

Effects of adenoviral-mediated coexpression of bone morphogenetic protein-7 and insulin-like growth factor-1 on human periodontal ligament cells

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Yang L, Zhang Y, Dong R, Peng L, Liu X, Wang Y, Cheng X. Effects of adenoviral-mediated coexpression of bone morphogenetic protein-7 and insulin-like growth factor-1 on human periodontal ligament cells. *J Periodont Res* 2010; 45: 532–540.
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Background and Objective: Bone morphogenetic protein-7 (*BMP-7*) and insulin-like growth factor-1 (*IGF-1*) are important in periodontal reconstruction. However, their synergistic effect in periodontal regeneration by gene delivery has not been reported. In this study, gene delivery of these two growth factors to human periodontal ligament cells (hPDLCs) was examined for its effects on cell proliferation and differentiation.

Material and Methods: Recombinant adenoviruses containing both human *BMP-7* and *IGF-1* cDNA created by introducing the internal ribosome entry site (IRES) sequence were used to transfer the genes into hPDLCs. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and cell cycle analysis were used to observe their effects on cell proliferation, while alkaline phosphatase activity measurement, RT-PCR and *in vivo* tests were conducted to investigate their effects on cell differentiation.

Results: The proliferation of hPDLCs transduced by adenoviruses coexpressing *BMP-7* and *IGF-1* was suppressed while their differentiation ability was enhanced. There was a synergism of *BMP-7* and *IGF-1* in up-regulating alkaline phosphatase activity and mRNA levels of collagen type I and Runx2. Implantation *in vivo* with scaffolds illustrated that the transduced cells exhibited osteogenic differentiation and formed bone-like structures.

Conclusion: The combined delivery of *BMP-7* and *IGF-1* genes using an IRES-based strategy synergistically enhanced differentiation of hPDLCs. It is suggested that this could be a new potential method in gene therapy for periodontal reconstruction.

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Key words: bone morphogenetic protein-7;
insulin-like growth factor-1; recombinant adeno-
virus; human periodontal ligament cell

Accepted for publication October 22, 2009

Periodontitis is a highly prevalent chronic disease, which leads to destruction of the tooth-supporting

tissues and eventually results in tooth loss (1). Various reconstructive procedures, including scaling, root

planning, autografting and guided tissue regeneration techniques, have been pursued in modern clinical

practice, but with limited successes (2).

Bone morphogenetic protein-7 (*BMP-7*), also known as osteogenic protein-1, has been suggested as a potential promotor of periodontal restoration. Recent investigations have demonstrated that *BMP-7* exists in the developing tooth and periodontal ligament (3). It has potential to stimulate alveolar bone regeneration around teeth (4), promotes rapid osseointegration of dental implants (5) and helps in maxillary sinus floor augmentation (6).

Several proteins have shown the ability to reinforce *BMP-7*, an important one of which is insulin-like growth factor-1 (*IGF-1*). Insulin-like growth factor-1, identified as a mitogenic protein (7), has been reported to stimulate cell proliferation and chemotactic migration (8), enhance cellular survival (9) and improve periodontal regeneration (10). Yeh *et al.* (11,12) reported that *IGF-1* synergistically enhanced alkaline phosphatase (ALP) activity in *BMP-7*-treated fetal rat calvaria cells. Moreover, the combination of *BMP-7* and *IGF-1* had better ability to promote the proliferation and matrix formation of articular chondrocytes than *BMP-7* or *IGF-1* alone (13,14). To date, however, there are no reports on the application of both *BMP-7* and *IGF-1* in periodontal tissue regeneration.

Since there are still some problems associated with the protein-delivery method, such as short biological activity and rapid diffusion of growth factors *in vivo*, a gene-delivery approach has been introduced. It is a unique technique, which involves delivering a therapeutic protein into target tissue by transgenes with the help of plasmids or viral particles (15–17). It has been reported that gene transfer of *BMP-7* to alveolar bone wounds or dental implant defects by adenoviral vectors successfully promoted osteogenesis (15,16). Therefore, this technique has great potential in periodontal regeneration.

Human periodontal ligament cells (hPDLs), the major cells in periodontal tissues, play a crucial role in periodontal homeostasis and regeneration. Generally, they are a mixture of heterogeneous cell types, and are

thought to possess osteoblast or cementoblast-like properties, such as generation of osteoblast-related extracellular matrix proteins and a high level of ALP activity (18,19). Now, the question is whether, given proper signal stimulation, these cells would differentiate into osteoblasts or cementoblasts.

Since the synergistic abilities of exogenous *BMP-7* and *IGF-1* in osteoblasts and chondrocytes have been verified by other investigators (15–17), in the present study, we investigate the synergistic effects of these two growth factors on hPDLs using a gene-delivery technique. For this purpose, recombinant adenoviruses encoding both human *BMP-7* and *IGF-1* cDNA were created by introducing the internal ribosome entry site (IRES) sequence in order to transfer the genes into hPDLs. We examined the effects of adenoviral-mediated coexpression of *BMP-7* and *IGF-1* on proliferation and differentiation of hPDLs.

Material and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Company (Grand Island, NY, USA). Chitosan (85% deacetylation degree) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Collagen type I was extracted and purified from tails of Sprague–Dawley rats.

Recombinant adenovirus construction

The AdEasy system (Stratagene, La Jolla, CA, USA) was used to construct recombinant adenoviruses based on the procedures of He *et al.* (20). Briefly, cDNA of human *BMP-7* (GenBank no. BC008584) with or without *IGF-1* (GenBank no. NM_000618, kindly provided by Dr Melissa Westwood, University of Manchester, UK (21)) was inserted into adenovirus pAdTrack-CMV to produce pAdTrack-B+I and pAdTrack-BMP,

respectively. The pAdTrack-B+I also included the IRES sequence between the *BMP-7* and *IGF-1* transgenes. The *PmeI*-digested shuttle vectors and the adenoviral backbone plasmid pAd-Easy-1 were cotransformed into *Escherichia coli* BJ 5183, and the recombinant adenoviral plasmids were obtained. After being purified and linearized with *PacI*, these plasmids were directly transfected into HEK 293 cells. The cytopathic effects and green fluorescent protein (GFP) expression of the transinfected cells were monitored. These final adenoviruses included Ad-GFP (empty vector, which served as a control), Ad-BMP (encoding *BMP-7* gene) and Ad-B+I (encoding both *BMP-7* and *IGF-1* genes). Viral titers were determined by the tissue culture infectious dose method.

Cell culture and gene transfer

Human periodontal ligament tissue was obtained from healthy premolars extracted for orthodontic reasons. Informed consent was obtained from all patients following ethical approval from the Ethics Committee, School of Stomatology, Wuhan University. The isolation of human periodontal ligament cells (hPDLs) was performed by a method described by Kobayashi (22). Low-passage cells (passage 2–4) were used for the study.

In the following experiments, four different groups were defined, as follows: group 1 was the control group, which contained only normal cells without adenovirus infection; and groups 2–4 were experimental groups, which contained cells infected with Ad-GFP, Ad-BMP and Ad-B+I, respectively. The hPDLs were transduced with adenoviruses at a multiplicity of infection (MOI) ranging from 10 to 50, and the optimal MOI was determined by monitoring GFP expression and cell morphology. Gene transduction was verified by RT-PCR with specific primers of *BMP-7* and *IGF-1* (Table 1). To detect protein expression, specific primary antibodies (rabbit anti-human *BMP-7* and rabbit anti-human *IGF-1*, PeproTech, Rocky Hill, NJ, USA), biotin-conjugated goat anti-rabbit immunoglobulin G and Cy3-conju-

Table 1. Primer sequence, product size and annealing temperature for PCR

RT-PCR primer set (GenBank accession no.)	Sequence	Product length (bp)	Annealing temperature (°C)	Cycles
<i>BMP-7</i> (BC008584)	Forward: 5'-GTCGACGGTACCATGCACGTGCGCTCAC-3' Reverse: 5'-GGTACCGGATCCGTGGCAGCCACAGGC-3'	1296	55	32
<i>IGF-1</i> (NM_000618)	Forward: 5'-GTATCTAGAATGGGAAAAATCAG-3' Reverse: 5'-TTAATTGATATCCTACATCCTGTAG-3'	462	54	30
<i>Collagen type I</i> (NM_000088)	Forward: 5'-TCCCAGAACATCACCTACCACTGC-3' Reverse: 5'-TGTATTCAATCACTGTCTTGCCCC-3'	202	60	32
<i>Bone sialoprotein</i> (NM_004967)	Forward: 5'-GAACCACTTCCCCACCTTTT-3' Reverse: 5'-TCTGACCATCATAGCCATCG-3'	201	55	38
<i>Runx2</i> (NM_004348)	Forward: 5'-CAGACCAGCAGCACTCCATA-3' Reverse: 5'-CAGCGTCAACACCATCATTC-3'	178	58	38
<i>β-Actin</i> (NM_001101)	Forward: 5'-GCGAGAAGATGACCCAGATCATGTT-3' Reverse: 5'-GCTTCTCCTTAATGTCACGCACGAT-3'	300	62	30

gated anti-biotin were chosen for immunofluorescence cytochemistry. Cells were counterstained with DAPI (4', 6-diamidino-2-phenylindole) to locate nuclei and examined under a fluorescence microscope.

MTT assay

Human periodontal ligament cells were plated in 96-well plates at a concentration of 10,000 cells per well in DMEM supplemented with 10% FBS. After incubation at 37°C in an atmosphere of 95% air–5% CO₂ overnight, cells in experimental groups were infected with different recombinant adenoviruses at a MOI of 20 for 4 h. The viabilities of hPDLs were determined by MTT assay at 24 h intervals after transduction (6 wells per group per day). Blue formazan was generated by cells incubated with MTT solution for 4 h, solubilized in DMSO and read by spectrometer at 490 nm.

Cell cycle analysis

Human periodontal ligament cells were plated in six-well plates at a concentration of 5×10^5 cells per well overnight. Then, samples in experimental groups were infected with different recombinant adenoviruses at a MOI of 20 for 4 h. After 72 h, all samples ($n = 4$) were collected for cell cycle analysis by flow cytometry, stained by propidium iodide. The proliferation index (PI) was calculated from equation 1, in which *S*, G0/G1 and G2/M

represent the percentage of cells in *S*, G0/G1 and G2/M phases of cell cycle, respectively.

$$PI = \frac{S + G2/M}{G0/G1 + S + G2/M} \times 100\% \quad (1)$$

Measurement of ALP activity

Human periodontal ligament cells were cultured and infected as described above. After 72 h, all samples ($n = 6$) were harvested and lysed by freeze–thawing three times. Total cellular ALP activity was measured by the colorimetric method of Kind and King (23). Light absorbance of the reaction mixture was measured at 520 nm. Alkaline phosphatase activity was normalized to total cell proteins as determined by Bradford assay.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed to determine the mRNA expression of collagen type I, Runx2 and bone sialoprotein (BSP). Cellular RNA from the four groups ($n = 3$) was extracted with TRIzol[®] reagent (Invitrogen, Carlsbad, CA, USA) 72 h after transduction. Total RNA (1.0 µg) was used as a template for first strand cDNA synthesis. The PCR was performed with Taq polymerase and specific primers within the exponential amplification range. The PCR products were visualized in a 1% (w/v) agarose gel stained by ethidium bromide. GEL IMAGE software (Syngene, Cambridge, UK) was used to analyze the density.

The levels of mRNA expression were quantified by comparing with the internal control (human *β-actin*). The specific primer sequences, product size and annealing temperature for each gene are shown in Table 1.

Transduced cells co-cultured with scaffolds

A porous chitosan–collagen scaffold was fabricated by the freezing and lyophilizing method described by Peng (24) and used in this study for cell culture. After 90% confluence, hPDLs were digested with 0.25% trypsin for 1 min and adjusted to 1×10^7 cells/mL. Cell suspension (100 µL) from each experimental group was mixed with one of the three recombinant adenoviruses (MOI = 20) for 30 min and then loaded onto a scaffold. After 4 h, culture medium was provided.

The *BMP-7* secreted into the culture medium was determined by using a commercial *BMP-7* ELISA kit (R&D Systems Inc, Minneapolis, MN, USA) according to the manufacturer's instructions on days 3, 6, 9, 15, 21 and 27 ($n = 3$). Briefly, the culture medium was replaced with non-serum medium 24 h before assay and then the supernatant was collected for evaluation.

In vivo test

Cell–scaffold complexes were created as described in the previous subsection. After 48 h, all the cell–scaffold complexes were implanted into the dorsal subcutaneous areas of six athymic

mice (BALB/c-nu; Hubei Medical Laboratory Animal Center). Before the transplantation, the mice were injected chloral hydrate intraperitoneally (400 mg/kg) for anesthesia. Each animal had four cell-scaffold complexes seated in the upper right, upper left, lower right and lower left of the dorsal subcutaneous area. Samples from four groups were implanted randomly in one of the four sites of the animal. All animal experimental procedures were carried out in accordance with the Experimental Animal Regulations of the Experimental Animal Board of Hubei Medical Laboratory Animal Center.

Eight weeks after transplantation, the mice were killed by cervical dislocation. Parts of the transplanted samples were immediately frozen, sectioned, stained with Hoechst 33342 to locate nuclei and examined under a fluorescence microscope. Other parts were fixed in 4% paraformaldehyde for 1 d, decalcified in 10% EDTA for 2 d and then dehydrated and embedded in paraffin. Samples were sectioned and stained using hematoxylin and eosin (HE). Immunohistochemical stain was also conducted, using *BMP-7* antibodies (rabbit anti-human *BMP-7*; PeproTech, Rocky Hill, NJ, USA). Specimens were examined with a light microscope and photographed by means of a mounted digital camera. Computer-assisted histomorphometry was performed on multiple sections using IMAGEJ software (Bethesda, MD, USA) to detect the area of new bone formation expressed as a percentage of the total area.

Statistic analysis

Data are presented as the means \pm SD. Statistical analyses were performed using one-way ANOVA followed by *post hoc* least significant difference multiple comparisons (LSD). A probability value of 0.05 was chosen to determine significant difference.

Results

Adenovirus transduction

All experimental hPDLCs were successfully infected with recombinant

adenoviruses. Green fluorescent protein was observed only in hPDLCs from experimental groups under an inverted fluorescence microscope. Approximately 90% of hPDLCs expressed GFP at a MOI of 20. A higher MOI resulted in an unacceptable level of cell death, whereas at a MOI less than 20 the GFP expression was reduced. Therefore, a MOI of 20 was the optimal value for transduction in this study.

Expression of *BMP-7* and *IGF-1* was detected by RT-PCR and immunocytochemistry at both mRNA and protein level. As shown in Fig. 1(1), both *BMP-7* and *IGF-1* cDNA (1296 and 462 bp, respectively) were detected in the Ad-B+I group. In Fig. 1(2), it can be seen that cells in the Ad-B+I group exhibited a strong positive immune reaction to both *BMP-7* and *IGF-1* antibodies, while those in the Ad-BMP group reacted only to *BMP-7* antibodies.

The proliferation of hPDLCs after transduction

The viabilities of transduced hPDLCs were measured by MTT assay (Fig. 2A). The difference between all four groups was not significant in the first 6 d ($p > 0.05$). However, on days 7 and 8, the control group showed better cell proliferation than the experimental groups ($p < 0.05$).

The cell cycle analysis was performed by flow cytometry after 72 h of transduction (Fig. 2B). The proliferation indexes of experimental groups were lower than that of the control group ($p < 0.05$), while there was no statistical difference among experimental groups ($p > 0.05$).

The differentiation of hPDLCs after transduction

The ALP activities of transduced hPDLCs are shown in Fig. 3A. Cells infected with Ad-B+I and Ad-BMP showed significantly higher ALP activities than those of the control group and the Ad-GFP group ($p < 0.05$). The Ad-B+I group exhibited a significantly higher ALP activity than the Ad-BMP group ($p < 0.05$).

The mRNA expression of collagen type I, Runx2 and BSP was assessed by

semi-quantitative RT-PCR (Fig. 3B–D). The Ad-B+I and Ad-BMP groups had higher mRNA expression of collagen type I, Runx2 and BSP than the control group and the Ad-GFP group ($p < 0.05$). Synergies were observed in the Ad-B+I group, because it had higher mRNA expression level of collagen type I and Runx2 than the Ad-BMP group ($p < 0.05$).

Bone morphogenetic protein-7 expression

An ELISA was performed to determine the *BMP-7* expression in the culture medium, and the results are presented in Fig. 4. Transduced hPDLCs in the Ad-BMP and Ad-B+I groups produced higher levels of *BMP-7* during the culture time than those cells in the control and Ad-GFP groups ($p < 0.05$). The maximal concentrations of *BMP-7* in the Ad-BMP and Ad-B+I groups were detected after 9 d, after which there was a moderate decline. After 27 d, *BMP-7* in supernatants maintained higher levels in these two groups than in the control and Ad-GFP groups.

In vivo study

Figure 5 is a coronal view of cell-scaffold complexes treated by *ex vivo* gene transfer in dorsal subcutaneous areas of mice after 8 wk. Green fluorescent protein was expressed in some cells of experimental specimens monitored by fluorescence microscopy in frozen sections (Fig. 5B1–D1), but was not found in the control samples (Fig. 5A1). Cells and extracellular matrix filled the pores of the scaffold, and tissue ingrowth with differentiation was noticed. Bone-like structures, immunohistochemically positive against *BMP-7* antibodies, were observed within the chitosan materials in the Ad-B+I and Ad-BMP groups, and osteoblast-like cells were embedded in these tissues. Similar structures were rarely found in the control and Ad-GFP groups (Fig. 5A2–D2 and A3–D3). In the tissue-formed areas, the percentage area of bone-like structure formation of the Ad-B+I group was $21.59 \pm 4.54\%$, higher than that of

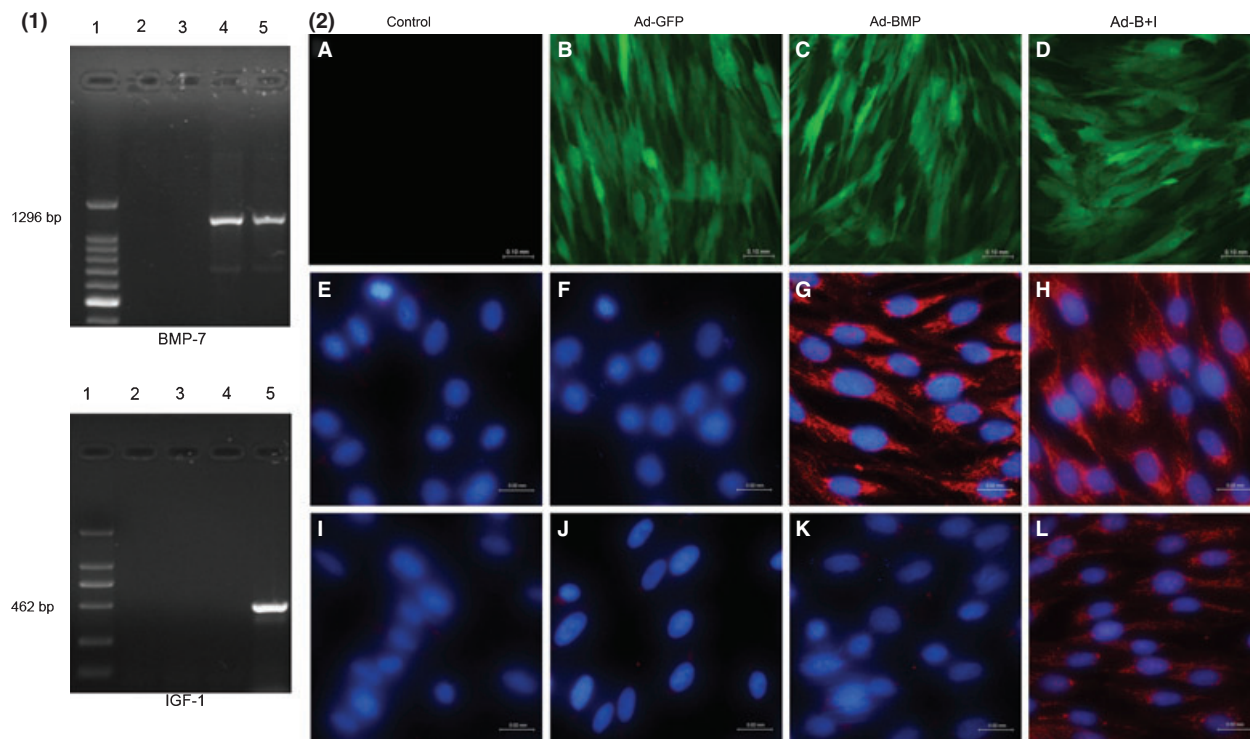


Fig. 1. Characterization of hPDLs transduced with recombinant adenoviruses coexpressing bone morphogenetic protein-7 (*BMP-7*) and insulin-like growth factor-1 (*IGF-1*). (1) The results of RT-PCR. Lane 1, DNA marker; lane 2, control group; lane 3, Ad-GFP group; lane 4, Ad-BMP group; and lane 5, Ad-B+I group. (2) Fluorescence microscopy of hPDLs after transduction. (A–D) Green fluorescent protein expression monitored by inverted microscope. Scale bars represent 0.1 mm. (E–H) and (I–L) Bone morphogenetic protein-7 and *IGF-1* expression, respectively, under immunofluorescence microscopy. Scale bars represent 0.02 mm.

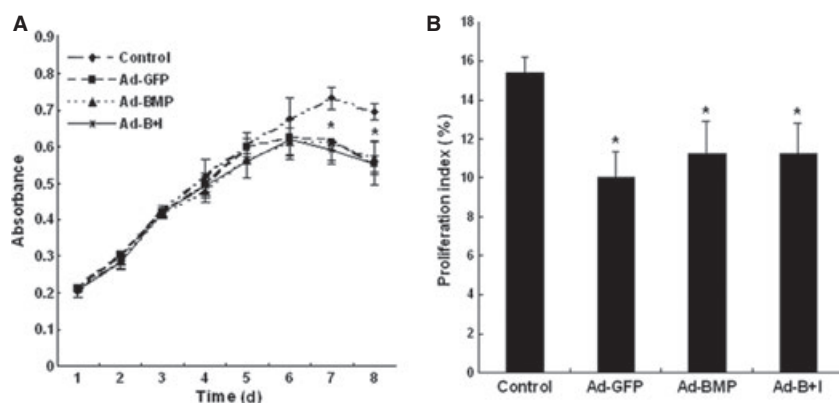


Fig. 2. The proliferation of hPDLs after transduction. (A) The cellular viability measured by MTT assay. (B) The proliferation index from cell cycle analysis by flow cytometry (* $p < 0.05$ vs. control group).

the Ad-BMP group, which was $17.02 \pm 7.22\%$ ($p < 0.05$).

Discussion

The limitations of recombinant growth factor administration include the need for high-dose bolus delivery,

transient biological activity and low bioavailability of factors at the wound site. Gene transfer offers promise as an alternative treatment strategy to deliver growth factors to periodontal tissues. It involves delivering a certain therapeutic protein into the target tissue by transgenes with the help of

plasmids or viral particles (15–17). This technique avoids the problems associated with the protein-delivery method by maintaining constant protein levels at the site of the defect (25).

In the present study, adenoviral vectors were chosen to be carriers for gene delivery. Adenoviral vectors are the most commonly used vectors for the transfer of genetic material into human cells. They have some advantages, in that they are capable of infecting a broad range of human cells efficiently in culture and *in vivo* and result in a high level of transient gene expression (26,27). Owing to removal of the *E1* gene (important for viral gene expression and replication), the current *E1*-deleted vectors are defective for replication and incapable of producing infectious viral particles in target cells (20,27). Moreover, the viral DNA does not normally integrate into the host genome and remains episomally in the nucleus of the infected cell; therefore, the virus does not interfere

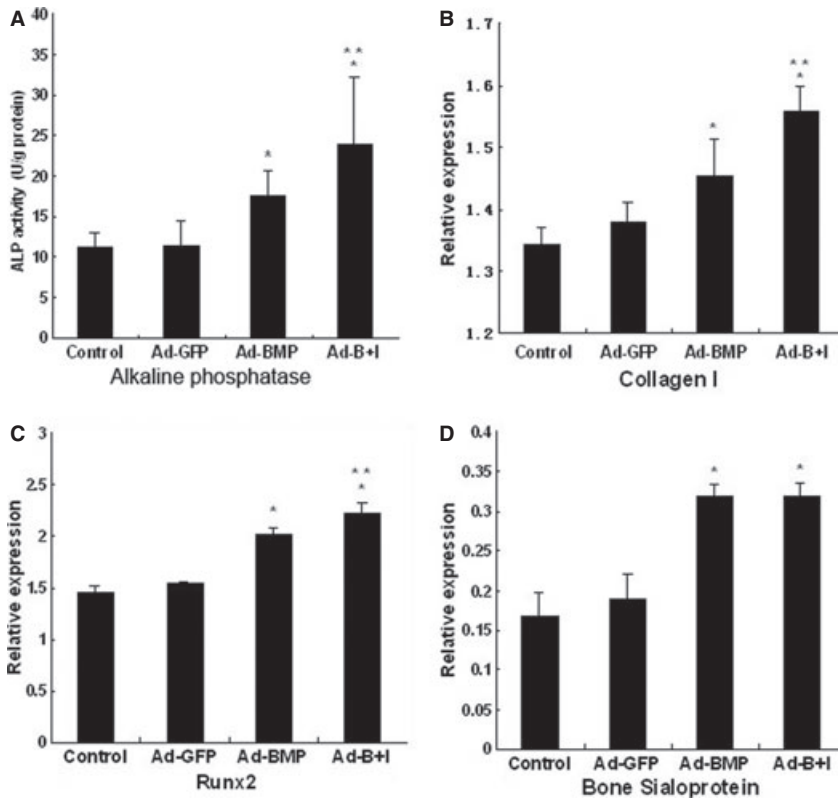


Fig. 3. The differentiation of hPDLCs after transduction. (A) Alkaline phosphatase activity of hPDLCs infected with adenoviruses. (B–D) mRNA expression of collagen type I, Runx2 and BSP (* $p < 0.05$ vs. control group and Ad-GFP group; ** $p < 0.05$ vs. Ad-BMP).

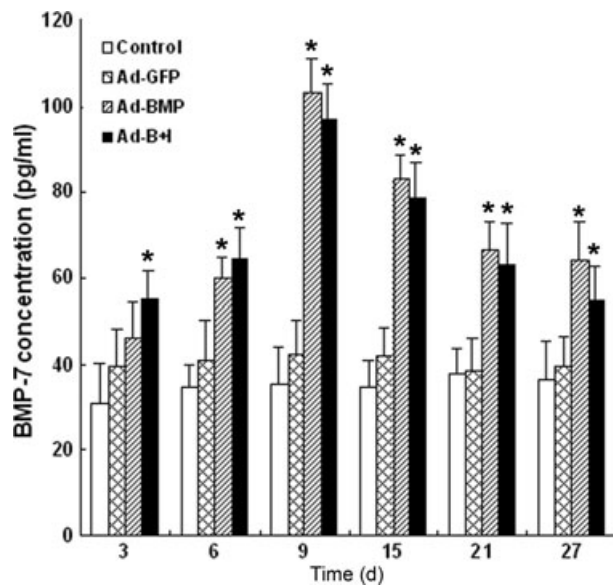


Fig. 4. The BMP-7 expression in supernatants of hPDLCs after transduction measured by ELISA (* $p < 0.05$ vs. control and Ad-GFP group).

with host genes to cause tumorigenesis (27,28). Research on adenoviral gene delivery of growth factor genes, such as BMP-7 and platelet-derived growth

factor (PDGF), to target cells derived from the periodontium has provided satisfactory results both *in vitro* and *in vivo* (15,16,29,30).

There are currently two methods to coexpress two or more heterologous genes in a single vector: either by using independent promoters or by introducing IRES sequences (31,32). In the first approach, promoter interference may occur between heterologous promoters (33), while the second method avoids such problems. Internal ribosome entry site sequences are RNA elements that promote ribosome binding and initiate translation at internal sites within a mRNA strand. Therefore, when two different genes are linked by the IRES sequence within a plasmid, both genes are transcribed as a single mRNA strand driven by the same promoter but are translated independently (34,35). In this study, this latter strategy was successfully employed to generate recombinant adenovirus with two growth factor genes. The cells transduced by Ad-B+I showed expression of both BMP-7 and IGF-1 at both mRNA and protein levels.

Alkaline phosphatase and collagen type I are recognized as early markers of osteogenic differentiation of osteoblastic differentiation. Runx2 is the primary transcription factor for osteoblastic differentiation and a master organizer that regulates transcription of numerous osteoblast phenotypic genes (36). Expression of bone sialoprotein (BSP) has been reported in the middle to late phases of differentiation, which relate to hydroxyapatite deposition and matrix mineralization (37). The present data demonstrated that hPDLCs transduced by Ad-BMP had higher expression of the above biochemical markers, which indicated osteogenic differentiation *in vitro*, and formed bone-like structures *in vivo*. This indicated that transduced hPDLCs had undergone osteoblastic cell differentiation and that the endogenous BMP-7 produced by the BMP-7 gene had stimulated the differentiation process. These findings are consistent with previous studies on other cells with adenoviral vectors transduction. For example, syngeneic dermal fibroblasts transduced with Ad-BMP-7 showed elevated ALP activity *in vitro* and rapid chondrogenesis with subsequent osteogenesis *in vivo* (15). Human oral keratinocytes

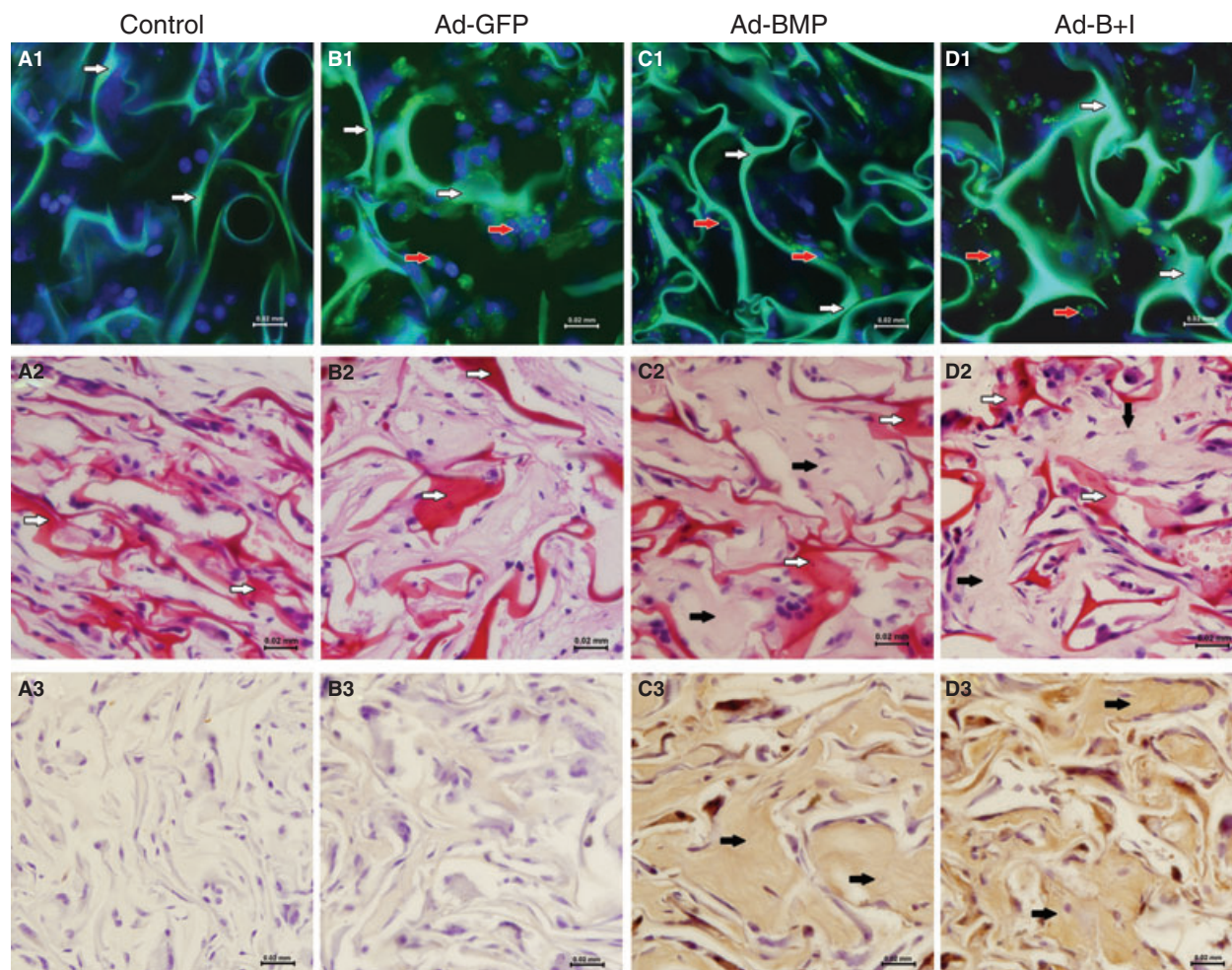


Fig. 5. *In vivo* test of hPDLCs with scaffolds after transduction. (A1–D1) Green fluorescent protein expression was monitored by fluorescence microscopy in frozen sections of specimens. (A2–D2) Sections were stained with hematoxylin and eosin. (A3–D3) Sections were immunohistochemically stained against *BMP-7* antibodies. White arrows indicate scaffold materials; red arrows point to cells expressing GFP; and black arrows indicate bone-like structures. (A1–A3), The control group; (B1–B3), Ad-GFP group; (C1–C3), Ad-BMP group; and (D1–D3), Ad-B+I group. Scale bars represent 0.02 mm.

and gingival fibroblasts infected with adenovirus encoding the *BMP-7* gene induced bone formation *in vivo* (38). These results suggest that *BMP-7* gene transfer can lead to osteogenesis by target cells.

The present study also showed that the ability of osteogenic differentiation of hPDLCs could be enhanced by co-expression of both the *BMP-7* gene and an enhancer gene, such as *IGF-1*. In the present study, hPDLCs in the Ad-B+I group had the highest ALP activity among the four groups, significantly higher than those in the Ad-BMP group ($p < 0.05$). The mRNA expression levels of collagen type I and Runx2 in the Ad-B+I group were also up-regulated compared with the Ad-

BMP group ($p < 0.05$). Moreover, the *in vivo* animal test also demonstrated that hPDLCs transduced by Ad-B+I induced osteogenesis, and the percentage area of bone-like structure formation in the Ad-B+I group was higher than that in the Ad-BMP group ($p < 0.05$). These data illustrated a significant synergistic effect of the *BMP-7* gene and the *IGF-1* gene in osteogenic differentiation of hPDLCs by adenoviral vector. The combination of the *BMP-7* gene and the *IGF-1* gene was more effective than the *BMP-7* gene alone. These results are in accordance with those from Yeh's study on fetal rat calvaria cells co-transfected with *BMP-7* and *IGF-1* genes using plasmids (12).

Since *IGF-1* is regarded as an important mitogenic protein (7), we expected that addition of the *IGF-1* gene would stimulate cell proliferation of hPDLCs in this study. However, to our surprise, the results from MTT assay and cell cycle analysis indicated that the proliferation of hPDLCs after transduction in the Ad-B+I group was suppressed compared with the control group ($p < 0.05$). Possible reasons may be as follows. Firstly, the cell proliferation of all experimental groups was lower than that of the control group, which might be because adenoviral transduction suppressed cell proliferation. Secondly, in the Ad-B+I group, the *IGF-1* gene was the second gene after the IRES sequence. Based

on the report from Mizuguchi *et al.* (32), the expression of the IRES-dependent second gene was only 20–50% of that of the first gene in most cases. Similarly, in this study, the expression of *IGF-1* in Ad-B+I may be reduced to a certain extent compared with that of *BMP-7*. In the present study, ELISA was performed to determine both *BMP-7* and *IGF-1* expression by the transduced cells. Bone morphogenetic protein-7 was detected in the culture medium of Ad-BMP and Ad-B+I groups, while, high background noise led to an unsatisfactory result for *IGF-1*. The low concentration of *IGF-1* secreted by cells of the Ad-B+I group might be one reason. In this case, it is reasonable to predict that the cell proliferation effect of *IGF-1* might be dramatically restricted. However, further experiments should be done to clarify this question.

In this study, porous chitosan–collagen scaffolds were employed for cell culture and the *in vivo* test. Collagen and chitosan are non-toxic natural polymers with good biocompatibility and can degrade in accordance with the time of tissue regeneration *in vivo* (39). Collagen is a major component of extracellular matrix. Chitosan, a partly deacetylated product of chitin, is structurally similar to glycosaminoglycans (24) and possesses an osteoconductive property (40,41). Recent investigations have demonstrated that chitosan accelerates wound healing and enhances bone formation as well (40,42). In addition, the chitosan–collagen scaffold can promote growth and differentiation of osteoblasts (43,44). Therefore, this composite scaffold is very attractive for tissue engineering, especially in periodontal regeneration (24,45).

Conclusion

The present study demonstrated the possibility to deliver a second gene, namely *IGF-1*, with the *BMP-7* gene by an IRES-based strategy to synergistically enhance the effects of *BMP-7*. The present findings illustrated that adenoviral-mediated coexpression of *BMP-7* and *IGF-1* promoted the dif-

ferentiation of hPDLs *in vitro* and induced formation of bone-like structure *in vivo*. This study provides a new potential method in gene therapy for periodontal reconstruction. However, further experiments at the cellular level, the animal level or even the clinical level are needed to validate the method.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (30371554) and the Natural Science Foundation of Hubei Province (2008CDB151). The authors would like to acknowledge Drs Tao Jiang and Yi Zhou for their constructive discussions.

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