Repair of mandibular defects by bone marrow stromal cells expressing the basic fibroblast growth factor transgene combined with multi-pore mineralized Bio-Oss

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Abstract. The aim of the present study was to evaluate the effect of combining Bio-Oss with bone marrow stromal cells (BMSCs) transfected with the basic fibroblast growth factor (bFGF) gene on bone regeneration during mandibular distraction of rabbits. BMSCs obtained from rabbits were transfected with bFGF gene-encoding plasmids and proliferation rate and the differentiation marker alkaline phosphatase activity were measured. Following seeding into Bio-Oss collagen and 9-day culture *in vitro*, the surface morphology of the Bio-Oss was assessed using scanning electron microscopy analysis. Three mandibular defects were induced in the lower border of the bilateral mandibular ramus in each New Zealand white rabbit (total n=6). Three scaffolds, group A (seeded with BMSCs/bFGF), B (seeded with BMSCs/pVAX1) and C (cell-free), which had been cultured *in vitro* under standard cell culture conditions for 18 days, were implanted into mandibular defects under sterile conditions. Animals were sacrificed by anesthesia overdose 12 weeks following surgery and the scaffolds were extracted for bone mineral density and histological analyses. Results indicate that bFGF was successfully transfected into BMSCs. Proliferation and osteoblast differentiation of BMSCs were stimulated by bFGF *in vitro*. No differences were identified in surface morphology for Bio-Oss loaded with variable groups of cells. At week 12 following implantation of Bio-Oss scaffolds, mineralization of BMSCs in Bio-Oss scaffolds was observed to be increased by bFGF. New bone and cartilage formation was revealed in hematoxylin and eosin-stained sections in Bio-Oss scaffolds and was most abundant in group A (BMSCs transfected with bFGF). In the current study, the bFGF gene was transfected into BMSCs and expressed successfully. bFGF promoted proliferation and differentiation of BMSCs *in vitro* and implantation of bFGF-expressing BMSCs combined with Bio-Oss enhanced new bone regeneration more effectively than traditional methods.

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Abbreviations: BMSCs, bone marrow stromal cells; bFGF, basic fibroblast growth factor; ALP, alkaline phosphatase; BMD, bone mineral density

Key words: basic fibroblast growth factor, bone marrow stromal cells, Bio-Oss, mandibular defect

Introduction

Mandibular defects remain a major challenge for dentists. Traditionally, augmentation of bony defects is performed using allografts, xenografts and autogenous bone (1). More recently, biomaterials, including commercially available Bio-Oss, have been utilized for restoration of mandibular defects (2). Bio-Oss is an inorganic sterilized bovine bone, composed of a calcium-deficient carbonate apatite (3). Previous studies have reported promising clinical outcomes for implantation of Bio-Oss in intra-bony periodontal and mandibular defects associated with dental implants (4). Bio-Oss is considered to exhibit superior biocompatibility and is highly suitable for use as a scaffold for osteogenesis and osteogenic cells. However, studies have also demonstrated that Bio-Oss has poor osteoinduction (5).

Bone marrow stromal cells (BMSCs) are a subset of plastic adherent nonhematopoietic stem cells, characterized by their ability to self-renew and differentiate into multiple cell types, including osteoblasts, adipocytes and chondrocytes (6). It has been widely accepted that tissue repair of bone defects is advanced by bone marrow stem cells that migrate to the site of damage and undergo differentiation promoting structural and functional repair (7). BMSCs are induced to differentiate into osteoblasts and restore bone defects. A previous study demonstrated that intravenous delivery of BMSCs led to migration to the injury site for restoration of bone or cartilage fracture (8). In particular, when combined with tridimensional scaffolds, BMSCs differentiate into osteoblasts and deposit extracellular matrix on the ceramic surface, promoting formation of new bones. However, currently this process is limited by poor proliferation of BMSCs (9).

Basic fibroblast growth factor (bFGF) is a potent mitogen for fibroblasts and other mesoderm-derived cells, including

osteoblasts and vascular endothelial cells (10). Previously, bFGF was identified to induce bone formation by stimulating proliferation and differentiation of bone marrow stromal cells (BMSCs), enhancing chondrogenic and osteogenic differentiation of BMSCs and stimulating these cells to deposit new mineralized bone (11,12).

To date, a limited number of studies have analyzed the potential of bFGF and BMSCs combined with Bio-Oss scaffolds for roles in the repair of mandibular defects. In the present study, we hypothesized that bFGF is likely to promote BMSC osteogenesis in conjunction with Bio-Oss scaffolds. To test the hypothesis, the potential effects of bFGF on the proliferation and differentiation of BMSCs were addressed *in vitro*. The effect of bFGF on the attachment of BMSCs to Bio-Oss scaffolds was also investigated *in vitro*. Furthermore, we examined whether bFGF enhanced osteogenesis of BMSCs attached to scaffolds *in vivo*.

Materials and methods

Animal experiments. All animal experiments were approved by the Animal Care and Use Committee of the Second Affiliated Hospital of Harbin Medical University (Harbin, China) and were performed in compliance with the NIH 'Guide for the Care and Use of Laboratory Animals'.

Isolation and culture of BMSCs. BMSCs were isolated by flushing the femurs and tibias of 9-month-old New Zealand white rabbits (Cyagen Biosciences, Guangzhou, China). Total bone marrow was washed, triturated using a 20-gauge needle and passed through a 40-μm nylon mesh cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) to produce a single cell suspension in phosphate-buffered saline (PBS). These cells were cultured in α-MEM (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA). Isolation was based on the plastic adherence and rapid proliferation of BMSCs. Following 48 h culture, nonadherent cells were removed by washing with PBS and adherent cells were maintained in fresh medium for propagation. Medium was changed every 2-3 days. Cells at passage 3 were used for the experiments. Culture was performed in a humidified incubator at 37˚C in a 5% CO_2 atmosphere.

Construction and transfection of the pVAX1-bFGF plasmid. Total RNA was isolated from BMSCs using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and cDNA was synthesized from 2 μg of purified total RNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Primer sequences used to amplify the target rabbit bFGF gene (XM_002717238) were as follows: forward, 5'-GAATTCATGGCAGCCGGGAGCATCA-3' and reverse, 5'-GGATTCTCAGCTCTTAGCAGACATTGG-3'. The amplified cDNA fragment was ligated with the plasmid vector ligase and cloned into the pVAX1 vector (Invitrogen Life Technologies). The pVAX1-bFGF plasmid was introduced into competent DH5α *E. coli* for replication and later extracted and purified using an EndoFree kit (Qiagen, Hilden, Germany). The recombinant plasmid was validated by restriction enzyme digestion and sequencing. Recombinant pVAX1-bFGF and empty pVAX1 vector were then transfected into BMSCs using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. In brief, Lipofectamine 2000 was mixed with recombinant pVAX1-bFGF plasmids at 10 μl:4 μg, diluted with DMEM (Gibco-BRL) and incubated for 20 min at room temperature. BMSCs were resuspended in DMEM. The mixture of Lipofectamine 2000/pVAX1-bFGF was added and cells were incubated at 37˚C, 5% CO_2 for 4 h. Following this, DMEM containing 10% FBS without antibiotics was added and the cells were incubated for 48 h. G418 (Roche Diagnostics, San Francisco, CA, USA) was used to select for stably transfected BMSCs. Restriction enzyme digestion and sequencing assays were used to test whether the transfection was successfully performed. To confirm the expression of bFGF in BMSCs, indirect immunofluorescence analysis was performed. Cells were fixed in 2% paraformaldehyde, permeabilized in ice-cold methanol and incubated with an antibody against bFGF (Cell Signaling Technology, Danvers, MA, USA) overnight at 4˚C, followed by incubation with goat anti-mouse FITC secondary antibody and mounting in medium containing DAPI. Cells were visualized with a Zeiss immunofluorescence microscope.

Cell proliferation assay. Cell proliferation assays were performed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, $2x10^3$ cells/well were plated in 96-well plates and cultured in growth medium. At the indicated time points, medium was aspirated and 100 μl serum-free DMEM and 10 μl 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-8) was added and incubated at 37˚C for 1.5 h. Absorbance was measured at 450 nm with a reference wavelength of 630 nm on a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). All experiments were performed 5 times.

Alkaline phosphatase (ALP) activity. BMSCs transfected with pVAX1-bFGF or empty vector were plated into 96-well culture plates ($2x10^4$ cells/well) and cultured for 24 h. Following this, cells were subjected to culture medium containing 1 mmol/l β-sodium phosphate, 50 μg/l L-ascorbic acid (both obtained from InterGen, Burlington, MA, USA) and 10 nmol/l dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) for osteogenic induction. The culture medium was renewed twice a week. ALP activity was measured at days 1, 7, 14, 21 and 28 using an ALP assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions.

Seeding of BMSCs into Bio-Oss collagen. Bovine spongious bone collagen graft (Bio-Oss collagen; Geistlich Pharma AG, Wolhusen, Switzerland) was cut to 6x4x2 mm under sterile conditions and prepared as scaffolds. Two groups of BMSCs, BMSCs/bFGF and BMSCs/pVAX1 were trypsinized and suspended in culture medium containing gelatum (20 mg/ml) and immediately loaded into Bio-Oss scaffolds ($2x10^5$ BMSCs/scaffold) using a pipette. Scaffolds were incubated in osteoblast-induction medium under standard cell culture conditions for 18 days prior to implantation. The surface morphological characteristics of Bio-Oss were investigated at day 9 using scanning electron microscopy.

Scanning electron microscopy analysis. BMSCs/bFGF or BMSCs/pVAX1 cells loaded in Bio-Oss were morphologically analyzed following incubation for 9 days using a scanning electron microscope (S2300; Hitachi, Tokyo, Japan). Samples were fixed with 2.5% glutaraldehyde for 2 h and washed 3 times with PBS. Osmium tetroxide (1%) was used for secondary fixation. Following washing, dehydration of the samples was performed for 30 min through a graded ethanol series (50, 70, 90 and 100% ethanol, each for 15 min). Then, samples were subjected to sputter coating with platinum and examined by scanning electron microscope at 10 kV.

Surgery and implantation of Bio-Oss. Six New Zealand white rabbits (9-12 months old; weight, ~2 kg; Cyagen Biosciences) were used for this study. Bio-Oss scaffolds groups were as follows (n=6 each group): A (seeded with BMSCs/bFGF); B (seeded with BMSCs/pVAX1); and C (cell-free). Animals were acclimatized for 1 week and maintained throughout at standard conditions: 25±2˚C temperature, 40-60% relative humidity and 12-h light/dark cycle. Prior to surgery, animals were anesthetized with 3% pentobarbital sodium (1 ml/kg body weight). Three mandibular defects (2.5x3 mm) were induced in the lower border of the bilateral mandibular ramus using a micromotor drill. Each rabbit recieved a scaffold from each group, which was implanted into mandibular defects under sterile conditions. Following this, muscles, soft tissues and skin were carefully repositioned and wounds were closed with 30 nylon sutures. Animals were sacrificed by anesthesia overdose 12 weeks following surgery and the scaffolds were extracted for analysis.

Bone mineral density (BMD) analysis. Extracted scaffolds were examined for bone mineralization by X-ray. BMD (g/cm^2) was measured using dual-energy X-ray absorptiometry (DEXA; Lunar Prodigy, GE Healthcare Biosciences, Pittsburgh, PA, USA). Scaffolds were scanned and analyzed using a specific animal program provided by the manufacturer. All experimental data were sampled 3 times.

Histological analysis. Samples for histological analysis were prepared by placing scaffolds in 4% paraformaldehyde solution for 24 h, followed by decalcification in a 10% EDTA solution for 3 weeks. Samples were dehydrated and embedded in paraffin and then cut into 5-μm sections and transferred to silicon-coated slides. For hematoxylin and eosin staining, sections were dewaxed, rehydrated in an ethanol gradient and rinsed in water. Sections were stained with hematoxylin and eosin for 10 and 5 min, respectively, then dehydrated by rinsing in an ethanol gradient. Sections were coverslipped and examined by light microscopy.

Statistical analysis. SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Data are expressed as mean ± SEM. Statistical analysis was performed using standard one-way ANOVA followed by LSD post hoc test. Bonferroni's correction was used to adjust for multiple comparisons. A two-tailed Student's paired t-test was also used to compare the difference in values between 2 groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Ectopic expression of bFGF stimulates BMSC proliferation and osteogenic differentiation in vitro. Stable bFGF-expressing and negative control pVAX1 (empty vector) BMSC lines were successfully established and confirmed by restriction enzyme digestion and sequencing. In addition, indirect immunofluorescence results revealed green fluorescence on the membrane of PVAX1-bFGF- but not PVAX1-transfected cells (Fig. 1A), indicating that bFGF was expressed in BMSCs. To investigate the effects of bFGF expression on BMSC cell growth, BMSCs transfected with recombinant plasmid pVAX1-bFGF or empty vector and untransfected BMSCs were plated and cultured with growth medium for specified times (1-6 days), followed by WST-8 assay. As demonstrated in Fig. 1B, bFGF was found to significantly stimulate growth of BMSCs, while no statistical significance between BMSCs and vector transfected cells BMSCs/pVAX1 was identified. Analysis of ALP activity, an early marker of osteoblast differentiation (13), demonstrated that bFGF enhanced ALP activity in BMSCs (Fig. 1C). Significant differences between BMSCs/bFGF and BMSCs/pVAX1 cells were apparent at day 14 and the peak value of ALP activity appeared at 32 h, following this, ALP activity decreased (Fig. 1C). Results indicate that osteoblast differentiation of BMSCs is stimulated by bFGF *in vitro*.

Surface morphology of the Bio-Oss. Morphology and microstructures of the cells seeded on the surfaces of Bio-Oss were examined by scanning electron microscopy. Images of the 2 groups seeded with BMSCs/bFGF or BMSCs/pVAX1 cells are presented in Fig. 2. At day 9, scanning electron microscopy revealed that the cells formed a dense multilayered arrangement. No difference in surface morphology between the 2 groups was observed.

Analysis of Bio-Oss implantations

BMD measurements. Following animal sacrifice at week 12, scaffolds were extracted and BMD (g/cm^2) was measured using DEXA. As demonstrated in Fig. 3, compared with the sham control cell-free scaffold group C, BMD in experimental groups A (BMSCs/bFGF) and B (BMSCs/pVAX1) was found to be significantly higher ($P<0.05$ and $P<0.01$, respectively). In addition, BMD in group A was markedly higher compared with B ($P<0.05$). Results indicate that bFGF promotes mineralization of BMSCs in Bio-Oss scaffolds.

Histological analysis. In addition, scaffolds were extracted for and underwent histological analysis. As revealed in Fig. 4, in groups A and B, new bone was observed to be growing towards and amalgamating with the Bio-Oss granules. By contrast, more compact mineralized areas were observed by toluidine blue staining in group A compared with B and C. In group C implants without seeded cells were used as controls, formation of a vascularized loose connective tissue was oberved, however, newly deposited bone was rare (Fig. 4C). Results indicate that bFGF enhanced the *in vivo* osteogenic potential of BMSCs.

Discussion

In the present study, BMSCs transfected with bFGF exhibited increased proliferation and differentiation compared with

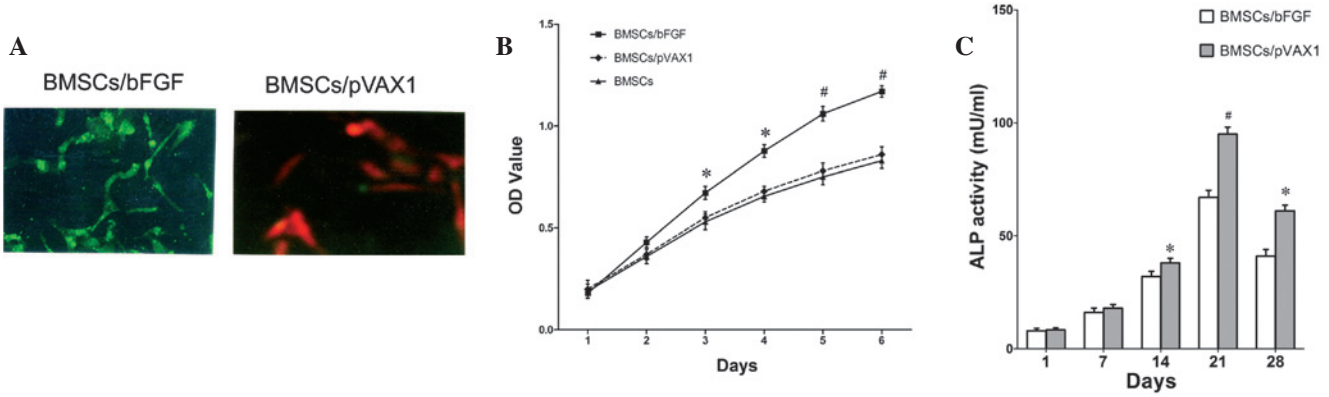

Figure 1. Effect of ectopic bFGF expression on proliferation and differentiation of BMSCs. (A) Confirmation of bFGF expression in BMSCs by indirect immunofluorescence. BMSCs transfected with (left, green) pVAX1-bFGF or (right, red) pVAX1 empty vector. (B) Ectopic bFGF expression promotes cellular growth of BMSCs. Cell proliferation was assessed by WST-8 assay, data are presented as the mean ± SEM of each time point from 5 samples. *P<0.05 and $^{\#}$P<0.01, vs. control BMSCs or BMSCs/pVAX1 cells. (C) Ectopic bFGF expression enhances ALP activity of BMSCs. ALP activity of BMSC transfected with bFGF or empty vector was measured at various days, data are presented as the mean ± SEM of each time point from 3 samples. *P<0.05 and $^{\#}$P<0.01, vs. control BMSCs/pVAX1 cells. BMSCs, bone marrow stromal cells; bFGF, basic fibroblast growth factor; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt; ALP, alkaline phosphatase.

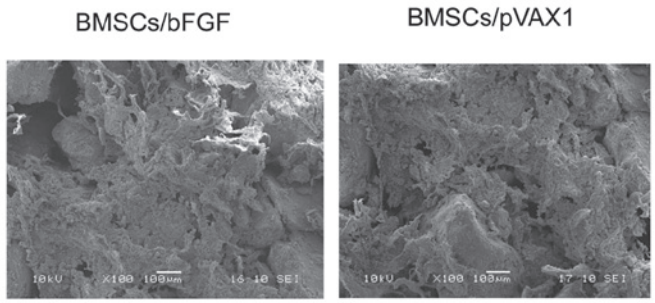

Figure 2. Scanning electron microscopy examination of the surface morphology of Bio-Oss seeded with BMSCs/bFGF or BMSCs/pVAX1 cells following 9 day incubation. bFGF-transfected or pVAX1-transfected BMSCs were seeded into Bio-Oss scaffolds following *in vitro* culture. At day 9, microscopy revealed that the cells formed a dense multilayered arrangement. Magnification, x100. BMSCs, bone marrow stromal cells; bFGF, basic fibroblast growth factor.

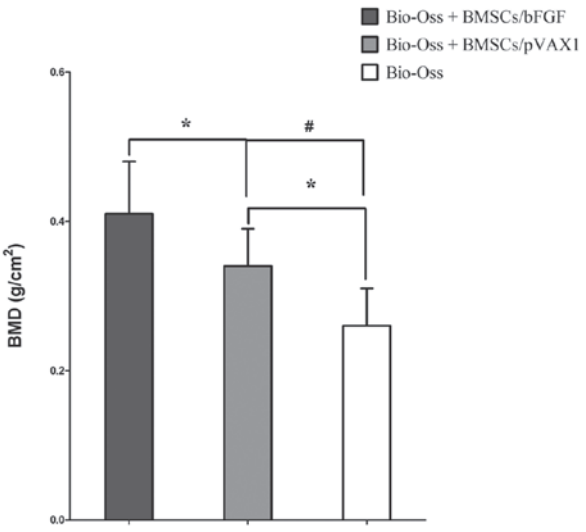

Figure 3. BMD (g/cm^2) of extracted Bio-Oss scaffolds was measured using DEXA. *P<0.05 and $^{\#}$P<0.01, vs. control scaffolds seeded with BMSCs/pVAX1 cells or cell-free scaffolds. All experiments were performed three times. BMSCs, bone marrow stromal cells; bFGF, basic fibroblast growth factor. BMD, bone mineral density; DEXA, dual-energy X-ray absorptiometry.

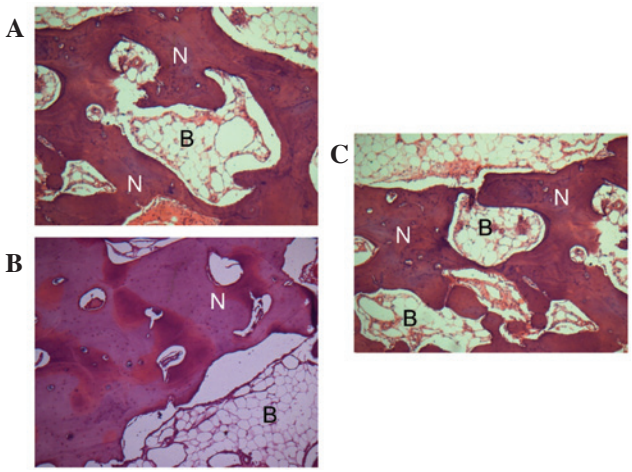

Figure 4. New bone and cartilage formation was identified in HE-stained sections in Bio-Oss scaffolds. Paraffin sections of bone tissue generated from Bio-Oss scaffolds seeded with osteoblast-induced BMSCs transfected with (A) bFGF, (B) empty vectors or (C) Bio-Oss scaffold only were stained with HE. Magnification, x200. N, newly formed bone; B, Bio-Oss; BMSCs, bone marrow stromal cells; bFGF, basic fibroblast growth factor; HE, hematoxylin and eosin.

controls *in vitro*. Following seeding into Bio-Oss scaffolds and transplantation into experimental mandibular defects of rabbits, BMSCs transfected with bFGF loaded in Bio-Oss markedly accelerated bone regeneration. Results indicate that bFGF increased osteogenesis of BMSCs and Bio-Oss loaded with BMSCs transfected with bFGF may prove to be a valid option for repairing mandibular defects.

BMSCs are osteogenic cell sources containing a phenotypically and functionally heterogeneous population of mesenchymal precursors which contribute to the physiological regeneration of bone, cartilage, adipose, muscle and other connective tissues (6,14). The osteogenic potential of BMSCs to produce bone-like mineralized tissue has been widely demonstrated *in vitro* and *in vivo* (15,16). BMSCs have been used in several *in vivo* animal model systems to generate bone tissue and have been demonstrated to enhance the ability of demineralized bone matrices to promote bone formation

within defect sites (17). bFGF is considered a growth factor with high osteoinductive activity in the FGF family (18). A previous study identified that the commitment of rat BMSCs to mineralization is enhanced by cytokine bFGF that affect the proliferation and differentiation of cells (19). bFGF has been demonstrated to upregulate markers of the mature osteoblastic phenotype, including ALP activity (20). In the present study, proliferation rate was observed to be significantly higher in BMSCs transfected with bFGF compared with untransfected BMSCs during various culture periods. Moreover, bFGF significantly stimulated ALP activity of BMSCs. ALP activity is widely used as a marker of early differentiation of osteoblast-like cells (13), indicating that bFGF stimulated differentiation of BMSCs *in vitro*. ALP activity in BMSCs transfected with or without bFGF was markedly decreased on day 28 compared with day 21, indicating that ALP activity peaked prior to initiation of calcification. These observations demonstrate the key role of bFGF in enhancing proliferation and differentiation of BMSCs.

Mandibular defects remain a major challenge in orthopedic surgery. Traditionally, autologous bone transplantation was considered the gold standard treatment method, however, damaged donor sites and insufficient donor tissue have prevented the large-scale application of this approach (21). In addition, allogeneic bone grafts are restricted in clinical applications due to immunological reactions in recipients (22). At present, a number of biodegradable polymer scaffolds have been used for experimental transplantation of BMSCs to regenerate bones (23). BMSCs are easily obtained from bone marrow and are therefore good candidates for innovative clinical applications when cultured with biomaterials. Bio-Oss is recognized to exhibit superior biocompatibility and high suitability for use as a osteogenesis scaffold, however, it is also known to exhibit poor osteoinductive properties (24). In addition, nutrient supply and cell viability at the centre of the scaffold remains a key problem in the use of Bio-Oss to restore bone defects (25). BMSCs are known to exhibit reduced proliferative capacities (26) and the maintenance of this ability is important for tissue engineering in Bio-Oss scaffolds. Therefore, specific pro-growth factors must be included in the Bio-Oss scaffold system. In the current study, bFGF was used to overcome reduced proliferative capacity and we investigated whether Bio-Oss, derived from bovine bone matrix, supported the osteogenic differentiation and bone-forming capacity of BMSCs *in vivo*. Results demonstrate that bFGF-transfected BMSCs seeded in Bio-Oss significantly accelerated bone regeneration compared with Bio-Oss alone or loaded with BMSCs transfected with empty vectors, indicating that bFGF may enhance bone regeneration of BMSCs *in vivo*. As the Bio-Oss scaffold is surrounded by fibrous tissues and macrophages appear, the material undergoes degradation and absorption and new bone grows and fills the entire space, indicating that Bio-Oss scaffold favors bone conductibility and biodegradability for BMSCs transfected with bFGF. Currently, bFGF is hypothesized to promote bone regeneration by accelerating BMSC proliferation. These cells are likely to differentiate into progenitor cells in implanted sites, by stimulating proliferation of osteoblasts and periodontal fibroblasts directly and by stimulating quiescent endothelial cells to induce morphogenesis and proliferation (27). In addition, bFGF has been reported to be important for wound healing by inducing angiogenesis, which in turn accelerates bone regeneration (28). However, the exact mechanism remains unknown and must be elucidated by further studies.

In the current study, the main constituents of the Bio-Oss scaffolds were calf decalcified bone with interspaces representative of the structure of human cancellous bones. A number of clinical studies have reported that Bio-Oss bone block is highly biocompatible and produces a desired synostosis by combining with the host bone (29,30). Therefore, this form of composite artificial bone is consistent with the organism bone in constituents and structure, in accordance with physiological requirements. In the present study, no inflammatory cell infiltration around the implanted material was observed during development. The material combined closely with the new bone tissues and the process of osteogenesis was rapid without development of surrounding fibrous tissue, demonstrating further that Bio-Oss composed of bFGF-BMSCs has excellent biocompatibility. Histological analysis demonstrated that osteogenesis was based on marked bone conductibility of the Bio-Oss bone block and the efficient bone inductivity of bFGF. Its bone conductibility mainly depends on the growth of new bones around the host bone, which takes the specific gap structure of Bio-Oss bone block as its support at any time in each group. The remaining space will be occupied by new bones as well, which develops a coordinating relationship. However, the effective existence of bone inductivity can be observed from the aggregation and differentiation of mesenchymal cells in the experimental group at an early stage. Moreover, it could keep the vigorous osteogenesis activity in long term, and its mode of osteogenesis is always multicentric in material exposure. This greatly accelerates the process of osteogenesis and has a significant difference from the control group. However, since the material used in this experiment is a compound form of various biological materials, there are some questions which are worthy of being discussed, such as what type of composite material functions most effectively, what appropriate proportion of each material should be used. These questions await further studies.

In conclusion, the results of the present study demonstrate that the bFGF gene was transfected into BMSCs and expressed and proliferation and differentiation of BMSCs was enhanced by bFGF *in vitro*.

In a mandibular defect model of rabbitts, the implantation of bFGF-modified BMSCs associated with Bio-Oss promoted new bone regeneration more effectively than traditional methods. These observations are likely to be useful for future studies on the use of BMSC-seeded Bio-Oss to repair bone defects, which may improve therapies for clinical mandibula reconstruction.

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