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Adenovirus-mediated bone morphogenetic protein-2 promotes osteogenic differentiation in human mesenchymal stem cells *in vitro*

HONG CAO[1*], ZHI-BO SUN[1*], LEI ZHANG[1], WEI QIAN[1], CHUN-YANG LI[2], XIAO-PENG GUO[3] and YING ZHANG[2]

Departments of [1]Orthopedic Surgery and [2]Reproductive Medicine, Renmin Hospital; [3]Department of Orthopedic Surgery, Taihe Hospital, Hubei University of Medicine, Shiyan, Hubei 442000, P.R. China

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Abstract. Delayed and failed bone union following fracture is a common clinical complication that requires treatment in orthopedics. Cell-based therapies and tissue-engineering approaches are potential therapeutic strategies for bone repair and fracture healing. However, the effect of adenovirus expressing bone morphogenetic protein-2 (Ad-BMP-2) on the osteogenic ability of human mesenchymal stem cells (hMSCs) has remained to be fully elucidated. Therefore, in the present study, hMSCs were transduced using Ad-BMP-2 to assess the effects of its application and to determine whether Ad-BMP-2 promotes the osteogenic differentiation of hMSCs. The purity of the hMSC cultures was assessed using flow cytometric analysis. In order to assess the osteogenic activity, alkaline phosphatase activity (ALP) was measured and to estimate the osteoblastic mineralization and calcification, von Kossa staining for phosphates was performed. Cells positive for Src homology 2 domain were determined to be hMSCs and the presence of CD34 was used to distinguish hematopoietic lineages. Following treatment, the Ad-BMP-2 and control group had significantly increased ALP levels ($P<0.05$). Compared to the blank group and the group transfected with adenoviral vector containing LacZ, the phosphate deposition in the Ad-BMP-2 group and the positive control group treated with dexamethasone was markedly increased. The results of the present study suggested that Ad-BMP-2 promotes osteogenic differentiation in hMSCs and may have a potential application in treating delayed union and nonunion following bone fracture.

Correspondence to: Ms. Ying Zhang, Department of Reproductive Medicine, Renmin Hospital, Hubei University of Medicine, 39 Chaoyang Middle Road, Shiyan, Hubei 442000, P.R. China
E-mail: 33556511@qq.com

*Contributed equally

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Introduction

Delayed or failed bone union is a common clinical complication that requires treatment in orthopedics, often requiring re-admission and surgery (1). Depending on the fracture site, ~5-10% of fractures may result in delayed union or nonunion (2,3). Previously, autogenous bone grafts or free vascularized bone grafts have been widely used for nonunion treatment. However, harvesting the grafts from the iliac crest is associated with donor site morbidity and particularly with chronic pain (4).

Cell-based therapies and tissue-engineered approaches have become potential therapeutic strategies for bone repair and fracture healing. Producing an optimal cell source for the generation of functional osteoblasts is critical to achieve clinical success with these therapeutic strategies. Mesenchymal stem cells (MSCs) are multipotent somatic stem cells that are able to differentiate into numerous of cell types, including chondrocytes, myocytes, osteoblasts and adipocytes (5). It has been demonstrated that MSCs may provide a source of cells for tissue engineering of bone tissue. The osteogenic potential of MSCs has already been applied in a number of clinical situations, including fracture nonunion, osteogenesis imperfecta, posterior spinal fusion, distraction osteogenesis and osteoarthritis (6). The number of human (h)MSCs in tissue and their proliferative activity has been observed to be reduced with increasing age of their donor (7), which leads to difficulties in preparing a sufficient number of hMSCs for cell therapy in elderly patients. In addition, hMSCs isolated from patients with osteoporosis exhibit a low proliferative activity and limited ability to differentiate into the osteogenic lineage (8,9).

It has been demonstrated that bone morphogenetic proteins (BMPs) have crucial roles in the process of new bone formation by inducing the differentiation of hMSCs into osteoblasts, and promoting osteoblast maturity and endochondral ossification (10). Among the BMPs studied (BMP-2, -7 and -9), BMP-2 has the highest osteoinductive potential (11). Due to the

efficient gene transfer achieved, adenoviral vectors are attractive vehicles for *in vivo* gene therapy. It has been demonstrated that BMP-2-expressing recombinant adenoviral vector gene (Ad-BMP-2)-modified tissue-engineered bone may efficiently promote osteogenesis and repair critical-sized bone defects in large animals (12). However, the effect of Ad-BMP-2 on the osteogenic ability of human mesenchymal stem cells has remained elusive.

Therefore, the present study assessed the possible application of Ad-BMP-2 in order to assess whether it promotes osteogenic differentiation of hMSCs. In the present study, the feasibility of using hMSCs in the treatment of delayed and nonunion complications of fracture repair *in vitro* was verified to potentially identify a novel method for treating delayed or failed bone union.

Materials and methods

hMSC isolation and culture. hMSCs were prepared as described previously (13) following the standard protocol by Roseti *et al* (14). Bone marrow aspirates (50 ml) were obtained from the iliac crest of 8 healthy volunteer donors (20-35 years of age) at Renmin Hospital, Hubei University of Medicine (Shiyan, China), and diluted to 1:3 with Iscove's modified Dulbecco's medium (IMDM; Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA). Following density gradient centrifugation (750 x g for 20 min), the mononuclear cell layer was obtained from the interface. The cells were washed twice with Hanks' balanced saline solution (Beyotime Institute of Biotechnology, Haimen, China), suspended in IMDM, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), L-glutamine and Hepes (25 mM), gentamicin (50 μg/ml) and 2% Ultroser™ G Serum Substitute (Pall Corp., Port Washington, NY, USA), plated in 75-cm^2 flasks at a density of $1.6x10^5$ cells/cm^2 and incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37˚C. After 2 days, when the cells had reached confluence, adherent cells were harvested by incubation for 10 min with 0.02% EDTA and 0.05% trypsin at room temperature. Hanks' balanced saline solution, without calcium and magnesium, supplemented with 10% FBS was used to wash the cells. Cells were resuspended in the aforementioned complete IMDM. The resulting cell population was referred to as primary culture (P0). Cells were plated at a density of 10^4 cells/cm^2 in 100-mm dishes to propagate this population (secondary culture; P1). The use of human bone marrow for this study was approved by the Human Research Ethics Board at Hubei University of Medicine. All patients provided informed consent.

hMSC characterization and phenotype. Following 14 days, the P0 cultures were trypsinized and passaged. Cell cultures were passaged weekly following P1 and grew exponentially. To assess the purity of the hMSC cultures, analysis of these cells was performed using a flow cytometer (CytoFLEX; Beckman Coulter Inc., Brea, CA, USA). Cells positive for the Src homology 2 domain (SH2) according to flow cytometric analysis with SH2-fluorescein isothiocyanate-conjugated antibody (cat. no. TA504381; Origene Technologies, Inc., Rockville, MD, USA) were determined to be hMSCs (15). No detectable contamination with hematopoietic cells was observed, as indicated by the absence of CD34, a marker of the hematopoietic lineage (16), as detected through the use of CD34-FITC-conjugated antibody (cat. no. ZM-0046; Zhongshan Goldenbridge Biotechnology, Ltd., Beijing, China). These data indicated that the population of hMSCs was morphologically homogeneous.

Treatment of cell cultures. Cells at P1 were randomly divided into four groups, as follows: i) Ad-BMP-2: The concentration of Ad-BMP-2 used in the culture was $1x10^{10}$ optical units/ml, following 24 h of incubation with Ad-BMP-2 at room temperature, followed by culture in regular culture medium. ii) Adenoviral vector containing LacZ (Ad-LacZ): $1x10^{10}$ optical units/ml Ad-LacZ replaced Ad-BMP-2. iii) Control: The positive control group was cultured with 1 nmol/l dexamethasone, 50 mg/l ascorbic acid and 10 mmol/l β-sodium phosphate (all from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with the medium changed every 3 days. iv) Blank, no specific treatment. The Ad-BMP-2 and Ad-LacZ vectors were constructed and donated by Li *et al* (17) at the Department of Trauma Orthopedics, Hubei University of Medicine.

Alkaline phosphatase (ALP) activity assay. To assess osteogenic activity, ALP activity was measured and scored 12 days following transduction. A 100-μl sample of the culture supernatant was incubated at 37˚C for 30 min with 100 μl p-nitrophenyl phosphate (1 mg/ml; Sigma-Aldrich; Merck KGaA) in 1 M diethanolamine buffer containing 0.5 mM $MgCl_2$ at pH 9.8. The addition of 50 μl 0.2 M sodium hydroxide stopped the reaction. Total protein content was determined using a Bio-Rad Protein Assay kit II (Bio-Rad Laboratories, Inc., Hercules, CA, USA), the absorbance was determined at 595 nm and the activity was calculated according to a series of bovine serum albumin standards. At the end of the experiment, ALP levels were normalized to the total protein content. Each sample was repeated in triplicate.

von Kossa staining. Cells were fixed with 4% paraformaldehyde and washed in phosphate-buffered saline. Cells were then treated with 5% silver nitrate solution at 37˚C in the dark for 30 min. Silver nitrate solution was then completely washed away with Hanks' balanced saline solution and the cells were exposed to bright light for 15 min to develop the color.

Statistical analysis. Values are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS software, version 12.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to assess the differences between the three groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization and phenotype of hMSCs. Following four days in culture, freshly harvested bone marrow cells were adherent (Fig. 1A). On day 14, a morphologically homogeneous population of fibroblast-like cells was observed to have >90% confluence (Fig. 1B).

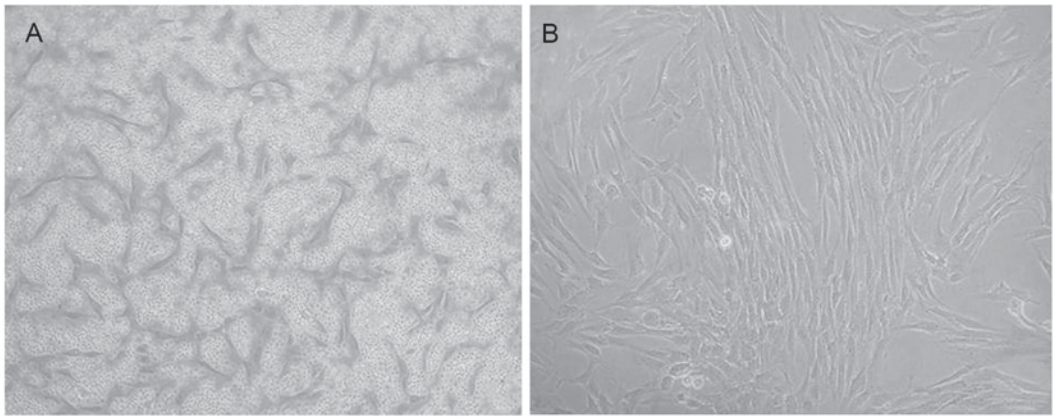


Figure 1. Morphological features of human mesenchymal stem cells imaged using inverted phase contrast microscopy (magnification, x100). Images were captured following (A) 4 days in culture, showing that freshly harvested bone marrow cells were adherent and (B) 14 days, showing a morphologically homogeneous population of fibroblast-like cells.

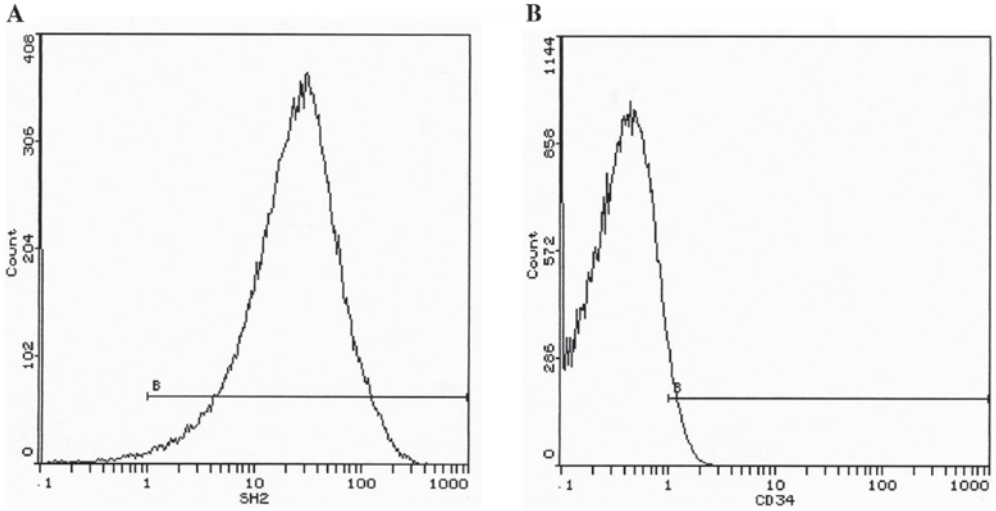


Figure 2. Flow cytometry was used to analyze the phenotype of human mesenchymal stem cells. (A) Cells positive for SH2 were determined. (B) Cells negative for CD34 were assessed to distinguish them from hematopoietic lineages. SH2, Src homology 2 domain.

The phenotype of hMSCs was analyzed using flow cytometry, and cells positive for SH2 were determined to be hMSCs (Fig. 2A), while CD34 was assessed to distinguish the cells from hematopoietic lineages (Fig. 2B).

Ad-BMP-2 induces osteoblast-like morphological changes in hMSCs. Following treatment with Ad-BMP-2, cells gradually transformed into polygons or irregular shapes, proliferation decreased and no colony formation was observed (Fig. 3A). The morphology in the positive control group was similar to that of the hMSCs treated with Ad-BMP-2. There was no evident change in the morphology of hMSCs in the Ad-LacZ group (Fig. 3B) and cells in the blank group, and colony formation was observed.

Ad-BMP-2 increases ALP activity in hMSCs. ALP is an enzyme present in osteoblasts and is pivotal for bone mineralization (18). The present study evaluated the osteoinductive effect of BMP-2 on hMSCs. Following treatment, the Ad-BMP-2 and positive control group demonstrated a significant increase in the level of ALP ($P<0.05$; Fig. 4).

Ad-BMP-2 increases mineralization and calcification of hMSCs. The mineralization and calcification of the bone matrix facilitates osteoblast formation and is therefore essential for the strength and rigidity of the skeletal system (19). To estimate the osteoblastic mineralization and calcification, von Kossa staining for phosphates at was performed day 14. Representative images of von Kossa stain were obtained by microscopy (Fig. 5). Compared with the Ad-LacZ and blank groups, the phosphate deposition in the Ad-BMP-2 and positive control groups was clearly increased (Fig. 5).

Discussion

The US Food and Drug Administration (FDA) define a nonunion as a fracture that does not heal within nine months and reports nonunion occurring in 1 out of 40 fractures (20). A lack of healing progression within three consecutive months is the clinical definition of a delayed union (21). Certain risk factors may predispose a patient to the development of a nonunion, including the type and site of fracture, fracture comminution with bone and soft tissue devascularization,

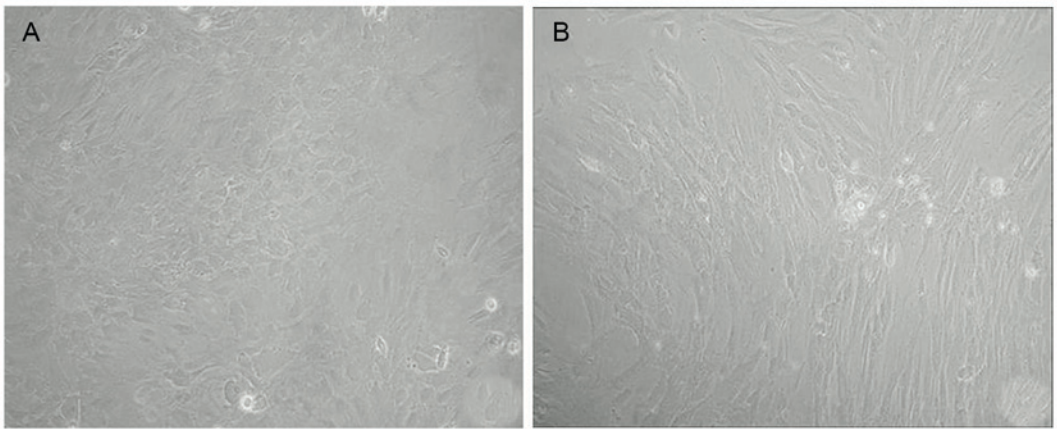


Figure 3. Morphological features of human mesenchymal stem cells treated with Ad-BMP-2 and Ad-LacZ (magnification, x100). (A) Following 24 h of incubation with Ad-BMP-2, cells gradually transformed into polygons or irregular shapes, the proliferation rate decreased and there was no clear colony formation. (B) Following 24 h of incubation with Ad-LacZ, cells were spindle-shaped and formed colonies. Ad-BMP-2, adenovirus expressing bone morphogenetic protein-2; Ad-LacZ, adenoviral vector containing LacZ.

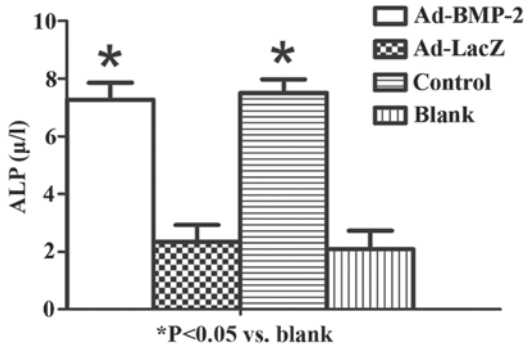


Figure 4. Expression of ALP activity was measured and scored 12 days after transduction to assess osteogenic activity. Following treatment of the four groups, a significant increase in the ALP activity was observed in the Ad-BMP-2 and control groups. *P<0.05 vs. blank. Ad-BMP-2, adenovirus expressing bone morphogenetic protein-2; Ad-LacZ, adenoviral vector containing LacZ; blank, no specific treatment; ALP, alkaline phosphatase.

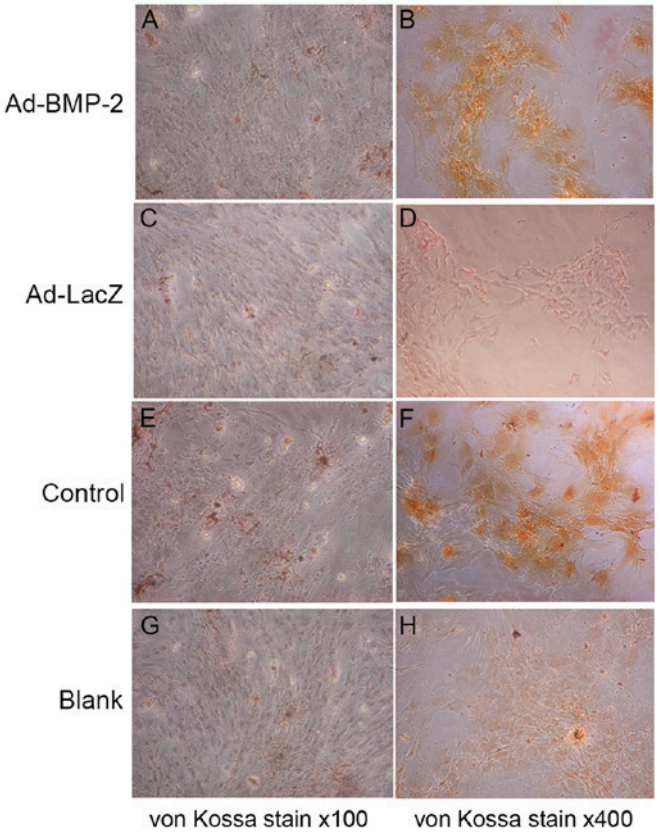


Figure 5. Estimation of the osteoblastic mineralization and calcification was performed using a von Kossa stain for phosphates at day 14. Representative images of von Kossa-stained cells were obtained via microscopy for each group. (A and B) Ad-BMP-2, (C and D) Ad-LacZ, (E and F) control and (G and H) blank (magnification, x100 in the left and x400 in the right panel). Compared to the Ad-LacZ and blank groups, the phosphate deposition in the Ad-BMP-2 and control groups was clearly increased. Ad-BMP-2, adenovirus expressing bone morphogenetic protein-2; Ad-LacZ, adenoviral vector containing LacZ; blank, no specific treatment.

instability, bone loss, presence of a chronic illness, infection and tobacco use. However, while it is known that these risk factors may predispose a patient to develop a nonunion, the underlying physiopathology remains to be fully elucidated (22).

Human marrow mesenchymal stem cells have become the primary cell source for bone tissue engineering (23). The theory of osteoblast modulation suggests that if a pluripotent cell is situated in the proper milieu it may convey an osteoblast phenotype (24). As described by Chamberlain *et al* (25), a several week incubation procedure that includes a mixed monolayer of hMSCs with ascorbic acid, dexamethasone and phosphate is the standard approach for differentiating hMSCs into osteoblasts *in vitro* (25), so the present study used this as a positive control. The methods typically used to incubate hMSCs include whole bone marrow culture, density gradient centrifugation and immunomagnetic separation. The primary hMSCs separated by the final two methods have relatively high purity, but the cells grow slowly and the culture cycle is long, making it difficult to meet clinical requirements (26,27). The primary cells incubated using the whole marrow method are mixed with hemopoietic stem cells. However, with Ad-BMP-2 and the extension of the incubation time, the suspension growth hemopoietic stem cells are removed through the exchange of cell medium. The present study assessed the use of Ad-BMP-2 with an extended incubation time using flow cytometry, confirming that the whole bone marrow culture also reach a

high purity, the cells multiply rapidly and the culture cycle is short, meaning it is suitable for clinical use.

To improve the osteogenic potential, two strategies have been developed. The first one is to enhance bone formation by incorporating bone-favor growth factors into the scaffold, known as growth factor-based bone tissue engineering. The second is cell-based bone tissue engineering, building up osteoinductive capability by growing living osteogenic cells on scaffolds *in vitro* (28). Numerous members of the whole BMP superfamily are associated with bone, cartilage and joint development. BMP-2 has been approved by the FDA for clinical practice as the most potent member of the BMP family in promoting bone and cartilage development. It is therefore a popular choice for MSCs-based bone tissue engineering. It has been demonstrated that the BMP-2-modified MSCs increase the ALP activity, cell proliferation and mineralization *in vitro* and heal critical-size bone defects, induce ectopic bone formation, repair fractures and trigger spinal fusion *in vivo* (29). BMP-2 serves an important role in fracture healing: During the process of bone tissue repair, BMP-2 transmits information between cells and intercellular substances through autocrine and paracrine signaling, regulating the secretion and proliferation of cells. The adenovirus commonly used as a gene delivery vector as it has a high transfection efficiency (30). However, the long-term overexpression of exogenous genes may lead to serious consequences, which are unpredictable and irreparable. However, the target genes that the adenovirus is able to mediate do not integrate into the chromosome, are only expressed in the cytoplasm and typically last for 4-8 weeks (31). Therefore, the requirement of gene therapy to be delivered quickly is satisfied while safety is guaranteed.

The flexibility to express the protein focally and locally, or in a disseminated fashion, as required is the most relevant advantage of gene therapy. Of note, gene therapy provides a possibility for intra-cellular production of proteins, thus facilitating therapeutic pathways to occur (32). Following treatment with Ad-BMP-2, hMSCs not only adopted osteoblastic features regarding their shape and growth patterns, but also had an increased expression of ALP. The present study also used a group transfected with Ad-LacZ to assess whether the expression of the adenoviral vector, which was identical to that in Ad-BMP-2, had any effect on osteogenesis. The results suggested that the stimulation of osteogenesis in the Ad-BMP-2 group was a result of the expression of BMP-2, not the adenovirus.

If active bone formation occurs, the level of ALP increases, as it is a byproduct of osteoblast activity (33). In the present study, the culture medium was changed to be serum-free prior to ALP detection. This eliminates any interference with the results due to ALP contained in serum. Osteoblastic mineralization and calcification are the most reliable evidence of the osteoblast. In the Ad-BMP-2 and positive control groups, ALP activity and the level of phosphate increased, indicating that Ad-BMP-2 has a function in promoting the osteogenesis of hMSC.

The present study confirmed the feasibility of transfecting hMSCs with Ad-BMP-2 to treat delayed or nonunion fractures *in vitro*. In addition, hMSCs may differentiate into chondrocytes, myocytes and adipocytes as well as osteoblasts. Therefore, it is necessary to induce osteoblastic differentiation prior to transplantation. The traditional method to induce osteoblast proliferation is to administer dexamethasone, ascorbic acid and β-sodium phosphate. The present study demonstrated that the osteogenic differentiation ability of hMSCs in the Ad-BMP-2 group was similar to that in the positive control group. The induction time was two weeks in the positive control group, while it was only 24 h in the Ad-BMP-2 group, suggesting that Ad-BMP-2 may reduce osteogenic differentiation time. As for clinical use, Ad-BMP-2 may significantly reduce the treatment cycle time and the risk of cell contamination with hematopoietic cells. Therefore, it has the potential to be a novel therapeutic method for treating delayed or nonunion fracture healing in the future.

Acknowledgements

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