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ORIGINAL ARTICLE

Combination of β-TCP and BMP-2 gene-modified bMSCs to heal critical size mandibular defects in rats

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OBJECTIVE: To investigate the effects of mandibular defects repaired by a tissue engineered bone complex with β-tricalcium phosphate (β-TCP) and bone morphogenic protein-2 (BMP-2) gene-modified bone marrow stromal cells (bMSCs).

MATERIALS AND METHODS: bMSCs derived from Fisher 344 rats were cultured and transduced with adenovirus AdBMP-2, AdEGFP gene *in vitro*. Osteogenic differentiation of bMSCs was determined by alkaline phosphatase staining, von Kossa assay and reverse transcription-polymerase chain reaction. Gene transduced or untransduced bMSCs were seeded on β-TCP scaffolds to repair mandibular full thickness defects with a diameter of 5 mm. Eight weeks post-operation, X-ray examination, micro-computerized tomography and histological and histomorphological analysis were used to evaluate the bone healing effects.

RESULTS: Alkaline phosphatase staining and mineralized nodules formation were more pronounced in AdBMP-2 group 14 days after gene transduction when compared with that of AdEGFP or untransduced group. The mRNA expression of osteopontin and osteocalcin also significantly increased 9 days after AdBMP-2 gene transduction. Mandibular defects were successfully repaired with AdBMP-2-transduced bMSCs/β-TCP constructs. The percentage of new bone formation in AdBMP-2 group was significantly higher than that of other control groups.

CONCLUSIONS: Bone morphogenic protein-2 regional gene therapy together with β-TCP scaffold could be used to promote mandibular repairing and bone regeneration.
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Introduction

Mandibular bone defects following trauma or ablative surgery result in the vast demand for new bone to replace and restore the original shape and function of the lost bone. Autogenous bone grafting for skeletal healing remains one of the most common surgical techniques in recent years. However, the main disadvantages associated with this method are potential donor site morbidity, insufficient donor availabilities and difficulty in achieving the desired bone profile (Alam *et al*, 2001). Advances in osseous tissue bioengineering over the past decade have enabled the *in vivo* regeneration of living bone by employing various growth factors, osteogenic cells and biocompatible scaffolds—or a combination of these approaches (Vacanti *et al*, 1988; Langer and Vacanti, 1993; Petite *et al*, 2000).

The bone morphogenic proteins (BMPs) are the most potent bone growth factors that have demonstrated powerful ability to induce orthotopic and heterotopic new bone formation (Cowan *et al*, 2007; Wikesjö *et al*, 2008). BMPs, members of the transforming growth factor-β superfamily, have many functions including chemotactic recruitment of particularly undifferentiated mesenchymal cells during bone healing (Lind *et al*, 1996) and induction of bone marrow stromal cells (bMSCs) differentiation to chondroblasts and osteoblasts. Among them, bone morphogenic protein-2 (BMP-2) is a potent bone stimulator and plays key roles in many steps during bone morphogenesis (Reddi, 1981; Yoon and Lyons, 2004). The effectiveness of BMP-2 in the treatment of bony defects has been evaluated thoroughly in different animal models. Although promising results have been achieved, even this biological factor has already successfully traversed clinical trials, several obstacles such as large dose requirements, high cost, short half-life and poor distribution still have to be circumvented. BMP regional gene

therapy has emerged as a possible alternative strategy to express BMPs in target cells for short or long time periods to stimulate osteogenesis maximally (Chen, 2001).

Recently, different gene therapy approaches for bone regeneration have been studied, plasmid gene delivery is low in efficiency, while retroviral vectors fail to infect non-dividing cells and have a risk for activation of oncogenes or inactivation of tumour suppressor genes (Verma and Somia, 1997). Adenovirus-mediated gene transduction, generally considered as one of the most efficient gene delivery approaches, using an *ex vivo* method, could avoid the possibilities of integration of the vector DNA into host chromosomes (Dai *et al*, 2005). Adenovirus-mediated transfer of BMP-2 had been adopted for the purpose of bone regeneration (Musgrave *et al*, 1999; Park *et al*, 2003; Chang *et al*, 2004; Dai *et al*, 2005), while it is unknown whether the critical size mandibular bony defects is responsive to BMP-2 regional gene therapy with a delivery carrier of β-tricalcium phosphate (β-TCP).

In this study, we investigated the transplantation of a tissue-engineered complex of β-TCP combined with adenoviral encoding BMP-2 (AdBMP-2)-transduced bMSCs to repair the critical size mandibular bone defects. The effects of the AdBMP-2-transduced bMSCs/β-TCP construct were compared with that of adenovirus with enhanced green fluorescent protein (AdEGFP)-transduced bMSCs/β-TCP construct, untreated bMSCs/β-TCP construct and plain β-TCP scaffold. A series of radiographic evaluation, micro-computerized tomography (micro-CT) and histological and histomorphological analysis were employed to measure bone healing for 8 weeks.

Materials and methods

Culture of rat bMSCs

Six-week-old male Fisher 344 rats weighing 125 ± 15 g were obtained from the Ninth People's Hospital Animal Center (Shanghai, China). The experimental protocol was approved by the Animal Care and Experiment Committee of Shanghai Jiao Tong University, School of Medicine (Shanghai, China). Rat bMSCs were isolated and cultured according to the protocol reported by Maniatopoulos *et al* (1988). Briefly, both ends of the femora were cut off at the epiphysis and the marrow was flushed out using Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) with 10% FBS (Hyclone, Logan, UT, USA) supplemented with 200 U ml^{-1} of heparin (Sigma, St. Louis, MO, USA). Cells were cultured in DMEM containing 10% FBS, 100 units ml^{-1} penicillin and 100 units ml^{-1} streptomycin, supplemented with 50 μg ml^{-1} ascorbic acid, 10 mM β-glycerophosphate and 10^{-8} M dexamethasone at 37°C in an atmosphere of 5% CO_2. The medium was changed after 48 h to remove non-adherent cells and renewed three times a week. When 90% confluence was reached, bMSCs were released from the culture substratum using trypsin/EDTA (0.25% w/v trypsin, 0.02% EDTA), and moved to dishes (10 cm in diameter) at a concentration of 1.0×10^5 cell ml^{-1} in 10 ml. The cells used in this study required three passages each, with about two doublings per subculture stage.

Gene transduction of bMSCs

The recombinant replication-defective AdBMP-2 and AdEGFP was used for gene transduction under a multiplicity of infection (MOI) of around 80 pfu $cell^{-1}$. The infected cells were maintained in DMEM with 10% foetal calf serum. Gene transfer efficiency was assessed 72 h after AdEGFP gene transfer under fluorescent microscopy. Gene transfer efficiency was determined by calculating the percentage of EGFP-expressing cells among all the cells present in 10 randomly selected 40× fields (Jiang *et al*, 2006).

Alkaline phosphatase staining

bMSCs transduced with AdBMP-2 and AdEGFP, and untransduced were evaluated for alkaline phosphatase (ALP) activity 14 days after transduction as per the manufacturer's instructions (ALP kit, Hongqiao, Shanghai, China). Briefly, the cells were fixed for 10 min at 4°C and incubated with a mixture of naphthol AS-MX phosphate and fast blue BB salt. Areas that stained purple were designated as positive.

von Kossa assay

bMSCs plated in triplicate in six-well plates were fixed in 70% ethanol 2 weeks after gene transduction. Cells were stained with von Kossa silver and placed under ultraviolet light for 10 min. Cells were then treated with 5% NaS_2O_3 for 2 min and washed with distilled water (Aghaloo *et al*, 2007).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA extraction was performed on days 3 and 9 after gene transduction using TRIzol Plus RNA purification kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 1.0 μg total RNA was used as template for the synthesis of cDNA with OligodT and AMV reverse transcriptase (TaKaRa, Shiga, Japan). The following PCR amplification reaction utilized Taq polymerase and specific primers. All primer sequences were synthesized commercially (Shengong Co. Ltd., Shanghai, China), and the specific primer sets are outlined in Table 1. The PCR products were visualized on 1% (w/v) agarose gel by staining with ethidium bromide and analysed densitometrically using Gel image software. The relative levels of mRNA expression were first normalized to the internal control (β-actin) and then quantified by comparison with those of untransduced bMSCs group. Each sample ($n = 3$) was assessed in triplicate ($n = 3$).

Preparation of bMSCs/β-TCP construct

The β-TCP scaffolds were purchased from Bio-Lu company (Shanghai, China). The average diameter of the pores was 450 μm. In this study, β-TCP disks of 5 mm diameter and 2 mm thickness were used. The method of cell seeding was essentially the same as that

Table 1 Nucleotide sequences for PCR primer

Genes	*Primer sequence (forward/reverse)*	*Product length (bp)*	*Annealing temperature (°C)*	*Accession number*
OCN	5′-AAAGCCCAGCGACTCT-3′ 5′-CTAAACGGTGGTGCCATAGAT-3′	232	55	NM_013414
OPN	5′-GACGGCCGAGGTGATAGCTT-3′ 5′-CATGGCTGGTCTTCCCGTTGC-3′	208	55	NM_012881
β-actin	5′-CCTGTGGCATCCACGAA ACT-3′ 5′-GAAGCATTTGCGGTGGACGA-3′	307	55	NM_017008

OCN, osteocalcin; OPN, osteopontin.

reported by Maniatopoulos *et al* (1988). Briefly, 72 h after gene transfer, bMSCs were released from the culture substratum using trypsin/EDTA (0.25% w/v trypsin, 0.02% EDTA) and were concentrated to 2×10^7 cells ml^{-1} in serum-free medium. Then bMSCs were seeded onto β-TCP scaffolds by simple dropping of the bMSCs suspension to a final saturation. The bMSCs/β-TCP construct was incubated for an additional 4 h to allow cell attachment *in vitro* before implantation.

In a parallel experiment, $3 \times 3 \times 3$ mm cuboids were prepared and seeded with bMSCs at an identical cell density. After 4 and 24 h of incubation, the constructs were fixed in 2% glutaric dialdehyde for 2 h, cut into two halves and then characterized by scanning electron microscopy (Philips Quanta-200, FEI, Eindhoven, The Netherlands).

Surgical procedure

For *in vivo* evaluation of mandibular repairing by these constructs, a total of 11 male Fisher 344 rats, aged 12 weeks, each with a weight of 250 ± 15 g were enrolled. The animals were anaesthetized by intraperitoneal injection of pentobarbital (Nembutal 3.5 mg/100 g). An incision was made in the skin, followed by plane-by-plane muscle dissection and incision of the periosteum. Bilateral non-healing full thickness defects of 5 mm diameter in the ascending ramus of the mandible were then created with a bur that was cooled continuously by 0.9% saline solution irrigation. The mandibular defects were randomly divided into four groups that received the following implants: (1) β-TCP alone ($n = 4$); (2) β-TCP with untreated bMSCs ($n = 6$); (3) β-TCP with bMSCs transduced with AdEGFP ($n = 6$) and (4) β-TCP with bMSCs transduced with AdBMP-2 ($n = 6$) (Figure 1). The wound was closed in layers using 4-0 resorbable sutures.

Radiographic evaluation and micro-CT

After 8 weeks, all the rats were killed by an intraperitoneally injected overdose of pentobarbital. Then the mandibles were explanted and fixated in 4% phosphate-buffered formalin solution. X-ray images of mandibles were made with a dental X-ray machine (Trophy, Croissy-Bearborg, France), from a distance of 7 cm (230 V, 8 mA) with an exposure time of 0.10 s. The morphology of the reconstructed mandibles was assessed using a micro-CT system (μCT-80; Scanco Medical, Bassersdorf, Switzerland). The CT settings were used as follows: pixel matrix, 1024×1024; voxel size, 36 μm; slice thickness, 36 μm. Mandibles were visualized in a pseudo-3D display.

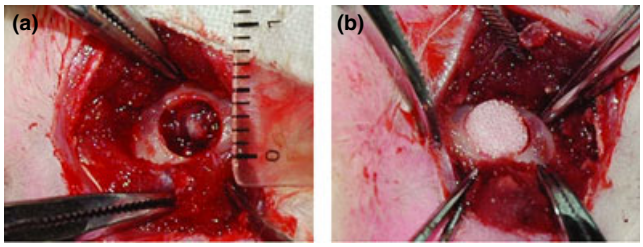

Figure 1 Surgical procedure. (**a**) A non-healing full thickness defect of 5 mm diameter in the ascending ramus of the mandible was made and then (**b**) the mandibular defect was filled with a β-tricalcium phosphate disk seeded with gene-modified bone marrow stromal cells

Histological and histomorphological analysis

After 3 days fixation, the specimens were decalcified in 10% EDTA for 2 weeks. Samples were embedded in paraffin parallel to the sectioned surface. Serial buccal and lingual sagittal cross sections were made for haematoxylin and eosin (H&E) staining and analysed histomorphometrically using Image Pro 5.0 system (Media Cybernetics, Silver Springs, MD, USA). The percentages of new bone area, residual scaffold and fibrous connective tissue were calculated using the average value of the three parallel slices selected from three equally divided paraffin samples defined at buccolingual directions. The mean value of the three measurements was calculated for each graft and was further used to calculate mean values for each group.

Statistical analysis

Statistically significant differences ($P < 0.05$) between the various groups were measured by ANOVA and SNK *post hoc* or Kruskal–Wallis non-parametric procedure, followed by Mann–Whitney *U*-test for multiple comparisons based on the normal distribution and equal variance assumption test. All statistical analyses were carried out using SPSS statistical software package (version 12; SPSS, Chicago, IL, USA). All the data are expressed as mean ± s.d.

Results

Cell culture and gene transduction

Cellular morphology for each group is shown in Figure 2. The bMSCs grew well after AdEGFP

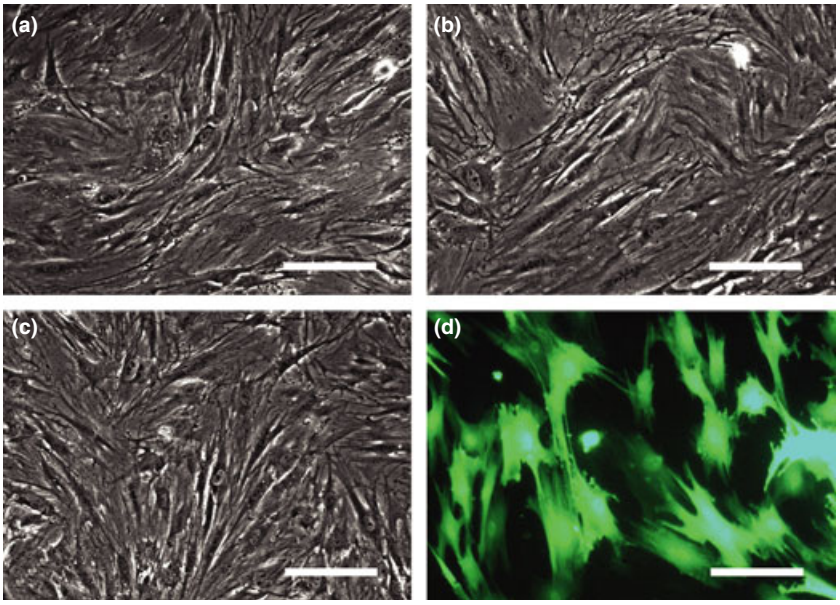

Figure 2 Cell culture and gene transduction. (**a**) Cellular morphology of untreated control cells. (**b**) Cellular morphology of adenovirus with enhanced green fluorescent protein (AdEGFP)-transduced cells. (**c**) Cellular morphology of adenovirus encoding bone morphogenic protein-2-transduced cells. (**d**) A multiplicity of infection of 80 pfu $cell^{-1}$ achieved high transfer efficiency about 60∼80% 72 h after transduced by AdEGFP. Bone marrow stromal cells emitted bright and intense green fluorescent. Scale bar = 50 μm

(Figure 2b) and AdBMP-2 (Figure 2c) transduction, without obvious observed cell death. Strong green fluorescence was detected in cells 72 h after AdEGFP transduction and the gene transfer efficiency reached 60∼80% (Figure 2d).

Osteogenic differentiation of bMSCs

Two weeks after gene transduction, ALP staining and calcium deposits demonstrated by von Kossa staining were greater and more intensive in AdBMP-2-transduced bMSCs (Figure 3c, f) than those in AdEGFP-transduced bMSCs (Figure 3b, e) and untransduced bMSCs (Figure 3a, d), which suggested that AdBMP-2 gene transduction promoted the bMSCs' differentiation into osteoblastic cells.

RT-PCR

Reverse transcription-polymerase chain reaction demonstrated that AdBMP-2-transduced bMSCs altered their genetic expression during the culture periods.

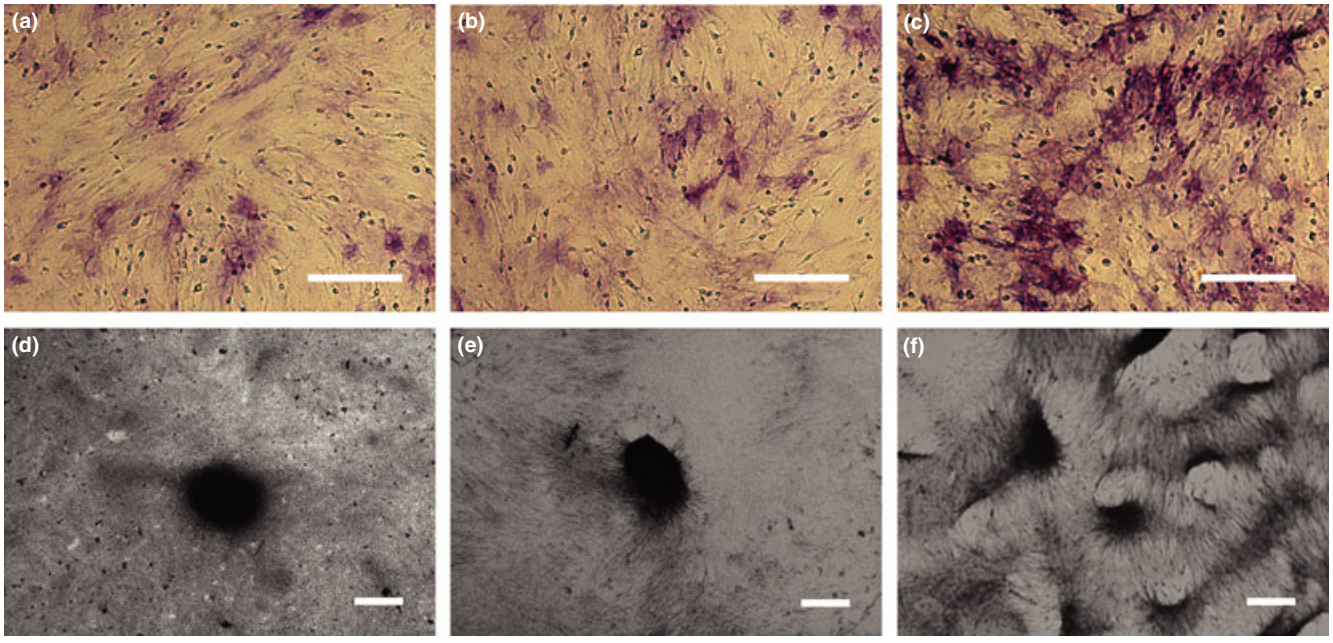

Figure 3 *In vitro* analysis of osteoblastic differentiation after bone marrow stromal cells (bMSCs) transduction with adenovirus encoding bone morphogenic protein-2 (AdBMP-2) and adenovirus with enhanced green fluorescent protein (AdEGFP). Alkaline phosphatase expression between (**a**) untransduced bMSCs, (**b**) AdEGFP-transduced bMSCs and (**c**) AdBMP-2-transduced bMSCs 14 days after gene transduction. von Kossa staining for calcium nodules between (**d**) untransduced bMSCs, (**e**) AdEGFP-transduced bMSCs and (**f**) AdBMP-2-transduced bMSCs 14 days after gene transduction. Scale bar = 100 μm

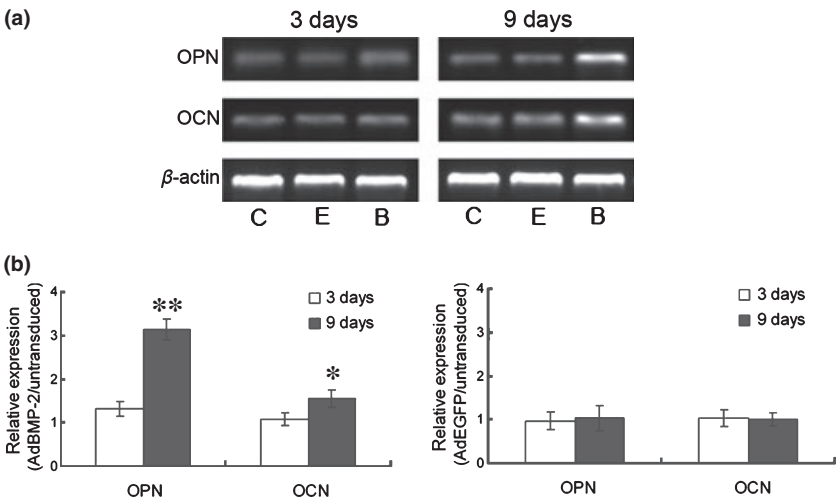

Figure 4 Effect of adenovirus encoding bone morphogenic protein-2 (AdBMP-2) and adenovirus with enhanced green fluorescent protein (AdEGFP) transduction on osteogenic gene marker expression. (**a**) Reverse transcription-polymerase chain reaction analysis revealed the expected 208 bp osteopontin and 232 bp osteocalcin DNA band, β-actin being an internal control. C: untransduced bone marrow stromal cells (bMSCs); E: AdEGFP-transduced bMSCs; B: AdBMP-2-transduced bMSCs. (**b**) DNA OD values of bMSCs transduced with AdBMP-2 were higher than those of the corresponding control groups. The significant differences were observed in the relative mRNA expression levels of osteopontin and osteocalcin when bMSCs were transduced with AdBMP-2 as compared with AdEGFP ($*P < 0.05$, $**P < 0.01$)

RT-PCR analysis revealed the expected 208 bp osteopontin (OPN) and 232 bp osteocalcin (OCN) amplified DNA band, β-actin being an internal control (Figure 4a). 3 days after transduction, the OPN and OCN mRNA expression from AdBMP-2-transduced bMSCs showed only a slight increase relative to that from AdEGFP-transduced bMSCs, but no significant differences were observed between them. However, following 9 days culture, the relative mRNA expression levels of OPN dramatically increased in AdBMP-2-transduced bMSCs compared with that of AdEGFP-transduced bMSCs, and the data also displayed a significant up-regulation of OCN mRNA expression (Figure 4b).

Scanning electron microscopy

Four hours after the bMSCs were combined with the implant, cells could be seen attaching to the inner surface of the scaffold *in vitro* (Figure 5a). After 24 h, cells were fully spreading and growing (Figure 5b). Nominal differences in cellular adhesion and proliferation were observed among bMSCs transduced with AdBMP-2, AdEGFP or untransduced. Additionally, these results suggested that β-TCP was a suitable biomaterial for the proposed *in vivo* studies as it facilitated bMSCs' initial attachment onto its surface, spreading and subsequent growth.

Radiographic evaluation and micro-computerized tomography

To evaluate the new bone formation, X-ray images were taken at 8 weeks after surgery. Representative photographs of each group are shown in Figure 6. Photographs showed radiopacities at the defect sites in β-TCP alone group (Figure 6a), untreated bMSCs/β-TCP group (Figure 6b) and AdEGFP-transduced bMSCs/β-TCP group (Figure 6c), but indicated a high increase in AdBMP-2-transduced bMSCs/β-TCP group (Figure 6d).

The morphology of the repaired mandibular defects was reconstructed using micro-CT. Bone formation was less pronounced for defects filled with untreated bMSCs/β-TCP (Figure 6f) and AdEGFP-transduced bMSCs/β-TCP (Figure 6g), but still advanced when compared with the implantation of β-TCP alone (Figure 6e). Substantial new bone formation was observed after 8 weeks in the critical size defects which received AdBMP-2-transduced bMSCs/β-TCP construct, with induced bone volume on the lateral side of the mandibles that equal to the cortical level on pseudo-3D displays (Figure 6h).

Histological examination and histomorphological analysis

Radiographic and micro-CT findings were confirmed through subsequent histological analysis of repaired

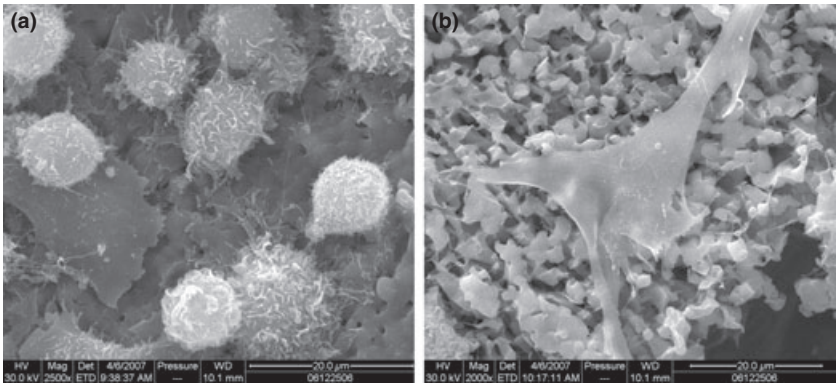

Figure 5 Scanning electron microscopic evaluation of the β-tricalcium phosphate scaffold microstructure and biocompatibility. (**a**) Four hours after the bone marrow stromal cells (bMSCs) were seeded onto the scaffold, cells could be seen attaching to the inner side of the scaffold. Scale bar = 100 μm. (**b**) One day after seeding, bMSCs were observed spreading along the scaffold surfaces. Scale bar = 100 μm

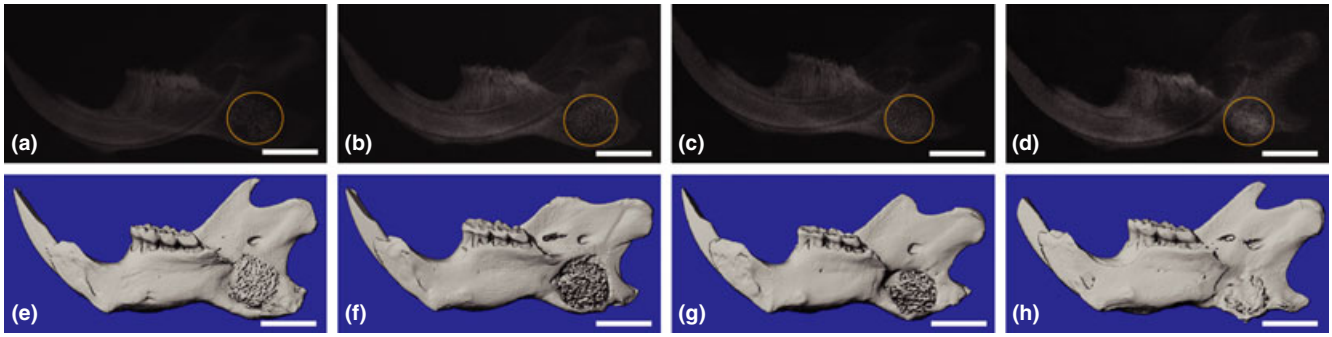

Figure 6 Radiographic evaluation and micro-computerized tomography of the repaired mandible. Representative photographs showed radiopacities at the defect sites in (**a**) β-tricalcium phosphate (β-TCP) alone group, (**b**) untreated bone marrow stromal cells (bMSCs)/β-TCP group, (**c**) adenovirus with enhanced green fluorescent protein (AdEGFP)-transduced bMSCs/β-TCP group but indicated highly increase in (**d**) adenovirus encoding bone morphogenic protein-2 (AdBMP-2)-transduced bMSCs/β-TCP group. Micro-computerised tomography images show the morphology of the newly formed bone in (**e**) β-TCP alone group, (**f**) untreated bMSCs/β-TCP group, (**g**) AdEGFPtransduced bMSCs/β-TCP group and (**h**) the new bone formed on the lateral side of the mandibles equivalent to the cortical level on pseudo-3D displays when the defects received AdBMP-2-transduced bMSCs/β-TCP construct. Scale bar = 0.5 cm

mandibular defects. Eight weeks after surgery, the implants were harvested, sectioned and evaluated histomorphologically. Samples with β-TCP alone group were found to contain more fibrous connective tissue in the area of defects, with only a little immature bone formation at the defect margins. Large amount of multinucleated giant cells (MNGCs) were seen in contact with the surface of β-TCP (Figure 7a, e). In the untreated bMSCs/β-TCP (Figure 7b, f) and AdEGFP-transduced bMSCs/β-TCP groups (Figure 7c, g), histological analysis showed a small amount of irregularly arranged woven bone tissue at the centre pores of β-TCP scaffold, and fibrous connective tissue was still frequently observed. In the defects filled with implantation of AdBMP-2-transduced bMSCs/β-TCP construct, mature newly formed bone tissue with few fibrous connective tissues infiltration was observed in the β-TCP pores at both centre and marginal area. Bone marrow also largely formed accompanied with the bony ingrowth (Figure 7d, h). The percentage of new bone area after 8 weeks was 4.72 ± 1.74% in β-TCP group, 10.12 ± 2.82% in untransduced bMSCs group, 12.22 ± 3.63% in AdEGFP-transduced bMSCs/β-TCP group and 31.83 ± 5.35% in AdBMP-2-transduced bMSCs/β-TCP group, respectively. The percentage of new bone area in AdBMP-2 group was significantly

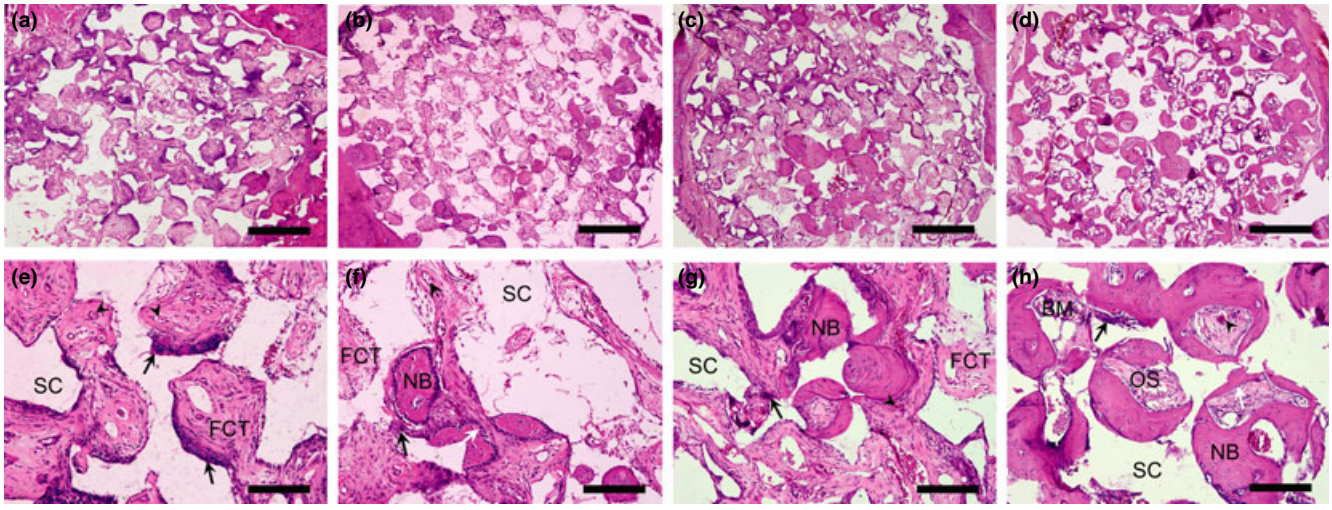

Figure 7 Representative histological images of repaired mandibles at 8 weeks post-operation. The microscopic view of repaired area in each group was shown: (**a**) In the β-tricalcium phosphate (β-TCP) alone group, more fibrous connective tissue was found in the area of defects with only a little immature bone formation occurred at the defect margins. (**b**) In the untreated bone marrow stromal cells (bMSCs)/β-TCP and (**c**) adenovirus with enhanced green fluorescent protein (AdEGFP)-transduced bMSCs/β-TCP group, histological analysis showed a small amount of irregularly arranged woven bone tissue at the centre pores of β-TCP scaffold and fibrous connective tissue was still frequently observed. (**d**) In the adenovirus encoding bone morphogenic protein-2 (AdBMP-2)-transduced bMSCs/β-TCP group, newly formed mature bone tissue with few fibrous connective tissues infiltration was observed in the β-TCP pores in both center and marginal area. Scale bar = 1 mm. The high magnified photomicrograph of each group: (**e**) β-TCP alone group, (**f**) untreated bMSCs/β-TCP group, (**g**) AdEGFP-transduced bMSCs/β-TCP group, and (**h**) AdBMP-2-transduced bMSCs/β-TCP group. BM: bone marrow; FCT: fibrous connective tissue; NB: new bone; OS: osteoid; SC: scaffold. White arrow shows the ingrowth osteoblast. Black arrow shows the multinucleated giant cells. Arrow head shows the blood vessels. Scale bar = 200 μm

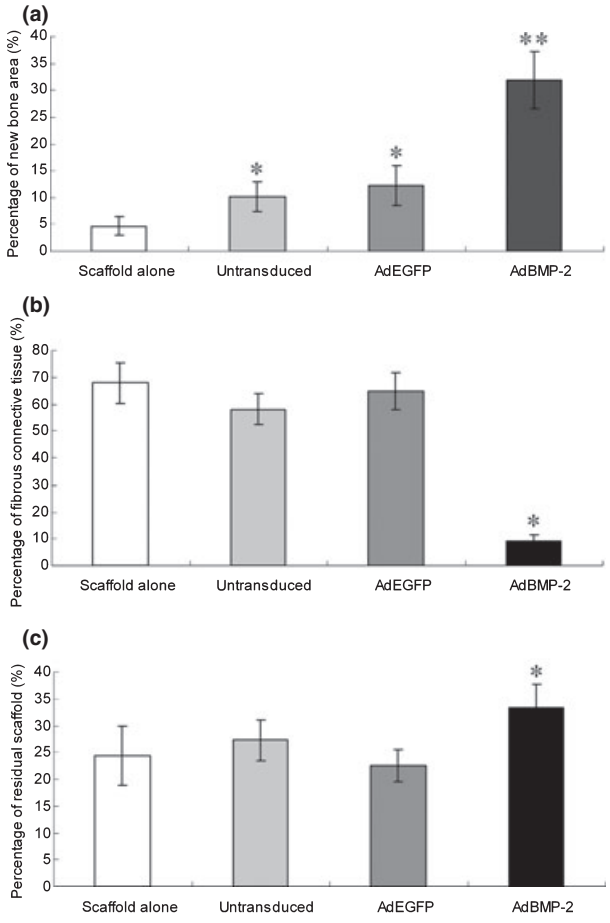

Figure 8 (**a**) Histomorphometirc analysis of the new bone area, (**b**) fibrous connective tissue and (**c**) residual scaffold for four groups at 8 weeks post-operation (*$P < 0.05$ *vs* β-tricalcium phosphate (β-TCP) alone group, **$P < 0.01$ *vs* β-TCP alone group)

higher than those of AdEGFP-transduced, untransduced and β-TCP alone groups 8 weeks post-operation (Figure 8a). The percentage of fibrous connective tissue was 67.89 ± 7.44% in β-TCP group, 58.13 ± 5.57% in untransduced bMSCs group, 64.78 ± 6.85% in AdEGFP-transduced bMSCs/β-TCP group and 9.04 ± 2.32% in AdBMP-2-transduced bMSCs/β-TCP group, respectively (Figure 8b). The percentage of residual scaffold was 24.38 ± 5.59% in β-TCP group, 27.32 ± 3.79% in untransduced bMSCs group, 22.53 ± 2.94% in AdEGFP-transduced bMSCs/β-TCP group and 32.23 ± 4.39% in AdBMP-2-transduced bMSCs/β-TCP group, respectively (Figure 8c). The lowest percentage of fibrous connective tissue and the highest percentage of residual scaffold were found in AdBMP-2-transduced bMSCs/β-TCP group.

Discussion

Using a critical size mandibular bony defects model in rats (Schliephake *et al*, 2008), we demonstrated that tissue-engineered bone with BMP-2 gene-modified bMSCs and a biodegradable β-TCP scaffold achieved an effective repair of the mandibular defects compared with that of AdEGFP-transduced bMSCs/β-TCP, untreated bMSCs/β-TCP construct and β-TCP scaffold alone.

Gene therapy, which involves the transfer of genes into individuals for therapeutic purposes, can be performed by either an *ex vivo* or an *in vivo* gene-transfer approach (Chen, 2001). *In vivo* gene therapy through direct injection of the recombinant adenoviral vector is a simple approach, but there are concerns about problems with transduction efficiency and the induction of an immune response to local expression of viral proteins. With the *ex vivo* method, the gene is transferred to cells *in vitro*, and re-implanted into a specific anatomic site. Main advantages of *ex vivo* approach include direct delivery of the osteoinductive gene to the desired site, providing a period of sustained BMP production and it is safer than *in vivo* techniques, as it avoids the inoculation of viral DNA complexes directly into the body (Scaduto and Lieberman, 1999; Dai *et al*, 2005).

Adenovirus vectors, although a transient gene expression system, have been used effectively to deliver BMP-2 to bMSCs both *in vitro* and *in vivo* (Chang *et al*, 2004). In this experiment, rat bMSCs were successfully transduced in the dishes and achieved a relatively high efficiency of approximately 70%, as demonstrated by EGFP expression in AdEGFP-transduced cells at an MOI of 80 pfu cell^{-1}. The length of adenovirus-mediated transgene expression *in vivo* varied from 2 to 11 weeks in different tissues (Roman *et al*, 1992). Our previous study demonstrated that the transduced bMSCs survived for at least 4 weeks after *in vivo* injection (Aghaloo *et al*, 2007). Gene transfer is highly effective, resulting in high over expression which, although transient, may well match the requirements of bone healing (Egermann *et al*, 2004).

Bone morphogenic proteins are powerful inducers of osteoblast differentiation *in vitro* and bone formation *in vivo* (Hughes *et al*, 1995; Wozney and Rosen, 1998). With our protocol, ALP staining, a marker of early osteogenic differentiation and commitment of bMSCs towards the osteoblastic phenotype, showed stronger expression in AdBMP-2-transduced bMSCs compared with that in AdEGFP-transduced bMSCs and untransduced bMSCs. Accordingly, more intensive mineralized nodules were observed in AdBMP-2-transduced bMSCs than that in other groups. These results suggested that cells infected by AdBMP-2 gene led to the bMSCs' differentiation into osteoblastic cells.

The development of bone formation with a defined sequence of gene expression and cell activity is a gradual process: an initial proliferation and matrix deposition, matrix maturation and finally, matrix mineralization phase (Owen *et al*, 1990). OPN, major non-collagenous protein (NCP) found in bone, is a non-specific early bone marker that is expressed bimodally, with an early peak during the proliferative phase and another after initial mineralization (Yao *et al*, 1994), and OPN was linked to pre-osteoblastic cell stages (Mark *et al*, 1987; Prince *et al*, 1987). Gene expression levels of OPN began to up-regulate at day 3 and dramatically increased at day 9 for AdBMP-2-transduced bMSCs. OCN, the most

specific late stage marker for osteoblast maturation (Holy *et al*, 2003), showed a significant increase at day 9. The RT-PCR analysis indicated that genetic modification of the bMSCs with BMP-2 gene successfully elevated the ability of bMSCs to synthesize genes associated with mineralization.

Porous β-TCP, a synthetic and biodegradable ceramic material with an interconnected pore diameter of 450 μm that facilitates vascular invasion and bone development, has been commonly used in various applications in oral and maxillofacial surgery, such as alveolar ridge augmentation and sinus floor augmentation (Horch *et al*, 2006; Wang *et al*, 2009). Some other scaffolds used in similar osseous tissue bioengineering (Park *et al*, 2003; Schliephake *et al*, 2008) have certain deficiencies; for example, the popular polylactides and glycolides could induce inflammation due to the acidity of their hydrolysis products (Athanasiou *et al*, 1996) and collagen derived from animal tissues might create concerns related to the quality, purity and predictability of their performance (Yang *et al*, 2004). Furthermore, these scaffolds are not generally considered osteoconductive. Coral hydroxyapatite was also used (Tang *et al*, 2008), but hydroxyapatite could not adequately degrade *in vivo*; the lingering degradation of the scaffold may impact the subsequent sufficient new bone formation as well as the tissue structure remodelling. β-TCP is an attractive material with good biocompatibility, resorbability and osteoconductive capacity. In this study, the cells could infiltrate and spread along the pore surface after being cultured *in vitro*. This can explain why we found newly formed bone mainly deposited on the periphery in β-TCP alone group, while in other tissue engineering groups when the scaffolds combined with cells, some new bone area presented in the centre of the defect sites.

Although the intrinsic reparative cells could creep towards the scaffold and this effect failed to be ruled out, only a little immature bone formation occurred at the defect margins, which indicated that the intrinsic bone repair process may not be powerful enough in the case of critical size defects, as shown in the β-TCP alone group. Regardless of new bone formation being influenced by recruitment of surrounding host cells, the results demonstrated effective repair of critical size mandibular bony defects using gene-modified tissue-engineered bone, whereas repair was incomplete using cell-loaded scaffolds at 8 weeks post-operation.

Biodegradable scaffold plays a pivotal role in bone tissue engineering. Ideal scaffold for bone regeneration should have a suitable degradation rate that can well match the speed of new bone formation. The degradation of β-TCP *in vivo* could be through two processes: extracellular liquid dissolution and cell-mediated resorption (Kurashina *et al*, 1997; Dong *et al*, 2002). In this study, the degradation of β-TCP might also be through both processes. Blood vessels largely formed in the defect area and MNGCs close to the surface of the scaffolds were detected. The abundant blood circulation of oral maxillofacial region could promote the dissolution process and break the scaffold into small particles, which could be further phagocytosed by MNGCs. In the current study, the lowest percentage of fibrous connective tissue and the highest percentage of residual scaffold were found in AdBMP-2-transduced bMSCs/β-TCP group. This phenomenon might be related to early bone formation and mineralization, which then slowed the cell-mediated resorption of the scaffold and prevented the fibrillar tissue infiltration.

Based on our findings, we may conclude that β-TCP scaffolds alone could not be successfully used for the repair of mandibular bony defects in rat; however, the combination of β-TCP scaffolds with bMSCs could promote new bone formation, and β-TCP scaffolds with AdBMP-2 gene-modified bMSCs could achieve the best effects in repairing mandibular critical sized bony defects. BMP-2 regional gene therapy and tissue engineering technique could be used to promote mandibular repair and bone regeneration.

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Author contributions

Dr J Zhao conducted the experiment and drafted the manuscript; Dr J-Z Hu and S-Y Wang executed the animal experiment; Dr X-J Sun and L-G Xia contributed in data collection and statistical analysis; Dr X-L Zhang contributed in cell culture; Dr X-Q Jiang provided the concept and organized the study; Dr Z-Y Zhang reviewed the manuscript.

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