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Mandibular Repair in Rats with Premineralized Silk Scaffolds and BMP-2-modified bMSCs

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Abstract

Prem mineralized silk fibroin protein scaffolds (mSS) were prepared to combine the osteoconductive properties of biological apatite with aqueous-derived silk scaffold (SS) as a composite scaffold for bone regeneration. The aim of present study was to evaluate the effect of pre mineralized silk scaffolds combined with bone morphogenetic protein-2 (BMP-2) modified bone marrow stromal cells (bMSCs) to repair mandibular bony defects in a rat model. bMSCs were expanded and transduced with adenovirus AdBMP-2, AdLacZ gene *in vitro*. These genetically modified bMSCs were then combined with pre mineralized silk scaffolds to form tissue engineered bone. Mandibular repairs with AdBMP-2 transduced bMSCs/mSS constructs were compared with those treated with AdLacZ transduced bMSCs/mSS constructs, native (nontransduced) bMSCs/mSS constructs and mSS alone. Eight weeks post-operation, the mandibles were explanted and evaluated by radiographic observation, micro-CT, histological analysis and immunohistochemistry. The presence of BMP-2 gene enhanced tissue engineered bone in terms of the most new bone formed and the highest local bone mineral densities (BMD) found. These results demonstrated that pre mineralized silk scaffold could serve as a potential substrate for bMSCs to construct tissue engineered bone for mandibular bony defects. BMP-2 gene therapy and tissue engineering techniques could be used in mandibular repair and bone regeneration.

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1. Introduction

Reconstruction of craniofacial bony defects caused by trauma and ablative oncologic procedures, or by congenital anomalies, is a frequent surgical challenge. Restoration of the original craniofacial bony structure is a prerequisite for the restitution of facial appearance and oral function. Although free revascularized autogenous bone grafting remains the standard procedure for indications requiring bone regeneration, the main disadvantages associated with this method are potential donor site morbidity, finite donor availability and difficulty in achieving the desired bone shape [1]. The use of allografts also has attendant limitations including disease transmission, immunogenic response, and nonunion [2,3]. Refinements in tissue engineering techniques during the past decade have enabled enhanced bone regeneration in many animal models. The useful tissue engineered bone complex combines osteoconductive scaffolds, cells and osteogenic growth factors [4].

Silk fibroin, an organic scaffold for tissue engineering, prepared in film, nanofiber, and porous matrix formats, which can be combined with stem cells, has been successfully used for the regeneration of cartilage [5] and bone [6-8]. Silk fibroin scaffolds offer significant advantages of predictable degradation rates [9,10], plasticity during processing to form desired shapes and sizes [11-13], and lower inflammatory response in comparison with collagens or synthetic polyesters such as PLGA [14,15]. Silks represent the strongest and toughest materials among current degradable polymers commonly used in biomaterials, and recently developed production processes enable the degradation rates of silk biomaterials to be controlled from weeks to years by controlling crystalline β -sheets [14]. Meanwhile, the porosity and pore size can be changed by manipulation of the concentration of silk fibroin protein used in the process and the size of the porogen (e.g., NaCl particles) [12,13].

In a biomimetic strategy, apatite coated metallic implant surfaces improved implant integration with the host bone and within three dimensional composites to fill bone defects, showing promise because of compositional and structural analogies to natural bone [16-20]. Similarly, silk scaffolds impregnated with apatite coatings also provided enhanced osteogenic environments due to the osteoconductivity of the bioceramic for expectant bone-related outcomes [21].

The bone morphogenic proteins (BMPs) are a family of growth factors that have demonstrated an impressive ability to induce orthotopic and ectopic new bone formation [22,23]. BMPs are pleiotropic signaling molecules critically involved at various stages in bone formation. However, local delivery of BMPs may have certain shortcomings, including short half-life, large dose requirements, high cost, the need of repeated applications, and poor distribution [24]. Fortunately, BMP-regional gene therapy can be employed to deliver both cells and osteoinductive factors to specific anatomic sites simultaneously in a more physiologic manner than exogenous protein release [25].

In the present study, the effectiveness of apatite-coated silk scaffolds loaded with AdBMP-2-transduced bone marrow stromal cells (bMSCs) was studied for the repair of 5 mm diameter rat mandibular defects. Three treatment groups and one control group were assessed: (1) apatite-coated silk, AdBMP-2-transduced bMSCs (AdBMP-2-transduced bMSCs/mSS, n=6); (2) apatite-coated silk, AdLacZ-transduced bMSCs (AdLacZ-transduced bMSCs/mSS, n=6); (3) apatite-coated silk and bMSCs (bMSCs /mSS, n=6); and (4) apatite-coated silk alone (mSS, n=6). A series of ELISA, real-time RT-PCR, radiographic, micro-computed tomographic, histological and immunohistochemical analyses were employed to determine if the apatite coated silk scaffold could be successfully used to host the BMP-2 gene modified bMSCs to achieve enhanced repairs in these critical sized mandibular defects.

2. Materials and methods

2.1 Culture of rat bMSCs

12-week-old male Fisher 344 rats with a weight of $250 \text{ g} \pm 15 \text{ g}$ were obtained from the Ninth People's Hospital Animal Center (Shanghai, China). All procedures concerning animal use were approved by the Animal Research Committee of the Ninth People's Hospital which is affiliated with Shanghai JiaoTong University Medical School (Shanghai, China). Rat bMSCs were isolated and cultured according to the protocol reported by Maniopoulos et al [26]. 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 of heparin (Sigma, St. Louis, MO, USA). Cells were cultured in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 units/ml streptomycin, supplemented with 50 $\mu\text{g/ml}$ ascorbic acid, 10 mM β -glycerolphosphate, and 10^{-8} M dexamethasone at 37°C in an atmosphere of 5% CO_2 . The medium was changed after 24 h to remove non-adherent cells and was then 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 were moved to dishes (10 cm in diameter) at 1.0×10^5 cell/ml in 10 ml. The cells used for this study required 2-3 passages each, with about 2 doublings per subculture stage.

2.2 Gene transduction of bMSCs

bMSCs were transduced with an adenovirus overexpressing BMP-2 (AdBMP-2) or LacZ (AdLacZ) at an overall multiplicity of infection (MOI, pfu/cell) of 80 pfu/cell. Cell morphology was evaluated under microscope (Leica DM 1RB, Germany), and gene transfer efficiency was determined by X-gal staining 3 days after transduction with AdLacZ by calculating the number of blue staining cells among all cells observed. The BMP-2 secreted into the culture medium was determined using a commercial BMP-2 ELISA kit (R&D Systems Inc, Minneapolis, MN, USA) according to the manufacturer's instructions at day 3, 6 and 9. Briefly, the culture medium was replaced with non-serum medium 24 h before assay, and then the supernatant was collected for evaluation.

2.3 Alkaline phosphatase staining

bMSCs transduced with AdBMP-2, AdLacZ or left untransduced were evaluated for alkaline phosphatase activity 14 days after transduction. Cells were fixed for 10 minutes at 4°C and incubated with a mixture of naphthol AS-MX phosphate and fast blue BB salt (ALP kit, Hongqiao, Shanghai, China) [27]. Areas stained purple were designated as positive.

2.4 von Kossa assay

bMSCs plated in triplicate for different groups in 6-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 minutes. Cells were then treated with 5% $\text{Na}_2\text{S}_2\text{O}_3$ for 2 minutes, and washed with distilled water [28]. Calcium nodules with a diameter greater than 1 mm were counted and analyzed.

2.5 RNA isolation and Real-time quantitative RT-PCR (RT-qPCR) analysis

Total cellular RNA extraction was performed on day 3, 6 and 9 after gene transduction using TRIzol Plus RNA purification kit (Invitrogen, USA) according to the manufacturer's instructions. The quality and quantity of the RNA obtained was analyzed on a Rneasy Mini kit (Qiagen, Germany). Reverse transcription was carried out using 1 μg of total RNA in a final volume of 20 μl using a PrimeScriptTM RT reagent kit (Takara Bio, Shiga, Japan) according to manufacturer's recommendations. Highly purified gene-specific primers for collagen 1 (coll1),

runx2 (Runx2), osteocalcin (OCN), osteopontin (OPN), and the calibrator reference gene, GAPDH, were synthesized commercially (Shengong, Co. Ltd. Shanghai, China), and the specific primers sets are outlined in Table 1. All RT-qPCRs of bone marker genes were performed with a ABI Prism 7300 real-time PCR system. For quantitative PCR, 10 µl SYBR Premix Ex Taq™, 0.4 µl of each forward and reverse primer, 0.4 µl of ROX Reference Dye and 0.4 µl cDNA template were used in a final reaction volume of 20 µl. Cycling conditions included an initial denaturation step of 10 s at 95°C followed by 40 cycles of 5 s at 95°C, 31 s at 58°C, and 30 s at 72°C. Data collection was enabled at 72°C in each cycle. CT (threshold cycle) values were calculated using the Applied Biosystems software. Analysis was based on calculating the relative expression level of the bone marker genes compared to the expression of untransduced controls on day 3, 6 and 9 (n=4), all values normalized to GAPDH.

2.6 Scaffold Preparation and cell seeding

The apatite-coated silk fibroin scaffolds were prepared by our previously published procedures [21]. Briefly, cocoons of *Bombyx mori* were boiled in an aqueous solution of Na₂CO₃, and then rinsed thoroughly with distilled water to extract the glue-like sericin proteins. The extracted silk fibroin was dissolved in LiBr solution then dialyzed in distilled water using a Slide-a-Lyzer dialysis cassette (MWCO 3,500, Pierce). The final concentration of silk fibroin aqueous solution was ca. 8 w/v%, which was determined by weighing the remaining solid after drying. After that polyaspartic acid solution (20 wt%) was added to this silk fibroin solution with mild stirring. Aqueous-derived silk fibroin scaffolds were prepared by adding granular NaCl (particle size; 850-1000 µm) into silk fibroin-polyaspartic acid solutions in disk-shaped containers. After 24 h, the containers were immersed in water and the NaCl extracted. The silk fibroin-polyaspartic acid scaffolds were then soaked in CaCl₂ and Na₂HPO₄ solution to grow apatite on silk fibers. After the soaking cycles, mineralized scaffolds were freeze-dried.

For cell seeding, 72 hours after gene transduction, bMSCs were released from the culture substratum using trypsin/EDTA (0.25% w/v trypsin, 0.02% EDTA) and concentrated to 2×10⁷ cells/ml in serum-free medium. Then bMSCs were seeded onto apatite-coated silk scaffold by pipetting the bMSCs suspension onto the materials. The bMSCs/mSS construct incubated for an additional 4 h to allow cell attachment *in vitro* before implantation. In a parallel experiment, 3 mm × 3 mm × 3 mm cuboids were prepared and seeded with bMSCs at the same cell density. The extent of cell attachment and growth was assessed 4 h and 7 days after cell seeding. The constructs were fixed in 2% Glutaric dialdehyde for 2 h, cut into two halves, and then characterized by scanning electron microscopy (Philips SEM XL-30, Amsterdam, Netherlands).

2.7 Surgical procedure

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. A non-healing full thickness defects of 5 mm diameter in the ascending ramus of the mandibles was then created with a bur that was cooled continuously by 0.9% saline solution irrigation. A total of 24 mandibular defects were randomly divided into four groups that received the following implants: (1) mSS with bMSCs transduced AdBMP-2 (n=6); (2) mSS with bMSCs transduced AdLacZ (n=6); (3) mSS with bMSCs (n=6); (4) mSS alone (n=6) (Fig. 1). The wound was closed in layers using 4-0 resorbable sutures.

2.8 Radiographic analyses and Micro-CT measurement

After 8 weeks post-operation, all the rats in each group were sacrificed by an intraperitoneal overdose injection of pentobarbital. Then the mandibles were explanted and fixed in 4% phosphate-buffered formalin solution. X-ray images of mandibles were made with a dental X-ray machine (Trophy, France), from a distance of 7 cm (230 V, 8 mA) with an exposure time

of 0.10 second. The morphology of the reconstructed mandibles was assessed using a micro-CT system (μ CT-80, Scanco Medical, Bassersdorf, Switzerland). The CT settings were used as follows: pixel matrix, 1024*1024; voxel size, 36 μ m; slice thickness, 36 μ m. After scanning, the micro-CT images were segmented using a nominal threshold value of 225 as reported previously [29], and a three-dimensional (3D) histomorphometric analysis was performed automatically. The parameters of bone volume fraction (Bone volume/total volume, BV/TV) and bone mineral densities (BMD) were used for comparison in this study.

2.9 Histology and immunohistochemistry

All the tissues from the original defect area of each group were fixed for 3 days, and decalcified in 10% EDTA for 2 weeks. Samples were embedded in paraffin and serial buccal and lingual sagittal cross sections were made. Three randomly selected cross sections from each implant were stained with haematoxylin and eosin. Then the area of new bone formation and remnant scaffolds were calculated using Image Pro 5.0 system (Media Cybernetics, USA). Immunohistochemical studies were performed using primary antibodies against β -galactosidase (1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), BMP-2 (1:100 dilution) (R&D Systems Inc, Minneapolis, MN, USA). Briefly, the tissue slides were deparaffinized and rehydrated, then submerged in hydrogen peroxide for peroxidase quenching. Before using the primary antibodies, the slides were incubated with 5% bovine serum albumin (BSA) to block non-specific binding. After 1 h incubation, the biotinylated secondary antibody (Boster Co., Ltd, China) was applied to the slides for 30 minutes at room temperature. Then streptavidin biotin complex (Boster Co.Ltd, China) was incubated for 20 minutes. Staining was performed by DAB substrate (Boster Co.Ltd, China) and the slides were counterstained with hematoxylin and mounted. Slides were assessed using a light microscope (Carl Zeiss, Inc. Germany).

2.10 Statistical analysis

Statistically significant differences ($p < 0.05$) between the various groups were measured using ANOVA and SNK post hoc. All statistical analysis was carried out using an SAS 6.12 statistical software package (SAS, Cary, NC, USA). All the data are expressed as mean $\pm$ standard deviation.

3. Results

3.1 Gene transduction and BMP-2 expression

In order to establish the optimal multiplicity of infection (MOI) for high adenoviral gene transfer efficiency, a set of preliminary experiments was performed using various doses of adenovirus. A MOI of 80 plaque forming units (pfu)/cell produced optimal effects in transfer efficiency without excessive cell death *in vitro*. Three days after transduction with 80 pfu/cell AdLacZ, X-gal staining showed around 70% bMSCs were stained blue (Fig. 2a). Cellular morphology was shown (as compared with untransduced control cells) after transduction with AdLacZ or AdBMP-2 (Fig. 2b-d). The amount of secreted BMP-2 into conditioned culture medium was determined using ELISA. bMSCs transduced with AdBMP-2 produced higher levels of BMP-2 during the entire culture period as compared with AdLacZ and untransduced bMSCs. The maximum concentration of BMP-2 in the culture media was detected after 6 days incubation and then followed a moderate decline (Fig. 2e).

3.2 Osteogenic differentiation of bMSCs

Two weeks after gene transduction, alkaline phosphatase staining was greater in bMSCs transduced with AdBMP-2 than in those with AdLacZ and untransduced bMSCs (Fig. 3a). In addition, von Kossa staining 2 weeks after gene transduction revealed a significant increase in

calcium nodules in AdBMP-2-transduced bMSCs when compared to the AdLacZ and untransduced groups (Fig. 3b, c).

3.3 RT-qPCR analysis of osteogenic markers

In order to compare differential gene expression between bMSCs transduced AdBMP-2, bMSCs transduced AdLacZ and untransduced bMSCs, real-time quantitative RT-PCR (RT-qPCR) analysis of osteogenic differentiation markers was carried out at defined time points (3, 6 or 9 days). The expression level of key osteogenic gene transcripts showed a dramatic difference in response to AdBMP-2 transduction. In bMSCs transduced with AdBMP-2, transcript level for collagen type I, an early marker of osteogenic differentiation associated with the formation of the extracellular matrix, showed an initial upregulation at day 6 which was slightly enhanced after day 9 (Fig. 4a). Runx2 showed moderate upregulation over day 9 (Fig. 4b). Osteopontin, another non-specific bone marker, showed sustained marked upregulation at day 6, persistently ascending to more than a 120 fold increase at day 9 (Fig. 4c). Osteocalcin, a late stage osteogenic marker showed a slight rise which was followed by a steep increase between days 6 and day 9 (Fig. 4d). In comparison, the transcripts of osteogenic markers in AdLacZ transduced bMSCs remained at basal levels over day 9. Taken together, these data support that the osteoinductive effect for AdBMP-2 transduction induced osteogenic differentiation in the rat bMSCs.

3.4 Growth of osteogenically induced bMSCs on scaffold

Cellular attachment and interaction within 3D scaffolds were evaluated by electron microscopy. Four hours after the bMSCs were combined with the scaffolds, cells could be seen attaching to the inner surface of the scaffold *in vitro* (Fig. 5a). For 7 days cultured in osteogenic media, bMSCs were suspended between the backbones of scaffolds and grew along the pores of the scaffolds. The cells grew tightly to each other and reached confluence with abundant fibril networks of extracellular matrix deposited on the scaffolds (Fig. 5b). Nominal differences in cellular adhesion and proliferation were observed between bMSCs transduced with AdBMP-2, bMSCs transduced with AdLac-Z and untransduced bMSCs. Additionally, these results suggested that the biomaterial was suitable for the proposed *in vivo* studies as it facilitated bMSCs initial attachment onto the surface, spreading and subsequent growth.

3.5 Radiographic analysis and Micro-CT measurement

To evaluate new bone formation and the development of bone unions within the defects, X-ray images were taken at 8 weeks after explantation of the mandible. Representative photographs of each group are shown in Fig. 6. A larger defined radio-opaque mass representing new bone formation and mineralization was observed in the AdBMP-2-transduced bMSCs group (Fig. 6a) when compared to the AdLacZ-transduced and untransduced groups and the radiopacity was close to that of the proximal normal mandible. New bone formation was also observed in AdLacZ-transduced (Fig. 6b) and bMSCs untransduced (Fig. 6c) groups, but to a lesser extent than the AdBMP-2-transduced bMSCs group. Defects filled with scaffolds alone failed to show any appreciable new bone formation (Fig. 6d).

The morphology of the newly formed bone was reconstructed using μ CT. Substantial formation of plate-like bone structures was visible in the centre of the defect site treated with the AdBMP-2-transduced bMSCs implants 8 weeks post-operation. The plate-like bone structures expanded and almost filled the mandibular defects (Fig. 7a-c). The implantation of the AdLacZ-transduced bMSCs (Fig. 7d-f) and untransduced bMSCs-seeded scaffolds (Fig. 7g-i) showed the formation of trabecular bone more scattered in the defect sites when compared to the AdBMP-2-transduced bMSC group, and less new bone formation was observed. No obvious

bone formation was visible for defects treated with scaffolds alone, except for limited formation mainly restricted to the defect margins 8 weeks post-operation (Fig. 7j-l).

The quantity of the newly formed bone in the defect sites was calculated by morphometrical analysis. Significantly more bone volume was observed in the AdBMP-2-transduced bMSCs group. The ratio of bone volume to total volume (BV/TV), as an indicator of the relative amount of newly formed bone, was significantly higher for the AdBMP-2-transduced bMSCs group when compared to all other groups (Fig. 8a). The local bone mineral densities (BMD) for AdBMP-2-transduced bMSCs group was also higher than the other three groups (Fig. 8b).

3.6 Histological analysis of bone regeneration

Histological evidence further supported the radiographic findings. Substantial bone formation in the AdBMP-2-transduced bMSCs implants, less bone formation in AdLacZ-transduced bMSCs or untransduced bMSCs-seeded scaffolds and no obvious bone formation was found in scaffold alone defects. Samples with AdBMP-2-transduced bMSCs group displayed a large amount of organized and mineralized bone tissue with typical lamellar bone morphology similar to native bone, as well as mature bone marrow coexisting in the repaired area. The scaffolds retained the pore structural integrity with substantial new bone ingrowth and little fibrillar configuration (Fig. 9a-c). In the AdLacZ-transduced (Fig. 9d-f) and bMSCs untransduced (Fig. 9g-i) groups, histologic analysis showed a small amount of irregularly arranged woven bone tissue with large bone lacuna interspersed among the defect sites. Defects filled with scaffolds alone were found with a large quantity of fibrous connective tissue in the area of defects with collapse of adjacent musculature infiltration. Remnant silk scaffolds hardly held their pore structure and separated into small pieces or fragments with multinucleated giant cells surrounding these pieces (Fig. 9j-l). The percentage of new bone area after 8 weeks was $57.79\% \pm 7.96\%$ in the AdBMP-2-transduced bMSCs group, $34.34\% \pm 6.67\%$ in the AdLacZ-transduced bMSCs group, $31.39\% \pm 8.29\%$ in the untransduced bMSCs group and $3.36\% \pm 0.95\%$ in the scaffold alone group, respectively. The percentage of new bone area in the AdBMP-2 group was significantly higher than those of AdLacZ-transduced, untransduced group and scaffold alone group 8 weeks post-operation (Fig. 10a). The percentage of remnant scaffold area was $12.52\% \pm 2.11\%$ in AdBMP-2-transduced bMSCs group, $11.06\% \pm 2.41\%$ in AdLacZ-transduced bMSCs group, $11.01\% \pm 1.71\%$ in untransduced bMSCs group and $6.63\% \pm 1.13\%$ in scaffold alone group respectively (Fig. 10b).

The origin of the implanted bMSCs present within the scaffold and/or new bone was determined by identification of the β -galactosidase encoded by the LacZ gene. β -galactosidase was apparent in the new bone matrix in the AdLacZ-transduced bMSCs group (Fig. 11b) 8 weeks post-operation, while negative staining was found in the other three groups. Immunohistochemistry displayed intensive BMP-2 staining in both the bone matrix and surrounding fibroblastic-like tissue for samples treated with the AdBMP-2-transduced bMSCs (Fig. 11e), whereas in AdLacZ-transduced bMSCs (Fig. 11f) and untransduced bMSCs (Fig. 11g) groups endogenous BMP-2 staining was present, but much weaker. No obvious positive staining was detected in the scaffold alone group (Fig. 11h).

4. Discussion

Osteoprogenitor cells, osteoconductive scaffolds and osteoinductive factors are the three main elements for forming tissue-engineered bone. Highly porous scaffolds which performed the role of a temporary matrix for anchorage dependent cells is an important factor in the success of tissue engineering. The biodegradability, distinguishing mechanical properties, and low inflammatory response of silk fibroin [30-32] make it one of the promising scaffolds for osteogenic applications. Calcium-phosphate (Ca-P) coatings which comprise a range of minerals found naturally in bone have been shown to reduce the fibrous encapsulation layer,

enhance direct bone contact and stimulate differentiation of bone marrow stromal cells along the osteogenic lineage [33-35]. Premineralized silk scaffolds, fabricated through a biomimetic synthetic approach by soaking silk fibroin in CaCl_2 and Na_2HPO_4 solution, promote osteogenic differentiation of bMSCs and more new matrix formation *in vitro* [21]. Combining the osteoconductive properties of bioceramics with the remarkable mechanical features of silk, this composite scaffold was hypothesized to be a useful carrier for osteogenic cells and growth factors that are relevant to bone regeneration.

To maximize the capacity of bMSCs to regenerate bone, exogenous BMPs are generally used to promote differentiation into osteoblasts. BMPs have been reported to be effective in enhancing bone formation in a variety of animal studies [24]. Among the BMPs, BMP-2 has the strongest and most significant biologic activities [36]. However, disadvantages of these exogenous proteins have limited the applications of BMPs. Gene therapy provides a possible alternative strategy to express BMPs protein in target cells for either short or long time periods to maximally stimulate osteogenesis but in localized regions of repair [37]. In our study, we chose the replication-deficient adenovirus vector and an *ex vivo* method for BMP-2 gene transfer because it is highly efficient at infecting both dividing and nondividing cells [38]. Previous studies showed that bMSCs are less efficient for adenoviral *ex vivo* gene transfer than other cell types [39], but in this experiment, rat bMSCs were successfully transduced at a relatively high efficiency of approximately 70% for AdLacZ and AdBMP-2 at a MOI of 80 pfu/cell. Meanwhile, bMSCs transduced with AdBMP-2 expressed and secreted a high-level BMP-2 protein 6 days after gene transduction *in vitro* followed by a moderate decline at day 9.

Alkaline phosphatase (ALPase) staining, a marker of early osteogenic differentiation and commitment of bMSCs toward the osteoblastic phenotype, as well as mineralized nodules, were determined after 2 weeks of cell culture. AdBMP-2-transduced bMSCs showed strong expression of ALPase and a significant increase in mineralized nodules when compared with AdLacZ and untransduced cells. These findings indicated that the rat bMSCs transduced with AdBMP-2 had been directed toward and specifically enhanced the osteogenic differentiation, which were further confirmed by RT-qPCR analysis of osteogenic markers. The expression pattern of key osteogenic gene transcripts showed increases after AdBMP-2 transduction. AdBMP-2-transduced bMSCs present a defined sequence of gene expression during the osteogenic differentiation and maturation of osteoblasts. Type I collagen, usually expressed at the beginning of osteoid matrix deposition, and Runx2, an attractive candidate of target genes for BMP signaling [39], were up-regulated. OPN, associated with the maturation stage of osteoblasts during attachment and matrix synthesis before mineralization and largely considered an intermediate or relatively earlier marker of osteogenic differentiation [40,41] showed a gradual upregulation with a marked increase at day 6, which was augmented through day 9. OCN which was reported to be the most specific and late marker of osteogenic differentiation [42] corresponding with matrix deposition and mineralization showed a slight upregulation at day 6 followed by a dramatic increase at day 9. In contrast, the rat bMSCs transduced with AdLacZ maintained a lower capacity to differentiate into osteoblasts within the 9-day time frame, even when cultured in the same osteoblastic differentiation media.

The potential for bMSCs to regenerate bone *in vivo* is highly correlated with the carrier [43]. Premineralized silk scaffolds have pores with a suitable diameter for cell seeding and growth. Cells were found attached along the material surface, secreting extracellular matrix after being cultured *in vitro*. These results suggest that apatite-coated silk scaffolds are non-cytotoxic and can facilitate bMSC adhesion. *In vivo* effects of AdBMP-2 transduced bMSCs-seeded scaffolds, AdLacZ transduced bMSCs-seeded scaffolds, bMSCs-seeded scaffolds and scaffolds alone were evaluated in a mandibular defect model in rats. The 4 mm defect in the rat mandibular ramus is a critical size nonhealing defect [44]. In our study, we used a diameter

of 5 mm in the mandibular defects model to investigate bone repair by implantation of the premineralized silk scaffolds seeded with BMP-2 modified bMSCs. Less bone formation was found for defects filled with AdLacZ transduced bMSCs-seeded scaffolds and untransduced bMSCs-seeded scaffolds, although they were more advanced when compared to the implantation of the scaffolds alone. The formation of a small amount of ossification with AdLacZ-transduced and untransduced groups is believed to be at least partially caused by the existence of dexamethasone in the current culture system [45,46] and invading reparative cells from the surrounding host tissue at the defect sites [47]. Ascorbic acid, beta-glycerophosphate, and dexamethasone added in culture media can stimulate bMSCs to differentiate to preosteoblasts, however, these *in vitro* manipulations with osteogenic media alone are not likely to be sustained after *in vivo* implantation [39]. Meanwhile, reparative cells might creep toward the implantation from the implant-host interfaces, but the result with the defects filled by scaffolds alone demonstrated low bone volumes per trabecular volume (BV/TV) which indicated the intrinsic bone repair process may not be powerful enough in the case of these critical size defects. Although untransduced and AdLacZ-transduced groups did reveal a minimal capability of new bone formation at the defect sites, the rat bMSCs were significantly stimulated to form new bone once they were transduced with AdBMP-2. The implantation of AdBMP-2 transduced bMSCs-seeded scaffolds into the defects resulted in well integrated with host bone, significantly higher BV/TV, and a substantial increase in local bone mineral density when compared to the other groups. Besides the quantitative analysis, the quality of newly formed bone is also an important factor to evaluate the effect of AdBMP-2 transduced bMSCs-seeded scaffolds on the healing of critical bone defects. In the AdLacZ-transduced and untransduced groups the new bone was composed of irregular trabeculae with large bone lacuna. However, in the AdBMP-2 transduced group the histologic analysis showed mature lamellar bone with Haversian systems and the presence of bone marrow therein. These promising results demonstrated apatite coated silk could successfully serve as three dimensional scaffolds for AdBMP-2 gene modified bMSCs and in turn promote new bone formation and maturation in rat mandibular defects.

As for the origin and role of the implanted bMSCs, the LacZ reporter gene expression in AdLacZ transduced group and stronger BMP-2-positive staining in the AdBMP-2 transduced group suggested that those donor cells had participated in the new bone formation. Since the expression of the target gene decreased over time with an adenovirus vector, the remaining implanted cells inside the defects were expected to be present more than those with positive β -galactosidase staining.

With regard to the degradation of silk fibroin, previous reports have shown that the degradation rate depends on many factors such as implantation site, processing conditions, pore size and silk fibroin concentration [14,48]. In our experiment, based on the histologic analysis, we observed multinucleated giant cells visible adjacent to the scaffolds and inflammation was required for silk degradation, due to the proteolytic requirements. Another interesting finding is that small pieces of separated silk scaffolds fragmented during the process were surrounded by the amount of multinucleated giant cells in the scaffold alone group. However, scaffolds seeded with cells still held their pore structural integrity. The percentage of remnant silk area significantly decreased in the scaffold alone group when compared to the cell-loaded groups. Similar observations using other biomaterials were found in other animal experiments [49, 50]. This phenomenon might be related to early bone formation and mineralization, which then slowed the cell-mediated resorption of the scaffold.

5. Conclusions

In summary, premineralized silk scaffolds alone did not result in the repair of mandibular bony defects in rats, however, they served as suitable scaffolds for bMSCs to increase new bone

formation, and the combination of these premineralized silk scaffolds with AdBMP-2 gene modified bMSCs further enhanced new bone formation and maturation in mandibular bone repair. BMP-2 gene therapy and tissue engineering techniques could be used in mandible repairs and bone regeneration.

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Fig. 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 premineralized silk scaffold seeded with gene modified bMSCs.

Fig. 2. Gene transduction and BMP-2 expression. (a) A multiplicity of infection of 80 pfu/cell achieved high transfer efficiency around 70% 3 days after AdLacZ transduction of rat bMSCs. Positive areas with X-gal staining are in blue. (b-d) Cellular morphology after transduction with AdLacZ, AdBMP-2, or untransduced control cells (a-d, 200×). (e) The amount of secreted BMP-2 into conditioned culture medium was determined using ELISA. bMSCs transduced with AdBMP-2 produced higher level BMP-2 during the entire culture period as compared with AdLacZ and untransduced bMSCs. The maximum concentration of BMP-2 in the culture media was detected after 6 days incubation and then followed by a moderate decline, * $p<0.05$.

Fig. 3.

In vitro analysis of osteogenic differentiation after bMSCs transduction with AdBMP-2, AdLacZ, or untransduced control cells. (a) Alkaline phosphatase expression 14 days after gene transduction (100×). (b) von Kossa assay comparing calcium nodules between AdBMP-2, and AdLacZ-transduced cells and untransduced control cells after 14 days culture (50×). (c) Quantitative analysis of calcium nodules present in the three groups. A significant increase is seen in the AdBMP-2 group when compared with the AdLacZ and untransduced control groups, * $p < 0.05$.

Fig. 4.

Real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) analysis of gene expression of osteogenic markers (a) collagen 1 (Col1), (b) runx2 (Runx2), (c) osteopontin (OPN) and (d) osteocalcin (OCN) in AdBMP-2, and AdLacZ-transduced bMSC relative to the expression of untransduced bMSC quantified before seeding on scaffolds, all values normalized to GAPDH.

Fig. 5.

Scanning electron microscopic evaluation of the premineralized silk scaffold microstructure and cells state. (a) 4 hours after the bMSCs were seeded onto the scaffold, cells could be seen attaching to the inner surface of the scaffold. Scale bar = 50 μm. (b) 7 days after seeding, the cells grew tightly to each other and reached confluence with abundant fibril networks of extracellular matrix deposited on the scaffolds. Scale bar = 200 μm.

Fig. 6.

Radiographic evaluation of the repaired mandible. Representative photographs showed large defined radiopacities at the defect sites in (a) AdBMP-2-transduced bMSCs group, an obviously lesser extent in (b) AdLacZ-transduced bMSCs group and (c) untransduced bMSCs group, and little calluses observed restrict to the defect margin in (d) scaffold alone group.

Fig. 7. Microcomputer tomography (μ CT) images of mandibular bony defects taken 8 weeks after implantation. Defects were treated with (a-c) AdBMP-2 transduced bMSCs/mSS constructs, (d-f) AdLacZ transduced bMSCs/mSS constructs, (g-i) untransduced bMSCs/mSS constructs, and (j-l) scaffold alone. Scale bar = 1 mm.

Fig. 8.

(a) Morphometric analysis of new bone formation and (b) local bone mineral density (BMD) analysis by μ CT of four groups at 8 weeks post-operation. Implantation of AdBMP-2 transduced bMSCs/mSS constructs achieved the most amount of new bone formation and the highest local bone mineral densities (BMD) than other three groups, **p<0.01, *p<0.05.

Fig. 9.

The whole and local photomicrograph of the histologic images of the implants represented the differences among four groups. The whole images of representative slices in (a) AdBMP-2 group, (d) AdLacZ group, (g) untransduced group and (j) scaffold alone group (a, d, g, j, 12.5 $\times$). The center photomicrograph of the defect sites in each group (b, e, h, k, 200 $\times$). The edge photomicrograph of the defect sites in each group (c, f, i, l, 200 $\times$). BL: blood clot; BM: bone marrow; FT: fibrous tissue; MC: muscle; NB: new bone; SC: scaffold. Black arrow shows the multinucleated giant cells. White arrow shows the ingrowth of osteoblasts. Arrow head represents blood vessels.

Fig. 10.

(a) Histomorphometrical analysis of the bone formation and (b) remnant scaffold for four groups at 8 weeks post-operation. The percentage of remnant scaffold dramatically decreased in the scaffold alone group, ** $p<0.01$, * $p<0.05$.

Fig. 11.

Immunohistochemical analysis of new bone formation in each group at 8 weeks post-operation. Immunostaining for β -galactosidase of (a) AdBMP-2 group, (b) AdLacZ group, (c) untransduced group and (d) scaffold alone group. Only AdLacZ group shows positive brown staining in bone matrix (arrow). BMP-2 staining demonstrates a strong BMP-2 expression in both the bone and surrounding fibroblastic-like tissue (arrows) in (e) AdBMP-2 group. In (f) AdLacZ and (g) untransduced groups weak endogenous BMP-2 staining were present. No obvious positive staining in (h) scaffold alone group (a-h, 400 $\times$).

Table 1

Nucleotide sequences for realtime RT-PCR primers

Genes	Primer sequence (forward/reverse)	Product size (bp)	Annealing temperature(°C)	Accession number
Col1	5'-TCCTGCCGATGTCGCTATC-3' 5'-CAAGTTCCGGTGTGACTCGTG-3'	234	58	XM_213440
Runx2	5'-GCTTCTCCAACCCACGAATG-3' 5'-GAACTGATAGGACGCTGACGA-3'	212	58	XM_346016
OCN	5'-AAAGCCCAGCGACTCT-3' 5'-CTAAACGGTGGTGCCATAGAT-3'	232	58	NM_013414
OPN	5'-GACGGCCGAGGTGATAGCTT-3' 5'-CATGGCTGGTCTTCCCGTTGC-3	208	58	NM_012881