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An ectopic study of apatite-coated silk fibroin scaffolds seeded with AdBMP-2 modified canine bMSCs

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Abstract

The present study was undertaken to evaluate ectopic new bone formation effects of apatite-coated silk fibroin scaffolds (mSS) seeded with adenovirus mediated bone morphogenetic protein-2 gene (AdBMP-2) transduced canine bone marrow stromal cells (bMSCs) in nude mice. In this study, bMSCs derived from canine were cultured and transduced with AdBMP-2, adenovirus mediated enhanced green fluorescent protein gene (AdEGFP) in vitro. Osteogenic differentiation of bMSCs was determined by alkaline phosphatase (ALP) activity analysis, and the transcript levels for BMP-2, osteopontin (OPN), osteocalcin (OCN) and bone sialoprotein (BSP) genes via real-time quantitative PCR (RT-qPCR) analysis. The ectopic bone formation effects of mSS seeded with AdBMP-2 modified bMSCs were evaluated through histological and histomorphological analysis 4, 8 and 12 weeks post-operation in nude mice. ALP activity was statistically increased in AdBMP-2 group, when compared with control groups. The mRNA expression of BMP-2, OPN, OCN and BSP also statistically up-regulated 6, 9 days after AdBMP-2 transduction. Significantly higher bone volume was achieved in AdBMP-2-transduced bMSCs/mSS constructs than that of AdEGFP-transduced bMSCs/mSS or bMSCs/mSS groups at 4, 8 and 12 weeks ($P < 0.01$). These results demonstrated that mSS seeded with AdBMP-2-transduced canine bMSCs can promote ectopic new bone formation and maturation in nude mice suggesting the potential of this silk scaffold based tissue engineered bone for further bone regeneration studies in canine models.

Keywords

bone marrow stromal cells; bone morphogenetic protein; gene therapy; canine; silk fibroin

INDUCTION

Large bone defect as a result of trauma, cancer treatment, and congenital disorders is a considerable clinical problem in dentistry. As such defects do not heal spontaneously and usually lead to severe functional impairment, several therapeutic approaches including

autologous bone grafts [1], allogenic bone grafts, distraction osteogenesis [2], guided bone regeneration (GBR) [3] and implantation of biomaterials have been investigated. However, all these treatment modalities have their own limitations such as donor site morbidity, pathogen transfer, multi-operations, membrane displacement or exposure, and deficiency of osteoinduction. As a viable alternative approach, tissue engineering method by creating a biological microenvironment that could induce transplanted cells residing in a biomedical three-dimensional scaffold to produce a desired extracellular matrix and thus regenerate osseous tissues [4] has been introduced.

Scaffolds, as one of the three key components of tissue engineering essential, ideally, should be degradable and provide appropriate mechanical properties, as well as elicit little to no host immune response. Silk fibroin, with controllable degradation rates [5], impressive mechanical properties, lower inflammatory response [6], versatile options for sterilization [7] and satisfactory processability, had been adopted in bone tissue engineering [8–14] aside from using as sutures for centuries [15]. Apatite-coated silk scaffolds, which combined the osteoconductive properties of bioceramics with the mechanical resilience of polymers, could promote cellular attachment and bone nodule formation in vitro [16]. Our previous study [17] even reported that the canine inferior mandibular border defects (2 cm×1 cm) could be repaired 12 months after operation with mSS and osteogenically induced autologous bMSCs. However, the timely reconstruction of defect in alveolar ridge is essential for successful rehabilitation of oral function using dental implants or dentures. Thus, efforts should continuously be made to speed up the healing process.

BMP-2, one of the most widely studied osteoinductive growth factors, is a potent bone stimulator and plays key role in many steps during bone morphogenesis [18], which has been shown to induce osteogenic differentiation of mesenchymal cells [19] and promote bone formation both in vitro and in vivo. BMPs regional gene therapy, with the core technique of transfer osteoinductive genes into seed cells, may provide with an alternative to BMPs protein therapy. Gene enhanced tissue engineering could overcome limitations associated with the one-time delivery of a bolus of protein by providing a sustained, local delivery of protein factors. To promote bone regeneration, both viral and nonviral vectors have been adopted to genetically modify cells for BMPs delivery. Adenovirus vector is a widely used gene transfer system for the induction of bone regeneration because of its high transduction efficiency, low toxicity, and unbounded transferring genes to both replicating and nonreplicating cells [20–24]. Studies have reported that adenovirus mediated transfer of BMP-2 gene therapy could successful promote heterotopic and orthotopic bone formation [25–34].

The aim of the present study was to investigate the ectopic osteogenic efficacy of mSS seeded with AdBMP-2 transduced canine bMSCs in nude mice. To address this goal, for the first time, we seeded mSS with AdBMP-2 modified canine bMSCs and evaluated its effects on ectopic bone formation in a nude mice model, so as to accumulate information for further bone regeneration studies in canine models.

MATERIALS AND METHODS

Experimental materials and animals

The apatite-coated silk fibroin scaffolds were prepared according to our previously published procedures [16]. Briefly, cocoons of *Bombyx mori* were boiled in an aqueous solution of Na₂CO₃, and aqueous-derived silk fibroin scaffolds were prepared by adding granular NaCl (particle size: 850–1000 µm) into silk fibroin-polyaspartic acid solutions. The alternate soaking process was used to grow apatite on the silk fibroin-polyaspartic acid scaffolds. First, silk fibroin-polyaspartic acid scaffolds were soaked in 200 mM CaCl₂

solution (buffered with 50 mM Tris-HCl, pH 7.4) for 20 min at 37 °C and washed twice with distilled water. The silk fibroin-polyaspartic acid scaffolds were then transferred to 120 mM Na₂HPO₄ solution, soaked for 20 min at 37 °C and washed twice with distilled water. After a total 8 soaking cycles, the mineralized scaffolds were freeze-dried.

18- to 24-month-old beagle dogs with an average weight of 12.5 kg and 6-week-old male athymic nude mice with a weight of 20 ± 2 g were enrolled in the experiments. The experimental protocol was approved by the Animal Care and Experiment Committee of Shanghai Jiao Tong University, School of Medicine (Shanghai, China).

Culture of canine bMSCs

Cell preparation has been previously described [17]. Around 4 ml bone marrow were harvested by needle aspiration from the iliac crests of dog and transferred into a pre-heparinized centrifuge tube. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), containing 100 U/ml of penicillin, 100 U/ml of streptomycin and 2 mM L-glutamine (L-glutamine, Sigma, USA). The first medium change occurred after 5 days to allow cell attachment and three times a week thereafter until confluence (12–14 days). After the first passage, the following 3 supplements for inducing osteogenesis were added: 10^{-8} M dexamethasone (dexamethasone, Sigma, USA), 50 µg/ml L-2-ascorbic acid (L-2-ascorbic acid, Sigma, USA) and 10 mM β-glycerophosphate (β-glycerophosphate, Sigma, USA). The cells were then incubated continuously at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cells at passage 2–3 were used for following experiments.

Gene transduction of bMSCs

AdBMP-2 and AdEGFP were used for gene transduction under a multiplicity of infection (MOI) of around 80 pfu/cell. Gene transfer efficiency was assessed 72 h after AdEGFP gene transfer under fluorescent microscopy (Leica TCS SP2, Heidelberg, Germany) by calculating the percentage of EGFP-expressing cells among all the cells present in 10 randomly selected 400 × fields [35].

Western blot analysis

Cell extracts were prepared from untransduced and transduced bMSCs on days 3 after transduction, and then lysed using a protein extraction reagent (Kangchen Bio-tech, Shanghai, China). Proteins were fractionated by electrophoresis on 8% and 12% polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Amersham Biosciences, Piscataway, NJ). The membranes were exposed to anti-BMP-2 (1:200 dilution) and anti-β-actin antibodies (1:5,000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were exposed to secondary rabbit anti-goat for BMP-2 and goat anti-mouse for β-actin immunoglobulin G antiserum conjugated to horse radish peroxidase, and developed with ECL plus chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ).

Cell proliferation analysis

Cells proliferation of AdBMP-2-, AdEGFP- transduced bMSCs or untransduced bMSCs were determined by cell counting utilizing a Coulter Counter (BECKMAN COULTER, Zz Coulter Particle Count and Size Analyzer, USA). A parallel set of 20,000 cells/well were cultured in 24-well plates (in triplicate). Gene transfer was performed on the second day, and cell numbers were counted for the next 6 days.

ALP staining and ALP activity assay

bMSCs transduced with AdBMP-2 and AdEGFP gene, and left untransduced were evaluated by ALP staining 14 days after transduction according to 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 [36]. Areas that stained purple were designated as positive.

ALP activity was measured as previously [37]. Briefly, cells were harvested in 200 µl lysis buffer 4, 7, 14 days after transduction, incubated for 4 hours at 37 °C. A 100 µl sample was mixed with 100 µl p-nitrophenyl phosphate (pNPP, Sigma, Saint Louis, USA) (1mg/ml) in 1M diethanolamine buffer containing 0.5 mM MgCl₂, pH 9.8 and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 50 µl of 0.2 M NaOH. Total protein content was determined by Bio-Rad Protein Assay (Kit II, Bio-Rad, U.S.), read at 595 nm and calculated according to a series of bovine serum albumin (BSA) standards. ALP levels were normalized to the total protein content at the end of the experiment. Each sample was assessed in triplicate.

RNA isolation and RT-qPCR analysis

Total cellular RNA extraction was performed on days 3, 6 and 9 after gene transduction using TRIzol Plus RNA purification kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's recommendations. RNA was further purified by utilizing a TURBO DNA-free kit (Ambion, Austin, TX, USA) to remove contaminating DNA. RNA integrity was verified by OD260/OD280 nm absorption ratio >1.95. Reverse transcription reactions was conducted on 1 µg of total RNA using a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan) according to manufacturer's instructions. The primers for RT-qPCR were designed as follows: BMP-2 (forward, 5'-TGA ACA CAG CTG GTC TCA GG-3'; reverse, 5'-CTG GAC TTA AGA CGC TTC CG-3'), OPN (forward, 5'-TTG CAG TGA TTT GCT TTT GC-3'; reverse, 5'-CAT CGT CAT GGC TTT CAT TG-3'), OCN (forward, 5'-AGC TCA ACC CCA ACT GTG AC-3'; reverse, 5'-GAT GAC AAG GAC CCC ACA CT-3'), BSP (forward, 5'-CGA CGC TGA GAA CTC TAC CC-3'; reverse, 5'-GTT GCT GCT GGT GCT GTT TA-3'), and the calibrator reference gene, GAPDH (forward, 5'-CGG GCG TTG ATG ACA AGT TTC CCG-3'; reverse, 5'-CTA CCC ACG GCA AAT TCC AC-3'). Highly purified salt-free optimized gene-specific primers were synthesized commercially (Shengong Co.Ltd., Shanghai, China). All RT-qPCR of bone marker genes were performed with a Bio-Rad iQ5 real-time PCR system. Cycling conditions included an initial denaturation step of 3 min at 95°C followed by 40 cycles of 10 s at 95°C, 30 s at 60°C, 10 s at 72°C. 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 of AdBMP-2-transduced bMSCs compared to the expression of AdEGFP-transduced bMSCs on days 3, 6 and 9, all values normalized to GAPDH. Each sample was assessed in triplicate and each assay was repeated three times.

Construction of cell-scaffold complexes

The sterilized mSS (5 mm diameter, 4 mm height) was pre-wetted in the culture medium for 24 h prior to cell seeding at 37°C. bMSCs 3 days after gene transduction were used for in vitro and in vivo experiments. For cell seeding, AdBMP-2, AdEGFP transduced bMSCs or untransduced bMSCs were resuspended in the culture media without serum at a density of 5×10^7 cells/ml and seeded on the top of the scaffold to generate cell-scaffold constructs. In a parallel experiment, after 1 day of incubation, these in vitro grown constructs were fixed in 2% glutaricdialdehyde and then characterized by scanning electron microscopy (SEM) (Philips Quanta-200, FEI, Eindhoven, Netherlands).

Surgical procedure

9 athymic nude mice were anesthetized by intramuscular injection of pentobarbital after light ether inhalation. Four subcutaneous pockets were created on the back of each mouse by blunt dissection, which were then implanted with mSS seeded with AdBMP-2-transduced bMSCs, mSS seeded with AdEGFP-transduced bMSCs, mSS seeded with untransduced bMSCs and mSS alone. The wound was closed using 4-0 resorbable sutures. 3 mice were sacrificed at 4, 8 and 12 weeks postoperatively.

Histological and histomorphological analysis

The implants were retrieved for analysis at 4 weeks, 8 weeks and 12 weeks time points after implantation. Then all specimens were fixed in 10% formalin solution (pH 7.4) for 2 days, decalcified in 10% EDTA, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H-E). The H-E staining result was observed and recorded with an OLYMPUS BX51 microscope. The area of new bone formation and the area of residual scaffold within the whole implant sections were quantified manually from randomly selected slides with the software tool-Image Pro 5.0 system (Media Cybernetics, Silver Springs, MD, USA). The extent of new bone formation or residual scaffold was displayed as a percentage of new bone area or residual scaffold per total area observed for the whole implant sections. For each implant, the percentage of new bone formation or residual scaffold was calculated by the mean value of 3 sections selected from each of the 3 equally divided parts cut parallel to the cross section with complete random sampling method, which was further used to calculate the mean value for each group.

Statistical analysis

Statistically significant differences ($P < 0.05$) between the various groups were measured using t-test or ANOVA and Student-Newman-Keuls (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.

RESULTS

Cell culture, gene transduction and BMP-2 expression

Cell clones formed 5–7 days after initial seeding and reached confluence after approximately 12–14 days. Examination with a microscope showed that bMSCs at passage 2 displayed the typical fibroblastic spindle shaped phenotype. Compared with untransduced control cells (Figure 1a), cellular morphology was similar 3 days after transduction with AdBMP-2 (Figure 1b) or AdEGFP (Figure 1c). Strong green fluorescence was detected in cells with AdEGFP transduction and the gene transfer efficiency reached 80% (Figure 1d).

Western blot analysis was performed to confirm the over expression of BMP-2 protein in AdBMP-2-transduced bMSCs (Figure 1e). A growth curve demonstrated a significant increase in cell proliferation in AdBMP-2-transduced cells ($P < 0.01$), when compared with either AdEGFP-transduced cells or untransduced cells at 4, 5, 6 and 7 days (Figure 1f).

ALP staining and ALP activity assay

In this study, we sought to evaluate the osteoinductive effect of BMP-2 on canine bMSCs. ALP staining was used to detect ALP activity, which is accepted as a hallmark of osteoblast phenotype [38, 39]. 14 days after gene transfer, ALP staining was more pronounced in bMSCs transduced with AdBMP-2 than that in untransduced and AdEGFP transduced bMSCs (Figure 2a, 2b, 2c). As shown in Figure 2d, AdBMP-2-transduced bMSCs presented

the highest activity at any given time, with a significant statistical difference to control groups ($P < 0.01$).

RT-qPCR analysis of osteogenic markers

At 3, 6 and 9 days after gene transduction, RT-qPCR analysis was carried out to compare differential gene expression between AdBMP-2-transduced bMSCs and AdEGFP-transduced bMSCs. bMSCs transduced with AdBMP-2 significantly increased the level of BMP-2 transcripts at 3, 6 and 9 days, when compared with AdEGFP-transduced bMSCs (Figure 3a). Besides, RT-qPCR analysis of transcripts showed significant upregulation of osteoblastic genes OPN, OCN and BSP at 3, 6 and 9 days, except OPN at 3 days (Figure 3b, 3c, 3d).

Cellular adhesion and spreading on scaffold

The attachment and spreading of bMSCs seeded on mSS with gene transduction or not was examined over 24 h in culture by SEM. Cells adhered to scaffolds closely, and began to spread with stretched filaments (Figure 4a, 4b, 4c). Nominal differences in cellular adhesion and spreading were observed among bMSCs transduced with AdBMP-2, AdEGFP or untransduced. Additionally, these results suggested that mSS was suitable for the proposed *in vivo* studies as it facilitated bMSCs' initial attachment and spreading onto its surface.

Histological findings

All nude mice survived the duration of the study and no infections developed. Decalcified sections from all groups stained with hematoxylin and eosin, and gross analysis of the sections via light microscopy was performed. There was increasing newly formed trabecular bone over the study course in all groups except that there was no new bone formation in mSS alone group, filling with abundant fibrous connective tissue instead.

4 weeks after surgery, there was substantial bone formation in AdBMP-2-transduced bMSCs/mSS group (Figure 5b, 5f, 5j), with less bone formation found in untransduced bMSCs/mSS group (Figure 5a, 5e, 5i) or AdEGFP-transduced bMSCs/mSS (Figure 5c, 5j, 5k). In AdBMP-2-transduced group, large irregularly arranged woven bone tissue with bone lacuna, few fibrous connective tissues infiltration and blood vessels occurrence were observed in the scaffold pores at both centre and marginal area. While, in the untreated bMSCs and AdEGFP-transduced bMSCs groups, only a small amount of woven bone tissue and rich fibrous connective tissue were detected. Silk scaffold degradation was not apparent, as indicated by the relatively smooth lattice surfaces, but lots of multinucleated giant cells, which could be foreign-body giant cells or osteoclasts when an osteoinductive foreign-body was implanted *in vivo*, were present on the surface of mSS.

Bone formation increased in the three cells containing groups over time (Figure 6). 8 weeks after surgery, the appearance of bone marrow was observed in AdBMP-2-transduced bMSCs group around the periphery (Figure 6b, 6f, 6j), as well as the reduction of fibrous connective tissues. Meanwhile, more irregularly arranged woven bone tissue was found in the scaffold pores in untransduced bMSCs (Figure 6a, 6e, 6i) or AdEGFP-transduced bMSCs group (Figure 6c, 6j, 6k). Silk scaffolds largely held their structural integrity in scaffold alone group with evident multinucleated giant cells infiltration (Figure 6d, 6h, 6l).

12 weeks postoperatively, AdBMP-2-transduced bMSCs group exhibited the most advanced bone formation, with a large amount of mineralized bone tissue and bone marrow observed (both peripheral and central, Fig. 7b, 7f, 7j), while, cancellous bones with bone lacuna were apparent in other two bMSCs containing groups (Figure 7a, 7e, 7i, 7c, 7j, 7k). Remnant silk

scaffolds began to lose their original pore structure and separate into fragments with multinucleated giant cells surrounded, especially in scaffold alone group (Figure 7d, 7h, 7l).

Histomorphological analysis

AdBMP-2-transduced bMSCs groups resulted in the highest level of new bone formation among the groups at any time point. The percentage of new bone area after 4 weeks was $9.67 \pm 1.65\%$ in untransduced bMSCs group, $37.50 \pm 5.75\%$ in AdBMP-2-transduced bMSCs group and $11.16 \pm 1.50\%$ in AdEGFP-transduced bMSCs group (Figure 8a), significantly higher in AdBMP-2-transduced bMSCs group ($P < 0.01$). Furthermore, the percentage of new bone area in AdBMP-2-transduced bMSCs group was $55.74 \pm 5.90\%$ at 8 weeks, $58.79 \pm 6.95\%$ at 12 weeks, respectively, also significantly higher than those of untransduced group or AdEGFP-transduced group ($P < 0.01$). Due to the nature of lack osteoinduction for the scaffold alone, there was no bone formation in that group.

During the whole course of observation, the percent of residual scaffolds decreased over time. The percentage of remnant scaffold area after 4 weeks was $19.37 \pm 2.99\%$ in AdBMP-2-transduced bMSCs group, $17.58 \pm 4.65\%$ in AdEGFP-transduced bMSCs group, $16.15 \pm 2.76\%$ in untransduced bMSCs group and $13.55 \pm 1.10\%$ in scaffold alone group (Figure 8b). The remnant scaffold percent was $16.35 \pm 5.13\%$ in AdBMP-2-transduced bMSCs group 8 weeks postoperatively and $14.69 \pm 3.70\%$ 12 weeks postoperatively, respectively. AdBMP-2-transduced bMSCs groups showed a relative higher percentage value of remnant scaffold among groups at any time point.

DISCUSSION

In this subcutaneous immune-deficient mice ectopic study, we demonstrated that AdBMP-2 gene transfer promoted the osteogenic proliferation and differentiation of canine bMSCs, and the tissue-engineered bone with AdBMP-2 modified canine bMSCs and mSS achieved significantly increased de novo bone formation compared with that of AdEGFP-transduced bMSCs/mSS or untreated bMSCs/mSS. The results suggested the potential of this silk fibroin based tissue engineered bone for further large bone regeneration studies in preclinical canine models.

Several growth factors, including BMPs, transforming growth factor β (TGF- β), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and fibroblast growth factor (FGF), contribute to bone formation and regeneration. Known as one of the most effective osteoinductive growth factors, it has been well established that BMP-2 can induce bone formation both in vivo and in vitro. Recently, different gene therapy approaches for bone regeneration have been studied, suggesting the use of viral vectors such as adenovirus [23, 34], retrovirus [40], and lentivirus [41], or nonviral vectors such as electroporation [42], gene activated matrix [43], sonoporation [44], and liposome [27, 45] as carriers for BMP-2 gene. Researches have demonstrated that a short-term expression of BMP-2 is sufficient to induce bone formation [46]. In consequence, an extensive application of transient vectors like adenovirus was reported for BMP gene transduction in previous literatures.

At the current working titre, data showed the transfer efficiency reached above 80% demonstrated by EGFP expression in AdEGFP-transduced cells and no obvious cell death was observed. The western blotting analysis and RT-qPCR confirmed the over expression of BMP-2 protein and BMP-2 mRNA in AdBMP-2-transduced bMSCs than control groups. Furthermore, a growth curve showed a significant increase in cell proliferation in AdBMP-2-transduced cells ($P < 0.01$) compared with AdEGFP transduced cells or

untransduced cells at 4, 5, 6 and 7 days ($P < 0.01$). The fact that AdBMP-2 gene transfer promoted bMSCs proliferation was consistent with previous reports [25].

ALP, selected to evaluate the preliminary differentiation, showed strong expression in AdBMP-2-transduced bMSCs when compared with AdEGFP and untransduced cells. The following RT-qPCR analysis further confirmed the enhanced osteogenic differentiation of bMSCs with AdBMP-2 transduction. OPN, OCN and BSP, which associated with osteoblasts maturation and matrix mineralization, showed a significant upregulation over AdEGFP control cells. Overall, the osteoinductive effect for AdBMP-2 transduction in canine bMSCs was in line with previous reports on rat bMSCs [23]. Given that adenovirus gene transfer with canine bMSCs as target cells could be effective for BMP-2 gene therapy, we further explored its ectopic osteoinductive properties with tissue engineering approaches in nude mice.

In our study, AdBMP-2-transduced bMSCs with mSS were implanted into the nude mice and bone formation was induced in vivo. On account of nude mice are immunodeficient, the implanted tissue-engineered bone with canine bMSCs were able to survive while the mice acted as incubators. The results of histomorphometric analysis showed much higher new bone formation percentage achieved 4 weeks, 8 weeks and 12 weeks postoperatively in AdBMP-2 group. It can be inferred that the generated BMP-2 was biologically active and functioned as an osteogenetic growth factor for the use of tissue engineering. Besides the quantitative analysis, the quality of newly formed bone was also an important factor of investigating the effect on ectopic new bone formation. At 12 weeks postoperatively, the new bone in the AdEGFP-transduced and untransduced groups was composed of trabecular bone with large bone lacuna, while in the AdBMP-2 transduced group mature lamellar bone and the presence of bone marrow therein were shown. Furthermore, the in vitro data demonstrated that AdBMP-2-infected bMSCs differentiated into osteoblast phenotype, therefore, we could speculate that these cells had the potential to promote new bone formation in vivo. The BMP-2 protein secreted by gene-transferred cells would affect the endogenous progenitor cells as well, thus, both implanted and endogenous cells might have been involved in the enhanced new bone formation in this study.

The duration of transgene over expression by adenoviral gene transfer is normally limited to a relatively short period of time. In our previous study, the adenovirus transduced bMSCs expressed BMP-2 around 4 weeks after in vivo injection [47]. Thus this short-term transgene expression may be considered sufficiently and reflects a comparatively confined expression manner which is advantageous for bone regeneration. However, controlling transgene expression is an important issue in gene enhanced bone tissue engineering, thus, the exogenous regulations of BMP expression (eg, tetracycline or doxycycline-regulate systems) could be explored to control bone formation by BMP-expressing mesenchymal stem cells [48, 49].

Based on the literature, silk is degradable but over longer time periods due to proteolytic degradation usually mediated by a foreign body response [5, 50]. The rate of silk fibroin scaffold degradation depends upon the original preparation method, structural characteristics and host immune system elements during degradation [51]. In the current experiment, according to the histological analysis, we noticed that foreign body responses were occurred and multinucleated giant cells presented adjacent to the scaffolds during the course of scaffolds degradation. Besides, the percentage of remnant scaffold decreased over time with the histomorphological analysis, and the value of remnant silk in AdBMP-2 transduced group was higher than that in rest groups at any given time point. We speculated that there were two ways in which degradation of silk scaffold could proceed, via a solution-mediated dissolution and via a cell-mediated process. The composites of bMSCs/silk scaffolds

induced early bone formation in the scaffold pores, particularly in AdBMP-2-transduced bMSCs group, which might have covered the surface and limited exposure to solution leading to slowing down of degradation [52]. The authors previously also have found that the percentage of residual silk scaffolds in scaffold alone groups was lower than that in a cell-loaded group [17]. The trend was also similar in previous studies by using some other biomaterials [52, 53].

CONCLUSIONS

In summary, AdBMP-2 gene transduction could enhance the proliferation and osteogenic differentiation of canine bMSCs and mSS seeded with AdBMP-2-transduced bMSCs could promote early new bone formation and maturation in nude mice, which encouraged the further studies for timely reconstruction of bone defects on large animal canine models.

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Figure 1.

Gene transduction and the effects on canine bMSCs proliferation. The cellular morphology of (a) untreated control cells, (b) AdBMP-2-transduced cells and (c) AdEGFP-transduced cells. (d) A multiplicity of infection of 80 pfu/cell achieved high transfer efficiency about 80% 3 days after transduced by AdEGFP, bMSCs emitted bright and intense green fluorescence (100 ×). (e) Western blot probed with antibodies against BMP-2 and β-actin 3 days after gene transduction. (f) Effects of AdBMP-2, and AdEGFP infection on bMSCs proliferation. A growth curve represented cellular proliferation after transduction with AdBMP-2 and AdEGFP. A significant increase was seen in AdBMP-2 group, when compared with AdEGFP group or untransduced group, $**P < 0.01$.

Figure 2.

ALP staining and ALP activity assay. ALP staining in (a) untransduced bMSCs, (b) AdBMP-2-transduced bMSCs and (c) AdEGFP-transduced bMSCs 14 days after gene transduction in vitro ($16\times$). (d) ALP activity 4, 7 and 14 days after gene transduction in vitro. The ALP activity was significantly higher in AdBMP-2-transduced bMSCs compared to control groups (** $P < 0.01$).

Figure 3. RT-qPCR analysis of gene expression of osteogenic markers (a) BMP-2, (b) OPN, (c) OCN and (d) BSP in AdBMP-2-transduced bMSCs relative to the expression of AdEGFP-transduced bMSCs, all values were normalized to GAPDH (** $P < 0.01$).

Figure 4. SEM evaluation of the attachment and spreading of cells on mSS at 24 h, (a) untransduced group, (b) AdBMP-2 group, (c) AdEGFP group. Black arrow shows the stretched pseudopodia.

Figure 5.

The whole and local photomicrograph of the histologic images of the implants among four groups 4 weeks postoperatively. The whole images of representative slices in (a) untransduced group, (b) AdBMP-2 group, (c) AdEGFP group and (d) scaffold alone group (a, b, c, d, 40 $\times$). The center photomicrograph of the implants in (e) untransduced group, (f) AdBMP-2 group, (g) AdEGFP group and (h) scaffold alone group (e, f, g, h, 200 $\times$). The edge photomicrograph of the implants in (i) untransduced group, (j) AdBMP-2 group, (k) AdEGFP group and (l) scaffold alone group (i, j, k, l, 200 $\times$). NB: new bone; S: scaffold; FT: fibrous tissue; Black arrow shows the multinucleated giant cells; Yellow arrow shows blood vessel.

Figure 6.

The whole and local photomicrograph of the histologic images of the implants among four groups 8 weeks postoperatively. The whole images of representative slices in (a) untransduced group, (b) AdBMP-2 group, (c) AdEGFP group and (d) scaffold alone group (a, b, c, d, 40 $\times$). The center photomicrograph of the implants in (e) untransduced group, (f) AdBMP-2 group, (g) AdEGFP group and (h) scaffold alone group (e, f, g, h, 200 $\times$). The edge photomicrograph of the implants in (i) untransduced group, (j) AdBMP-2 group, (k) AdEGFP group and (l) scaffold alone group (i, j, k, l, 200 $\times$). NB: new bone; S: scaffold; FT: fibrous tissue; BM: bone marrow; Black arrow shows the multinucleated giant cells.

Figure 7.

The whole and local photomicrograph of the histologic images of the implants among four groups 12 weeks postoperatively. The whole images of representative slices in (a) untransduced group, (b) AdBMP-2 group, (c) AdEGFP group and (d) scaffold alone group (a, b, c, d, 40 $\times$). The center photomicrograph of the implants in (e) untransduced group, (f) AdBMP-2 group, (g) AdEGFP group and (h) scaffold alone group (e, f, g, h, 200 $\times$). The edge photomicrograph of the implants in (i) untransduced group, (j) AdBMP-2 group, (k) AdEGFP group and (l) scaffold alone group (i, j, k, l, 200 $\times$). NB: new bone; S: scaffold; FT: fibrous tissue; BM: bone marrow; Black arrow shows the multinucleated giant cells; Yellow arrow shows blood vessel.

Figure 8. Histomorphometrical analysis of (a) the bone formation and (b) remnant scaffold at 4 weeks, 8 weeks, 12 weeks post-operation. ** $P < 0.01$.