have been identified [7], and some of them, like BMP2 and BMP7, have been experimentally confirmed to play an essential role in osteogenesis [8,9]. Bone morphogenetic protein-1 (BMP-1) is an important bone morphogenetic factor. The research interest in BMP-1 is in proteolytic removal of the C-propeptides to make the major fibrillar collagen types I–III stable, and it is essential for the assembly of mature collagen monomers into fibrils [10]. Deficiency of BMP-1 delays cleavage of type I procollagen C-propeptide and hampers the processing of the small leucine-rich proteoglycan [11]. Also, it is importance for bone formation and stability, and the molecular and cellular bases of BMP-1-dependent osteogenesis has been defined [12]. Wang et al. [13] knocked out the BMP-1/mammalian tolloid-like 1 (TLL1) gene of mice and found the crucial roles of BMP-1/TLL1 in root formation and dentin mineralization. Many studies have also reported that BMP-1 is involved in bone diseases [14,15], but the effects of BMP-1 on osteogenesis of BMSCs have been unclear.

In the present study, BMP-1 was overexpressed in a recombinant lentivirus carrying the BMP-1 gene, and the transfection efficiency was verified. Then, cell viability of BMSCs, gross examinations, ALP activity, Ca²⁺ concentration, and the expression of collagen I were assessed. The role of BMP-1 in the osteogenesis of BMSCs was verified.

Material and Methods

Cell cultures

Rabbit BMSCs were extracted according to published methods with some modification [16]. Briefly, the bilateral tibia of New Zealand rabbits was excised and dissected on a clean bench. The medullary cavity was flushed out with low-glucose

Dulbecco's modified Eagle's medium (DMEM) containing 200 U/mL heparin. After filtration using a mesh, it was centrifuged at 800 rpm for 5 min. The upper-fat droplets and the supernatant were discarded, and 20 mL of a low-sugar DMEM (containing 10% fetal bovine serum, 100 U/mL penicillin, and 0.27 g/L of L-glutamine) was added. It was gently blown into a single-cell suspension and uniformly inoculated into 2 Petri dishes having a diameter of 10 cm. The culture solution in each dish was added to 12 mL and placed in an incubator at 37°C and 5% CO₂. Half of the culture solution was replaced 48 h after incubation. Then, the entire culture solution and floating cells were removed after another 48 h. The remaining adherent cells were defined as BMSCs [17]. Then, every 2 days, the entire culture solution was changed once, and cell growth was observed under an inverted microscope.

BMP-1 transfection

Lentivirus vectors were constructed with the assistance of Genechem (Shanghai, China). Lentivirus packaging was performed using 293 cells according to the manufacturer's instructions and then screened for the appropriate multiplicity of infection (MOI=10). All cells were divided into 3 groups. The BMP-1 group was transfected with Lenti-BMP-1, the GFP group was transfected with blank lentivirus vectors, and the Control group remained untransfected. The effects of transfection and the cell conditions were observed by fluorescence microscopy.

Transfection efficiency verified by Western blotting

The proteins of the 3 groups were extracted, and Western blotting (WB) was used to verify the transfection efficiency. We took 30 µg of protein from the Control group, GFP group, and BMP-1 group, added 5×protein loading buffer corresponding to 1/4 volume of a protein sample, boiled it for 5 min in a boiling water bath, and then performed SDS-PAGE. Proteins were separated by electrophoresis (5% laminating gel, 10% separation gel, laminated glue voltage 80 V, separation gel voltage 100 V, electrophoresis time 120 min). The protein was transferred to a polyvinylidene difluoride (PVDF) membrane by wet transfer method (constant pressure 100 V, transfer film 150 min, transfer buffer methanol concentration 20%). After the transfer, the PVDF membrane was stained in Ponceau red staining solution for 2–5 min to observe the protein transfer effect. The upper right corner of the PVDF membrane was labeled, and the PVDF membrane was transferred to a TBST (Tween-20 concentration 0.05%) blocking solution containing 5% BSA and blocked at room temperature for 1.5 h. The antibody BMP-1 (ab205394) was diluted 1: 1000, and the antibody β-actin (ab8226) was diluted 1: 500 in a blocking solution. The PVDF membrane was placed in a dish, and a dilution of the primary antibody was added for overnight incubation at 4°C. The next day, the membrane was washed with TBST

for 10 min×4 times. The PVDF membrane was placed in a dish containing diluted secondary antibody and incubated for 1 h on a decolorizing shaker. After incubation, the membrane was washed with TBST for 10 min×3 times. In a dark room, the liquid on the membrane was gently blotted with a filter paper, and allowed to react for 2–3 min in the enhanced chemiluminescence reaction mixture, and then developed by tableting.

Cell sheets preparation

The harvested BMSCs were inoculated into a petri dish at a density of 1×10^5 cells/cm². Incubation was continued for 2 days (inoculation of cells adherent) with low-glucose DMEM containing 10% fetal bovine serum. Then, the culture solution was changed to a high-glucose DMEM-inducing culture solution containing an osteogenic inducer containing 10% fetal bovine serum, dexamethasone 8 mol/L, β -glycerophosphate 10 mmol/L, and ascorbic acid 50 mg/L. The cultivation was continued at 37°C with 5% CO₂. The culture solution was changed once every 2–3 days. After 2 weeks of continuous culture, a translucent milky cell sheet was observed at the bottom of the culture dish. The gross examination of cell sheets was observed in a general view, and the thickness of the cell membrane was measured under a microscope.

Cell viability

Cell viability assays were performed using the Cell Counting Kit-8 (CCK-8) kit. The cell suspension (100 μ L/well) was seeded in a 96-well plate and pre-cultured in an incubator (37°C, 5% CO₂). After inoculation of cells, 10 μ L of CCK solution was added on days 2, 3, 4, 5, 6, 7, 8, 9, and 10, and after 4 h of culture, the absorbance value was measured at 450 nm. The cell viability of each group was calculated by a standard curve.

Alkaline phosphatase activity

The activity of ALP was detected according to the instructions of the alkaline phosphatase assay kit (Beyotime). Cell sheets of the BMP-1 group, GFP group, and Control group were obtained and minced. The cells were rinsed with PBS and lysed with 0.2% Triton X-100. The samples were centrifuged, and the supernatant was removed. The reaction solution was added and allowed to react at 37°C for 30 min. The absorbance of light was measured by an enzyme-linked detector (405 nm wavelength) for quantitative detection of ALP activity. The alkaline phosphatase activity in the sample was calculated with reference to a standard curve. Each sample was measured 3 times.

Calcium concentration examinations

The calcium concentration was detected according to the instructions of the calcium concentration assay kit (Beyotime).

Cell sheets of the BMP-1 group, GFP group, and Control group were obtained and minced. The lysis buffer was added in a ratio of 100–200 μ L per 20 mg of tissue. The sample was homogenized until fully cleaved. The supernatant was collected after centrifugation at 10 000–14 000 g for 3–5 min at 4°C, then 50 μ L of standard (for standard curve) or sample was added to each well in a 96-well plate. We added 150 μ L of the test solution and incubated it at room temperature for 5–10 min in the dark. The absorbance was measured at 575 nm. The calcium concentration in the sample was calculated with reference to a standard curve. Each sample was measured 3 times.

Histochemical analysis

Hematoxylin-eosin staining

The cultured sheets were randomly selected and fixed with 4% paraformaldehyde. After gradient alcohol dehydration and conventional paraffin embedding, the sheets were cut into 5- μ m-thick sections. Tissue structure was observed using conventional hematoxylin-eosin staining (H&E). At the same time, we selected the “Z” axis profile of the sheets. The thickness of the cell membrane was measured under a microscope at randomly selected points and averaged.

Immunohistochemistry

Cell sheets were fixed with 4% paraformaldehyde and cut into 5- μ m paraffin sections. The sections were heated at 60°C for 1 h, and then rehydrated with a gradient of xylene and absolute ethanol, and washed with distilled water for 1 min. The sections were placed in citrate (pH 6.0) antigen retrieval solution for high-pressure repair for 2 min, followed by washing with PBS 3 times for 5 min each time, after which 3% H₂O₂ was added dropwise, and then the sections were placed in a wet box, allowed to stand at room temperature for 30 min, and washed again with PBS. After the goat serum blocking solution was added dropwise, the sections were placed in a wet box, allowed to stand at room temperature for 1 h, and decanted without washing. The primary antibody diluted 1: 500 was added to the tissue and left overnight in a 4°C wet box. The next day, after rewarming for 30 min, the primary antibody was aspirated and washed with PBS. The biotinylated secondary antibody working solution was added to the tissue and placed in a wet box at room temperature for 30 min. We discarded the secondary antibody and washed the sections 4 times in PBS for 5 min each time. HRP-labeled streptavidin working solution was added to the sections, and the sections were placed in a wet box at room temperature for 20 min. Freshly prepared DAB coloring solution was added dropwise to the sections and sealed with neutral gum. The color development time was controlled under the microscope, and the positive color was brown.

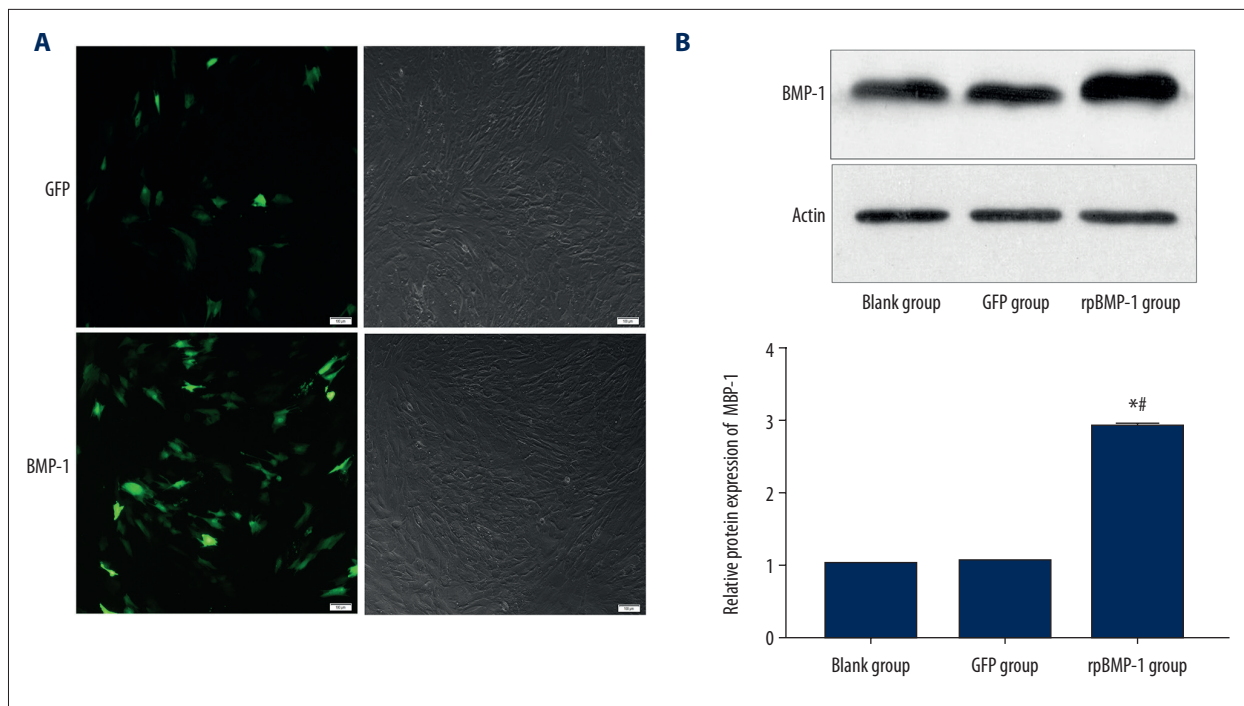


Figure 1. Transfection efficiency was assessed by fluorescence microscopy and Western blotting. BMP-1 and GFP genes were transfected successfully (A). The expressions of BMP-1 protein was increased after transfection (B). * $p < 0.05$ compared with the Control group, # $p < 0.05$ compared with the GFP group.

Statistical analysis

All data are expressed as mean values with standard deviation (mean \pm SD). Differences in the data among each group were analyzed by one-way analysis or two-way analysis of variance (ANOVA) using SPSS version 19.0. $P < 0.05$ was considered statistically significant.

Results

Transfection efficiency

After transfection of the recombinant plasmid of BMP-1 or GFP, we verified the transfection efficiencies. The fluorescence pictures in Figure 1A show that the BMP-1 or GFP had been successfully transfected. Also, the WB results in Figure 1B show that the expressions of BMP-1 proteins in the BMP-1 group were markedly upregulated compared with the GFP group and Control group.

Gross examination of BMSCs sheets

After inoculation into the culture dish, the cells gradually grew over the bottom of the culture dish, the layer became thicker, and the transmittance of the cells was gradually reduced as assessed under the microscope. The gross examination of BMSCs sheets is shown in Figure 2A, demonstrating that the

sheet in the BMP-1 group (60.37 ± 8.28) was markedly thicker than in the GFP group (23.81 ± 0.77) (Figure 2B).

Cell viability, ALP activity, and calcium concentration of BMSCs

The cell viabilities of 3 groups at the different timepoints are shown in Figure 3A. From day 4, the cell viability of the BMP-1 group significantly increased relative to the GFP group and Control group. There was no difference between the GFP group and Control group.

During incubation of the cell sheets, we examined the osteogenic-related indicators ALP, Ca^{2+} , and type I collagen at different timepoints. As shown in Figure 3B, the ALP activity of the BMP-1 group was significantly upregulated compared with the GFP and Control group at all timepoints, which means the overexpression of BMP-1 promotes the differentiation of pre-osteoblasts into mature osteoblasts. There was no difference between the GFP group and Control group.

The Ca^{2+} concentrations in the 3 groups were assessed, and the results are shown in Figure 3C. From the day 10, the Ca^{2+} concentration of the BMP-1 group showed significant increases compared with the GFP and Control group, which means that the mineralization of the BMP-1 group was significantly higher than in the other 2 groups.

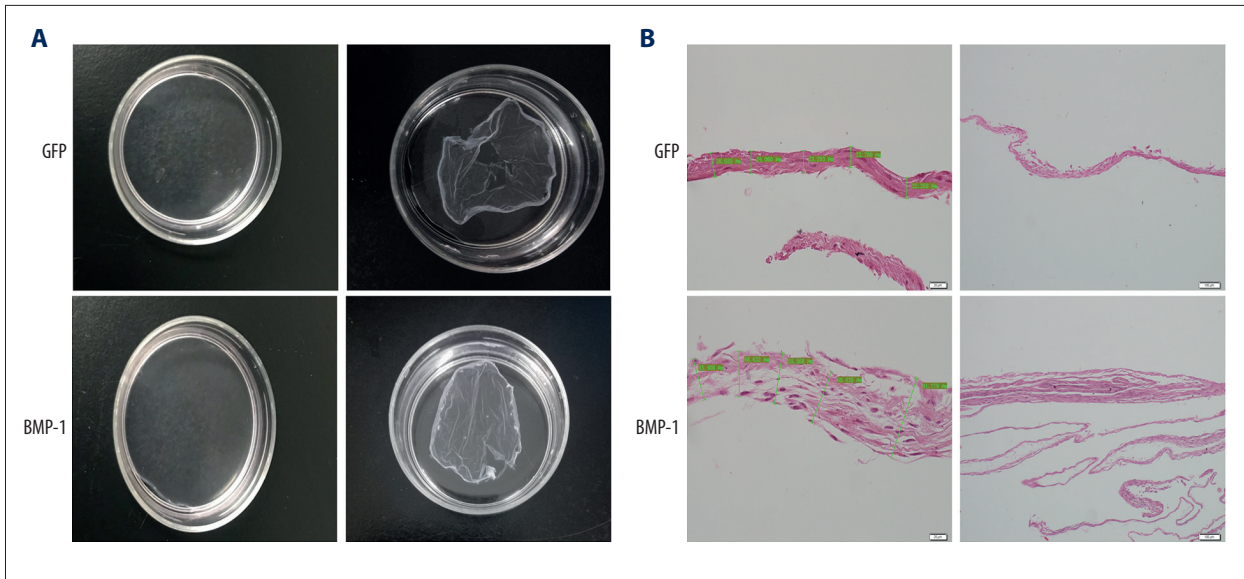


Figure 2. Gross examination of BMSCs sheets. The cells layered multiply and the transmittance of the cells were gradually reduced, as seen under the microscope (A). The sheets were thicker in the BMP-1 group compared with the GFP group (B).

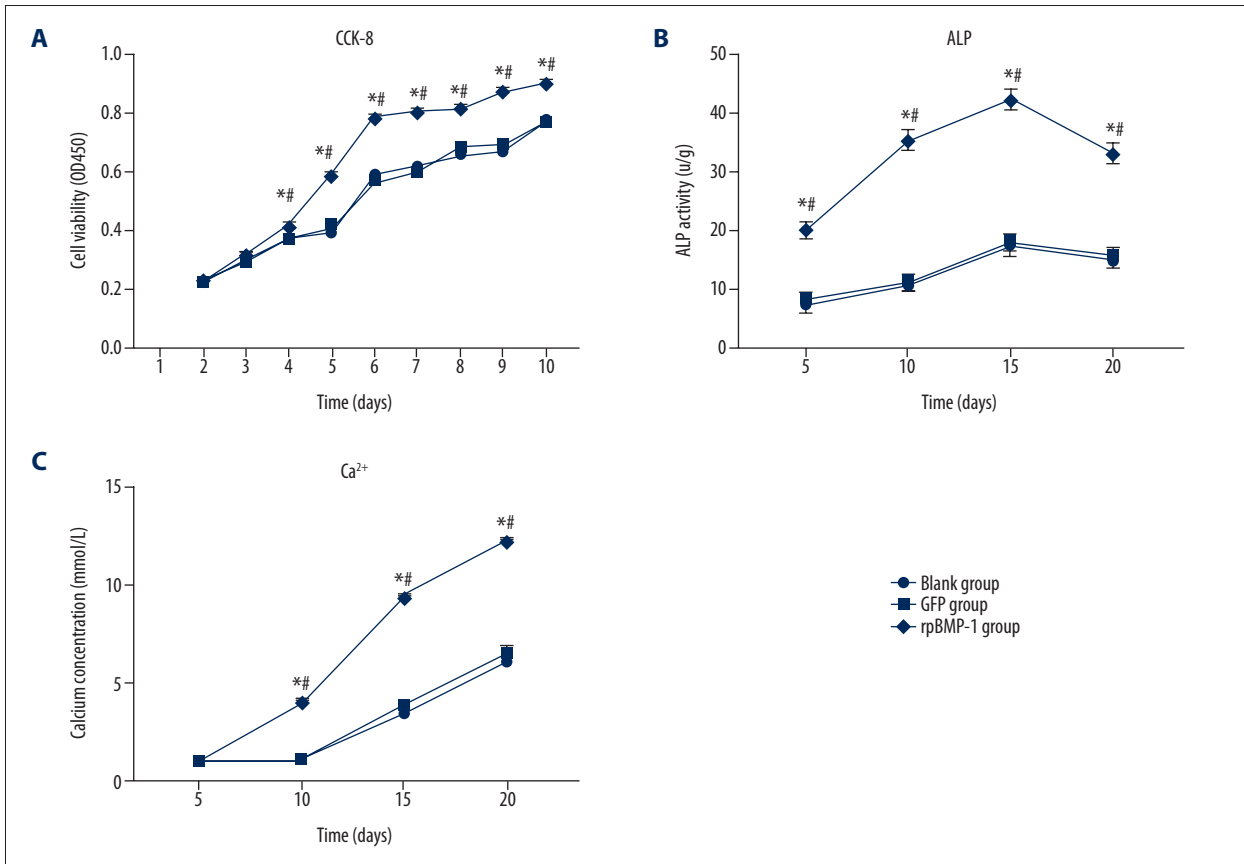


Figure 3. Cell viability, ALP activity, and Ca²⁺ concentration of BMSCs were assessed. The cell viability (A), ALP activity (B), and Ca²⁺ concentration (C) of the BMP-1 group were significantly higher compared with the GFP group and Control group. The assays were repeated 3 times. * p<0.05 compared with the Control group, # p<0.05 compared with GFP group.

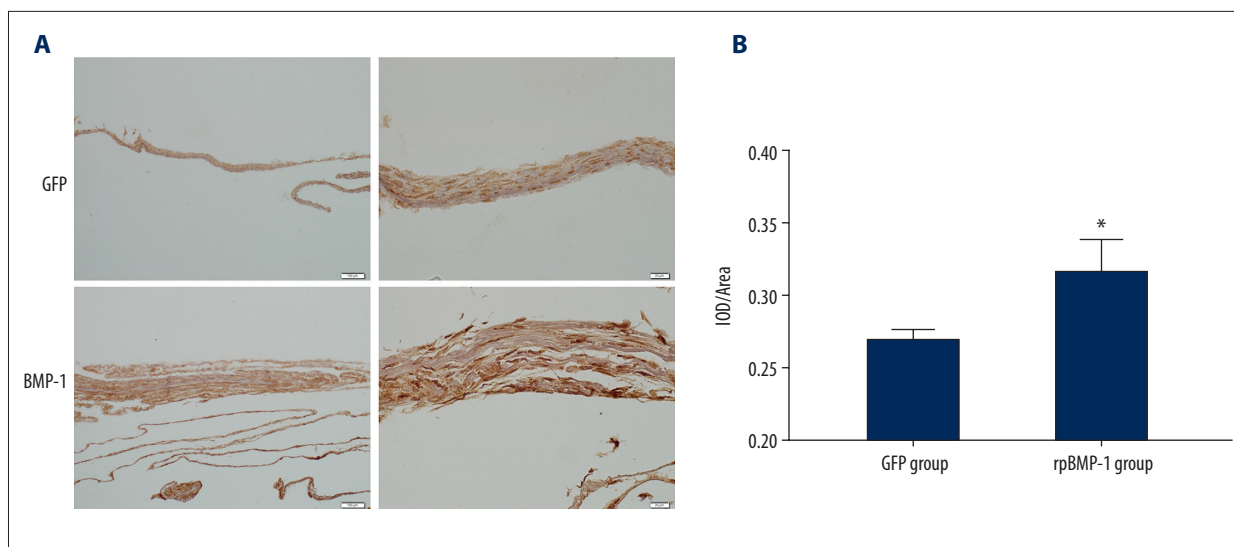


Figure 4. (A, B) Collagen I expression of BMSCs sheets. After transfection of BMP-1, the expression of collagen I was markedly increased compared with the GFP group. * $p < 0.05$ compared with the GFP group.

Collagen I expression of BMSCs sheets

Type I collagen is the most crucial fibrous collagen component in bone matrix. It is a specific and important indicator of osteogenesis. The expression of type I collagen was detected by immunohistochemistry, and the results are presented in Figure 4. The pictures show that the expression of type I collagen of BMP-1 group was markedly increased compared with the GFP group.

Discussion

Due to its multiple-differentiation potential, BMSCs are extensively used in cell-based tissue engineering research [18]. BMSCs have been used in cell substitution therapy and tissue engineering, especially in tissue-engineered bone [19,20]. There are also many studies that enhanced the osteogenic efficiency of BMSCs by gene editing [21,22]. In the present study we overexpressed BMP-1 by gene editing and determined its role in the osteogenesis of BMSCs. We extracted BMSCs from rabbit tibia. Then, BMP-1 was overexpressed using lentiviral vectors, and the fluorescence results and WB results showed that the transfection efficiency was high. We compared the gross examinations of cell sheets in the Control group, the GFP group, and the BMP-1 overexpression group, then we examined the osteogenesis-related indicators, ALP, GA^{2+} concentration, and COL I expression. In general, we demonstrated that overexpression of BMP-1 promoted the osteogenesis of BMSCs.

BMPs (also known as cytokines) regulate osteogenesis during the differentiation of BMSCs to osteoblasts [23,24]. BMP-2, BMP-4, and BMP-7 have been widely studied, showing that

recombinant human BMP-4 more strongly promotes spinal fusion than does recombinant human BMP-2 [25,26]. BMP-1 is not a typical BMP and has received little research attention. Some clinical studies showed that mutation of BMP-1 can lead to osteogenesis imperfecta [27], and osteogenesis imperfecta patients with BMP-1 mutations tend to have recurrent fractures, generalized bone deformity, or osteopenia [28]. Moreover, the importance of BMP-1 for bone formation and stability has been established [12]. We hypothesized and demonstrated that overexpressed BMP-1 can promote the osteogenesis of BMSCs.

Lentivirus is a type of retrovirus that has a broad host range and is capable of infecting both dividing cells and non-dividing cells. Compared to other virus tools, lentivirus has the following advantages: a) it can achieve long-term stable expression of the target gene by integrating the foreign gene into the host cell genome, and is not lost as the cell divides and passes; b) it is very safe and has no pathogenicity; and c) it has low immunogenicity and can be directly injected into living tissue to cause an immune reaction, which is suitable for animal experiments. In addition, some studies showed that lentiviral vectors are superior to other vectors [29]. Thus, in the present study, we used lentiviral vectors for transfection. Regarding the other transfection vectors, one study used the Adeno-associated virus (AAV) virus vector system to construct the AAV-BMP-4/7 vector, making the screening of recombinant vectors more straightforward, and they successfully built the recombinant AAV-BMP-4/7 virus that carried the BMP-4/7 fusion gene in a relatively short time [30]. This may provide a new tool for BMPs-related gene research.

As in other studies on BMSCs, obtaining an adequate amount of BMSCs was difficult. The content of BMSCs with osteogenic

potential is only 0.001–0.01% of the total amount of bone marrow nucleated cells. Further separation and purification are required to obtain a sufficient concentration of BMSCs, and the proliferation and differentiation abilities of BMSCs are reduced by *in vitro* culture and expansion [31]. A study suggested that mesenchymal stem cells (MSCs) can be isolated from virtually any tissue and can form cartilage, bone, and fat [32]. However, some researchers pointed out that MSCs are rarely applied to clonal populations, and the effects on bone formation cannot be proven by BMP treatment or genetic engineering [33].

Many studies focus on treating cells with the forced expression of bone morphogenetic proteins by gene editing, but this genetic technic has limits. Stephan et al. [34] analyzed the maximal effects of BMP-2 on osteochondral remodeling, and found that BMP-2 could not induce complete healing despite stable expression. How to ensure the efficiency and stability of gene editing technology application is an important issue. The combination with bone tissue engineering technology had shown potential. Eguchi et al. [35] showed that composite materials

containing recombinant human BMP-2 increased the generation of bridging callus and the callus mass in the bone defect. However, the immunogenicity of the carrier material and the toxicity of degradation products are also factors that influence the technical effect. The use of gene editing technology and improvement of the combination of gene editing with bioengineering technology warrant further research.

Conclusions

This study confirmed that the overexpression of BMP-1 can promote the osteogenesis of BMSCs. We showed that BMP-1 has the biological ability to induce BMSCs to form bone, which provides a potential method of cell-based tissue engineering.

Conflict of interests

None.

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