hBMP-7 induces the differentiation of adipose-derived mesenchymal stem cells into osteoblast-like cells

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ABSTRACT. The aim of this study was to investigate the differentiation potential of adipose-derived mesenchymal stem cells (ADMSCs) into osteoblasts by human bone morphogenetic protein-7 (hBMP-7) induction. ADMSCs were isolated from the subcutaneous adipose tissue of a rabbit, and then transfected with the pcDNA3.1 vector alone and pcDNA3.1-hBMP-7 (hBMP-7), respectively. Untransfected ADMSCs were used as the control group. After transfection, the morphology and green fluorescent protein (GFP) fluorescence intensity of ADMSCs

were observed by fluorescent microscopy. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to detect the growth of ADMSCs at 1, 3, and 5 days, respectively. Transmission electron microscopy was performed to observe the ultrastructural morphology of ADMSCs. In addition, ADMSCs were stained with quinalizarin and toluidine blue to reflect the content of osteoblasts and chondrocytes, respectively. Finally, the expression of collagen I and osteocalcin in ADMSCs was detected by western blot. ADMSCs were successfully isolated. Obvious GFP fluorescence and high expression of hBMP-7 demonstrated the successful transfection of hBMP-7. Specific morphological characters with a metabolically active ultrastructure were exhibited on the ADMSCs transfected with hBMP-7. In addition, the growth rate of ADMSCs transfected with hBMP-7 was significantly higher than that of the cells in the vector and control groups. Successfully induced osteoblast-like cells were identified by an obvious erythrine area and high expression of collagen I and osteocalcin in ADMSCs transfected with hBMP-7. Thus, ADMSCs can be successfully differentiated into osteoblast-like cells by hBMP-7 induction *in vitro*.

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells that exhibit a high capacity of self-renewal and maintenance of multipotency. MSCs can differentiate into multiple cell types (Beyer Nardi and da Silva Meirelles, 2006), including cartilage (Bernardo et al., 2007), muscle (Yoon et al., 2005), bone (Holtorf et al., 2005), and adipose tissue. Growth factors and cytokines can accelerate the rate of cell differentiation (Potapova et al., 2007). In addition, due to the relative ease in establishing *in vitro* cultures and achieving good proliferation, MSCs have been developed as an attractive model cell to study differentiation mechanisms (Bianco et al., 2001).

Bone regeneration in calvarial defects remains a challenge for surgeons. Along with the substantial progress in effective tissue engineering methods performed on bone regeneration, the application of stem cells has increased (Grayson et al., 2015). MSCs have been shown to differentiate into osteoblasts, and this property has been applied for promoting the bone regeneration of calvarial defects (Koob et al., 2011). In addition, MSCs originating from the local bone marrow, periosteum, endosteum, mineralized bone, or systemic circulation have been shown to play important roles in osteoblastic differentiation and the secretion of factors during the spontaneous healing of a bone trauma or defect (Wang et al., 2013). Adipose-derived mesenchymal stem cells (ADMSCs) can also differentiate into various kinds of cells, including adipogenic, osteogenic, chondrogenic, myogenic lineages, and osteoblasts (Mizuno et al., 2002; Zuk et al., 2002; Semyari et al., 2016). The growth factor bone morphogenetic protein (BMP) was originally discovered owing to its ability to induce the formation of bone and cartilage (Sánchez-Duffhues et al., 2015). BMPs have been reported to play important

roles in early development and bone inductive activity, which could orchestrate the tissue architecture throughout the body via a group of pivotal morphogenetic signals (Bleuming et al., 2007). However, studies on the induction of ADMSCs into osteoblasts by BMPs are still limited.

In this study, full-length human BMP-7 (hBMP-7) was transfected into ADMSCs, and the growth state and morphological changes of the ADMSCs were observed. In addition, the proliferation of ADMSCs was measured, and the effect of hBMP-7 on osteoblasts differentiation was evaluated. Our findings may reveal the process of the hBMP-7-induced differentiation of ADMSCs into osteoblasts, and could provide a new perspective for bone regeneration in a clinical setting.

MATERIAL AND METHODS

Isolation and culture of ADMSCs

Subcutaneous adipose tissues of about 5 cm^3 were obtained from the nape of the neck of a male New Zealand rabbit (weighing 2.5-3.0 kg) provided by the Animal Experimental Center of China Medical University, Shenyang, Liaoning, China. The obtained tissue was washed with phosphate buffered saline, and cut into small pieces of 1-3 mm^3. After digestion with 0.1% type-I collagenase (Invitrogen, Carlsbad, CA, USA) at 37°C for 1 h, the precipitate was collected by centrifugation at 400 g for 10 min and was re-suspended by NH_4Cl (0.16 M) at room temperature for 15 min to hemolyze the red blood cells. The solution was then filtered through a 200-mm filter, and cultured in Dulbecco's modified Eagle medium with 10 mM glucose and 5% fetal bovine serum at 37°C. After culturing for 48 h, the culture medium was initially replaced daily until the cells grew to fill the bottom of the container. Then, the cells were digested with 0.25% pancreatic enzyme and 0.02% ethylenediaminetetraacetic acid, and finally generated, using a ratio of 1:2. The study was approved by the Experimental Animal Ethics Committee at China Medical University.

Transfection of hBMP-7

The encoding sequence of hBMP-7 cDNA (1.3 kb) was amplified from pBluescript-hBMP-7 (provided by Dr. Yu Bing, Fourth Military Medical University, Shanxi, China). The segment was connected with the pcDNA3.1 plasmid vector (Invitrogen), and transfected into *Escherichia coli* DH5α. After accurate sequencing, the recombinant plasmid pcDNA3.1-hBMP-7 (hBMP-7) was extracted using a large-quantity plasmid extraction kit (Omega Bio-tek, Inc., Doraville, GA, USA).

The second generation of ADMSCs was seeded at a density of 0.5 x 10^6 cells/well in 6-well plates, and then transfected with pcDNA3.1 (vector) or hBMP-7 using Lipofectamine 2000, according to the manufacturer instructions (Invitrogen). Untransfected ADMSCs were used as the control group. At 24 h post-transfection, the cells were inoculated onto fresh culture lysates and diluted at 1:20 for passage 2. After 48 h, the transfected cells were cultured for 14 days, and geneticin (G418, Gibco-BRL, 500 mg/mL) was used to select geneticin-resistant transfected cells. The green fluorescent protein (GFP) fluorescence intensity and morphology of ADMSCs in the different groups were observed on a BX50 fluorescence microscope (Olympus, Tokyo, Japan).

Reverse transcription-polymerase chain reaction (PCR)

Total RNA from the ADMSCs of each group was isolated by TRIzol reagent (Invitrogen). RevertAid First-Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) was used for reverse transcription. The specific primers for reverse transcription-PCR were as follows: hBMP-7 forward, 5'-GGT CTA GAA TGC ACG TGC GCT CAC TGCG-3'; hBMP-7 reverse, 5'-CC GGT ACC TTA GTG GCA GCC ACA GGC CCGG-3'. β-actin was used as an internal control. The sequences of the β-actin primers were 5'-CTC CTT AAT GTC ACG CAG GAT TTC-3' (forward primer) and 5'-GTG GGG CGC CCC AGG CAC CA-3' (reverse primer). The PCR program included 94°C for 5 min, then 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 2 min. PCR products were resolved and documented by 1% agarose gel electrophoresis.

3-(4,5, Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The proliferation of ADMSCs in the different groups was detected by an MTT assay following the manufacturer instructions (Amresco, Solon, OH, USA) at 1, 3, and 5 days, respectively. In brief, 250 mg MTT was added to the ADMSCs at 0.5×10^5 cells/well in 96-well plates, and the optical density was detected at 570 nm using an ultraviolet spectrophotometer (Bio-Rad, Hercules, CA, USA).

Quinalizarin and toluidine blue staining, and transmission electron microscopy (TEM)

After transfection, quinalizarin and toluidine blue staining were performed on the ADMSCs of different groups and observed using a fluorescent microscope (Olympus) to determine the contents of osteoblasts and chondrocytes. In addition, the ultrastructural morphology of ADMSCs in the different groups was observed using TEM (Philips CM 120, Eindhoven, Netherlands). After the cells were fixed, they were dehydrated by acetone, soaked in propylene oxide at 24°C for 24 h, and embedded in araldite. Subsequently, the embedded specimens were cut into ultrathin slices, stained with lead citrate, and examined under TEM.

Western blot

After isolation of the cellular lysates from ADMSCs, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 for 2 h, and then specific diluted primary antibodies against collagen I and osteocalcin (1:100, Neumark, USA) were added. The membrane was then incubated overnight at 4°C, washed in Tris-buffered saline with Tween-20 three times, and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Zhongshan Jinqiao, Beijing, China) for 1 h at 25°C. Finally, after the samples were washed, the gel image was analyzed semi-quantitatively using an ultraviolet gel imager (Shanghai Tianneng Co., Ltd., Shanghai, China).

Statistical analyses

All data are expressed as means ± standard deviations. Comparison between different

groups was determined with the Student t-test. A P value < 0.05 was considered to indicate a significant difference. Statistical analysis was performed on SPSS version 12.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Morphology of ADMSCs

No GFP fluorescence of ADMSCs was found in the control group (Figure 1A) or vector group (Figure 1B). However, obvious GFP fluorescence was observed in the hBMP-7 group, which indicated the successful transfection of hBMP-7 into ADMSCs (Figure 1C). After 48 h of culture, the ADMSCs were considered to be successfully isolated, and those of the control (Figure 1D) or vector group (Figure 1E) exhibited a typical elliptical or fibroblastic morphology. Furthermore, specific morphological characters, including numerous mitotic figures, an increased cell volume with polygonal, oval, or irregular shape, a large and oval nucleus with a prominent nucleolus, and emerged vacuoles in the cytoplasm, were exhibited on the cells in the hBMP-7 group (Figure 1F). hBMP-7 was expressed in the hBMP-7 group of ADMSCs, whereas no expression of hBMP-7 was found in the vector or control group (Figure 2).
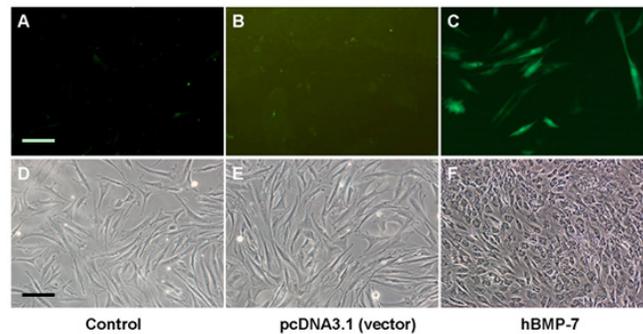

Figure 1. Green fluorescent protein (GFP) fluorescence and morphology observation (200X) of adipose-derived mesenchymal stem cells (ADMSCs) transfected in the control group (**A**, **D**), vector group (**B**, **E**) and hBMP-7 group (**C**, **F**). Scale bar, 200 mm.
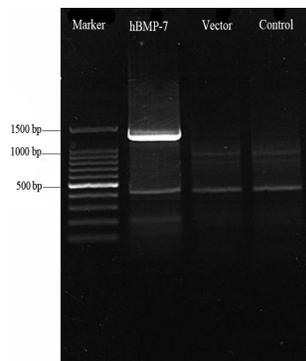

Figure 2. Expression of hBMP-7 in ADMSCs in different groups was examined by RT-PCR.

The growth of ADMSCs was improved by hBMP-7

The proliferation evaluation of ADMSCs using MTT assays revealed that the ADMSCs transfected with hBMP-7 exhibited rapid growth, and the growth rate was higher than that of the vector and control groups at 1, 3, and 5 days (Figure 3). In addition, ADMSCs transfected with the vector showed a similar growth rate to those of the control group.
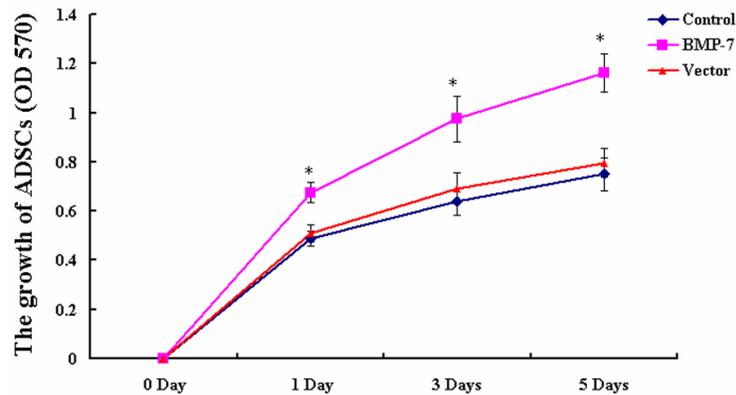

Figure 3. Proliferation of ADMSCs transfected with pcDNA3.1 or hBMP-7. Untransfected ADMSCs were used as control group. *P < 0.05 compared with vector and control group.

Ultrastructural observation of ADMSCs transfected with hBMP-7

The ultrastructural observation of ADMSCs in the vector (Figure 4A) or control group (Figure 4B) demonstrated obvious immature characteristics, including an oval-shaped nucleus with a prominent nucleolus, chromatin with a diffuse and scattered distribution, and few organelles. In addition, the Golgi apparatuses, mitochondria, or endoplasmic reticula were poorly developed in these two groups. By contrast, the ADMSCs in the hBMP-7 group had denser chromatin with plenty of well-developed organelles, which indicated that these cells were metabolically activated (Figure 4C).
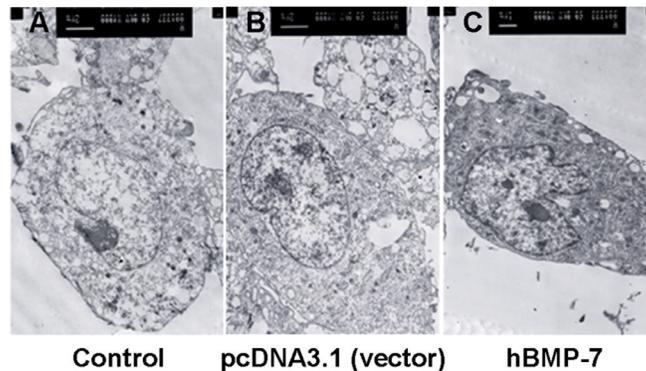

Figure 4. Ultrastructural observation of (**A**) control group (untransfected ADMSCs); (**B**) ADMSCs transfected with pcDNA3.1; and (**C**) ADMSCs transfected with hBMP-7. Original magnification: 7500X.

Formation of osteoblast-like cells in ADMSCs transfected with hBMP-7

After staining with quinalizarin, no erythrine was observed in the control (Figure 5A) and vector groups (Figure 5B). However, an obvious erythrine area was observed in the ADMSCs transfected with hBMP-7 (Figure 5C), which reflected high contents of osteocalcin. Furthermore, we assessed the distribution of chondrocytes in ADMSCs by toluidine blue staining, and found that the number of chondrocytes in the control (Figure 5D) and vector groups (Figure 5E) was lower than that in the hBMP-7 group (Figure 5F).
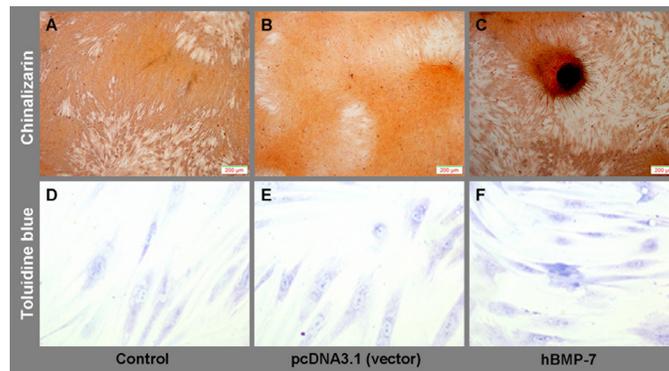

Figure 5. Chinalizarin and toluidine blue staining of (**A**, **D**) untransfected adipose-derived mesenchymal stem cells (ADMSCs), (**B**, **E**) ADMSCs transfected with pcDNA3.1 and (**C**, **F**) ADMSCs transfected with hBMP-7.

hBMP-7 induced high collagen I and osteocalcin expression

Both collagen I and osteocalcin were expressed in the three different groups of ADMSCs (Figure 6A). In addition, compared with the vector and control groups, the levels of collagen I and osteocalcin in the hBMP-7 group were higher (Figure 6B). The levels of collagen I and osteocalcin in the vector and control groups were relatively low, with no significant difference observed between these two groups (Figure 6B).
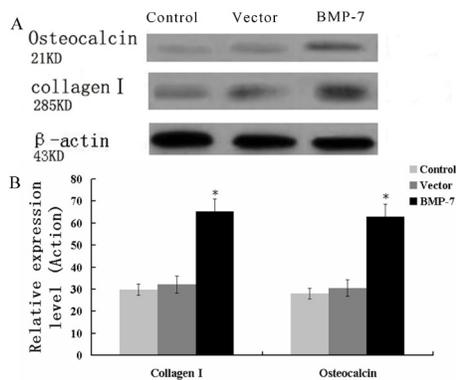

Figure 6. The expression (**A**) and relative expression levels (**B**) of collagen I and Osteocalcin in adipose-derived mesenchymal stem cells (ADMSCs) transfected with pcDNA3.1and hBMP-7. Untransfected ADMSCs were used as control group. *P < 0.05 compared with vector and control group.

DISCUSSION

Recently, stem cells have been widely used in tissue-engineering strategies for bone defects and repair (Seong et al., 2010). An applicable cell source with an abundant supply, easy accessibility, and no immunogenic reaction with osteoplastic potential is required for the successful cell-based treatment of bone (Yamada et al., 2013). ADMSCs can differentiate into adipogenic, osteogenic, chondrogenic, myogenic lineages, and osteoblasts (Mizuno et al., 2002; Zuk et al., 2002; Semyari et al., 2016). The technique of ADMSCs isolation from the subcutaneous adipose tissue of rabbits, *in vitro* culture, and expansion has been previously reported (An et al., 2010). In the current study, we evaluated the effect of hBMP-7 on ADMSCs differentiation to generate osteoblast-like cells, and found that ADMSCs transfected with hBMP-7 exhibited robust proliferation and were capable of producing osteoblast-like cells.

BMPs are closely associated with cell growth and bone formation (Kung and Ries, 2007). We have also demonstrated the prominent growth rate of ADMSCs transfected with hBMP-7 in comparison with the vector or control, similar to previous descriptions of adipose stem cells (Knippenberg et al., 2006). As a specific osteoblastic growth factor in the activation of the bone repair process, BMP-7 is also the initiating signal molecule for the osteoblastic differentiation of MSCs. The stimulation effects of hBMP-7 in osteoblastic differentiation and its effectiveness in bone repair have been verified (Boon et al., 2011). As reported, BMP-7 is beneficial for the attachment, proliferation, and differentiation of pre-osteoblasts, and thus plays important roles in bone tissue regeneration (Mantripragada and Jayasuriya, 2014). In addition, ADMSCs have the ability to form osteoblasts and maintain a differentiated osteoblast phenotype for a long time *in vitro* (Lu et al., 2008).

In this study, hBMP-7 was successfully transfected into ADMSCs. In addition, specific morphology, obvious quinalizarin staining, a significantly increased growth rate, and higher collagen I and osteocalcin expression in the hBMP-7 group suggested the formation of osteoblast-like cells. During this process, transfected ADMSCs may react with cell membrane receptors via hBMP-7, thereby inhibiting adipose cell differentiation and inducing osteoblastic differentiation (Liu et al., 1999).

Moreover, ADMSCs show chondrogenic potential, with the ability to maintain the differentiated chondrocyte phenotype for a long time both *in vivo* and *in vitro* (Erickson et al., 2002), and exhibit a strong capacity to form chondrocytes (Kessler et al., 2008). In the current study, the number of chondrocytes in the control and vector groups was lower than that in the hBMP-7 group, suggesting that the differentiation of ADMSCs induced by BMP-7 toward chondrocytes was effective. However, in another study, ADMSCs were shown to exhibit poor chondrogenic capability, which was attributed to the presence of preadipocytes (Liu et al., 2013). Thus, more studies are needed to verify the induction potential of hBMP-7.

In cell regeneration, ADMSCs are capable of multilineage differentiation, and show several advantages such as their abundant supply, high yield, easy accessibility, and non-immunogenic property (Romagnoli and Brandi, 2014). However, their clinical application remains limited. For example, it is unclear how the plasmid replicates and breaks apart in ADMSCs, and whether the induced osteoblasts possess the same biological abilities in the body. Therefore, further research on ADMSCs induction and its potential application *in vivo* are still needed.

In conclusion, the transfection of hBMP-7 could successfully induce the differentiation of ADMSCs toward osteoblasts with high proliferation capability. ADMSCs may be used as an abundant and effective source for bone regeneration.

ACKONWLEDGMENTS

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