Enhancement of osteogenesis of rabbit bone marrow derived mesenchymal stem cells by transfection of human BMP-2 and EGFP recombinant adenovirus via Wnt signaling pathway

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Abstract. Bone marrow mesenchymal stem cells (BMSCs) are considered the most important seed cells in bone tissue engineering. The present study aimed to investigate the potential of rabbit BMSCs in osteogenesis after the transfection of human BMP-2 and EGFP recombinant adenovirus. Rabbit BMSCs were isolated and the surface stem cell makers, including CD29, CD44 and CD45 were detected by flow cytometry. The expression of BMP-2 mRNA and protein in BMSCs were detected by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. After an induction with osteogenic medium, the alkaline phosphatase (ALK) activity at 7 days, the type I collagen at 14 days, and the calcium nodules at 21 days were performed using an ALK activity kit, immunohistochemical staining and alizarin red S staining, respectively. The expression levels of proteins related to the Wnt signaling pathway were detected by western blot analysis. The positive rates of CD29, CD44 and CD45 were 62.92±1.99, 93.55±0.99 and 0.21±0.12%. The expression of BMP-2 mRNA and protein was significantly upregulated in Ad-BMP-2/EGFP transfected BMSCs. Furthermore, Ad-BMP-2/EGFP induced ALP activity, promoted the production of type I collagen and calcium nodule formation in rabbit BMSCs. The levels of β-catenin, cyclin D1, Runx2 and c-myc were upregulated in Ad-hBMP-2/EGFP transfected BMSCs, while the level of GSK3β was significantly decreased. Results also indicated that the overexpression of BMP-2 by Ad-hBMP-2/EGFP enhanced the osteogenic differentiation ability of cultured rabbit BMSCs via stimulating the Wnt signaling pathway with the accumulation of β-catenin and suppression of GSK3β. The Ad-hBMP-2/EGFP transfected

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rabbit BMSCs are expected to be a good seed cell in bone tissue engineering.

Introduction

The bone defect caused by various factors is a difficult problem in orthopedics (1,2). At present, the main method to solve this problem is bone grafting, which is the most common tissue graft following blood transfusion (3). In recent years, with the rapid development of tissue engineering and bioengineering, bone tissue engineering has been favored in the clinical application of bone defect repair (4,5).

Bone tissue engineering is mainly composed of three aspects: Seed cells, cytokines and scaffold materials (6). In the study of bone tissue engineering, bone marrow mesenchymal stem cells (BMSCs) are the most studied seed cells, which have capable of self-renewal and of multi differentiation into a variety of bone cells under certain conditions (7-9).

Bone morphogenetic protein (BMP) is an acidic protein found in bone matrix. It belongs to the TGF-β superfamily (10,11). It is a multifunctional transforming growth factor group, which consist of at least 20 members. Among them, BMP-2 is one of the most widely studied BMPs in bone tissue engineering, and it is also known to induce osteogenesis (12). BMP-2 is the most commonly used cytokine at present. It can induce pluripotent stem cells BMSCs differentiated into osteoblasts and promote the expression of type I collagen, which can lead to endochondral ossification and promote the formation of new bone (12). Type I collagen is not only an important component of extracellular matrix in bone tissue, but also is one of the markers of osteoblast because it is mainly synthesis and secretion by mature bone cells, and can provide autologous scaffolds for the BMSCs growth, thus playing an important role in maintaining the normal structure and function of bone (13,14). In addition, alkaline phosphatase (ALP) has the potential to improve osteogenic differentiation of BMSCs and is also one of marker enzymes of osteoblast (15).

The efficient osteogenic induction of BMP-2 has been confirmed by many experimental and clinical studies (11,12). BMP-2 can stimulate expression of other growth factor receptors to accelerate bone formation. Kubota *et al* (16) implanted

the freeze-dried bone that combined with recombinant human BMP-2 (hBMP-2) into the chest for ectopic osteogenesis, and found that the expression of FGF-4 and FGF receptors increased, and the formation of new bone and mineralization were accelerated. The methods of preparing BMP by purified bone matrix or genetic engineering synthesis are more commonly used, but it was limited by complicated procedures, and low yield (17,18). There are many problems in the method of directly adding growth factor including BMP-2 into the bone grafts in bone tissue engineering *in vivo*, such as low titer, which remains to further resolved or develop alternative methods. With the rapid development of transgenic technology, the method of adenovirus vector-mediated gene transfection in target cells is considered as an effective way to induce osteogenesis, as the persistent expression of target gene *in vivo* is the main factor to promote the healing of bone defect (19-22).

In the present study, we isolated and identified the rabbit BMSCs, transfected hBMP-2 and EGFP genes by adenovirus vector into rabbit BMSCs, and detected the osteogenesis by ALP level, type I collagen expression and calcium nodules formation assays. As the important role of Wnt signaling pathway in osteogenesis (23-25), we also explored whether Ad-hBMP-2/EGFP affects the osteogenic ability of rabbit BMSCs via stimulating the Wnt signaling pathway.

Materials and methods

Reagents. Low glucose DMEM (L-DMEM), trypsin, and fetal bovine serum were purchased from HyClone; GE Healthcare Life Sciences, (Logan, UT, USA). MTT, vitamin C, β-phosphoglycerol, dexamethasone purchased from Biosharp, Inc., (Seoul, South Korea). Mouse anti-rabbit CD29/CD44/CD45 monoclonal antibody (Abcam, Cambridge, MA, USA), mouse anti-rabbit IgG1 (eBioscience; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and PerCP biotin-conjugated goat anti-mouse IgG (H+L; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Horseradish (HRP)-conjugated Goat anti-mouse IgG and DAB color kit were purchased from ZSGB-Bio, (Beijing, China). Mouse anti-rabbit type I collagen was purchased from Wuhan Bashan Biotechnology Co., Ltd., China. All antibodies were purchased from Bioworld Technology, Inc., (St. Louis Park, MN, USA). TRIzol and HiFi-MMLV Reverse Transcription cDNA Synthesis kit were purchased from Invitrogen; Thermo Fisher Scientific, Inc. SYBR-Green PCR Master Mix was purchased from ABI, USA. Alizarin red was purchased from Sinopharm Chemical Reagent Co., Ltd., (Shanghai, China). The specific PCR primers were designed and synthesized by Invitrogen; Thermo Fisher Scientific, Inc.

Isolation and culture of rabbit BMSCs. New Zealand rabbits were anesthetized with auricular vein intravenous pentobarbital sodium with dose of 40 mg/kg. A 5.0 ml bone marrow was extracted syringe containing 0.2 ml 600 U/ml heparin by puncturing with no. 12 myeloid puncture needle at greater trochanter of the femur under aseptic condition. The bone marrow was diluted with L-DMEM (1:1), centrifuged at 1,500 rpm/min for 5 min. After removed the supernatant, cells were resuspended in 3 ml L-DMEM and centrifugal separation with an equal volume of Percoll (1.073 g/ml) at 2,500 rpm/min for 20 min. The middle

floc mist cell suspension layer was taken, then 5 ml L-DMEM medium was added, mixed, and centrifuged at 1,500 rpm/min for 5 min. The isolated BMSCs were collected and cultured with complete culture medium containing 15% fetal bovine serum at 37°C, 5% CO_2, saturated humidity. After 48 h, cell medium was exchanged, and maintained until reached 80% confluence for passages. BMSCs at passage 3 was used for experiments. The present study was approved by the Animal Care and Use Committee of the Qujing No. 1 Hospital, China.

BMSCs phenotype detection. The third generation of BMSCs ($1.5x10^6$) was trypsin digestion, centrifuged at 1,500 rpm/min for 5 min, PBS washed 2 times, and resuspended in 100 μl PBS. Cells were incubated with primary mouse anti-rabbit CD29, CD44, or CD45 antibody at room temperature for 30 min, washing with PBS twice, and then resuspended in 100 μl PBS. The corresponding second goat anti-mouse PerCP labeled secondary monoclonal antibody was incubated the cells for 30 min in the dark. After PBS washing three times, cells were resuspended in 300 μl PBS and analysis of positive rates of CD29, CD44 and CD45 were performed by FACSAriaTM III flow cytometry (BD, USA).

Transfection of BMP-2 and EGFP gene into BMSCs by adenovirus vector. The third generation of BMSCs were growth at Petri dish. At 70-80% confluence, the culture medium was replaced to serum-free L-DMEM. and BMSCs were infected with Ad-BMP-2/EGFP with multiplicity of infection (MOI) 50, 100, or 200. After 24 h, BMSCs were cultured with complete culture medium containing 15% fetal bovine serum. After 48 h, the expression of green fluorescent protein in BMSCs was observed under inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan) to detect the infection efficiency and determine the optimum MOI. Empty adenovirus (negative control (NC)) and Ad-EGFP were set as control.

The expression of hBMP-2 gene was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). According to the hBMP2 gene sequence in the NCBI database, the specific PCR primers were designed and synthesized. GAPDH as the internal reference. The primer sequence used as follow: GAPDH, Forward primer: 5-CCAGAACATCCCTGC CTC-3, Reverse primer: 5-TAGCCAAATTCGTTGTCATAC CA-3; hBMP2, Forward primer: 5-ACTACCAGAAACGAG TGGGAA-3, Reverse primer: 5-GCATCTGTTCGGAAAACC T-3. RNA from BMSCs in Ad-BMP-2/EGFP, Ad-EGFP and NC groups (empty adenovirus) (72 h post transfection) were extracted by TRIzol, and the concentrations and purity were detected by UV spectrophotometer. RNA samples with A260/A280 ratios in range of 1.8 to 2.1 were used for subsequent experiments. CDNA synthesis was performed according to the instructions of the reverse transcription kit. The synthetic CDNA is stored at -20°C. qPCR was performed using ABI 7500 Real-time PCR instrument (USA) with SYBR-Green PCR Master Mix kit. The PCR reaction conditions: 50°C 2 min, 95°C 10 min, 95°C 15 sec 40 cycles; 60°C 1 min. hBMP2 expression was analyzed using $2^{-\Delta\Delta Ct}$ methods (26).

The expression of hBMP-2 and Wnt-related pathway proteins were detected by western blot. Protein was extracted from
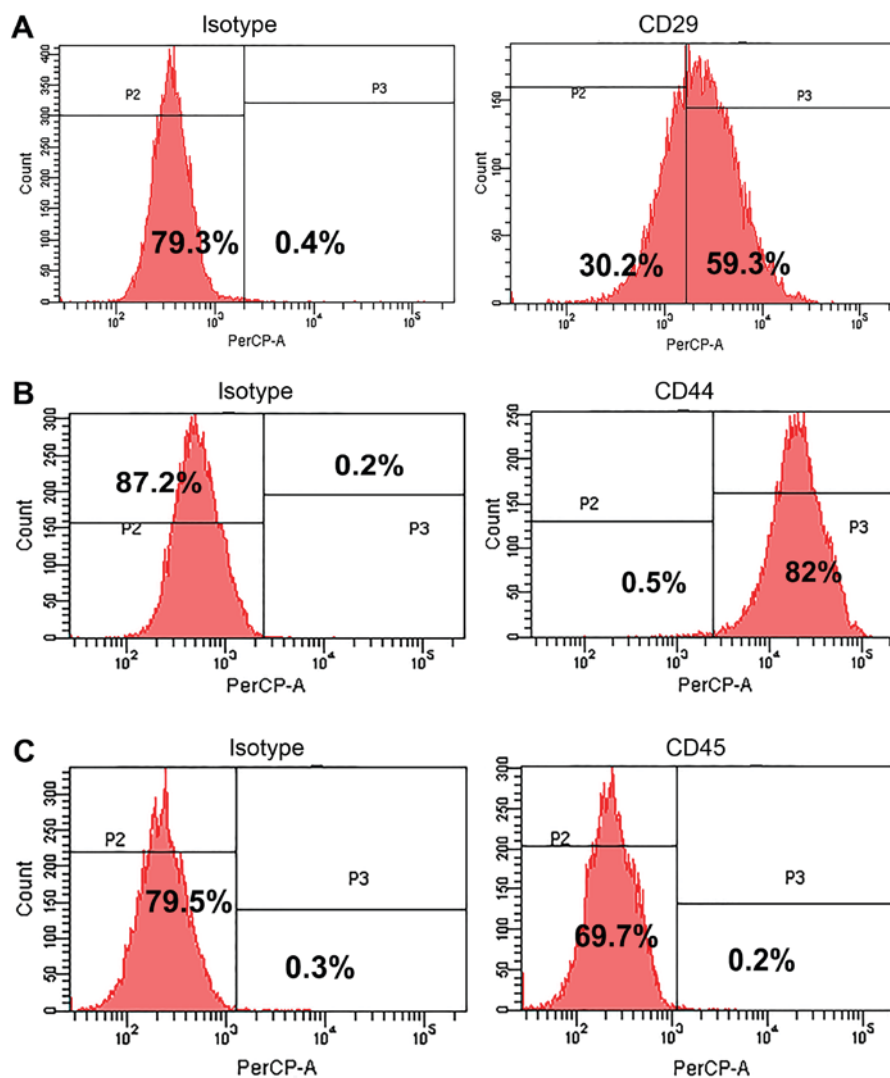
Figure 1. Detection of stem cell surface markers on isolated BMSCs by flow cytometry. (A) CD29; (B) CD44; (C) CD45. A represent image was shown. The positive rates of CD29, CD44, and CD45 were 62.92±1.99, 93.55±0.99, and 0.21±0.12%, respectively.

BMSCs in Ad-BMP-2/EGFP, Ad-EGFP and NC groups (48 h post transfection) with RIPA. The concentration was quantified by BCA protein concentration assay. 30 μg proteins were boiling 10 min, centrifugal 10 sec, and separated by 10% SDS-polyacrylamide gel electrophoresis at 80 V, 60 min, and 120 V, 30 min, and electrophoresis into PVDF at 90A for 50 min. The blots were blocked in 5% non-fat milk overnight at 4˚C, incubated with primary BMP-2 monoclonal antibody, Anti-β-catenin, anti-p-β-catenin, anti-GSK3β, anti-p-GSK3β, anti-Runx2, anti-cyclin D1 or anti-c-myc for 1 h, and HRP-conjugated secondary antibody for 1 h at room temperature. ECL was used to incubate the blots for 1 min, and blots were imaged by gel imager (JS-780; Shanghai Peiqing Science & Technology, Co., Ltd., Shanghai, China).

Detection of ALP, type I collagen, and calcium nodules for osteogenic differentiation. After transfection, osteogenic medium (L-DMEM containing 10^{-8} mol/l dexamethasone, 50 mg/l vitamin C, 10 mmol/l beta glycerophosphate, 15% fetal bovine serum) was used to induce osteogenic differentiation of BMSCs. After induction of 7 days, the ALP activity was examined by ELISA kit according to the kit instructions, and OD

value was measured by MLDEL680 ELISA detector (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 520 nm. After induction of 14 days, the cells were fixed with 4% paraformaldehyde, permeabilized with 1% Triton-X100, and blocked with 1% BSA. Then, cells were incubated with primary mouse anti-rabbit type I collagen antibody overnight at 4˚C, HPR-conjugated secondary goat anti-mouse antibody at room temperature for 1 h. DAB colored, stained, dehydrated, transparent, lining, and then the expression of type I collagen was observed under light microscope. After induction of 21 days, alizarin red S staining was performed. BMSCs were immobilization, rinse, and incubated with 0.1% (mass/volume) alizarin red solution for 5 min at room temperature, rinsed 3 times, and the calcium nodules was observed under microscope.

Statistical analysis. All data were presented as mean ± standard deviation (SD) at least three experimental repeats. The statistical analysis was performed using SPSS v.16.0 (IBM Corp., Armonk, NY, USA). Multigroup comparisons of the means were carried out by one-way analysis of variance (ANOVA) test with post hoc contrasts by Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.
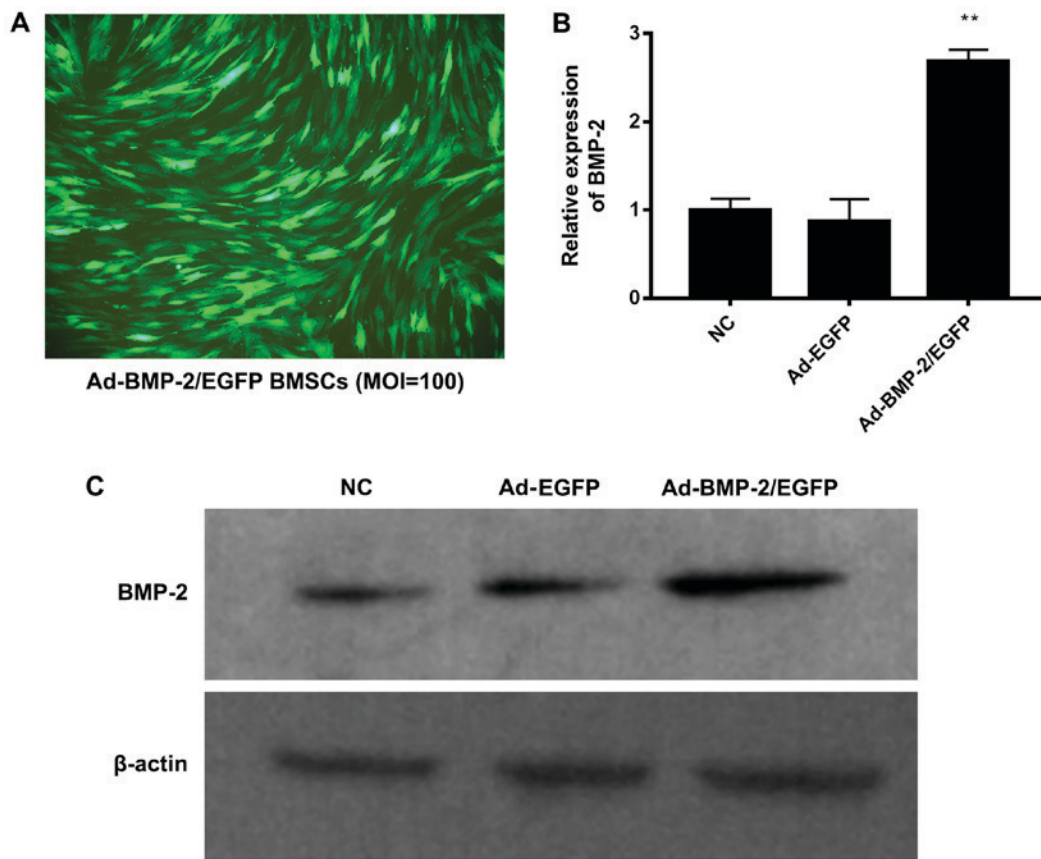
Figure 2. Transfection of Ad-BMP-2/EGFP in rabbit BMSCs. (A) Transfection efficient was detected after transfection for 48 h (MOI 100) by fluorescence microscope (magnification, x100). The rabbit BMSCs were transfected with empty adenovirus (NC), Ad-EGFP or Ad-BMP-2/EGFP. (B) Expression of BMP-2 mRNA was performed by reverse transcription-quantitative polymerase chain reaction. (C) Expression of BMP-2 protein was performed using western blot analysis. **P<0.01 vs. NC.

Results

Identification of the isolated rabbit BMSCs. The surface markers were detected to identify the rabbit BMSCs. The isolated cell at passage 20 were labeled with anti-CD29, anti-CD44, and anti-CD45 and examined by flow cytometry (Fig. 1). The results showed that CD44 and CD29 were positive expression with a positive rate of 93.55±0.99% (Fig. 1B) and 62.92±1.99% (Fig. 1A), respectively, but CD45 was negative expression and its positive rate was only 0.21±0.12% (Fig. 1C). The results suggested the isolated rabbit cells were BMSCs.

Transfection of rabbit BMSCs with Ad-BMP-2/EGFP. The rabbit BMSCs were transfected with empty adenovirus, Ad-EGFP or Ad-BMP-2/EGFP. After transfection of 48 h, it was observed that the transfection efficiency was more than 95% with good cell growth state (MOI:100) by fluorescent microscopy (Fig. 2A). When MOI was 50, the transfection efficient was about 60%. When MOI was 200, the transfection efficient was not significantly increased compared to MOI 200, but there were floating cells and cell morphology was changed from long spindle into fan-shaped distribution, which may be due to adenovirus cytotoxicity (data not shown). Thus, the optimal MOI was 100, which was used in the followed experiments.

The rabbit BMSCs were transfected with empty adenovirus (NC), Ad-EGFP or Ad-BMP-2/EGFP. After transfection of
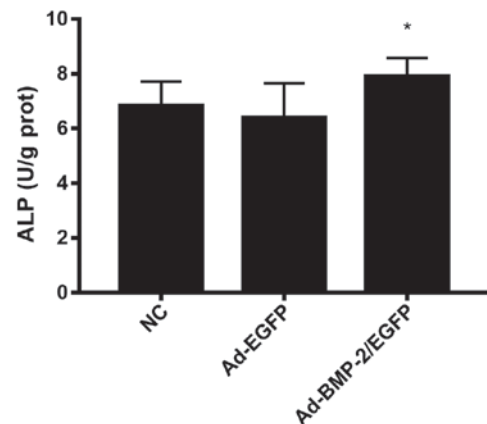

Figure 3. ALP level in Ad-BMP-2/EGFP transfected rabbit BMSCs. After induction with osteogenic medium (L-DMEM containing 10^{-8} mol/l dexamethasone, 50 mg/l vitamin C, 10 mmol/l beta glycerophosphate, 15% fetal bovine serum) for 7 days, ALP measurement was performed. *P<0.05 vs. NC. ALP, alkaline phosphatase; BMSCs, bone marrow mesenchymal stem cells.

48 h, the expression of BMP-2 mRNA and protein was detected by RT-qPCR and western blot (Fig. 2B and C). The BMP-2 mRNA was significantly upregulated in Ad-BMP-2/EGFP transfected BMSCs, compared with NC and Ad-EGFP transfected BMSCs (Fig. 2B). Consistently, the BMP-2 protein was significantly upregulated by Ad-BMP-2/EGFP (Fig. 2C). Thus, the BMP-2 was upregulated in rabbit BMSCs.
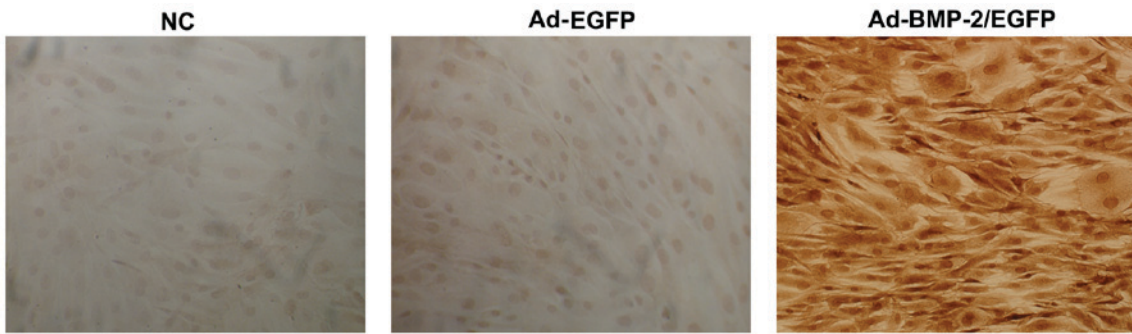
Figure 4. Type I collagen level in Ad-BMP-2/EGFP transfected rabbit BMSCs. After induction with osteogenic medium (L-DMEM containing 10⁻⁸ mol/l dexamethasone, 50 mg/l vitamin C, 10 mmol/l beta glycerophosphate, 15% fetal bovine serum) for 14 days, immunohistochemical detection of type I collagen was performed. BMSCs, bone marrow mesenchymal stem cells.
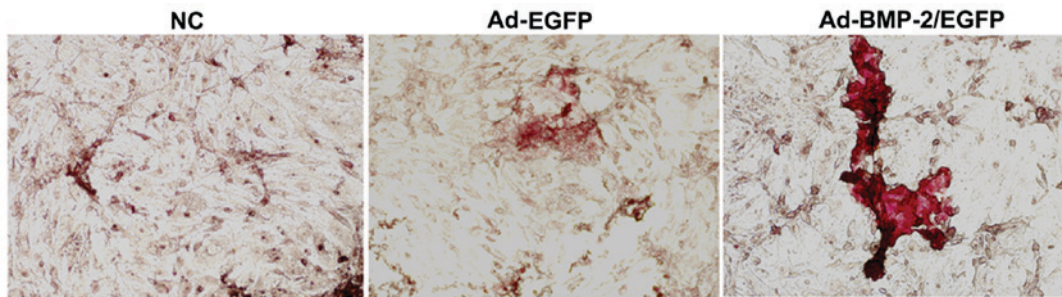

Figure 5. Formation of mineralized nodules in Ad-BMP-2/EGFP transfected rabbit BMSCs. After induction with osteogenic medium (L-DMEM containing 10⁻⁸ mol/l dexamethasone, 50 mg/l vitamin C, 10 mmol/l beta glycerophosphate, 15% fetal bovine serum) for 21 days, alizarin red S staining was performed (magnification, x100). BMSCs, bone marrow mesenchymal stem cells.
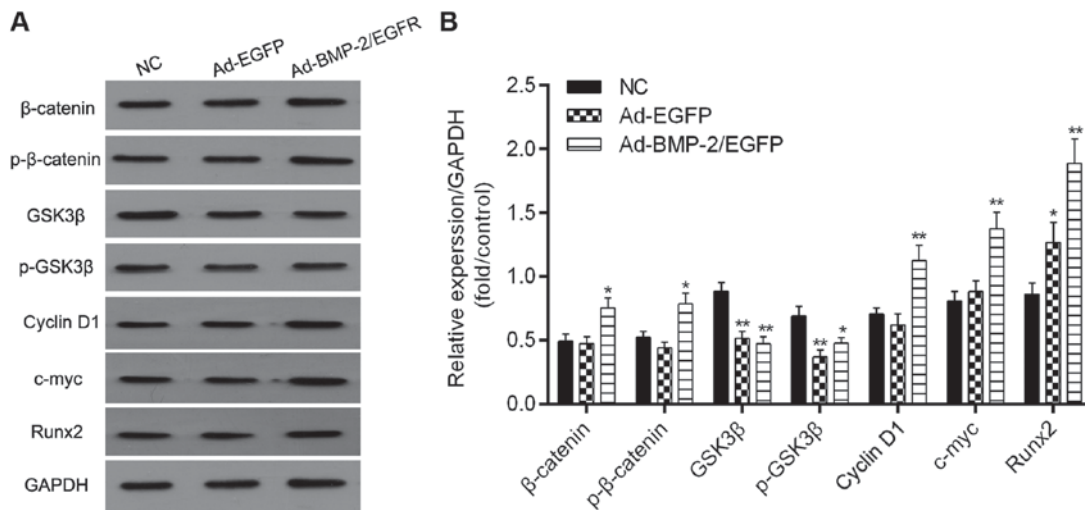

Figure 6. Detection of Wnt signaling marks. After induction with osteogenic medium (L-DMEM containing 10⁻⁸ mol/l dexamethasone, 50 mg/l vitamin C, 10 mmol/l beta glycerophosphate, 15% fetal bovine serum) for 21 days. (A) The expression of β-catenin, p-β-catenin, GSK3β, p-GSK3β, cyclin D1, c-myc and Runx2 were detected using western blot analysis. (B) Protein levels of β-catenin, p-β-catenin, GSK3β, p-GSK3β, cyclin D1, c-myc and Runx2 quantified by densitometry. *P<0.05 and **P<0.01 vs. NC.

Ad-BMP-2/EGFP promotes osteogenesis of rabbit BMSCs. To detect the osteogenesis of rabbit BMSCs after transfection, ALP measurement was performed in cells after induction with osteogenic medium (L-DMEM containing 10⁻⁸ mol/l dexamethasone, 50 mg/l vitamin C, 10 mmol/l beta glycerophosphate, 15% fetal bovine serum) for 7 days (Fig. 3). Ad-BMP-2/EGFP induced ALP compared with NC and Ad-EGFP groups.

Immunohistochemical detection of type I collagen in BMSCs were performed after 14 days of induction (Fig. 4). In Ad-BMP-2/EGFP transfected cells, brown yellow staining was shown, suggesting positive expression of type I collagen. The rabbit BMSCs transfected with NC and Ad-EGFP showed a negative expression of type I collagen. Thus, upregulation of BMP-2 in rabbit BMSCs promoted production of type I collagen.

After induction with osteogenic medium for 21 days, the alizarin red S staining was performed (Fig. 5). In Ad-BMP-2/EGFP transfected cells, obvious mineralized nodules were formed with red staining, in contrast, no calcium nodules were formed in NC and Ad-EGFP group. Thus, overexpression of BMP-2 enhanced osteogenic differentiation ability of cultured rabbit BMSCs.

Expression of Wnt signaling-related proteins. The expression levels of Wnt signaling-related proteins including β-catenin, p-β-catenin, GSK3β, p-GSK3β, cyclin D1, c-myc and Runx2 were examined by western blot (Fig. 6). A significant increase in the protein expression levels of β-catenin, cyclin D1, Runx2 and c-myc were found in Ad-hBMP-2/EGFP transfected BMSCs, and the levels of GSK3β and p-GSK3β were significantly decreased. Thus, these results suggested that activation of Wnt signaling was responsible for the Ad-hBMP-2/EGFP-induced bone formation.

Discussion

In the present study, we isolated and identified the rabbit BMSCs, which was highly expressed CD44 and lowly expressed CD45. The positive rate of CD29 is only 62.92±1.99%, suggesting these cells are not CD29-dependent. Those cells showed a strong osteogenic differentiation ability after Ad-BMP-2/EGFP transfection. The rabbit BMSCs provides enough BMP-2 protein for osteogenic differentiation, which is expected to become the ideal seed cells in bone tissue engineering.

In recent years, the researches of stem cells have provided new ideas for bone tissue engineering (27,28). It has been confirmed that BMSCs has significant differentiation ability of osteoblasts *in vitro* in the treatment of specific chemical substances including β-phosphoglycerol, dexamethasone and vitamin C; cytokines and mechanical mechanics stimulation (28,29). Many factors including cytokines can induce BMSCs to differentiate into osteoblasts. The most important one of the cytokines are BMPs (28,30). BMP is a major regulatory factor for the differentiation of BMSCs into osteoblasts among a number of factors that activate or inhibit the osteoblastic signaling pathways. The increase in BMP contents promotes the differentiation of BMSCs into osteoblasts. Ishikawa *et al* (31), used hBMP2 to incubate the *in vitro* cultures of BMSCs, and found hBMP-2 enhanced cell proliferation, while maintain osteogenic differentiation in the process of passage.

The mainstream approach used in bone tissue engineering is to release BMP directly from biomaterials to the site where bone or cartilage tissue needs to be regenerated. If large bone remodeling is required for prolonged induction of BMSCs, the researchers trend to choose a controlled release carrier or scaffold. Simmons *et al* (32) used sodium alginate hydrogel as bio-scaffold to combine with BMP-2 and/or transforming growth factor beta 3 (TGF-β3), to induce the osteogenic differentiation of BMSCs in SCID mice. They found that the effective protein concentration of BMP-2 in the osteogenesis is associated with the biological scaffold materials. The nature BMP induced BMSCs ossification in a dose-dependent manner. Roostaeian *et al* (33) used hBMP-2 to induce the osteogenesis of rabbit BMSCs, and found that the best suitable concentration of BMP-2 is 50 ng/ml. Wang *et al* (34) found that, in mouse BMSCs after treatment of BMP-2, ALP level and mineralization

was only induced in a very low extent, but the osteogenic differentiation ability of BMSCs was significantly increased when combined use of bone foaming medium with BMP-2. In our study, the BMP-2 and EGFP genes can be efficiently introduced into rabbit BMSCs by recombinant adenovirus vector, which increased ALK, type I collagen, and calcium nodules, thus accelerated the osteogenesis.

Zhu *et al* (35) infected the goat BMSCs with recombinant adenovirus containing BMP-7 gene and used in the treatment of segmental bone defect. They found BMP-7 can significantly promote new bone formation, and the biomechanics of new bone meet the physiological requirements after operation for 5 months (35). Xu *et al* (36) used adenovirus mediated BMP2 gene transfection goat BMSCs to treat the bone defect, and found the maximum compressive strength was increased after 24 weeks, but with a persistent humoral immune response. The role of rabbit BMSCs with recombinant adenovirus containing BMP-2 gene will studied in the future.

Li *et al* (37), demonstrated that BMP2 activated the Wnt signaling pathway by upregulating β-catenin and cyclin D1, but downregulating GSK3β. Previously, Qian *et al* (38) reported that in the BMP2-induced diabetic BMSCs, the levels of GSK3β and p-GSK3β were significantly reduced, and the levels of Runx2, OSX, cyclin D1 and c-myc were significantly increased. In our study, Ad-hBMP-2/EGFP induced significant increases in the protein expression levels of β-catenin, cyclin D1, Runx2 and c-myc in rabbit BMSCs, and decreases in the levels of GSK3β and p-GSK3β, which is in agreement with previous studies.

In conclusion, the transfected BMSCs can provide the source of BMP-2 protein and also provide BMP-2 self-target cells, which might promote osteogenesis of rabbit BMSCs via activation of Wnt signaling pathway. Our results provided the novel evidence to support rabbit BMSCs as the best seed cell in bone tissue engineering, and a foundation for the treatment of bone defect in animal model by Ad-hBMP-2/EGFP as it induces the differentiation of rabbit BMSCs into osteoblasts. However, further studies are needed to validate this observation that Ad-hBMP-2/EGFP induced osteogenesis of BMSCs *in vivo*. Moreover, it will be necessary to further explore the functions and mechanisms of Ad-hBMP-2/EGFP in osteogenesis of BMSCs.

Authors' contributions

CCW, FW, SR, JR, JSW, LXS, ZW, TL and QL made substantial contributions to the conception and design of the present the study. CCW, FW, SR and JR performed the experiments. CCW wrote the paper. CCW and QL revised the manuscript

critically for important intellectual content. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Care and Use Committee of Qujing No. 1 Hospital and experiments were performed in accordance with the policies and principles in the Guide for Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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