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Bone Morphogenetic Protein-2 regulates *in vitro* osteogenic differentiation of mouse adipose derived stem cells

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Abstract. – OBJECTIVE: The aim of this study is to investigate the feasibility and efficiency of Bone Morphogenetic Protein-2 (BMP-2) in regulating *in vitro* osteogenic differentiation of mouse adipose derived stem cells (ADSCs).

MATERIALS AND METHODS: Mouse ADSCs were isolated from adipose tissues of C57/BL6 mice (age of 4-6 w) and cultured. Surface antigens of passage 3 (P3) ADSCs, including CD31, CD34, CD90, CD105 and CD133, were analyzed using flow cytometry. Overexpression of BMP-2 was achieved through gene transfection of ADSCs. *In vitro* osteogenic differentiation of transfected and non-transfected ADSCs cultured in specific induction media was evaluated by Alizarin Red staining. In addition, expression of osteoblast-specific gene, Runx2, was analyzed by quantitative RT-PCR (qRT-PCR).

RESULTS: Abundant ADSCs could be isolated from adipose tissue. P3 ADSCs expressed stem cell-specific molecular markers, CD90 and CD105 but did not express CD31, CD34 or CD133. BMP-2 could efficiently transfect mouse ADSCs. Alizarin Red staining revealed that more calcified nodules were formed in BMP-2 transfected ADSCs. qRT-PCR further confirmed higher level of Runx2 expression in BMP-2 transfected ADSCs ($p < 0.05$).

CONCLUSIONS: BMP-2 can promote *in vitro* osteogenic differentiation of mouse adipose stem cells.

Key Words:
Bone Morphogenetic Protein-2 (BMP-2), Adipose derived stem cells (ADSCs), *In vitro* study, Osteogenic differentiation.

Introduction

The repair of critical size bone defects resulting from trauma or tumor excision remains a challenge in clinical practice[1]. The emergence of bone tissue engineering brings new hope for critical-sized bone defect repair[2]. Stem cells, served as a crucial seed cell in bone tissue engineering, have displayed lower efficacy in induced osteogenic differentiation and affected the progress of bone tissue engineering[3]. Bone morphogenetic protein 2 (BMP-2) has exhibited powerful anti-apoptotic and differentiation-promoting effects[4,5]. Therefore, the feasibility and efficacy of BMP-2 in regulating *in vitro* osteogenic differentiation of mouse adipose derived stem cells (ADSCs) were investigated in the present study.

Materials and Methods

Animals, Major Materials and Instruments

SPF C57BL/6 mice (age of 4-6 weeks, a weight range of 20-25 g) were obtained from the Animal Centre of Benbu Medical College, Anhui, China). Major reagents used were listed as follows: Dulbecco's Modified Eagle's medium (DMEM), β-glycerophosphate (Sigma, St. Louis, MO, USA), collagenase, vitamin D3 (Sigma, USA), fetal bovine serum (FBS), dexamethasone (Sigma, USA), 0.25% trypsin (Sigma, USA), alizarin Red dye solution (Sigma, USA), paraformaldehyde (Sigma, USA), Phycoerythrin (PE)-conjugated anti-mouse antibody CD34 and CD105, and FITC anti-mouse CD31, CD90, CD133 antibodies (eBioscience, San Diego, CA, USA). Major instruments included the following items: super clean bench (Suzhou Purification Group, Suzhou, China), inverted microscope (Olympus, Tokyo, Japan), refrigerator (Haier, Yulin, China), flow cytometry (Beckman Coulter,

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Fullerton, Canada), RT-PCR kit (Takara, Otsu, Shiga, Japan), a lentivirus and plasmid transfection system (Genechem Biotechnology Co., Ltd., Shanghai, China), lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, CA, USA).

Isolation, Amplification and Flow Cytometry of Mouse ADSCs

Ten SPF 6-8 week-old C57BL/6 mice were sacrificed by cervical dislocation and soaked in 75% alcohol for 10 min. Anatomical dissection was performed to harvest bilateral inguinal adipose tissue, which were cut into 2-3 mm^3 pieces and digested in 0.2% collagenase for 2h. After centrifugation at 1500 rpm for 5 min, the supernatant was discarded and cell pellets were resuspended in DMEM low glucose media. Subsequently, cells were counted and adjusted to a cell density of $3 \times 10^4/cm^2$, which were than inoculated in a 25 cm^2 flask and cultured in an incubator with 5% CO_2 at 37°C for 48h. When reaching 80% confluence, cells was digested with 0.25% trypsin for 1 min for passages with one passage performed every 48h.

Third-passage (P3) ADSCs were collected and centrifuged with the supernatant discarded. These cells were resuspended in 4% FBS to a cell density of 2.0×10^5 per Eppendorf (EP) tube. CD31, CD34, CD90, CD105, CD133 antibodies and isotype controls (in 1:50 dilution) were added to the cells, incubated on ice for 30 min, rinsed, centrifuged at 1500 rpm for 5 min and resuspended in 0.5 ml 4% FBS for flow cytometry.

BMP-2 Gene Transfection of ADSCs

Primers were designed and synthesized based on features of restriction sites in lentiviral vector and BMP-2 gene sequence. After cell recovery, 293T cells were transfected with a lentiviral vector carrying BMP-2 and a plasmid and packaging of lentivirus were achieved. P3 ADSCs were digested, counted, and 1×10^6 ADSCs collected, which was cultured with harvested lentivirus in polybrene (8 μg/ml). Subsequently, mouse ADSCs were infected with the virus based on multiplicity of infection (MOI) value and immunofluorescence microscopy was performed to detect the expression of GFP-BMP-2.

Osteogenic Induction of BMP-2 Transfected and Nontransfected ADSCs and Alizarin Red Staining

P3 BMP-2 transfected and non-transfected ADSCs were seeded on six-well culture plates at a concentration of 5.0×10^3 cells/cm^2. The culture media were replaced with osteogenic solution (10 mM β-glycerophosphate, 10 nM vitamin D3, 0.1 μM dexamethasone) 24h later. Half amount of solution was refreshed every three days and cells were cultured for a total of three weeks. Formation of calcified nodules was observed under microscope.

Cell induction was terminated after three weeks. Cells were washed with phosphate buffered saline (PBS) for three times, fixed in 70% ice cold ethanol, rinsed three times with distilled water and stained with 3 ml Alizarin Red at room temperature for 10 min. Afterwards, cells were repeatedly washed with distilled water, observed and photographed under microscope for the formation of red calcified substances.

Detection of Osteoblast-Specific Runx2 Gene Using RT-PCR

The primers for Runx2 gene is listed as follows: 5'GGA CTG TGG TTA CCG TCA T 3', 5'GGA GGA TTT GTG AAG ACT GTT 3'. At three-week cell culture, 1×10^6 BMP-2 transfected and non-transfected ADSCs were collected respectively, and mRNA were isolated for qRT-PCR analysis.

Statistical Analysis

All data were expressed as mean ± SD. Statistical analysis was performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). Differences of means between groups were analyzed using *t*-test. $p < 0.05$ was considered significant different.

Results

Isolation, Cell Culture and Flow Cytometric Analysis of ADSCs

Five hours after ADSCs seeding, adherence of some round cells was observed. After 24h cell culture, a mixed cell morphology was observed in P0 ADSCs under microscope, which displayed as polygonal, flat or elongated shape with floating red blood cells (Figure 1A). As the duration of cell culture extended, groups of cell clones were visible. P3 ADSCs were arranged in a swirling pattern and exhibited a fibroblast-like morphology (Figure 1B). Flow cytometry of P3 ADSCs revealed that stem cell specific surface markers, CD90 and CD105, endothelial cell-specific molecular markers, CD31, as well as molec-

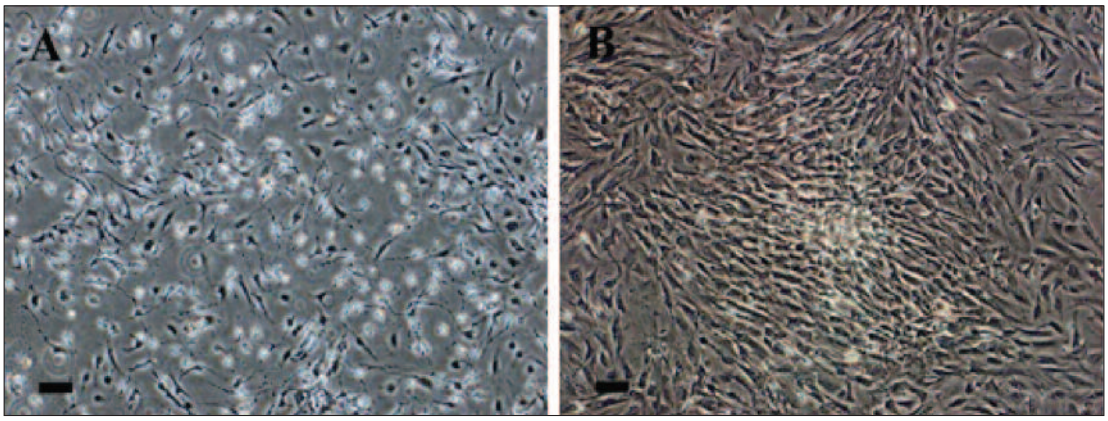

Figure 1. Morphology of P0 and P3 ADSCs (GFP-ADSCs). ***A,*** P0 ADSCs exhibited a mixture of various shapes. ***B,*** P3 ADSCs appeared to adopt a more uniformed, fibroblast-like shape. (Scale bar: 100 μm).

ular markers of hematopoietic system, CD34 and CD133 were expressed in these cells but absent in isotype control group (Figure 2).

BMP-2 Transfection of ADSCs

In the present study, a transfection system was constructed for the transferring of BMP-2 gene into ADSCs. Immunofluorescent staining demonstrated that transfected P3 ADSCs (Figure 3A) expressed GFP-BMP-2 (Figure 3B). However, BMP-2 gene was not expressed (Figure 3D) in control group (Figure 3C).

Osteogenic Induction and Alizarin Red Staining

Induced ADSCs were adopted a radial arrangement and gathered into groups following one week of osteogenic induction. As the dura-

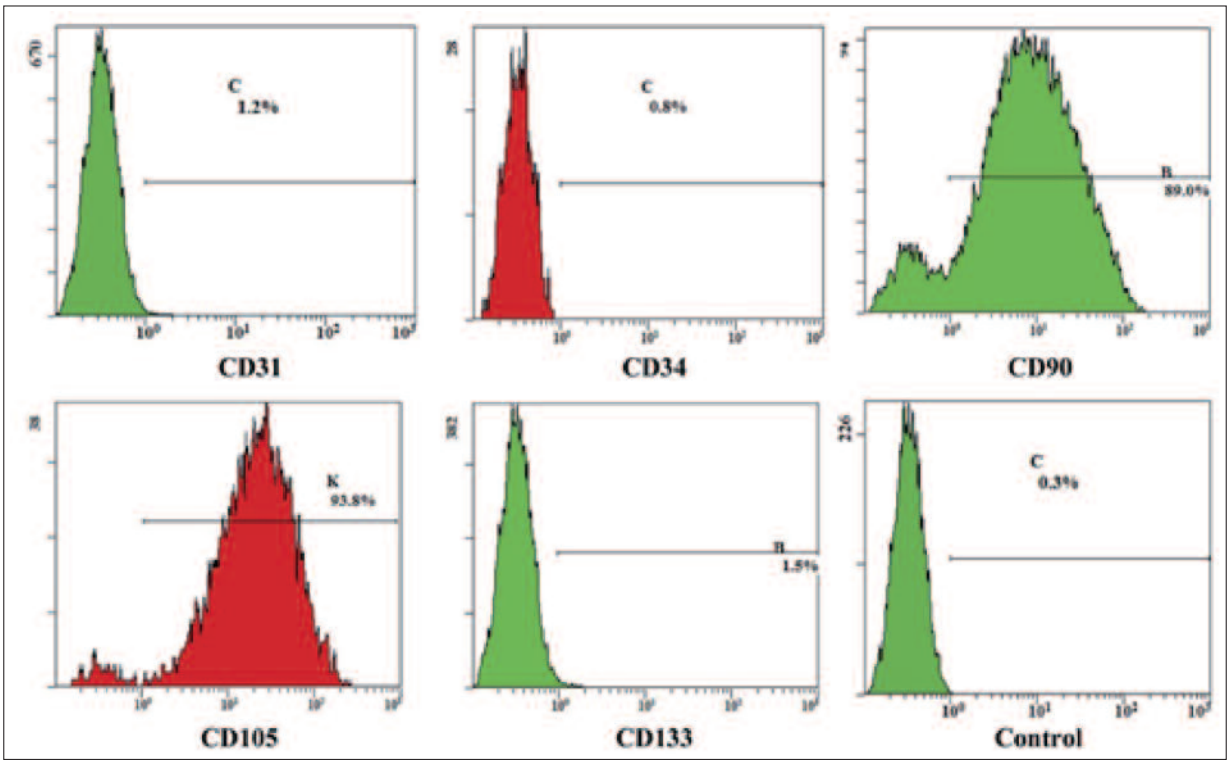

Figure 2. Flow cytometry analysis of surface antigen of GFP-ADSCs. CD90 and CD105 were positive, whereas CD31, CD34 and CD133 were negative in P3 ADSCs and the isotype control.

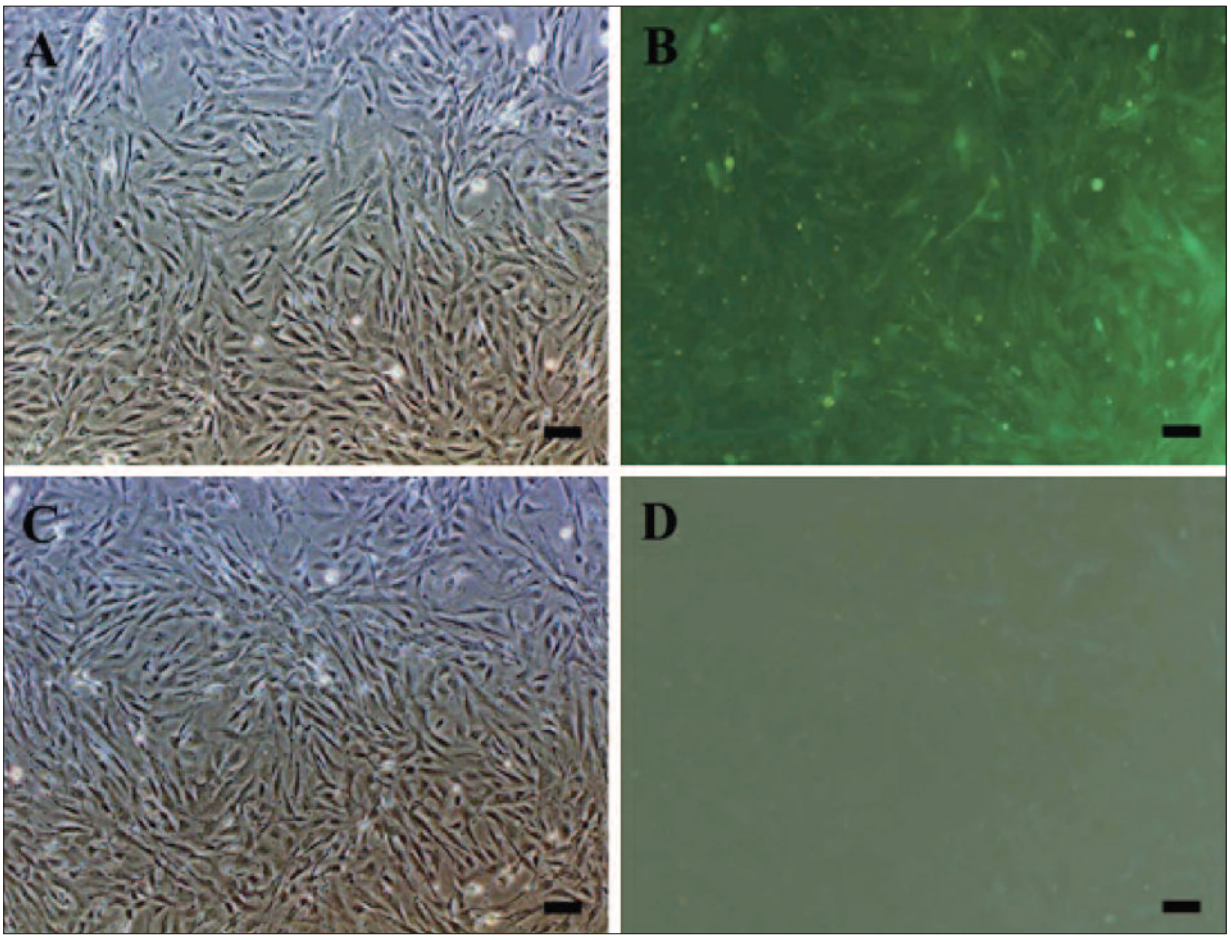

Figure 3. BMP-2 gene transfection of ADSCs. ***A,*** P3 ADSCs in bright field. ***B,*** Immunofluorescence staining showed overexpression of GFP-BMP-2 in ADSCs. ***C,*** non-transfected P3 ADSCs (*control*). ***D,*** GFP-BMP-2 gene expression was absent in ADSCs of controls. (Scale bar: 100 μm).

tion of cell culture extended, groups of cells gradually increased. Alizarin Red staining after three week of osteogenic induction showed that sporadic red calcified nodules were observed in both BMP-2 transfected and non-transfected ADSCs. However, the number of calcified nodules were significantly higher in BMP-2 transfected ADSCs cells (Figure 4C) than in non-transfected ADSCs (Figure 4B). No red calcified nodules were observed in control group (Figure 4A).

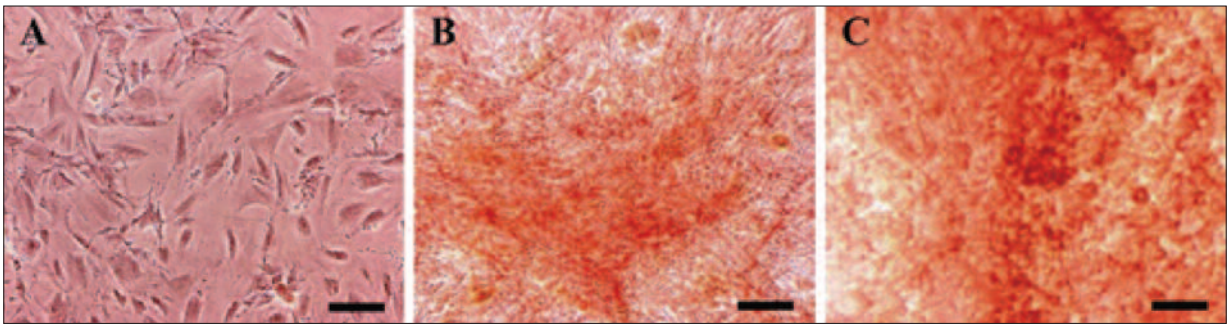

Figure 4. Osteogenic differentiation and Alizarin Red staining of ADSCs. ***A,*** The control group. ***B,*** Fewer calcified nodules were observed in ADSCs BMP-2 non-transfected. ***C,*** Many calcium calcified nodules were present in BMP-2 transfected ADSCs. (Scale bar: 100 μm).

qRT-PCR Analysis of Runx2 Gene Expression

qRT-PCR analysis found that Runx2 level was significantly higher in BMP-2 transfected ADSCs than in non-transfected ADSCs.

Discussion

The development of bone tissue engineering has brought new hope for the repair of critical-sized bone defects resulting from trauma or tumor excision[2]. Currently, bone marrow – derived stem cells (BMSCs) have been the most commonly used seed cells in bone tissue engineering. Although bone defect using BMSCs has achieved certain effect, several disadvantages of using BMSCs have been shown in that BMSCs harvest requires bone marrow aspiration, relatively fewer source of the cells are available and the procedure is difficult to be tolerated in elderly patients. As a result, the clinical application of BMSCs has been limited[6]. In contrast, abundant source of adipose tissue is available, which contains a large number of mesenchymal stem cells (ADSCs) that can be easily isolated and proliferated *in vitro*[7]. In the present report, large quantities of ADSCs were harvested from adipose tissue of mice in a short period of time and amplified rapidly with one passage in 48h. Some researchers have studied surface molecular markers in both ADSCs and BMSCs and found that 90% of these markers are identical[8]. In the present work, flow cytometry revealed that P3 ADSCs expressed stem cell-specific molecular markers, CD90 and CD105, but express neither endothelial marker, CD31, nor hematopoietic stem cell markers, CD34 and CD133. These results suggested that the surface molecular markers of ADSCs were similar to those of BMSCs. As we all know, human has an abundant source of subcutaneous adipose tissue and adipose stem cells can be easily isolated through liposuction. Another study[9] shows that adipose stem cells, unlike embryonic stem cells, do not express major histocompatibility II (MHC-II), suggesting that ADSCs have the advantages of low immunogenicity and are not ethically and legally restricted. Therefore, ADSCs are expected to become ideal seed cells for bone tissue engineering and bring new hope for the repair and regeneration of critical-sized bone defects.

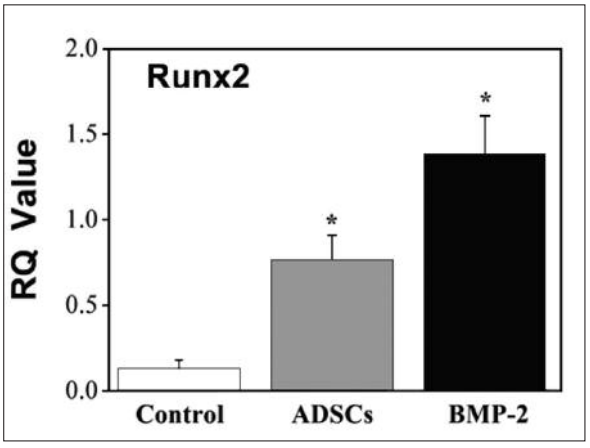

Figure 5. qRT-PCR analysis of Runx2 gene expression in ADSCs transfected and non-transfected with BMP-2. Significantly higher level of Runx2 transcription was observed in BMP-2 transfected ADSCs than non-transfected ADSCs.

Researchers have shown that multipotent stem cells can be induced to differentiate into various types of cells under certain conditions, including osteocytes, chondrocytes, adipocytes, endothelial cells, etc[6,7]. The present report also confirmed that ADSCs could differentiate into osteocytes through osteogenic induction. ADSCs have been shown to exhibit higher proliferation and multipotent differentiation capacities than BMSCs[9]. However, some other papers have shown that differentiation potential of stem cells is reduced after implantation, significantly limiting the clinical application of these cells. Many researchers have attempted to enhance the differentiation efficiency of stem cells through exogenous modifications (such as supplementation of growth factors, pretreatment with cytokines and gene modifications)[10]. BMP-2 is considered as a bone adhesion molecule that regulates the adhesion and differentiation of osteoblasts[11]. Although BMP-2 had been reported in earlier researches to be absent in tissues other than bone and lungs, recent investigations have revealed that expression levels of BMP-2 are increased in many other tissues[12] and BMP-2 has been shown to exhibit potent differentiation promoting effect during bone repair.

In this article, over expression of BMP-2 was achieved in ADSCs transfected by lentiviral vectors. Lentiviral vectors exhibits various advantages over other viral systems in that they are low immunogenic, can transduce various cell types, enable stable expression of target genes in transfected cells, and can be concentrated to high titers[13]. In the present study, BMP-2 was overexpressed in ADSCs through lentiviral transfection.

Alizarin Red staining after three week of osteogenic induction showed the presence of red calcified nodules in both BMP-2 transfected and non-transfected ADSCs, with significantly more calcified nodules in BMP-2 transfected ADSCs. qRT-PCR analysis demonstrated that the level of osteogenic-specific gene, Runx2, was significantly higher in BMP-2 transfected ADSCs than non-transfected ADSCs. However, molecular mechanism by which BMP-2 promotes osteogenic differentiation of ADSCs is not entirely clear. Yang et al[13] have confirmed that BMP-2 protein may promotes the survival, osteogenic induction and differentiation of ADSCs via activation of integrin/p38/Smad5 signaling pathway by interaction with members of integrin family (β1, αvβ5 and αvβ4).

Conclusions

Abundant ADSCs can be isolated from adipose tissue. P3 ADSCs expressed stem cell-specific molecular markers, CD90 and CD105 but did not express CD31, CD34 or CD133. BMP-2 can efficiently transfect mouse ADSCs. Alizarin Red staining revealed the formation of more calcified nodules in BMP-2 transfected ADSCs. qRT-PCR further confirmed higher level of Runx2 expression in BMP-2 transfected ADSCs. Taken together, BMP-2 can significantly promote *in vitro* osteogenic differentiation of mouse adipose stem cells, providing novel insights and theoretical evidences for repair of critical-sized bone defects in clinical practice.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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