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Synergistic effects of recombinant Lentiviral-mediated BMP2 and TGF-beta3 on the osteogenic differentiation of rat bone marrow mesenchymal stem cells *in vitro*

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ABSTRACT

Background: Bone marrow mesenchymal stem cells (BMSCs) are considered good candidates for seed cells in bone engineering. The study aim to investigate the synergistic effects of human bone morphogenetic protein 2 (hBMP2) and transforming growth factor beta3 (hTGF-beta3) modified BMSCs on inducing osteogenic differentiation *in vitro*.

Methods: Lentivirus (LV) carrying hBMP2 and/or hTGF-beta3 genes were constructed and used to transduce rat BMSCs. The expression of osteogenic molecules was detected by qRT-PCR and western blotting.

Results: Targeted genes were PCR-amplified and confirmed by DNA sequencing and BLAST analysis. BMSCs infected by vectors effectively resulted in the overexpressions of hBMP2 and hTGF-beta3 and higher levels of hBMP2 and hTGF-beta3 in the culture supernatant. The co-transduction of hBMP2 and hTGF-beta3 induced BMSCs osteogenic differentiation more effectively than the transduction of hBMP2 or hTGF-beta3 individually. The expression levels of osteopontin (OPN), osteocalcin (OCN), and osteoprotegerin (OPG) in LV-hBMP2 + LV-hTGF-beta3 group (BMSCs transfected by vectors respectively carrying hBMP-2 gene and hTGF-beta3 gene) and LV-hBMP2-hTGF-beta3 group (BMSCs transfected by vector carrying hBMP2 and hTGF-beta3 fusion gene) were significantly higher than in LV-BMP2 (BMSCs transfected by vector carrying hBMP2 gene) and LV-TGF-beta3 (BMSCs transfected by vector carrying hTGF-beta3 gene) groups ($P < 0.05$). The hBMP2 and/or hTGF-beta3 overexpression upregulated alkaline phosphatase (ALP) activity.

Conclusion: The present study showed that hBMP2 and/or hTGF-beta3 genes can be successfully overexpressed in BMSCs. Our study proved that the two cytokines (hBMP2 and hTGF-beta3) could induce bone differentiation synergistically, which foresees the use of the combination of these two cytokines as a therapeutic strategy in the future.

1. Introduction

Bone defects and injuries caused by trauma, infection, tumors, bone necrosis, congenital malformations, and other diseases are serious clinic complications, and the obstruction of bone tissue regeneration in old people and patients with chronic diseases is the major limitation for bone recovery [1]. Bone formation and repair highly depend on the number and activity of bone-forming cells regulated by appropriate stimulation by regulatory factors. The development of gene therapy using gene transfer to express the gene of interest to promote osteogenic differentiation has opened up new methods for treating bone defects [2]. Bone marrow mesenchymal stem cells (BMSCs) are considered good candidates for seed cells in bone engineering [3], because BMSCs have great differentiation potential, being able to differentiate into a variety of cell types including osteoblasts, chondrocytes, and other kinds of parenchymal cells under certain internal environments [4].

Growth factors play a crucial role in tissue engineering, for several growth factors have been indicated to facilitate bone repair through the rapid development of gene therapy technology. Two classic growth factors involved in bone regeneration are human bone morphogenetic protein 2 (hBMP2) and transforming growth factor-beta3 (hTGF-beta3) [5]. The hBMP2, a member of the TGF-beta superfamily, plays an

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important role in inducing bone differentiation and bone formation. Experimental studies have shown that hBMP2 is the only growth factor capable of singly inducing bone formation [6–8]. The hTGF-beta3 was found to more strongly induce chondrogenesis and to increase the expression of cartilage relevant genes, including collagen-II and aggrecan, in BMSCs [9–11]. The hTGF-beta3 was also shown to regulate the early stages of osteoblastic differentiation [12,13], and to elicit endochondral bone differentiation by up-regulating hBMP2 to induce bone formation [14].

Bone engineering aimed at increasing hTGF-beta3 expression in BMSCs has been reported to promote bone defect repair [15,16]. Additionally, the BMSCs engineered to express both hTGF-beta3, and hBMP2 gene showed high osteogenic differentiation efficiency, as well as a good biocompatibility, which could well induce cartilage regeneration and promote calvarial bone repair [17,18]. However, quantitative studies investigating bone formation following hBMP2 and hTGF-beta3 infusion have proved inconclusive.

In the present study, we constructed lentiviral vectors (LV) carrying hBMP2 and/or hTGF-beta3 genes to investigate the synergistic effects of hBMP2 and hTGF-beta3 modified BMSCs on inducing osteogenic differentiation *in vitro*. We demonstrate the significant up-regulation of hBMP2 and hTGF-beta3 expression, and a significant increase in osteogenic molecules including osteocalcin (OCN), osteopontin (OPN), and osteoprotegerin (OPG). The transduction of hBMP2 and hTGF-beta3 together was more effective than either growth factor alone. Moreover, our data suggested that hBMP2 and hTGF-beta3 overexpression in BMSCs upregulated alkaline phosphatase (ALP) activity. Our study proved that the two cytokines (hBMP2 and hTGF-beta3) could induce bone differentiation synergistically, which foresees the use of the combination of these two cytokines as a potential treatment.

2. Materials and methods

2.1. Animals

Twenty male *SD* rats (8–10 weeks old, weighing 220 ± 20 g) were purchased from the Laboratory Animal Center of the Second Affiliated Hospital of Harbin Medical University [license: SCXK (Heilongjiang, China) 2002-0002]. All rats were housed in microisolator or cages in the barrier facility of Fujian Medical University. All experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of Fujian Medical University.

2.2. BMSCs preparation and identification

Rat BMSCs were collected from the tibia and femur of *SD* rats by flushing with a 25-gauge needle. BMSCs were cultured in DMEM medium supplemented with 10% v/v fetal bovine serum medium (Gibco) at 37 °C with 5% CO_2 for 8 days. The cells were plated in 6-well plates at a density of $1.5 \times 10^5/cm^2$/ml in DMEM medium supplemented with 10% heat-inactivated fetal calf serum. After 3 days, the medium was gently removed and replaced with fresh. After another 2–3 days, non-adherent cells and loosely adherent aggregated proliferating BMSCs were harvested and confirmed morphologically using an inverted microscope (Nikon, Japan) and histological analysis. The third generation of BMSCs were affixed to microscope slides, then fixed in 4% paraformaldehyde, and dehydrated in a graded series of ethanol baths. The sections were stained with hematoxylin-eosin to assess morphologic changes.

The BMSC phenotype as characterized by the expression of CD29, CD44, CD90, CD31, CD34, and CD45 was determined by flow cytometry. Freshly harvested BMSCs (1.5×10^6 cells) were incubated with PE-Cy5-conjugated antibodies (anti-CD29, anti-CD44 anti-CD90, anti-CD31, anti-CD34, and anti-CD45; eBioscience) for 30 min at 4 °C according to the manufacturer's instructions. Stained cells were washed twice in PBS with 1% w/v BSA. FACS analysis was performed using FACScan flow cytometry (ThermoFisher). Three replicate experiments were performed, and the mean expression levels were calculated.

2.3. Construction and identification of lentiviral vectors (LV) carrying hBMP2 and/or hTGF-beta3 genes

Plasmids pIRES2-EGFP-hBMP2, pIRES2-EGFP-hTGF-beta3, and pIRES2-EGFP-hBMP2-hTGF-beta3 were purchased from GeneChem Co., Ltd. (Shanghai, China). For the overexpression of hBMP2 and/or hTGF-beta3, gene ORFs were PCR-amplified and verified by sequencing. The following primers were synthesized: hBMP2-Age I-F 5′-GATCGCTAGC GCCACC ATGCAAGCCAAACACAAACA-3′ and hBMP2-Age I-R 5′-GAA TTCCTAGCGACACCCACAACCCT-3′; hTGF-beta3-Age I-F 5′-GATCGCT AGC GCCACCATGAAGATGCACTTGCAAAG-3′ and hTGF-beta3-Age I-R 5′-GGATCCTCAGCTACATTTACAAGACT-3′; and hBMP2-TGF-beta3-Age I-F 5-GATCGCTAGCGCCACCATGCAAGCCAAACACAAACA-3′ and hBMP2-TGF-beta3-Age I-R 5′-GGATCCTCAGCTACATTTACAAGACT-3′. After double digested with Nhe I and Bam HI, Nhe I and EcoRI, and Nhe I and Bam HI, the fragments of LV-hBMP2, LV-hTGF-beta3, and LV-hBMP2-hTGF-beta3 were detected by agarose gel electrophoresis. Additionally, interested genes were PCR-amplified and confirmed by gel electrophoresis. These oligonucleotides were annealed and inserted downstream of the ubiquitin promoter in the lentiviral vector PCDH-CMV-EGFP (GeneChem Co.). A control vector containing no insertion was supplied by GeneChem Co. 293 T cells at 80% confluency were then triple transfected with pHelper 1.0 and pHelper 2.0 helper plasmids (GeneChem Co.) together with PCDH-CMV-EGFP-BMP2, PCDH-CMV-EGFP-TGF-beta3, or PCDH-CMV-EGFP-BMP2-TGF-beta3 plasmid using Lipofectamine 2000 (Invitrogen, USA). Forty-eight h post-transfection, lentiviruses were harvested and centrifuged to remove cell debris, then filtered and concentrated through Centricon Plus-20 Centrifugal Filter Units followed by ultracentrifugation (Millipore, USA). The titer of concentrated lentivirus was tested following the instructions of the qPCR Lentivirus Titration Kit (LV900, Applied Biological Materials Inc.).

2.4. Transduction of BMSCs with LV expression vectors

BMSCs were harvested and seeded into 6-well plates at 1×10^6cells/well in DMEM medium with 10% fetal bovine serum at the 5th day after primary culture. Cells were then divided into five groups, they were LV-control group, LV-hBMP2 group, LV-hTGF-beta3 group, LV-hBMP2 + LV-hTGF-beta3 group, and LV-hBMP2-hTGF-beta3 group, and respectively transfected by LV empty vector, LV carrying hBMP2 gene, LV carrying hTGF-beta3 gene, two kinds of LV respectively carrying hBMP2 gene and hTGF-beta3 gene, LV carrying hBMP2 and hTGF-beta3 fusion gene. LV was added at a different multiplicity of infection (MOI, respectively 5, 10, 15, 20) into the culture medium of respective cells containing 0.8 μg/ml polybrene (Sigma, USA). An equal amount was then added after a further 24 h. Half of the medium was replaced every other day. The infection efficiency was determined by fluorescent microscopy and FACS analysis to evaluate the optimal MOI conducted in the following studies.

2.5. Protein analysis

BMSCs were grown in 6-well plates and infected with LV when the cells reached 70% confluency; cells were then incubated for a further 7 days. Enzyme-linked immunosorbent assays (ELISA) were performed to detect the levels of hBMP2 (DY355, R&D Systems, Inc.) and hTGF-beta3 (DY243, R&D Systems, Inc.) in the culture supernatant. Cells were homogenized in RIPA buffer (R0278, Sigma-Aldrich), and a total of 30 μg of protein was loaded onto gels and subjected to SDS-PAGE. After the proteins were transferred to membranes, primary antibodies targeting hBMP2 (ab6285; Abcam, UK), hTGF-beta3 (15537; Abcam), osteoprotegerin (ab73400; Abcam), β-actin (Hc201-01, TransGen) were

added to detect the protein levels. After incubated by HRP-conjugated goat anti-mouse IgG (Hs201-01, TransGen), the bands were detected using the BeyoECL Plus Chemiluminescent Kit (Beyotime Institute of Biotechnology, Haimen, China), and the level of protein expression was quantified as the relative density value compared to β-actin using Quantity One software (Bio-Rad, USA). The experiment was repeated at least three times.

2.6. Quantitative real-time RT-PCR (qRT-PCR)

Total cellular RNA was isolated using TRIzol (Invitrogen), and reverse transcription was performed using the PrimeScript® RT reagent Kit (Takara) according to the manufacturers' instructions. The resulting first-strand cDNA for measuring the expression of hBMP2, hTGF-beta3, OPN, OCN, beta-actin, and IDO was obtained from 800 ng total RNA and used as a template for quantitative real-time PCR, which was carried out using the primers listed: hBMP2 (F): AATTCTGGGAGGGCTT GGTT, (R): CTGTTTCAGGCCGAACATGC; hTGF-beta3 (F): TACTGCTT CCGGTGAGACTG, (R): CAGGGACCCTCTGTTGAGTG; OPN (F): AATT CTGGGAGGGCTTGGTT, (R): GTTGTTTGGCTGGCACAGTT; OCN (F): TCCTTTGGGGTTTGGCCTAC, (R): CCAGCCTCCAGCACTGTTTA; beta-actin (F): GTTGACATCCGTAAAGAC, (R): GGACAGTGAGGCCAGGATA. Quantitative real-time PCR was conducted using corresponding primers and cDNA templates mixed with reagents from a SYBR Premix Ex Taq II kit (DRR081, Takara). The data were analyzed and quantified by using the ΔΔCt method, and transcript levels of each gene were normalized to that of GAPDH in the same sample.

2.7. Quantification of alkaline phosphatase activity

ALP activity was assessed from cell lysates on day 14 post-transduction using a LabAssay ALP colorimetric assay kit (Wako Pure Chemical Industries, Ltd.). Total protein levels were determined by the BCA Protein assay kit (Beyotime Institute of Biotechnology) following standard protocols. ALP activity was calculated as the amount of phosphorylated nitrophenol release in n/μmol/min and was further normalized to the cell protein input. Each sample was assessed in triplicate.

2.8. Statistical analysis

Data are presented as averages ± standard deviation. Statistical analysis was performed using SPSS software 17.0. The unpaired two-tailed Student's *t*-test was used to determine the statistical significance of between-group differences. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Identification of BMSCs

BMSCs were visible as spindle-shaped, fibroblast-like cells under an inverted microscope (Fig. 1A). Flow cytometry analysis revealed significant increases in the expressions of cell surface antigens such as CD29, CD44, and CD90, and the marked decreased expressions of markers of hematopoietic cells such as CD31, CD34, and CD45 (Fig. 1B).

3.2. Identification of LV carrying hBMP2 and/or hTGF-beta3 genes

The double digestion of LV-hBMP2, LV-hTGF-beta3, and LV-hBMP2-hTGF-beta3 with Nhe I and Bam HI, Nhe I and EcoRI, and Nhe I and Bam HI produced bands of 7384 bp and 368 bp, 7384 bp and 1251 bp, and 7384 bp and 1632 bp, respectively (Fig. 2A). Interested genes were PCR-amplified and confirmed by gel electrophoresis (Fig. 2B). The optimal multiplicity of infection was 15 according to the results of fluorescent microscopy shown in Fig. 2C (transduction rate: MOI5: 22.8%; MOI10: 46.2%; MOI15: 83.7%; MOI20: 65.5%).

3.3. Characteristics and expressions of hBMP2 and hTGF-beta3 of gene transduced BMSCs

To overexpress hBMP2 and/or hTGF-beta3, LV-hBMP2, LV-hTGF-beta3, and LV-hBMP2-hTGF-beta3 were used to transfect BMSCs. The over-expression of hBMP2 and hTGF-beta3 induced morphologic changes in BMSCs. Following the infection of LV carrying hBMP2 and/or hTGF-beta3 genes, BMSCs became larger and more rounded in shape, as shown in Fig. 3A. Western blotting showed that hBMP2 expression was significantly higher in LV-hBMP2-hTGF-beta3 and LV-hBMP2 groups, while hTGF-beta3 expression was significantly higher in LV-hTGF-beta3 and LV-hBMP2-hTGF-beta3 groups compared with the control group ($P < 0.05$, Fig. 3B). Thus, the infection of LVs encoding hBMP2 and hTGF-beta3 resulted in overexpression of these cytokines. Compared with the control group, the supernatant hBMP2 levels in LV-hBMP2-hTGF-beta3 and LV-hBMP2 groups significantly increased, and the supernatant hTGF-beta3 levels in LV-hTGF-beta3 and LV-hBMP2-hTGF-beta3 groups significantly increased ($P < 0.05$, Table 1).

3.4. High expression of osteogenic molecules

The expression of osteogenic molecules including OCN, OPN, and OPG was detected by qRT-PCR and western blotting at day 7 after infection, as shown in Fig. 4. OCN (Fig. 4C) and OPN (Fig. 4B) mRNA expression in LV-hBMP2, LV-hTGF-beta3, LV-hBMP2 + LV-hTGF-beta3, and LV-hBMP2-hTGF-beta3 groups was significantly higher than in the control group ($P < 0.05$). OCN mRNA expression was particularly high in LV-hBMP2-hTGF-beta3 and LV-hBMP2 + LV-hTGF-beta3 groups, especially the former ($P < 0.05$), while OPN mRNA expression was significantly higher in the LV-hBMP2 + LV-hTGF-beta3 group compared with other groups ($P < 0.05$). OPN mRNA expression was significantly lower in the LV-hTGF-beta3 group than in LV-hBMP2-hTGF-beta3 and LV-hBMP2 groups ($P < 0.05$), but there was no significant difference between expression in LV-hBMP2-hTGF-beta3 and LV-hBMP2 groups.

Western blotting revealed that OPG protein expression was significantly higher in LV-hBMP2 + LV-hTGF-beta3 and LV-hBMP2-hTGF-beta3 groups than in LV-hBMP2 and LV-hTGF-beta3 groups ($P < 0.05$) at day 7 post-infection (Fig. 4A). There was no OPG expression in the control group.

3.5. Quantification of ALP activity

The ALP activity of LV-hBMP2 + LV-hTGF-beta3, LV-hBMP2-hTGF-beta3, LV-hBMP2, and LV-hTGF-beta3 groups was significantly higher than in the control group ($P < 0.001$). Moreover, ALP activity in LV-hBMP2-hTGF-beta3 and LV-hBMP2 + LV-hTGF-beta3 groups was significantly higher than in LV-hBMP2 and LV-hTGF-beta3 groups ($P < 0.05$, Fig. 5 and Table 2). This suggested that hBMP2 and/or hTGF-beta3 overexpression upregulates ALP activity.

4. Discussion

In the present study, we investigated the synergistic effects of hBMP2 and hTGF-beta3 gene-modified BMSCs on inducing osteogenic differentiation *in vitro*. We demonstrated that the overexpression of hBMP2 and hTGF-beta3 in BMSCs significantly increased hBMP2 and/or hTGF-beta3 mRNA and secreted protein expression, indicating that LV-mediated hBMP2 and/or hTGF-beta3 transduction in BMSCs was functional. The transduction also led to significant increases in the expressions of osteogenic molecules including OCN, OPN, and OPG, and the upregulation of ALP activity. Although transduction of hBMP2 or hTGF-beta3 could also induce osteogenic differentiation, the co-

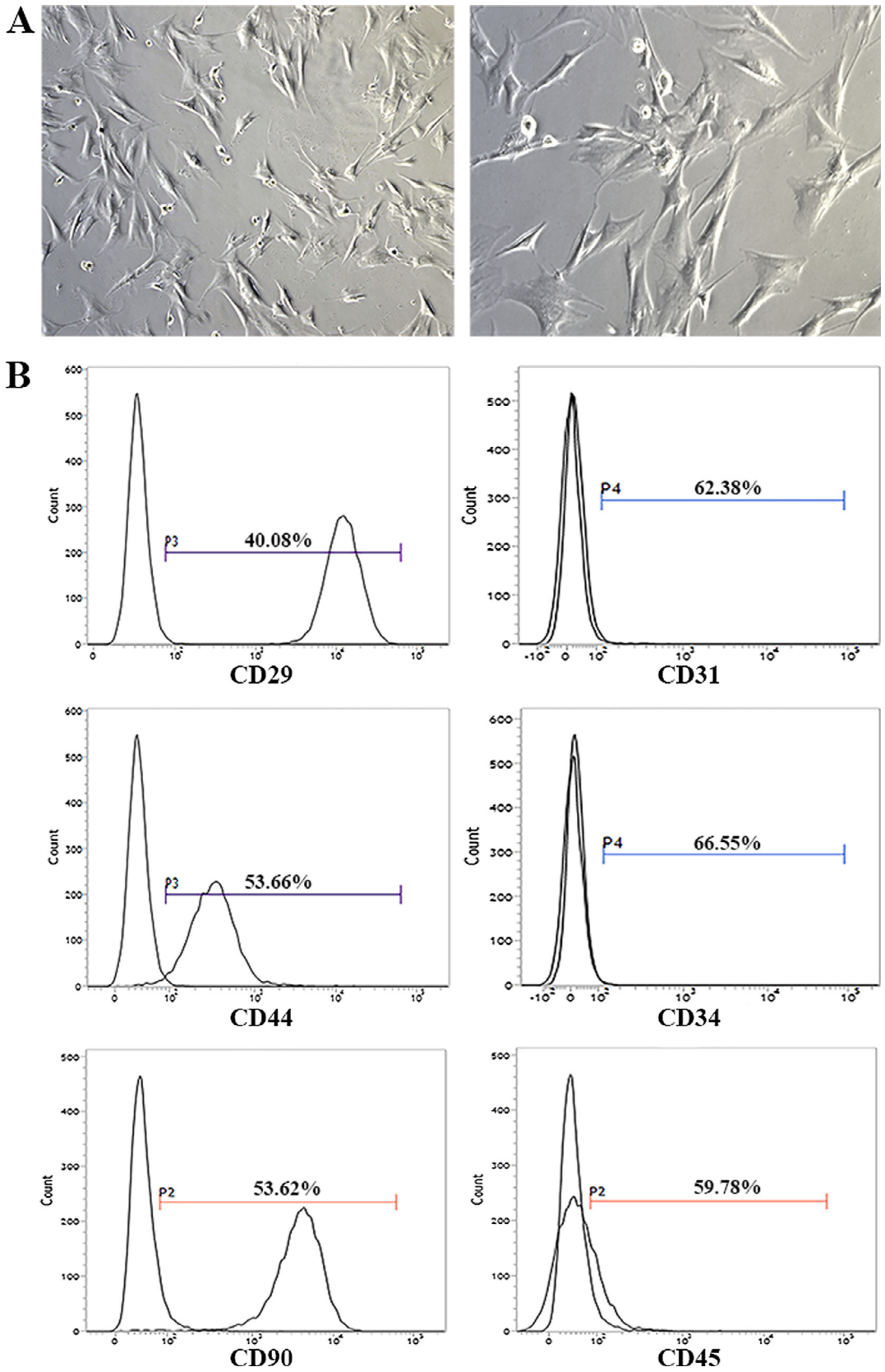

Fig. 1. Features and immunophenotypes of cultured BMSCs. (A) Seperated and cultured BMSCs were spindle-shaped, fibroblast-like cells [original magnification 200× (left) and 400× (right)]. (B) Significant increases in the expressions of cell surface antigens such as CD29, CD44, and CD90, and the marked decreased expressions of markers of hematopoietic cells such as CD31, CD34, and CD45 were detected.

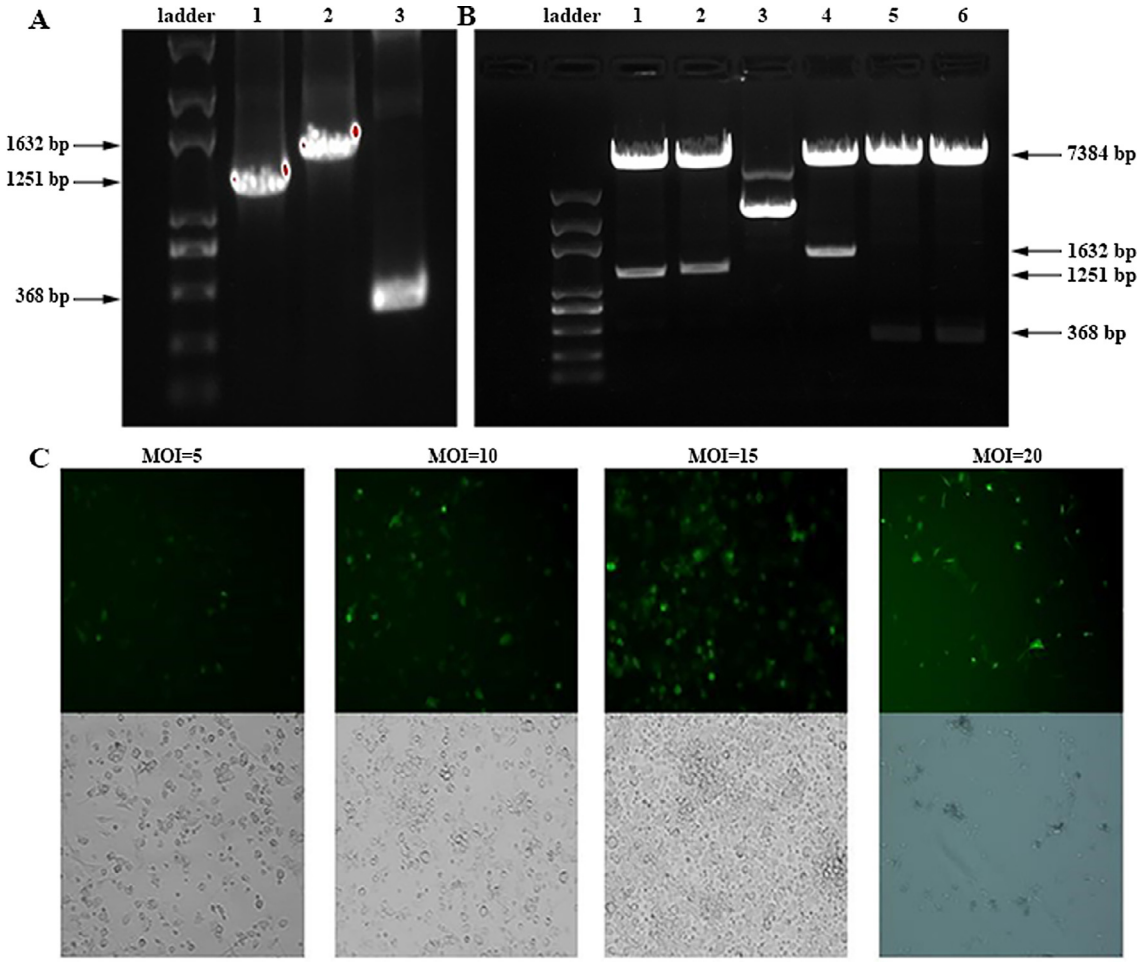

Fig. 2. The PCR products separated by agarose gel electrophoresis and the optimal MOI identified by fluorescent microscopy. (A) PCR identification of LV carrying hBMP2 and/or hTGF-beta3 genes. Lane 1: LV-TGF-beta3, Lane 2: LV-BMP2-TGF-beta3, Lane 3: LV-BMP2. (B) Result of enzyme digestion of LV carrying hBMP2 and/or hTGF-beta3 genes. Lane 1,2: LV-TGF-beta3, Lane 3,4: LV-BMP2-TGF-beta3, Lane 5,6: LV-BMP2. (C) LV were added at different multiplicity of infection (MOI, respectively 5, 10, 15, 20) into culture medium of respective BMSCs, and the infection efficiency was determined by fluorescent microscopy and FACS analysis. The optimal MOI was 15.

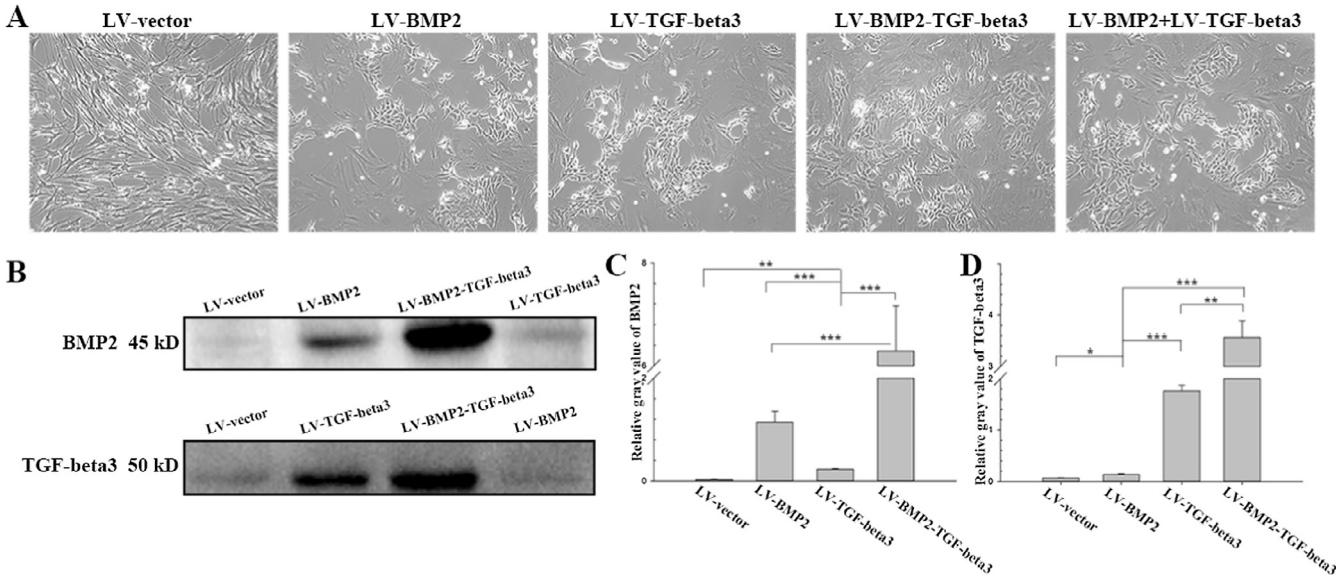

Fig. 3. Characteristics and expressions of BMP2 and TGF-beta3 of gene transduced BMSCs. (A) Morphology of BMSCs infected by BMP2 and/or TGF-beta3 at day 7 (original magnification 200 ×) showed no significant differences among groups. (B–D) BMP2 expression significantly increased in LV-BMP2-TGF-beta3 and LV-BMP2 groups, while TGF-beta3 expression significantly increased in LV-TGF-beta3 and LV-BMP2-TGF-beta3 groups detected by western blotting. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Table 1
The supernatant expressions of hBMP2 and hTGF-beta3 in each group (pg/mL, Mean ± SD).

Group	hBMP2	hTGF-beta3
LV-hBMP2-hTGF-beta3	633.6 ± 78.2[abcd]	778.8 ± 54.5[abcd]
LV-hBMP2 + LV-hTGF-beta3	361.2 ± 37.6[ac]	495.3 ± 37.2[ab]
LV-hTGF-beta3	165.2 ± 16.5[abd]	546.8 ± 33.6[ab]
LV-hBMP2	457.2 ± 56.8[ac]	212.5 ± 16.9[acd]
LV-vector	117.3 ± 4.7[bcd]	146.3 ± 12.4[bcd]

[a] Compared with LV-vector group, $P < 0.05$.
[b] Compared with LV-hBMP2 group, $P < 0.05$.
[c] Compared with LV-hTGF-beta3 group, $P < 0.05$.
[d] Compared with LV-hBMP2 + LV-hTGF-beta3 group, $P < 0.05$.

transduction of both cytokines was more effective at achieving this.

Transgenic technology has proven an efficient method of gene therapy, and both viral and nonviral vectors have been used to transduce or transfect seed cells [2,19,20]. Effective viral vectors carriers such as BMP2, 4, or 6, Osterix, Shh have achieved notably high transduction efficiencies and levels of gene expression [21,22]. Besides several safety problems such as the risk of gene overexpression, immune reaction, and tumorigenesis, more basic issues related to the safe use of gene therapy are not solved such as the need for short-term vs. long-term gene expression, and the identification of gene to be used for efficient bone healing. One approach is to identify the best genes to target in BMSCs [23,24].

Our findings that the overexpression of hBMP2 and/or hTGF-beta3 upregulates ALP activity and induces the osteogenic differentiation of BMSCs are in agreement with those of previous studies [14,25–28], which showed that the *ex vivo* overexpression of hTGF-beta3 stimulated

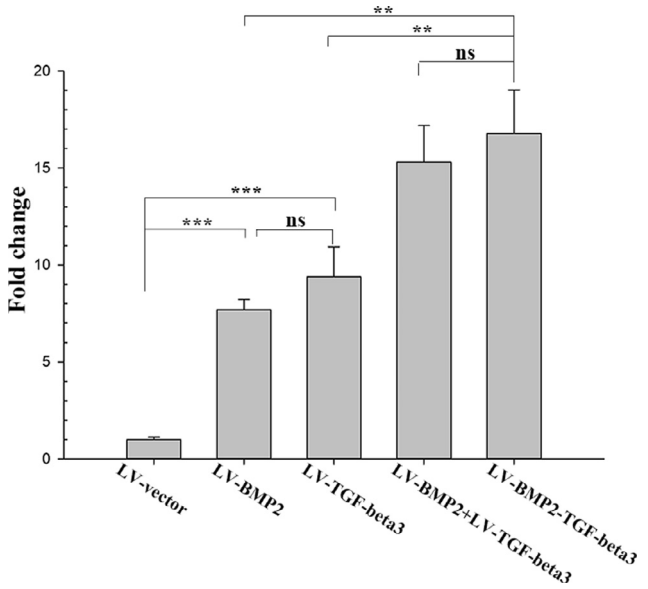

Fig. 5. The ALP activity of each group. ALP activity in both LV-BMP2-TGF-beta3 and LV-BMP2 + LV-TGF-beta3 groups were significantly higher than in either LV-BMP2 or LV-TGF-beta3 group. **: $P < 0.01$, ***: $P < 0.001$, ns: $P > 0.05$.

the secretion of BMP2 [14,26]. Moreover, hTGF-β3 previously promoted bone formation by increasing endogenous hBMP2 levels and participated in the reprogramming of progenitor cells into active secreting osteoblasts [14]. Additionally, Shen et al. reported that hBMP2 enhanced the hTGF-beta3-mediated chondrogenesis of MDSCs [11].

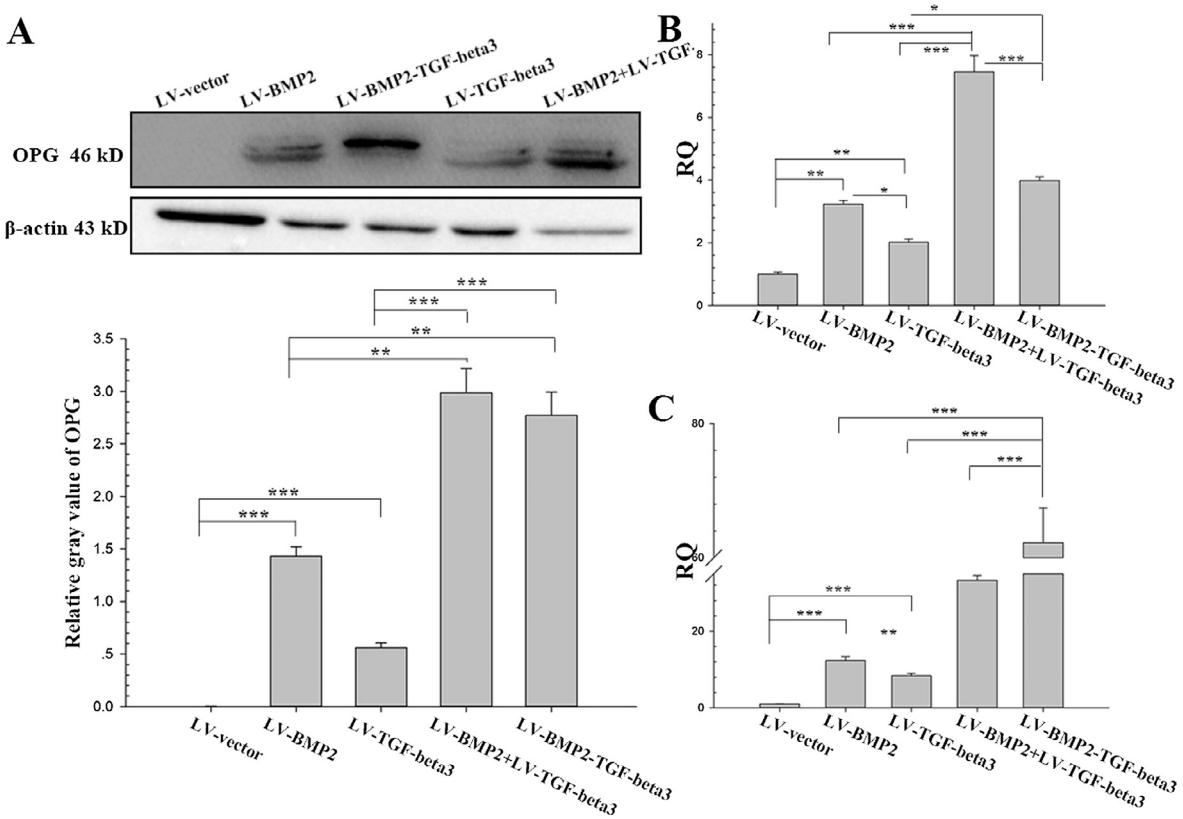

Fig. 4. Expressions of osteogenic molecules in each group. (A) OPG expression was significantly higher in LV-BMP2 + LV-TGF-beta3 and LV-BMP2-TGF-beta3 groups than either LV-BMP2 or LV-TGF-beta3 group. (B) OPN mRNA expression significantly increased in the LV-BMP2 + LV-TGF-beta3 group compared with other groups. (C) OCN mRNA expression significantly increased in LV-BMP2-TGF-beta3 and LV-BMP2 + LV-TGF-beta3 groups, especially the former. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Table 2
The ALP activity of each group (Mean ± SD).

Group	ALP activity
LV-BMP2-TGF-beta3	15.731 ± 2.410[abc]
LV-BMP2 + LV-TGF-beta3	14.271 ± 2.368[abc]
LV-TGF-beta3	9.850 ± 3.603[a]
LV-BMP2	7.420 ± 0.002[a]
LV-vector	0.699 ± 1.449[bcd]

[a] Compared with LV-vector group, $P < 0.05$.
[b] Compared with LV-BMP2 group, $P < 0.05$.
[c] Compared with LV-TGF-beta3 group, $P < 0.05$.
[d] Compared with LV-BMP2 + TGF-beta3 group, $P < 0.05$.

While short-term hBMP2 expression was necessary and sufficient to irreversibly induce bone formation by BMSCs [29], suggesting this may be adequate for cell-mediated hBMP2 therapy for bone regeneration. Further study is required to investigate the effect of MDSCs co-infected with hBMP2 and hTGF-beta3 genes on the repair of bone defects *in vivo*.

Moreover, this study found that the MDSCs transfected by LV carrying hBMP2-hTGF-beta3 gene and LV respectively carrying hBMP2 and hTGF-beta3 gene expressed hBMP2 and hTGF-beta3 mRNA and secreted protein at the nearly same level, and performed a similar function on stimulating osteogenic molecules and upregulating ALP activity. Although we did not measure the exact transfection rates of cells expressing hBMP2 and/or hTGF-beta3 gene after transfected by both LV-hBMP2 and LV-hTGF-beta3, the above results may suggest the transfection-positive cells were simultaneously transduced by both LV. Generally, the transfection efficiency of LV may partially depend on the target gene length, while the length of hBMP2-hTGF-beta3 gene is approximately equal to the length of hTGF-beta3 gene due to the short hBMP2 gene, which may lead to similar efficiency of LV carrying fusion gene transfection comparing to dual LV transfection.

The lentivectors for the human proteins are suitable for a possible future translational approach, and this study proved experimentally that changes occurred with the overexpression of the human cytokines in rat BMSCs cells which imply the combination of BMP2 and TGF-beta3 be potential means employed for bone regeneration. Although some reference has also reported the fact that the human cytokines could also work on rat setting [30–32], the viability, effectiveness, and security are still subject to proof by human cells and clinical trials.

In conclusion, this study showed that hBMP2 and/or hTGF-beta3 genes could be successfully infected and overexpressed in BMSCs. Our study proved that the two combined cytokines (hBMP2 and hTGF-beta3) could induce bone differentiation synergistically as a novel treatment.

5. Grants

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6. Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

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