Cell sheets of co-cultured BMP-2-modified bone marrow stromal cells and endothelial progenitor cells accelerate bone regeneration *in vitro*

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Abstract. Bone tissue engineering provides a substitute for bone transplantation to address various bone defects. However, bone regeneration involves a large number of cellular events. In addition, obtaining sufficient source material for autogenous bone or alloplastic bone substitutes remains an unsolved issue. In previous studies, it was confirmed that bone marrow stromal cells (BMSCs) and endothelial progenitor cells (EPCs) had the capacity to promote bone regeneration. Additionally, bone morphogenetic protein-2 (BMP-2) has been demonstrated to be an active inducer of osteoblast differentiation. Therefore, the aim of the present study was to produce an effective integration system, including a scaffold, reparative cells and growth factors, that may enhance bone regeneration. Firstly, bone marrow-derived BMSCs and EPCs were isolated and identified by flow cytometry. Cell proliferation ability, secreted BMP-2 levels and alkaline phosphatase (ALP) activity were highest in the cell sheets containing BMP-2-modified BMSCs and EPCs. In addition, the expression levels of osteogenesis-associated genes, including runt related transcription factor 2 (Runx2), distal-less homeobox 5 (Dlx5), ALP and integrin-binding sialoprotein (Ibsp), and osteogenesis-associated proteins, including Runx2, Dlx, ALP, Ibsp, vascular endothelial growth factor, osteonectin, osteopontin and type I collagen, gradually increased during the co-culture of ad-BMP-2-BMSCs/EPCs. The levels of these genes and proteins were increased compared with those observed in the BMSC, EPC and BMP-2-modified BMSC groups. Finally, scanning electron microscopy observation also demonstrated that the BMP2-modified BMSCs were able to combine well with EPCs to construct a cell sheet for bone formation. Collectively, these results describe an adenovirus (ad)-BMP2-BMSCs/EPCs co-culture system that may significantly accelerate bone regeneration compared with a BMSCs/EPCs co-culture system or ad-BMP2-BMSCs alone.

Introduction

Large bone defects caused by acute injuries, trauma, metabolic or genetic bone diseases, spinal degenerative diseases, fall fractures in patients with osteoporosis, tumors and congenital deformities are very common in clinical orthopedic cases (1,2). Accumulating evidence has suggested that there are >3,000,000 patients with bone defects in China; in addition, the number of bone defects is increasing 10% each year with increases in population aging. Autogenous bone transplantation, which has the advantages of biocompatibility and the lack of immunogenicity, or alloplastic bone substitutes would be the gold standard for these patients (3,4). Nevertheless, the clinical practice of these therapies has been largely limited due to the lack of sources for autogenous bone and by significant complications, including infection, bleeding, pain and fracture. Therefore, it is of critical importance to identify suitable substitutes or alternative materials for bone transplantation.

In previous reports bone marrow stromal cells (BMSCs) have been widely applied for the treatment of various diseases, including graft-versus-host disease, osteogenesis imperfecta and myocardial infarction (5,6). In addition, multiple studies have clearly demonstrated that BMSCs have great potency for promoting the regeneration of bone defects in animal models and in humans, due to their high capacity for self-renewal and multipotentiality for differentiation; therefore, they are now being considered for use in a wide range of tissue engineering applications, and in cell or gene therapy as an alternative

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strategy and promising option (7,8). Although it is accepted that the treatment of bone defects using BMSCs or genetically modified BMSCs may effectively promote bone regeneration in human and animal models, the size of the regenerated bone has been a limiting factor for complete bone repair, primarily due to a lack of vessels in the grafts, which prevents sufficient nutritional support to the entire bone graft (9). However, endothelial progenitor cells (EPCs), a subpopulation of pluripotent hematopoietic stem cells, may proliferate and migrate to sites of damaged endothelium and differentiate into vascular endothelial cells (10). For example, exogenous EPCs were implanted into various ischemic tissue models, including areas of myocardial infarction and ischemic hindlimbs, to facilitate neovascularization (11,12). Additionally, it has been demonstrated that EPCs may contribute to new bone formation in fracture healing (13,14). Therefore, EPCs may serve a critical role in vessel regeneration and functional recovery following bone injuries. With regard to the respective characteristics of BMSCs and EPCs, the present study aimed to construct a cell sheet that combined BMSCs and EPCs for the study of bone regeneration.

Bone morphogenetic proteins (BMPs) are potent osteoinductive growth factors that induce ectopic bone formation (15). Of these, BMP-2 is one of the most potent osteoinductive cytokines and has been demonstrated to initiate the differentiation of mesenchymal stem cells into osteoblasts and chondrocytes in several animal models (16,17). Recombinant human BMP-2 was first approved by the United States of America Food and Drug Administration in 2002 to be used as a bone graft substitute in the field of bone surgery, in procedures including the fixation of open tibial fractures, spinal fusion surgery, or oral and maxillofacial surgery (18). Therefore, the present study examined whether BMP-2 exhibited a synergistic stimulatory effect on bone formation in a co-culture of BMSCs and EPCs.

Materials and methods

BMSCs and EPC isolation and culture. All experiments in the present study were performed in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals (19) and approved by the Ethics Committee of The First Hospital of Kunming Medical College (Kunming, China). Adult male Sprague-Dawley (SD) rats ($n=20$; age, 5 weeks; weight, ~220 g) were purchased from Charles River Laboratories. Rats were housed in standard translucent ventilated laboratory rat cages, maintained in climate-controlled rooms (21-24°C; 50-55% humidity) with diurnal lighting (12-h light/dark cycle; lights on at 06:00). Bone marrow collected from male SD rats aged 5 weeks was flushed out from the femurs and tibias with Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences) containing 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 g/ml streptomycin using a 1 ml syringe under sterile conditions. To obtain the BMSCs, the bone marrow was placed on top of Ficoll solution (cat. no. B340217P; Bioplorer; https://www.biomart.cn/info-supply/61288999.htm) and centrifuged at 150 x g at 4°C for 25 min. The opaque white layer on the surface of the Ficoll solution was carefully collected using Pasteur pipettes and resuspended in DMEM. The collected cells were analyzed by

flow cytometry, and CD44 antigen (CD44)$^+$ and transferrin receptor protein 1 (CD71)$^+$ cells were isolated using fluorescence-assisted cell sorting (FACS), as described subsequently. Then, the isolated cells were seeded into tissue culture flasks at a final concentration of $1x10^6$ cells/ml in DMEM. After 24 h incubation in a 37°C humidified atmosphere with 5% CO$_2$, non-adherent cells were removed, and the adherent fraction was cultured in fresh medium. Cells used for subsequent experiments were passaged ≤10 times.

The isolation of EPCs involved the use of Percoll, rather than Ficoll, for density gradient centrifugation, 300 x g at room temperature for 10 min. Similar to the BMSCs, the collected EPCs were analyzed by flow cytometry, and **platelet endothelial cell adhesion molecule (CD31)$^+$**, prominin-1 (CD133)$^+$ and vascular endothelial growth factor receptor (VEGFR)$^+$ cells were isolated using FACS, as described subsequently. Isolated EPCs were washed twice with PBS and then suspended at a density of $1x10^6$ cells/ml in endothelial cell growth medium (EGM) media (PromoCell GmbH) supplemented with 2% FBS and growth factors, including 50 ng/ml VEGF, 1 ng/ml basic fibroblast growth factor and 2 ng/ml insulin-like growth factor 1. Non-adherent cells were removed after 24 h incubation in 5% CO$_2$/95% air at 37°C in a humidified atmosphere, and fresh EGM media was added to the culture dishes. After 5-7 days, the adhered cells at 90% confluence were separated for subsequent passages.

Flow cytometry analysis of cells. To analyze the expression of surface markers characteristic of BMSCs and EPCs, FACS was performed using specific fluorochrome-conjugated monoclonal antibodies corresponding to each cell type. Briefly, BMSCs and EPCs were harvested at passage 3, and $1x10^6$ cells were washed with 10% FBS/PBS and centrifuged at 300 x g for 5 min at room temperature to pellet the cells. The collected cells were blocked using Innovex Fc Receptor Blocker (Newcomer Supply, Inc.) for 1 h at 4°C and washed using ice-cold PBS and centrifuged at 300 x g at 4°C for 5 min. Subsequently, primary antibodies purchased from BIOSS, including fluorescein isothiocyanate/phycoerythrin (FITC/PE)-conjugated rat anti-CD44 (cat. no. bs-4916R-FITC), FITC/PE-conjugated rat anti-CD71 (cat. no. bs-1782R-FITC) and FITC/PE-conjugated rat anti-receptor-type tyrosine-protein phosphatase C (CD45) (cat. no. bs-10599R-FITC) at a concentration of 2 mg/ml for the identification of BMSCs; and FITC/PE-conjugated rat anti-CD31 (cat. no. bs-0195R-FITC), FITC/PE-conjugated rat anti-CD133 (cat. no. bs-0395R-FITC) and FITC/PE-conjugated rat anti-VEGFR (cat. no. bs-0170R-FITC) were added to the cells at a concentration of 2 mg/ml and incubated with anti-mouse compensation beads for 30 min in the dark for the identification of EPCs. Subsequently, unbound antibody was removed by washing with 2 ml of 10% FBS/PBS, and pellets were resuspended in 500 μl PBS and examined by flow cytometry, with 10,000 events recorded for each condition. Flow cytometry data was analyzed using BD CellQuest™ Pro software Version 5.1 (BD Bioscience).

BMP2 gene transfer. BMP2 adenovirus (ad-BMP2) was purchased from Sangon Biotech Co., Ltd. The optimal virus concentration for gene transfer was evaluated by examining a range of multiplicities of infection (MOI), according to the

manufacturer's protocol. For the transduction of BMSCs, ad-LacZ (control) or ad-BMP2 adenovirus was added to the cells at a MOI of 100 in serum-free DMEM. After 4 h, FBS was added to a final concentration of 2%, and cells were cultured for an additional 24 h, using a protocol as described previously (20).

Co-culture of BMSCs and EPCs. Following the third passage of BMSCs and 24 h after adenoviral transduction, a mixture of EPCs and ad-lacZ or ad-BMP-2-transduced BMSCs were co-cultured at EPC:BMSC ratio of 5:1, according to the optimized conditions as previously described (9). This EPC:BMSC mixture was seeded into 6-well plates at a density of $3.6x10^5$ cells/well. When the cells grew to 80-90% confluence, the cell culture medium was shifted to cell sheet-inducing medium (α-minimum essential medium supplemented with 10% FBS and 1% penicillin/streptomycin).

Proliferation assay. The proliferative capacity of the cells was measured using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. Briefly, 2,000 cells/well were seeded into 96-well plates and transfected with different adenoviral particles as aforementioned. At the indicated time points (days 3 and 7), 10 μl CCK-8 reagent was added to each well and cells were cultured for an additional 2 h, followed by measurement of the optical density (OD) at an absorbance wavelength of 450 nm using an enzyme immunoassay analyzer (Bio-Rad Laboratories, Inc.).

Assessment of BMP-2 release in vitro. BMP-2 levels in BMSC: EPC-conditioned cell medium were measured using a human-specific BMP-2 Quantikine ELISA kit (cat. no. DBP2000; R&D Systems, Inc.) according to the manufacturer's protocols.

Alkaline phosphatase (ALP) activity. To determine ALP activity, cells from the different treatment groups were lysed with 50 μl CelLytic M and 100 μl substrate solution consisting of 3.33 mM $MgCl_2$ (VWR International, LLC) and 500 mM 2-amino-2-methyl-1-propanol (Sigma-Aldrich; Merck KGaA) in distilled water with a pH 7.4, and repeatedly frozen-thawed 3 times to disrupt the cell membranes. Then, aliquots of the cell lysates were incubated with 0.5 μg/ml p-nitrophenol-phosphate (Sigma-Aldrich; Merck KGaA) for 30 min at 37°C, and the results were quantified at 405 nm using a microplate reader (BioTek Instruments, Inc.). A standard curve for total ALP activity was generated using increasing concentrations of the ALP reaction product, 4-nitrophenol (0-1 nmol/μl; Sigma-Aldrich; Merck KGaA).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. The expression levels of runt related transcription factor 2 (Runx2), distal-less homeobox 5 (Dlx5), ALP and inte-grin-binding sialoprotein (Ibsp) were analyzed by RT-qPCR in cell cultures after 0, 1, 3, 7 and 14 days of growth. Total RNA was isolated using RNeasy kit (Qiagen China Co., Ltd.), and its quantity and purity were estimated using a NanoDrop™ 2000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Only samples with an A_{260}/A_{280} nm ratio

Table I. Sequences of primers used in the present study.

Gene	Primer sequences
Runx2	Forward, 5'-CGTCCACTCTGAATACCTGT-3' Reverse, 5'-TTGCCATTTTCAGTTTTGCAT-3'
Dlx5	Forward, 5'-CTCGGCTTCCTGGTACCCAA-3' Reverse, 5'-TCCATTGTTCAAACATCCCCGTA-3'
ALP	Forward, 5'-TCAAAGCAGCATCTTACCAGT-3' Reverse, 5'-TGCCACAGTCAATACCGGAA-3'
Ibsp	Forward, 5'-TCAACTCAGGAAGGTGCAAT-3' Reverse, 5'-CAGCCCTGATTTACGATGACC-3'
GAPDH	Forward, 5'-CAAAGTGGACATTGTTGCCAT-3' Reverse, 5'-TCACCCCATTTGATGTTAGCG-3'

Runx2, runt related transcription factor 2; Dlx5, distal-less homeobox 5; ALP, alkaline phosphatase; Ibsp, integrin-binding sialoprotein.

between 1.8 and 2.0 were used. A total of 1 μg total RNA sample was used as a template for conversion into cDNA using a SuperScript® First-Strand Synthesis System kit (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. qPCR was performed using a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.); the reactions contained cDNA, primers (Table I) and SYBR® Premix Ex Taq™ (Takara Bio, Inc.). Post-PCR melting curves confirmed the specificity of single-target amplification, and the fold change in the gene of interest relative to GAPDH was determined. All reactions were performed in triplicate. qPCR was performed with SYBR® Premix Ex Taq™ in accordance with the manufacturer's protocols (Takara Bio, Inc.) in a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc). The thermocycler conditions were as follows: Initial denaturation at 95°C for 10 sec, followed by 40 cycles of 95°C 10 for sec and 34 sec at 60°C. Each cDNA sequence was examined in triplicate, and a dissociation melt curve protocol was conducted following each PCR procedure, to determine the specificity of the products. The relative amount of expressed mRNA was also calculated using the $2^{-\Delta\Delta Cq}$ method (21).

Western blot analysis. Total protein was extracted from all samples with radioimmunoprecipitation assay lysis buffer (0.5 mM HEPES, pH 7.4, 5.0 M NaCl, 10% Triton X-100, 10% glycerol, 0.2 M Na_3VO_4, 0.5 M NaF and 0.1 M NaPP) and quantified using the Bicinchoninic Acid assay method (Promega Corporation). A total of 30 μg protein per sample was electrophoresed on an 8-10% denaturing SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (EMD Millipore) at a constant current of 200 mA for 60 min. The membrane was blocked with 5% non-fat milk in TBS with 0.1% Tween-20 (TBST) for 2 h at room temperature and incu-bated overnight with the following primary antibodies: Rabbit anti-rat Runx2 (1:1,500; cat. no. ab92336; Abcam); rabbit anti-rat Dlx (1:1,000; cat. no. ab109737; Abcam); rabbit anti-rat ALP (1:500; cat. no. sc-365765; Santa Cruz Biotechnology, Inc.); rabbit anti-rat Ibsp (1:1,000; cat. no. ab52128; Abcam); rabbit anti-rat VEGF (1:1,000; cat. no. ab53465; Abcam);
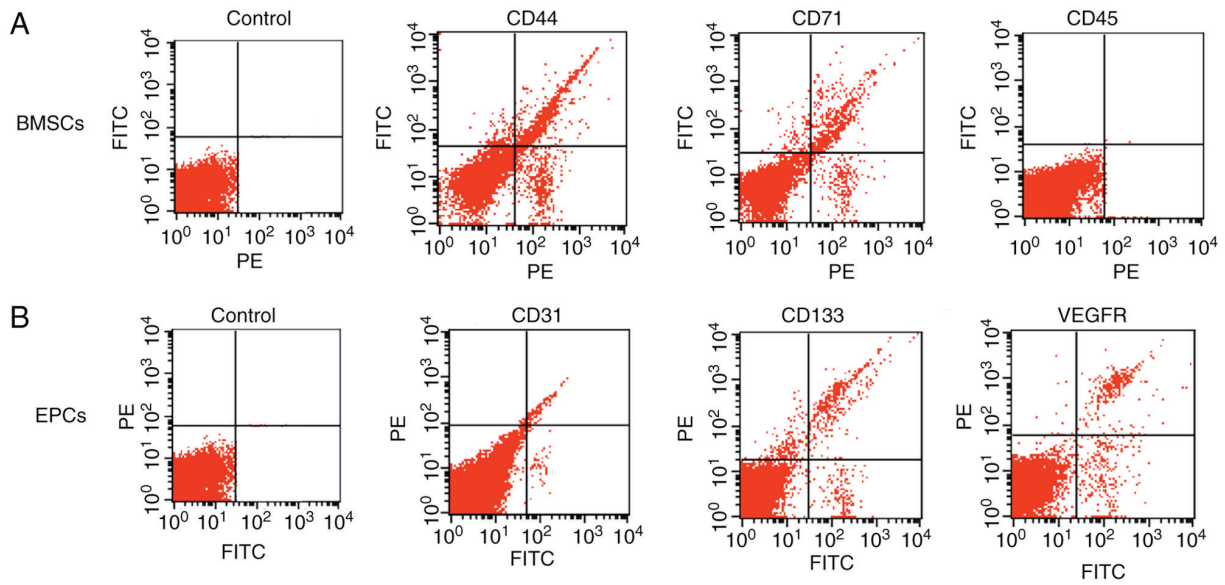
Figure 1. Characterization of BMSCs and EPCs. (A) Flow cytometry analysis of Ficoll-separated BMSCs surface markers, including CD44, CD71 and CD45. (B) Flow cytometry analysis of Percoll-separated EPCs surface markers, including CD31, CD133 and VEGFR. BMSCs, bone marrow stromal cells; EPCs, endothelial progenitor cells; CD44, CD44 antigen; CD71, transferrin receptor protein 1; CD45, receptor-type tyrosine-protein phosphatase C; CD31, platelet endothelial cell adhesion molecule; CD133, prominin-1; VEGFR, vascular endothelial growth factor receptor; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

rabbit anti-rat osteonectin (also known as SPARC; 1:2,000; cat. no. ab245733; Abcam); rabbit anti-rat osteopontin (1:2,000; cat. no. ab8448; Abcam); and rabbit anti-rat type I collagen (1:1,500; cat. no. 84336; Cell Signaling Technology, Inc.) for 1.5 h at room temperature. Following extensive washing with TBST, the membrane was exposed to the corresponding horse-radish peroxidase (HRP)-conjugated secondary antibodies (1:3,000; cat. no. ab97080; Abcam) for 1 h at room temperature and detected using the Phototope-HRP Western Detection kit (Thermo Fisher Scientific, Inc). Expression levels of each protein were normalized to that of GAPDH.

Scanning electron microscopy (SEM). SEM was used to examine the surface and microstructure of the cell sheets. Briefly, the cells from different groups were washed with PBS twice, fixed with 4% glutaraldehyde solution at room temperature for 2 h and dehydrated in increasing concentrations of ethanol (50, 70, 80, 90, 95 and 100%). Subsequently, the samples were freeze-dried, coated with a gold layer using a sputter coater, and imaged by SEM.

Statistical analysis. All experimental values are presented as the mean ± standard deviations, and all analyses were performed using the SPSS v17.0 software (SPSS, Inc.) One-way ANOVA followed by Tukey's post-hoc test was used to compare data between >2 groups, and one-tailed t-test between two groups by using SPSS software (version 19.0; IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification and isolation of BMSCs and EPCs. The present study assessed the phenotypes of BMSCs, EPCs and cells isolated from BMSCs and EPCs by flow cytometric analysis. Ficoll density gradient-separated BMSCs were identified to express high levels of CD44 and CD71, but did not express CD45 (Fig. 1A). In addition, Percoll density gradient-separated EPCs were identified to express high levels of EPC surface markers, including CD31, CD133 and VEGFR (Fig. 1B). CD44$^+$ and CD71$^+$ cells were isolated from pure BMSCs and CD31$^+$, CD133$^+$ and VEGFR$^+$ cells were isolated from pure EPCs using FACS. The isolated BMSCs and EPCs were cultured for subsequent experiments. The results from the flow cytometry analysis suggested that the isolated cells were classic BMSCs and EPCs, respectively.

ad-BMP-2-BMSCs/EPCs system accelerates cell proliferation, promotes persistent BMP-2 secretion and activates ALP activity in the BMSCs and EPCs co-culture system. A CCK-8 assay was used to quantify levels of cell proliferation. It was demonstrated that the OD values after 7 days were increased compared with those after 3 days in all groups (Fig. 2A). In addition, the cells in the BMP-2-modified BMSCs + EPCs group grew at an increased rate compared with those in the other groups. BMP2 secretion and ALP activity in the BMSCs and EPCs co-culture system were markedly increased in a time-dependent manner (Fig. 2B and C). Collectively, these data concluded that the BMSCs and EPCs co-culture system may promote bone cell proliferation and differentiation.

ad-BMP-2-BMSCs/EPCs system promotes the mRNA expression of Runx2, Dlx5, ALP and Ibsp. Next, the expression levels of osteoblast-associated genes and proteins, including Runx2, Dlx5, ALP and Ibsp, were examined to evaluate osteoblast differentiation and bone formation in each group. As indicated in Fig. 3, the expression levels of Runx2 (Fig. 3A), Dlx5 (Fig. 3B), ALP (Fig. 3C) and Ibsp (Fig. 3D), which are essential
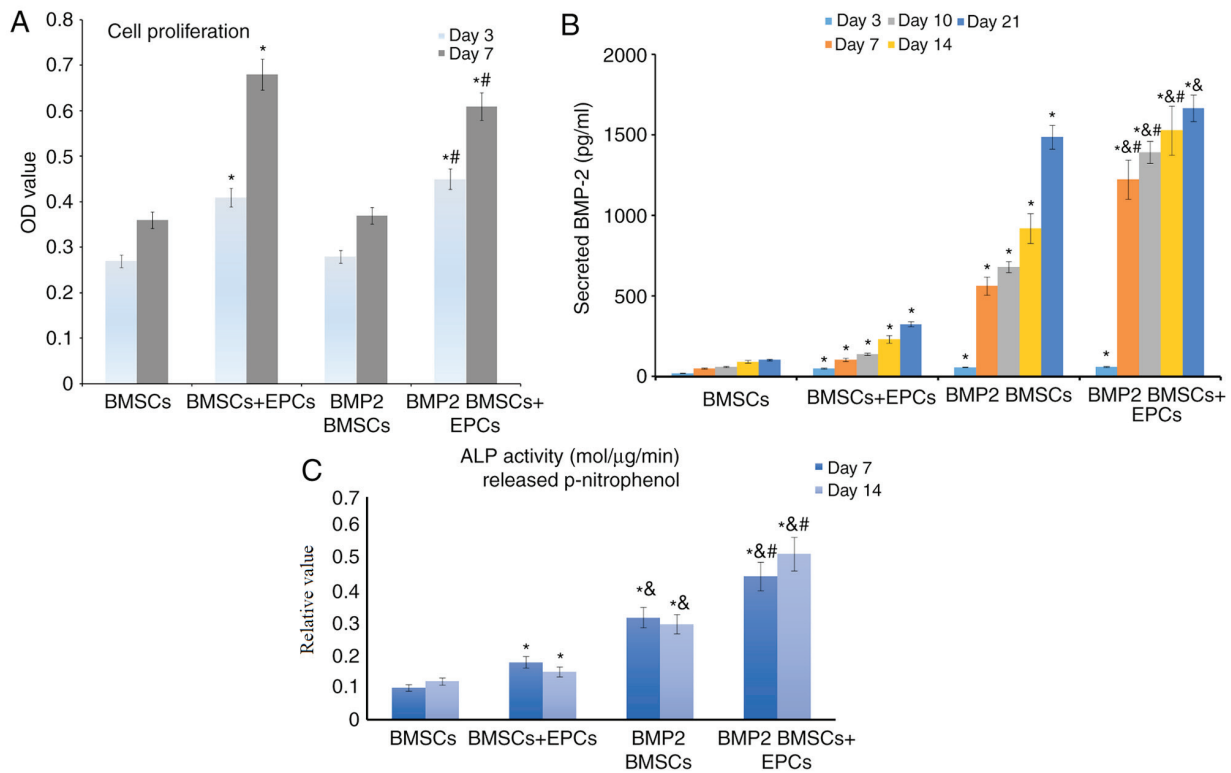
Figure 2. Effects of the ad-BMP-2-BMSCs/EPCs system on cell activity. (A) The proliferation rate of cells in different groups was detected using Cell Counting Kit-8 assay. (B) BMP-2 release was determined using ELISA. (C) The effects of culture conditions on ALP activity were examined. $^*P<0.05$ vs. BMSCs group; $^{\&}P<0.05$ vs. BMSCs + EPCs group; $^{\#}P<0.05$ vs. BMP2 + BMSCs group. BMSCs, bone marrow stromal cells; EPCs, endothelial progenitor cells; BMP2, bone morphogenic protein 2; ALP, alkaline phosphatase.

in bone formation, increased with time in all groups. In addition, the most notable changes in expression of these genes were in the BMP-2-modified BMSCs/EPCs group. Concomitantly, the expression levels of these genes in BMP-2-modified BMSCs were significantly increased compared with those in the BMSCs and the BMSCs + EPCs group at all times. Therefore, these data indicated that BMP-2 may facilitate bone formation in a co-culture of BMSCs and EPCs.

ad-BMP-2-BMSCs/EPCs system promotes the protein expression of Runx2, Dlx, ALP, Ibsp, VEGF, osteonectin and collagen I. As demonstrated in Fig. 4, Runx2, Dlx, ALP, Ibsp, VEGF, osteonectin and collagen I protein levels were markedly increased in the BMP-2-modified BMSCs + EPCs group compared with the other groups. These results suggested that BMP-2-modified BMSCs may affect the expression of Runx2, Dlx, ALP, Ibsp, VEGF, osteonectin and collagen I, which may contribute to bone formation in a co-culture of BMSCs and EPCs.

SEM observation of cell sheets. To examine the osteoblast-oriented differentiation of the BMP2-modified BMSCs in culture conditions with EPCs, SEM was employed to observe the morphological appearance and ultrastructure of the BMP2-modified BMSCs/EPCs construct. It was clearly observed that the BMP2-modified BMSCs presented as ball-shaped, and they adhered well to the surface of EPCs through their pseudopods (Fig. 5). Concomitantly, the BMP2-modified BMSCs secreted extracellular matrix, expanded well and extended multiple cell processes into

the pores of the EPCs (Fig. 5). Therefore, from these results it was concluded that tissue engineering approaches using BMP2-modified BMSCs in combination with EPCs may be a suitable cell delivery strategy for treating bone defects.

Discussion

Bone defects or nonunion, which are serious consequences for conditions including surgical resections, infection, trauma, spinal degenerative diseases, fall fractures in patients with osteoporosis, tumors or other systemic problems that negatively affect the bone healing process, remain a major clinical challenge in orthopedic surgery (22,23). Presently, ideal bone tissue engineering should not only mimic local tissue architecture but also support robust osteogenic differentiation of cells (24,25). BMSCs have been confirmed to exhibit osteogenetic potential (26,27). In addition, EPCs have also been demonstrated to contribute to enhanced bone formation to bridge bone defects during bone repair (28,29). Therefore, BMSCs and EPCs are considered attractive cell sources for tissue engineering applications and key candidates for cell therapy in bone defects. In the present study, surface markers were used to identify, isolate and culture the BMSCs and EPCs cells from the bone marrow of SD rats. The results revealed that the isolated and cultured cells exhibited typical characteristics of BMSCs and EPCs. Following verification of the phenotypes of the BMSCs and EPCs, the BMP2 gene was transferred into BMSCs. BMP2, a multifunctional growth factor belonging to the transforming growth factor β superfamily,
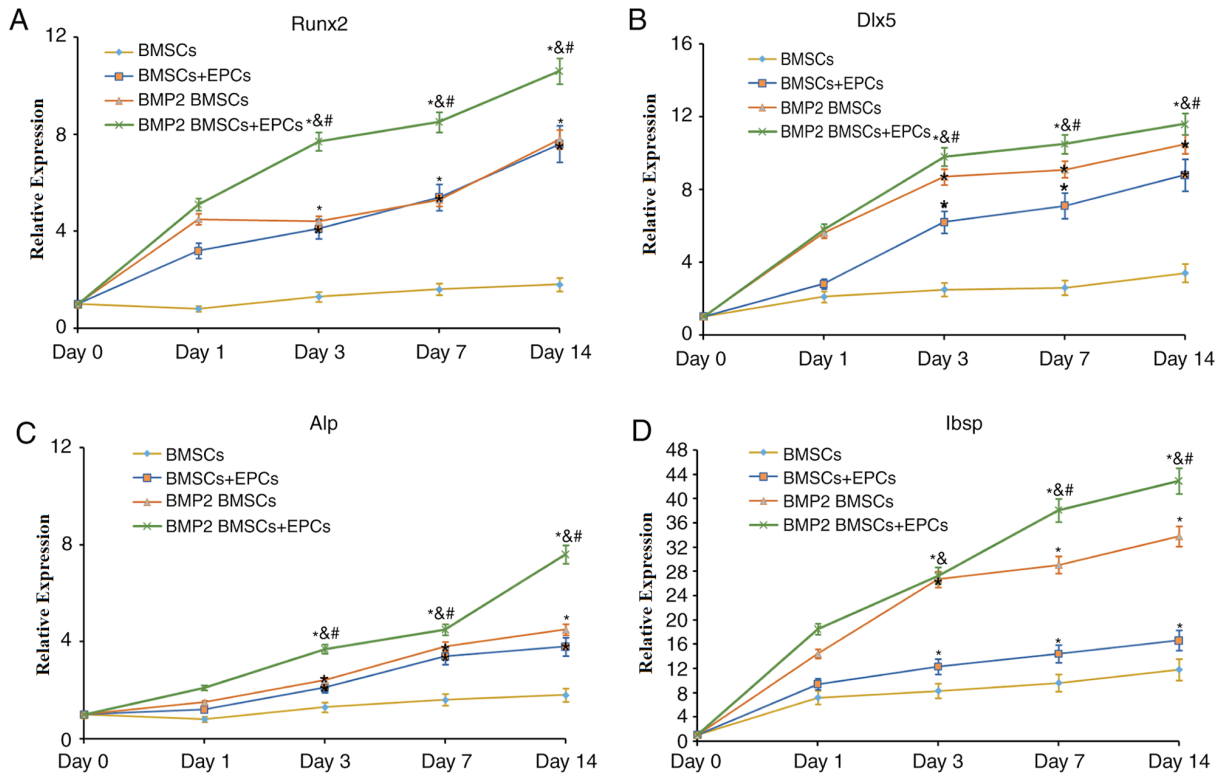
Figure 3. mRNA expression of osteogenesis-associated genes. (A) Runx2, (B) Dlx5, (C) ALP and (D) Ibsp mRNA expression levels in BMSCs, BMSCs + EPCs, BMP2 BMSCs and BMP2 BMSCs + EPCs cultures were analyzed using reverse transcription quantitative polymerase chain reaction. *P<0.05 vs. BMSCs, &P<0.05 vs. BMSCs + EPCs and #P<0.05 vs. BMP2 BMSCs. Runx2, runt related transcription factor 2; Dlx5, distal-less homeobox 5; ALP, alkaline phosphatase; Ibsp, integrin binding sialoprotein.
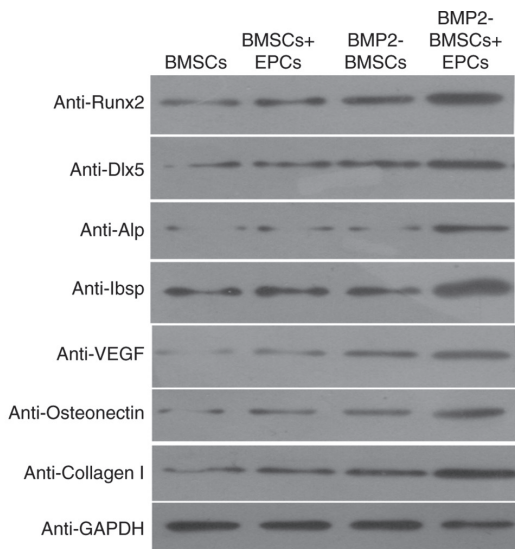

Figure 4. Protein expression of osteogenesis-associated genes. Runx2, Dlx5, ALP, Ibsp, VEGF, osteonectin and collagen I protein levels in BMSCs, BMSCs + EPCs, BMP2 BMSCs and BMP2 BMSCs + EPCs cultures by western blot analysis. Runx2, runt related transcription factor 2; Dlx5, distal-less homeobox 5; ALP, alkaline phosphatase; Ibsp, integrin binding sialoprotein; VEGF, vascular endothelial growth factor; collagen I; type I collagen BMSCs, bone marrow stromal cells; EPCs, endothelial progenitor cells; BMP2, bone morphogenic protein 2.
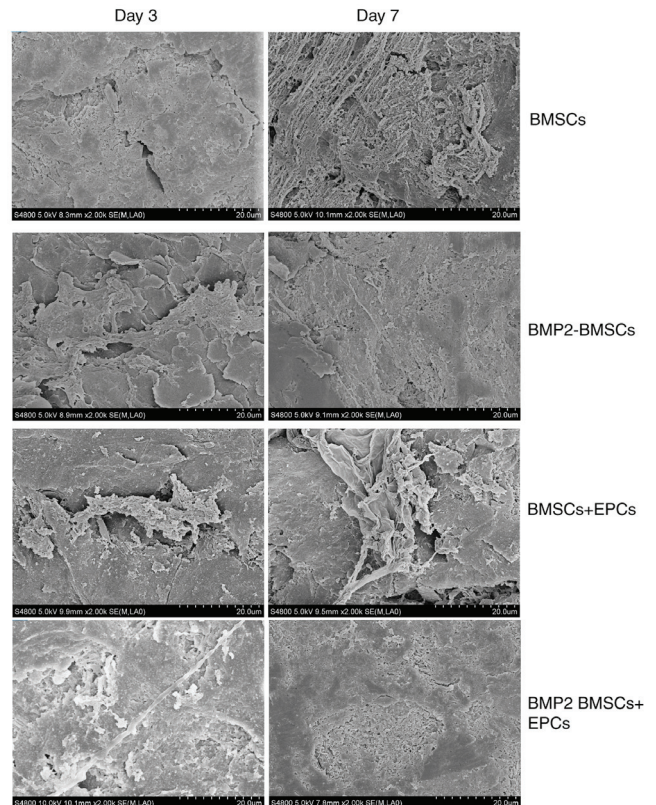

Figure 5. Morphology of the BMP2-modified BMSCs adhering on EPCs surface at days 3 and 7. Images were captured using scanning electron microscopy. Magnification, x10,000. BMP2, bone morphogenic protein 2; BMSCs, bone marrow stromal cells; EPCs, endothelial progenitor cells.

is a potent osteoinductive molecule that induces osteogenic differentiation of immature osteoblasts and non-committed

cells, stimulates bone formation and accelerates callus remodeling and fracture healing (15,30,31). Therefore, to additionally investigate the effects of BMP2 in a mixed culture of BMP2-modified BMSCs and EPCs, a co-culture system of BMP2-modified BMSCs and EPCs was constructed at a ratio of 1:5. Concomitantly, a series of biochemical experiments were performed. It was identified that the levels of cell proliferation in the BMP2-modified BMSCs + EPCs group were remarkably increased compared with those in the other groups. Additionally, the secreted BMP2 levels and ALP activity were markedly increased in the BMP2-modified BMSCs + EPCs group as compared with the other groups. Cell proliferation is an important factor for the evaluation of cell growth ability; therefore, the data from the present study implied that BMP2 may accelerate cell proliferation in the cell sheet system. It is known that BMP2 is a crucial factor for the regulation of angiogenesis and bone formation, and serves as a marker for angiogenesis (31,32). Therefore, the increase in its secretion observed in the present study indicated that the capacity of bone formation was notably increased in the BMP2-modified BMSCs + EPCs group. Furthermore, ALP is an indicator of bone tissue maturation (33,34), suggesting that BMP2 may quickly promote bone maturation. In addition, the expression levels of bone formation-associated genes and proteins were examined using RT-qPCR and western blot analysis, respectively. Tissue engineering offers a novel approach to regenerate diseased or damaged tissues, including bone, which is a natural organic-inorganic composite consisting of collagen fibrils containing embedded, well-arrayed, nanocrystalline and plate-like inorganic materials (24). During the bone formation process, a number of osteogenesis-associated genes and proteins serve important regulatory roles. In the present study, the levels of the osteogenesis-associated genes and proteins examined were all augmented in the BMP2-modified BMSCs + EPCs group. Furthermore, the SEM results indicated the successful formation of a composite scaffold of BMP2-modified BMSCs and EPCs, which implied that the BMP2-modified BMSCs and EPCs constructs may have the ability to facilitate bone regeneration.

Taken together, the data from the present study indicated that BMSCs and EPCs were successfully isolated, and demonstrated that BMP2-modified BMSCs combined with EPCs may promote cell proliferation, promote BMP2 secretion and enhance ALP activity. In addition, the expression levels of osteogenesis-associated genes and proteins were markedly increased in cell sheets of the BMP2-modified BMSCs + EPCs group. Finally, SEM observation also revealed that the correct combination of BMP2-modified BMSCs and EPCs may integrate to generate cell sheets. Therefore, the use of cell sheets of BMP2-modified BMSCs and EPCs may be a promising and effective clinical treatment modality for patients with bone defects.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JE and XH performed the molecular and animal experiments, data acquisition and revised the article; SW, YZ and XD analyzed and interpreted the data. BL performed the molecular experiment during the revision and edited the revision; LL and XZ designed the concept and wrote the manuscript. All of the authors agree to publish the manuscript.

Ethics approval and consent to participate

All experiments in the present study were performed in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Ethics Committee of The First Hospital of Kunming Medical College (Kunming, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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