Published in final edited form as:
*J Periodontol*. 2003 February ; 74(2): 202–213. doi:10.1902/jop.2003.74.2.202.

# Gene Therapy of Bone Morphogenetic Protein for Periodontal Tissue Engineering

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## Abstract

**Background—**The reconstruction of lost periodontal support including bone, ligament, and cementum is a major goal of therapy. Bone morphogenetic proteins (BMPs) have shown much potential in the regeneration of the periodontium. Limitations of BMP administration to periodontal lesions include need for high-dose bolus delivery, BMP transient biological activity, and low bioavailability of factors at the wound site. Gene transfer offers promise as an alternative treatment strategy to deliver BMPs to periodontal tissues.

**Methods—**This study utilized ex vivo BMP-7 gene transfer to stimulate tissue engineering of alveolar bone wounds. Syngeneic dermal fibroblasts (SDFs) were transduced ex vivo with adenoviruses encoding either green fluorescent protein (Ad-GFP or control virus), BMP-7 (Ad-BMP-7), or an antagonist of BMP bioactivity, noggin (Ad-noggin). Transduced cells were seeded onto gelatin carriers and then transplanted to large mandibular alveolar bone defects in a rat wound repair model.

**Results—**Ad-noggin treatment tended to inhibit osteogenesis as compared to the control-treated and Ad-BMP-7-treated specimens. The osseous lesions treated by Ad-BMP-7 gene delivery demonstrated rapid chrondrogenesis, with subsequent osteogenesis, cementogenesis and predictable bridging of the periodontal bone defects.

**Conclusion—**These results demonstrate the first successful evidence of periodontal tissue engineering using ex vivo gene transfer of BMPs and offers a new approach for repairing periodontal defects.

### Keywords

Alveolar bone loss/therapy; bone regeneration; dental cementum; periodontal regeneration; proteins; bone morphogenetic

Periodontal diseases are characterized by an inflammatory reaction of gingival tissues in response to bacterial biofilms that accumulate on tooth root surfaces resulting in loss of alveolar bone, cementum, and periodontal ligament (PDL). The field of tissue engineering offers great promise for periodontal repair to restore, maintain, or enhance tissues and organs.[1,2] Various reconstructive procedures have been pursued to re-engineer lost tooth support such as bone autografts, allografts, or cell occlusive barrier membranes. In general, these therapies are limited in both predictability and extent of healing response.[3]

Bone morphogenetic proteins (BMPs) are multifunctional growth factors belonging to the transforming growth factor β (TGF-β) superfamily (see reference [4] for review). BMPs are

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powerful regulators of cartilage and bone formation during embryonic development and regeneration in post-natal life. Some BMPs also participate in the development and repair of extraskeletal tissues and organs such as the brain, kidney, and nerves.[5] A striking and discriminatory feature of some of these proteins is their ability to induce *de novo* endochondral osteogenesis in ectopic sites (e.g., skin or muscle).[6] BMP activities are not only modulated through gene expression and protein processing, but by the interaction with antagonists such as noggin.[7] Noggin expression is essential for proper skeletal development and unregulated BMP activity in the noggin null mutant results in excess cartilage and failure of the initiation of joint formation.[8] Furthermore, application of noggin to embryonic tooth bud explants can switch tooth type from incisors to molars, hence demonstrating a potential critical role of noggin in tooth development.[9]

Several studies have demonstrated the expression of BMPs in tooth development and periodontal repair.[10–12] BMP-7 potently stimulates alveolar bone regeneration around teeth,[13] endosseous oral implants,[14] and in maxillary sinus floor augmentation procedures.[15] However, to date human trials have failed to equal results reported in preclinical studies.[16] The reasons for this are unclear; however, a major limitation with growth factor delivery to periodontal wound sites is the extremely short biological activity of the factors in vivo. This phenomenon is presumably due to proteolytic degradation, rapid diffusion, and the solubility of the delivery vehicle in the hostile wound healing environment.[17] Therefore, DNA delivery systems may be an alternative strategy for growth factor application in tissue engineering.[16,18,19] Ex vivo gene transfer has demonstrated success in a variety of orthopedic indications including non-union fractures and craniofacial defects.[20–22] In the present study we show that targeted, local gene delivery of BMP-7 can repair lost periodontal supporting structures.

## MATERIALS AND METHODS

### In Vitro Adenoviral Gene Transfer of BMP-7 and Noggin

For gene transfer studies, recombinant adenoviruses AdCMVGFP (adenovirus encoding green fluorescent protein [GFP] driven by the cytomegalovirus promoter [CMV]) and AdCMVBMP-7 (adenovirus encoding murine BMP-7 or osteogenic protein-1 [OP-1]) were constructed as previously described.[23,24] The AdCMVnoggin (ade-novirus encoding human noggin devoid of the heparin binding site) construct was generously donated (gift of A.N. Economides, Regeneron Pharmaceuticals, Inc. Tar-rytown, New York).[25] Titers of the virus stocks were determined on embryonic kidney 293 cells by plaque assay and expressed as the number of plaque forming units (pfu) per ml as previously described.[26,27]

### Cell Culture and Gene Transfer

Primary cultures of rat syngeneic dermal fibroblasts (SDFs) were utilized as previously described for ex vivo gene transfer.[21] Low passage cells (P4–P6) were plated at a density of 50,000 cells/cm$^2$ in Dulbecco's modified Eagle medium (DMEM)[†] supplemented with 10% fetal bovine serum[‡] (FBS), antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin[†]), and 2 mM glutamine. After incubation overnight (O/N) in a humidified atmosphere of 5% $CO_2$ in air at 37°C, the cells were transduced with 1 of 3 different recombinant adenoviruses (Ad-GFP, Ad-BMP-7 or Ad-noggin) at a multiplicity of infection (MOI) range of 2 to 200 in serum-free DMEM for 5 hours while shaking. Next, the same volume of DMEM supplemented with 4% FBS was added and the cells were incubated O/N. After 24 hours, the medium was changed to DMEM containing 10% FBS for an additional 24 hours. For the final 24 hours the

[†]Gibco BRL Life Technologies, Inc., Grand Island, NY.
[‡]Gemini Bio-Products, Woodland, CA.

cells were transferred to serum-free DMEM and then the medium was collected for analysis by Western blotting.

### Alkaline Phosphatase Activity and Cell Proliferation

The SDFs were infected with recombinant adenoviruses Ad-GFP, Ad-BMP-7, or Ad-noggin at MOI = 200 for 24 hours under the same conditions described above. Then the medium was changed to DMEM containing 10% FBS during the experimental period. The beginning of adenovirus transduction was designated as day 0. After 10 days of culture, the cells were collected and then lysed using a solution containing 0.2% NP-40, 10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.4. DNA content as a measure of cell growth was measured using a fluorochrome[§] via a fluorometer.[‖] The alkaline phosphatase (AP) activities of the cells at each designated time point were measured with an AP kit[§] according to the manufacturer's instructions.

### Northern Blotting

The total cellular RNA of the SDFs transduced by the adenoviruses was extracted using a modified guanidine thiocyanate procedure with Trizol reagent.[28] Ten micrograms of total RNA was denatured, fractionated on 6% formaldehyde/1.2% agarose gels, transferred onto nylon membranes,[¶] and immobilized.[#] The blots were then hybridized with $^{32}$P-labeled probes (BMP-7/OP-1 cDNA (kind gift of H. Opermann, Stryker Biotech, Hopkinton, Massachusetts; noggin cDNA was a kind gift of A.N. Economides, Regeneron) generated from randomly primed cDNA[**] and exposed to film with intensifying screens at −70°C for 6 to 24 hours. To evaluate the relative loading of RNA samples, 18S was used as a standard.

### Western Blotting

The serum-free media (above) were dialyzed against distilled water containing proteinase inhibitors and lyophilized. The lyophilized samples were dissolved in 60 μl SDS-PAGE loading buffer (2% SDS, 4M urea, 5% β-mercaptoethanol, 7% glycerol, 10 mM Tris-HCl, 0.002% bromophenol blue pH 6.8). Fifty microliters of each sample was fractionated by SDS-PAGE on 15% gels for BMP-7 detection, while 1 μl of each sample was fractionated by the same gel for noggin detection. The fractionated proteins in the gels were then electrophoretically transferred to membranes.[††] Rabbit anti-BMP-7 antibody (kind gift of D. Rueger, Stryker Biotech, Hopkinton, Massachusetts) was used at a dilution of 1:4,000 and secondary antibody of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G was used at a dilution of 1:35,000. Rat anti-human noggin antibody (RP57-16; gift of Regeneron) was used at a concentration of 20 ng/ml and secondary antibody of horse-radish peroxidase-conjugated goat anti-rat immunoglobulin G was used at a dilution of 1:12,000. Immunoreactivity was determined using an ECL kit.[‡‡]

All animal procedures were performed under the University of Michigan Unit for Laboratory Animal Medicine Guidelines.

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[§]Sigma Diagnostics, Inc., St. Louis, MO.
[‖]Hoefer Scientific, San Francisco, CA.
[¶]Duralon UV, Stratagene, Inc., La Jolla, CA.
[#]Stratalink, Stratagene, Inc.
[**]Rediprime, Amersham, Arlington Heights, IL.
[††]Immuno-Blot, Bio-Rad Laboratories, Hercules, CA.
[‡‡]Amersham Pharmacia Biotech, Inc. Piscataway, NJ.

## Ex Vivo Gene Transfer to Periodontal Alveolar Bone Wounds

We utilized a modified periodontal alveolar bone defect model as described by King et al.[29] In brief, 25 Lewis rats[§§] (approximate weight 250 to 300 g) had osseous defects created under general anesthesia using ketamine[||||] and xylazine.[¶¶] The osteotomies were created by initially preparing an extraoral 2 cm superficial skin incision at the lower border of the mandible bilaterally. The superficial fascia and underlying masseter muscle were separated with sharp dissection and the ligamentary attachment of the masseter muscle to bone was severed at its inferior base, and both the masseter and periosteum were elevated from the bone to expose the buccal plate of the mandible. The oral mucosa on the superior wall of the surgically created osteotomy was identified, and its attachment to the intraoral keratinized gingival margin was maintained during defect preparation. The bone overlying the mandibular first molar was removed with a high speed handpiece under saline irrigation while visualizing with a surgical microscope. The buccal and distal roots of the first molar tooth and the buccal root of the second molar tooth were carefully denuded of periodontal ligament, overlying cementum, and superficial dentin. The defects measured approximately 0.3 × 0.2 cm in dimension. One million SDFs were plated in 100 $cm^2$ culture dishes in DMEM containing 10% FBS, supplemented with 100 units/ml penicillin/streptomycin, and 2 mM glutamine. One million cells were then transduced with 200 MOI of Ad-GFP, Ad-BMP-7 or Ad-noggin as described above for 24 hours. At the time of surgery, $10^6$ transduced SDFs were seeded into gelatin sponges[##] measuring approximately 3 × 2 × 2 mm and then transplanted ex vivo to the periodontal wounds. Treatment was randomly assigned to Ad-GFP control-treated, Ad-noggin-treated, or Ad-BMP-7 SDFs on one side of the arch while another treatment was randomly assigned to the contralateral side (n = 4 to 7 animals/group). The transduced SDFs seeded in the gelatin sponges were gently placed into the mandibular osteotomy defects. The internal wounds were approximated with resorbable 5-0 chromic gut sutures and the external skin incisions closed with surgical staples. The animals were administered supplemental antibiotics (ampicillin 268 μg/ml of drinking water) given daily for up to 14 days. The study design is summarized in Figure 1.

## Descriptive Histology

Rats were sacrificed at 3, 10, 21, and 35 days after surgery and block biopsies placed into Bouin's fixative,[***] decalcified with 10% vol/vol acetic acid, 4% vol/vol formaldehyde, 0.85% NaCl for 2 to 3 weeks, and then embedded in paraffin. The specimens were cut into 4 to 5 μm coronal sections and stained with hematoxylin and eosin (H & E) or toluidine blue for visualization by light or polarized light microscopy.

## Immunohistochemistry

Immunodetection of noggin and BMP-7 in the periodontal lesions following gene transfer were performed using immunohistochemistry. The sections were deparaffinized with xylene, rehydrated with gradient alcohol, and rinsed with phosphate buffer saline (PBS). Antigen retrieval was performed by soaking the sections with 10 mM sodium citrate, pH 6 at 90° to 95° C for 15 minutes, washed twice with PBS, followed by 0.1% trypsin digestion in PBS at 37° C for 10 minutes. The sections were washed twice with PBS. Endogenous peroxidase was blocked with 0.3% $H_2O_2$ in PBS for 30 minutes at room temperature (RT) and washed twice again with PBS. Nonspecific binding was blocked using 2% goat normal serum for 30 minutes at RT. The sections were incubated with polyclonal rabbit anti-human BMP-7 (1:2,000) for 1

[§§]Charles River, Wilmington, MA.
[||||]Ketaset, Fort Dodge Animal Health, Fort Dodge, IA.
[¶¶]AnaSet Lloyd Laboratories, Shenandoah, IA.
[##]Gelfoam, Upjohn, Kalamazoo, MI.
[***]Polysciences, Warrington, PA.

hour at RT and then washed 3 times with PBS. Goat anti-rabbit secondary antibody (1:500) was incubated for 30 minutes at RT using a commercial kit[†††] according to the manufacturer's protocol. For noggin staining, the sections were treated in the same manner except the antigen retrieval step was omitted. Rat biotinylated anti-human noggin (50 μg/ml, RP57-21, [gift of Regeneron]) was incubated for 1 hour at RT and washed 3 times with PBS. Streptavidin/peroxidase complex[‡‡‡] was incubated for 8 minutes. The sections were then washed 3 times with PBS. DAB staining was performed using a DAB substrate kit.[‡‡‡] The sections were counterstained with Gill's hematoxylin # 1[§§§] for 90 seconds, rinsed in running water for 5 minutes, dehydrated through a series of alcohols and xylene, and then mounted with mounting medium.[§§§]

### Histomorphometry

Computer-assisted image analysis was utilized to determine the ability of gene transfer to affect periodontal tissue repair. Specimens were captured using a microscope[‖‖‖] fitted with a camera[¶¶¶] for analysis using software.[###] Images of coded specimens were captured at 2, 4, 10, and 20 × magnification for histomorphometric analysis. A single masked, calibrated examiner (OA) examined all of the slides and demonstrated a pre- and post-study calibration inter- and intra-examiner error of <5% compared to a standard (WG). Several parameters of periodontal regeneration were measured and included: 1) length of new cementum on the distal root of the first molar was measured as the total length of mineral-like tissue on the denuded root surface in mm; 2) length of new bridging bone measured from the borders of the original osseous defect (mesially-distally) in mm; and 3) density of new bone associated with the osseous defect and gelatin carrier in $mm^2$. Mean values were generated for each of the groups evaluated. The coded specimens were then analyzed using an analysis of variance and a Fisher's probability of least significant difference (PLSD) multiple comparison procedure to measure statistical differences among groups.

## RESULTS

### Expression of BMP-7 and Noggin Following Ad-BMP-7 and Ad-Noggin Gene Transfer

To examine whether Ad-BMP-7 and Ad-noggin transduced cells produce and secrete immunoreactive BMP-7 and noggin protein, cell layers and conditioned media from transduced SDFs were collected and probed for BMP-7 and noggin by Northern and Western blot analyses, respectively. To choose the appropriate media collection time for maximal protein release, a time course by flow activated cell sorting (FACS) was performed using the control virus Ad-GFP. FACS revealed initial GFP fluorescence as early as 6 to 8 hours after Ad-GFP exposure, with >95% of the cells exhibiting GFP protein by 20 to 24 hours after transduction using a multiplicity of infection (MOI) = 200 (data not shown). Two days following gene transfer, cell lysates were assessed for the expression of BMP-7 and noggin genes by Northern analysis. A dose response of increasing level of expression relating to MOI (2 to 200) was noted (data not shown). The conditioned medium harvested after 3 days was evaluated for noggin and BMP-7 protein production. Figure 2A demonstrates a Western blot of protein that was concentrated and lyophilized from media that was probed with monospecific BMP-7 antibody or anti-noggin antibody. Ad-BMP-7 and Ad-noggin transduced cells produced immunoreactive protein that exhibited the same electrophoretic mobility as purified rhBMP-7 and rhnoggin, respectively on β-mercaptoethanol reduced samples with maximal protein expression at MOI = 200.

[†††]Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA.
[‡‡‡]Vector Laboratories.
[§§§]Permount, Fisher Scientific, Fair Lawn, NJ.
[‖‖‖]Nikon Eclipse E800, Nikon, Inc., Melville, NY.
[¶¶¶]SPOT-2, Diagnostic Instruments, Inc., Sterling Heights, MI.
[###]Image Pro Plus, Media Cybernetics, Silver Spring, MD.

Interestingly, transduction of SDFs with Ad-BMP-7 also initiated the release of immunodetectable noggin protein in these cultures (Fig. 2A, middle panel) at both MOI = 20 and 200. Figure 2B illustrates the ability of Ad-BMP-7 to induce the expression of the osteoblastic differentiation marker alkaline phosphatase. Cell cultures were maintained for 10 days and demonstrated a nearly 4-fold increase in AP activity suggesting that BMP gene transfer promotes conversion of these fibroblasts to an osteoblastic phenotype. By 10 days it was noted that some of the cells were still producing protein via gene transfer; however, the percentage of cells at this time was less than 10% as shown by fluorescence of Ad-GFP-treated cells (Fig. 2C). Evaluation of DNA content as a measure of cell proliferation revealed no significant differences among groups ($P > 0.05$).

## Immunodetection of BMP-7 and Noggin in Periodontal Defects Following Gene Transfer Via Adenoviruses

To determine the transduction of the transplanted SDFs in vivo in the periodontal wounds, immuno-histochemical detection of BMP-7 and noggin was performed at 3, 10, and 35 days after surgery. It was noted that expression of BMP-7 and noggin was found at 3 days following gene delivery, however, was essentially undetectable by 10 and 35 days after surgery (data not shown). Figure 3 shows high level expression of BMP-7 and noggin in Ad-BMP-7 and Ad-noggin treated specimens, respectively. In addition, as noted in Western blotting experiments, Ad-BMP-7 gene transfer elicited noggin protein production that was detectable immunohistochemically in some specimens. Ad-GFP or Ad-noggin treatment did not affect BMP-7 protein production in vivo.

## BMP-7 Gene Delivery Promotes Periodontal Tissue Engineering In Vivo

To determine the role in vivo of BMP and noggin gene delivery on periodontal engineering, SDFs transduced by Ad-GFP, Ad-noggin, or Ad-BMP-7 were utilized in large mandibular alveolar bone defects. The lesions were compared at 10 and 35 days following gene transfer. Figure 4 illustrates a coronal view of defects treated by ex vivo gene transfer. Minimal to no evidence of osteogenesis was seen in Ad-GFP and Ad-noggin groups at this early timepoint. There was a preponderance of macrophage-like cells coating the gelatin implants containing cells transduced with Ad-noggin (Fig. 4, middle panel). Interestingly, in the Ad-BMP-7 group, cells were visualized more closely associated with the root surface (Fig. 4f). Furthermore, defects treated with Ad-BMP-7 demonstrated cartilage (Fig. 4e, asterisk) and limited areas of bone (Fig. 4e, open arrows) in the majority of the defects examined. The cartilage was found to be localized lateral to the gelatin carrier in all of the specimens exhibiting chondroblast-like cells. We also found that much of the cartilage persisted to day 21 (Fig. 5) but was completely gone by day 35. To our knowledge, this is the first evidence of endochondral ossification in the alveolar process following BMP exposure.

At 35 days following ex vivo gene transfer extensive bone formation, bone bridging, and cementogenesis with fiber insertion could be seen (via polarized light microscopy) in most of the defects treated by Ad-BMP-7 (n = 6; Figs. 6 and 7), while minimal osteogenesis and cementogenesis and lack of fiber insertion was noted in the Ad-GFP (n = 7; Fig. 6a and b) and Ad-noggin-treated (n = 4; Fig. 6c and d) animals. Moreover, in none of the GFP or noggin-treated specimens was complete bridging of the defects noted, while 4 of the 6 animals treated with BMP-7 gene delivery demonstrated complete bridging of the osseous defects. Histomorphometric analysis showed no significant differences among the groups in terms of initial defect sizes (mean range ± SD of groups: 2.86 ± 0.37 to 2.95 ± 0.27 mm; $P > 0.05$). Masked histomorphometry at 35 days revealed significant improvements in all parameters of periodontal wound healing for lesions treated by BMP-7 gene delivery (Fig. 8). There was nearly a 4-fold increase in new cementum length and a 9-fold increase in bone volume in defects treated with Ad-BMP-7 versus control (Ad-GFP) treatment ($P < 0.01$; Fig. 8). There was a

modest reduction in bone area and length of new bone by noggin gene delivery as compared to GFP treatment, while no difference seen for cementogenesis. Interestingly, ectopic bone formation was noted in all specimens treated with Ad-BMP-7 and was located immediately lateral to the carrier in all directions. No cartilage was seen in any of the defects at 35 days. Minimal evidence of inflammation was seen at all sites at this time period as well.

## DISCUSSION

To date, predictable periodontal regeneration has been elusive in the field of periodontology. The complex microenvironment of the periodontal wound creates many challenges in the repair of periodontal osseous lesions.[30] The field of tissue engineering has provided alternative methodologies to repair tissue defects combining transplantation biology with scaffolding devices for delivery of cells, genes, or proteins.[1] Our approach of ex vivo gene transfer of BMPs to periodontal wounds can be considered as an extension of the tissue engineering concept. The BMPs have demonstrated much success in regenerating periodontal[31] and peri-implant[32] bone defects (see reference [33] for review). In most of the periodontal studies, both alveolar bone and cementum were predictably regenerated.

Despite many of the positive results of using BMPs for periodontal regeneration, several limitations persist. Depending on the carrier or dose applied, varying degrees of ankylosis has been noted.[34] Furthermore, drug instability at the site of delivery contributes to the need of pharmacologic dosing, which is limited by local and systemic toxicity.[35] The therapeutic delivery of BMPs requires a well-characterized delivery system to safely target the factors to the wound. Recent studies of pharmaco-kinetic release profiles for rhBMP-2 typically consist of an initial burst followed by a secondary release phase characterized by a $t_{1/2}$ of 1 to 10 days.[36,37] A few human trials using BMPs reported to date have utilized superphysiological doses of BMPs that result in "dose-dumping" of potent morphogens in the wound site.[15,38,39] This use of bolus delivery can lead to systemic toxicity,[40] likely through cytokine diffusion into the bloodstream.[41]

This approach using ex vivo gene transfer of SDFs provided a more robust response in the stimulation of osteogenesis as compared to our previous efforts using direct gene transfer[42] (and unpublished data). Indeed, our group and others have demonstrated more potent effects in stimulating osteogenesis using ex vivo techniques.[20,21,43] Several groups have shown positive results for bone repair with direct nonviral gene transfer in immunocompetent animals using parathyroid hormone with or without BMP-4.[44,45] The delivery of naked DNA via polymer matrix sponges termed "gene-activated matrices" or GAMs are effective for uptake of genes in orthotopic wounds. This approach and others such as microseeding[46] offer potential for in vivo gene transfer to periodontal wounds.

Additional studies will be needed to determine the feasibility of BMP-gene therapy in other more relevant larger animal model systems possessing a pathologically-exposed tooth surface and kinetic healing profile more similar to the human situation.[47] Although this model does not represent a critical size defect for periodontal regeneration, it serves as a reasonable screening model to examine wound healing kinetics. Indeed, King and colleagues have successfully and extensively used this model to measure the tissue healing responses of BMPs.[29,48,49]

A major consideration in using adenovirus for gene transfer is the significant cytotoxic T-cell-mediated immune response that occurs when delivered in vivo.[50] Despite the inflammatory cell infiltrate that was notable 3 days after gene transfer, the majority of inflammation in our model was decreased by 10 days and was still successful in evoking a potent chondrogenic and osteogenic response. Other investigators have attempted to circumvent the problems of the

immune response inhibiting Ad-BMP-induced bone formation by using immunodeficient animals for study in long bone,[20,43] ectopic[51] and mandibular ramus[52] defects. By using the ex vivo gene transfer approach we were better able to reproducibly regenerate these bony defects while studying the effects of gene therapy in immuncompetent animals. Krebsbach et al. have shown that transduced SDFs elicit the formation of newly formed bone that is a chimera of both donor and recipient cells.[21] BMP transduced fibroblasts from gingiva, dermis, periodontal ligament, and dental pulp all produce bone upon implantation in orthotopic and ectopic sites[53] (and unpublished data). BMP transduced fibroblasts also form the "mineralizing tissue" in diffusion chambers implanted in vivo that appears identical to that produced by calvarial periosteal digest cells.[54] These are the definitive data that BMP transduction converts dermal fibroblasts to osteoblasts. These data suggest that non-osteogenic cells can be genetically modified to not only serve as a reservoir for paracrine effects of BMP delivery, but also promoting the differentiation of fibroblasts into osteoblasts.[54] By the use of real time PCR with a detection limit of about 2 picograms viral DNA/$10^6$ cells, the implanted cells and virus remain at the site of the implant, albeit at low levels. After about 3 weeks, the presence of virus genome is not detectable at the graft site. Our immunohistochemical data suggest the BMP-7 protein is detectable for <7 days. Although the gene expression profile is relatively short, the high level production of BMP-7 in the transplanted fibroblasts (~0.2–0.5 μg/$10^6$ cells) is sufficient to elicit a potent osteogenic effect. Although doses released by the cells within the periodontal defect are difficult to measure, it is speculated the levels of BMP released in this model are significantly less than those delivered by conventional protein delivery approaches. Future studies utilizing other gene therapy vectors may extend the expression profile of osteotropic factors in vivo.

Our data also shows that noggin, the competitive inhibitor of BMP-activity tended to decrease several parameters of periodontal bone regeneration although did not reach the $P < 0.05$ significance level (Fig. 8). Noggin is an inhibitor of several BMP molecules including BMP-2, −4 and, to a lesser extent, BMP-7. Noggin knockout mice die at the time of birth and demonstrate coarse and thickened bones suggestive of the inhibitory role of noggin on periosteal bone growth. Recently noggin has been also shown to inhibit membranous ossification in titanium bone chambers.[55] Although noggin did not appear to have a major inhibitory effect on blocking osteogenesis as compared to control in our study, this finding was likely due in part to the nature of the model used (i.e., limited healing with the control at 35 days). Therefore, investigations examining the effects of noggin on periodontal bone and cementum formation during periodontal repair in smaller defects are warranted. Our finding that BMP-7 transduced fibroblasts stimulate noggin production in this model suggests that the antagonism of BMP-action via noggin may in part explain the persistence and maintenance of the periodontal ligament space during osteogenesis. Indeed, diffuse ankylosis despite administering high levels of BMPs to defects is rare with the exception of submerged bone defects.[31,34] It has been previously shown that BMP-7 and noggin have a coordinated expression pattern during skeletogenesis[56] and that other BMPs stimulate noggin production.[57] We have also found that noggin gene expression is upregulated in cultured PDL fibroblasts (unpublished data). Thus, the PDL/tooth signal to prevent ankylosis and retain the PDL compartment may emanate from noggin. Future studies examining noggin gene and protein expression following BMP delivery need to be carefully explored.

We conclude from this study that: 1) ex vivo gene transfer of Ad-BMP-7 to SDFs releases BMP-7 protein that stimulates periodontal wound healing including bone, PDL, and cementum; 2) ex vivo gene transfer of Ad-noggin tends to decrease periodontal bone repair; and 3) the use of BMP-7 gene transfer offers an alternative approach for the delivery of growth factors for periodontal tissue engineering.

## Acknowledgements

This study was funded by NIH/ NIDCR grants DE 11960, DE 13397 (WVG) and DE 12466 (RBR). The authors appreciate the helpful discussions with Drs. Martha Somerman and Renny Franceschi and the excellent histology assistance from Mr. Christopher Strayhorn. We also thank Mr. Chris Jung for his assistance with the figures.

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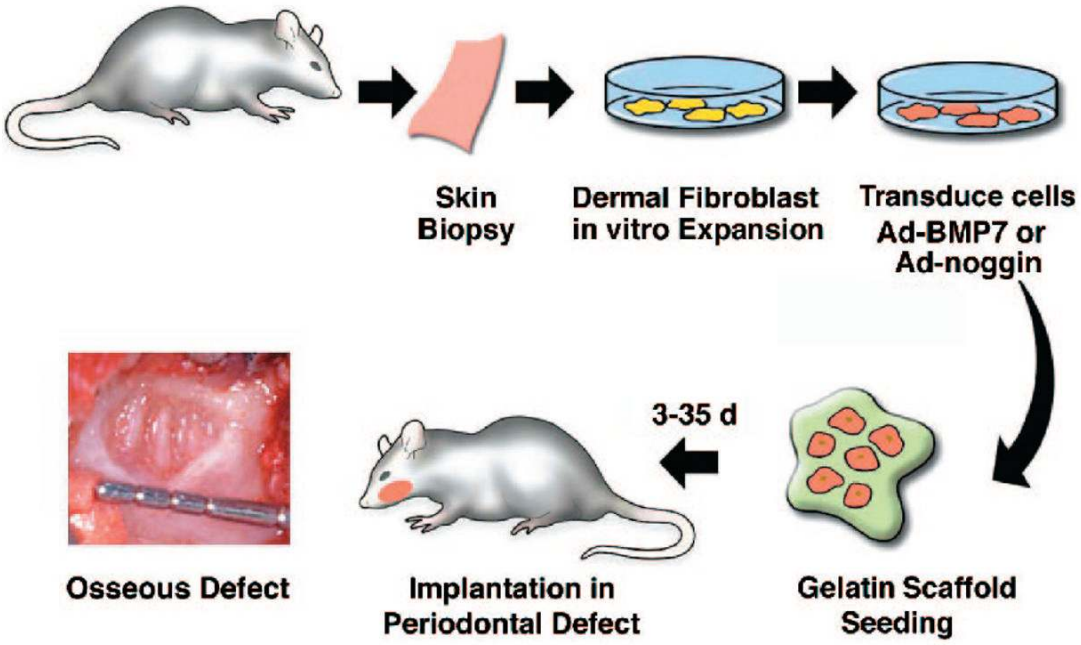


**Figure 1.**

Ex vivo gene transfer to periodontal alveolar bone wounds. Syngeneic dermal fibroblasts (SDFs) were expanded in culture following biopsy, then transduced with recombinant adenoviruses (Ad-GFP, Ad-BMP7, or Ad-noggin). After 24 hours, cells were seeded into gelatin sponges and placed into large mandibular osseous defects created in 25 Lewis rats. Block biopsies were harvested and analyzed using conventional histology, immunohistochemistry, and histomorphometry.

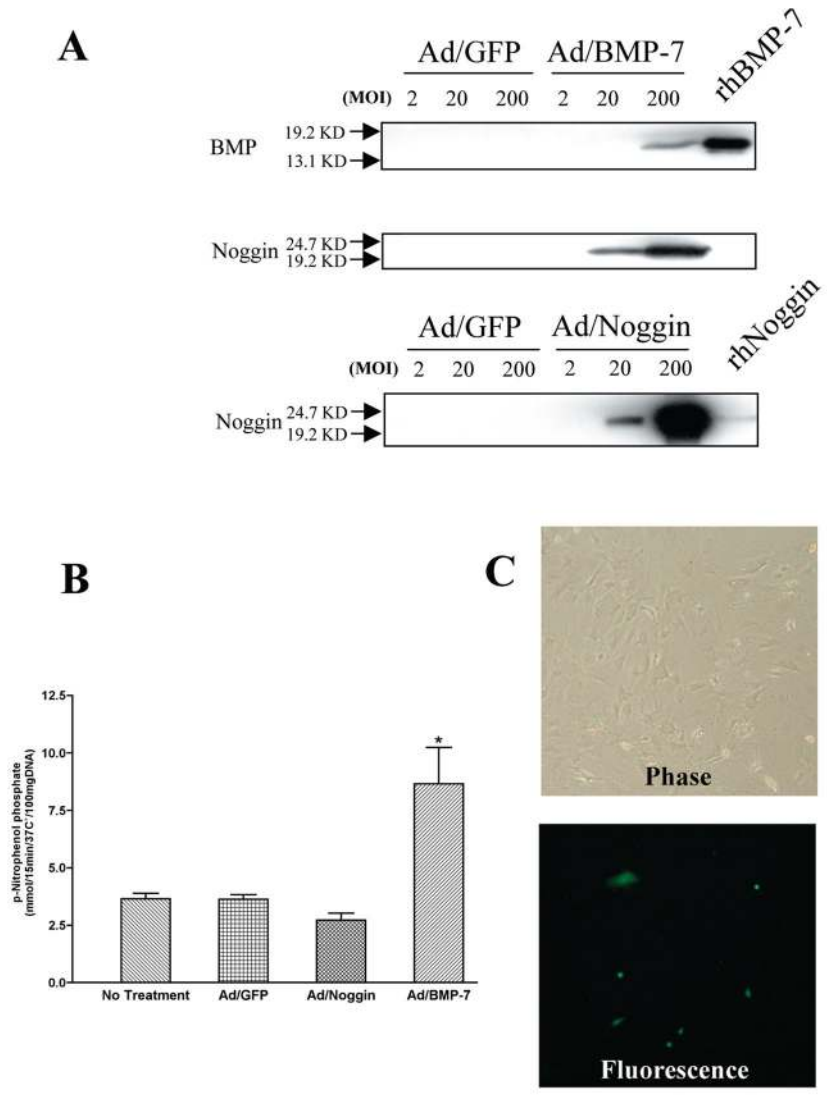


**Figure 2.**

In vitro production of BMP-7 and noggin by adenoviruses encoding BMP-7 and noggin. Following 24 hours of serum starvation, SDFs were transduced with Ad-BMP-7, Ad-noggin or Ad-GFP at a MOI ranging from 2 to 200. Conditioned media were collected after 48 hours, dialyzed, concentrated, and lyophilized. The lyophilized samples were resuspended in reducing sample buffer and analyzed by Western blotting with anti-BMP-7 or anti-noggin polyclonal antibodies. Five nanograms of either BMP-7 or noggin were loaded as controls. Note that Ad-BMP-7 treatment resulted in immunodetectable BMP-7 and noggin (**A,** top and middle panels). Ad-noggin gene transfer resulted in noggin protein release into the medium at MOI = 20 and 200 (**A,** bottom panel). **B** illustrates SDFs transduced with Ad-GFP, Ad-BMP-7, or Ad-noggin at MOI = 200 for 24 hours. After 10 days, the cells were lysed and alkaline phosphatase (AP) activities of the cells were measured with an AP kit. Note the significant increase in AP activity in Ad-BMP-7 SDFs, while no treatment (NT), Ad-GFP and Ad-noggin-treated cells revealed no increase in AP activity. (n = 3 plates/experiment; *P <0.01). **C** shows the same cells evaluated in B under fluorescence microscopy for the production of GFP. It was seen that at this timepoint that approximately 5 to 10% of the cells demonstrated positive GFP fluorescence (10× original magnification; UV fluorescence).

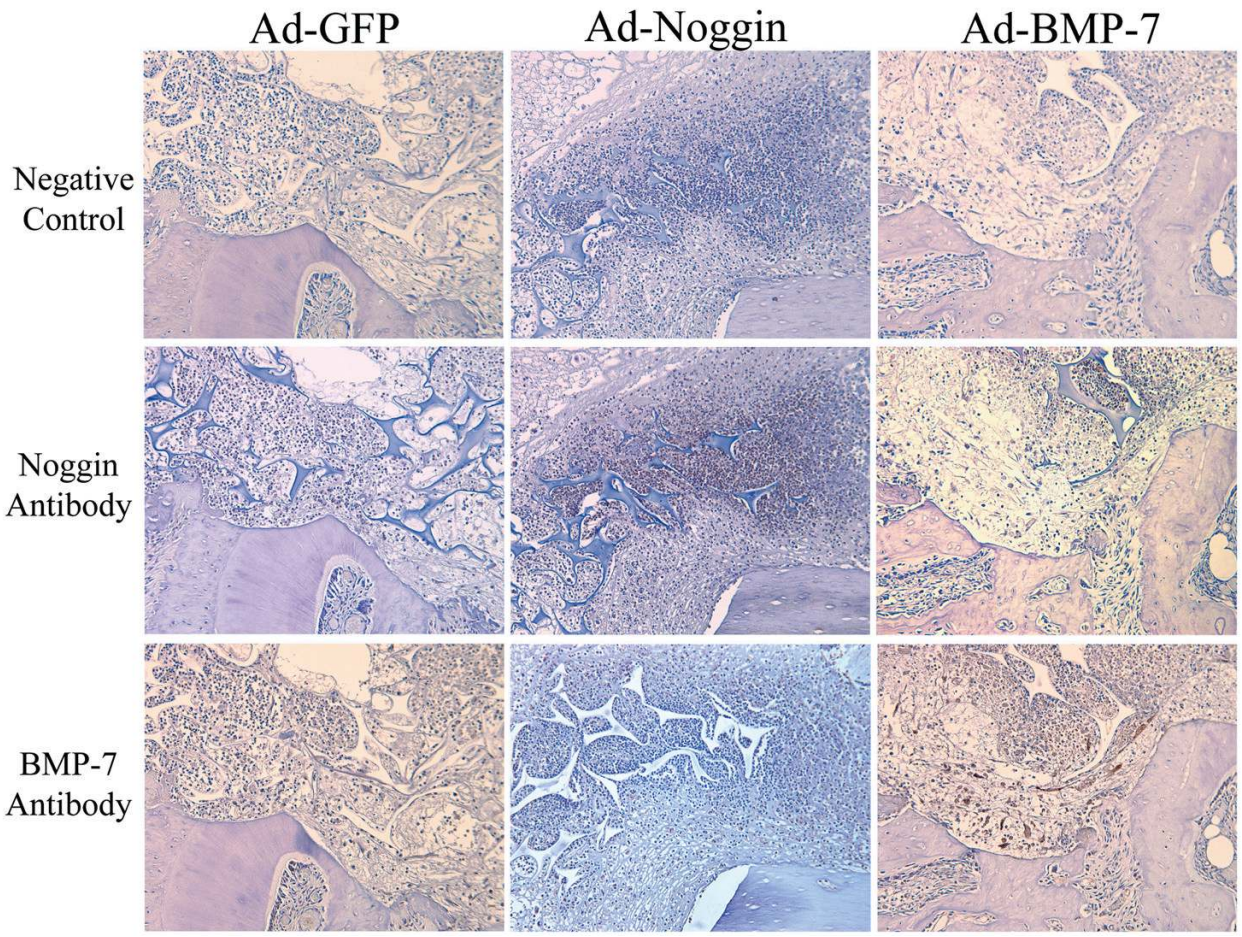


**Figure 3.**

Immunolocalization of BMP-7 and noggin 3 days following gene delivery of Ad-BMP-7 and Ad-noggin to periodontal alveolar bone wounds. Three days following ex vivo gene transfer, block biopsies were harvested, fixed, decalcified and immunoprobed for the expression of BMP-7 and noggin immunoreactivity. Note increased BMP-7 immunoreactivity within the gelatin carrier in specimens treated with Ad-BMP-7. In addition, specimens treated with Ad-noggin expressed immunoreactivity for noggin protein. At subsequent timepoints (days 10 and 35) no differences in BMP-7 or noggin staining could be seen among the groups. No significant staining for noggin or BMP-7 could be ascertained in Ad-GFP-treated specimens (n = 2 to 4 specimens/group; hematoxylin counterstain; 10× original magnification; coronal orientation to sections).

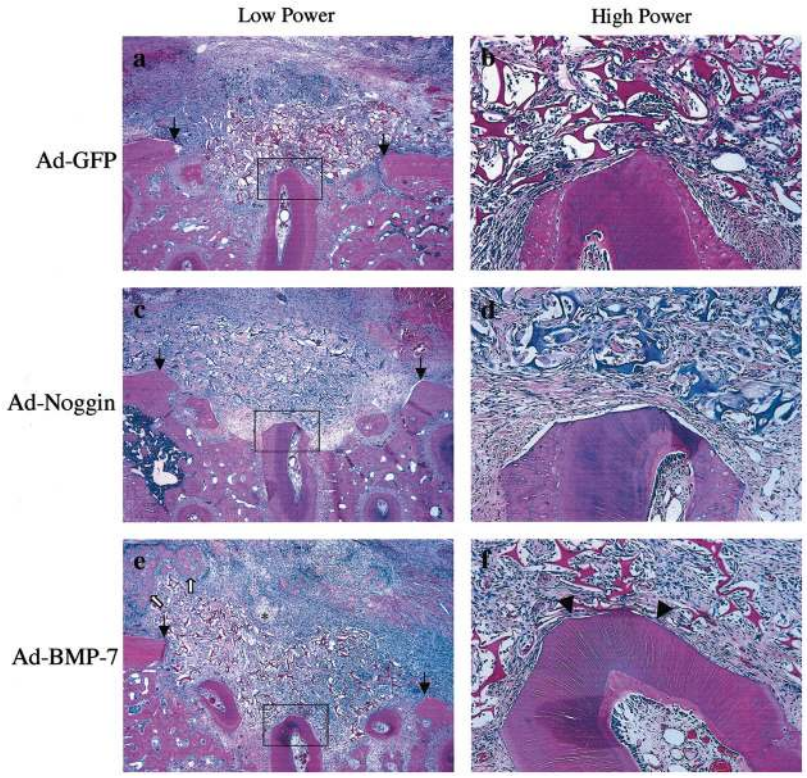


**Figure 4.**

Descriptive histology of periodontal wound repair 10 days following adenoviral gene transfer of BMP-7 and noggin. 10 days following ex vivo gene transfer to periodontal wounds, block biopsies were taken and descriptive histology was performed to assess the wound healing following gene delivery. **Panels a, c,** and **e** show coronal slide orientations of Ad-GFP, Ad-noggin and Ad-BMP-7 treatments at low power (4×) magnification, respectively, and **panels b, d** and **f** show high power (10×) magnification of corresponding treatment groups. Note minimal healing overall in all of the groups treated (black arrows depicting margins of the osseous lesions). However, in Ad-BMP-7 treated specimens cartilage could be seen in 3 of 4 specimens (asterisk; panel e) with islands of bone lateral to the carrier matrix (panel e, open arrows) In the Ad-BMP-7-treated specimens, cells were visualized closely associated with the root surface (panel f, arrows).

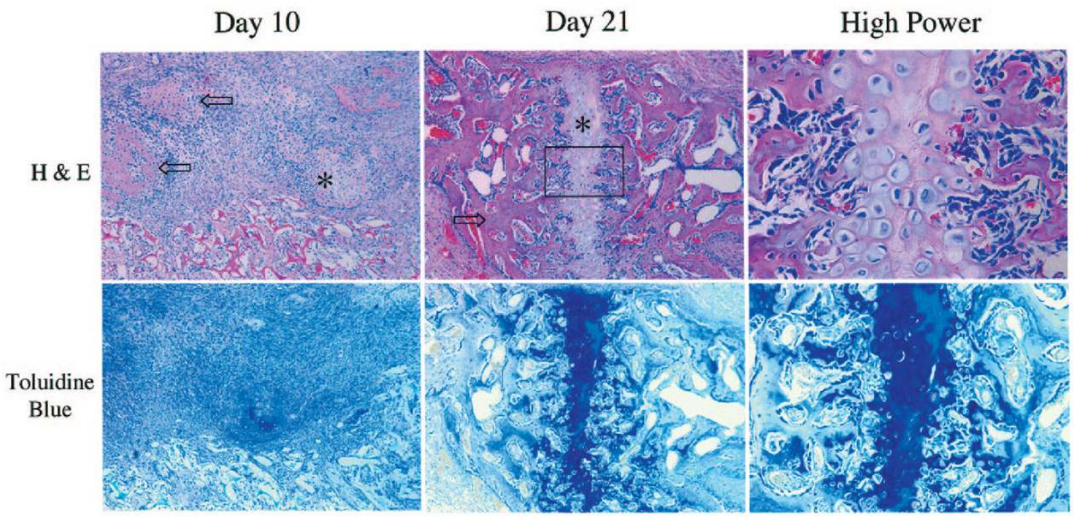


**Figure 5.**

Ad-BMP-7-mediated periodontal bone regeneration forms via a cartilage intermediate. Specimens evaluated at 10 and 21 days following gene transfer with Ad-BMP-7 demonstrated evidence of islands of cartilage and chondroblast-like cells (day 10 specimens) or associated with bone-like tissue (day 21 specimens, middle and last columns). Note presence of immature woven bone (open arrows, day 10 top panel) and an island of cartilage, (top and bottom panels). At day 21, more mature cartilage associated intimately with newly formed bone that was consistent with chondroblasts and a cartilaginous matrix (last column) (H & E and toluidine blue staining 10× original magnification, days 10 and 21; 20× original magnification, high power).

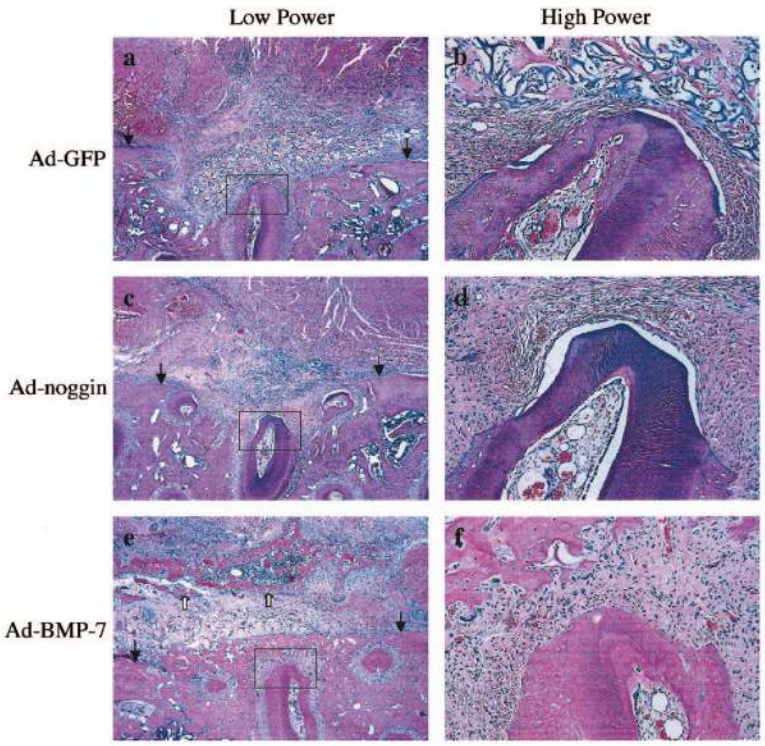


**Figure 6.**

Ex vivo gene transfer of BMP-7 stimulates periodontal tissue regeneration. Block biopsies harvested 35 days following gene transfer of Ad-GFP, Ad-noggin, or Ad-BMP-7. **Panels a, c,** and **e** demonstrate low power magnification (×4) of typical specimens harvested following treatment. The black arrows demarcate the borders of the original osseous lesions. Note minimal bridging of the osseous defect in the Ad-GFP-treated specimen, with essentially no bridging of the bone defect in the Ad-noggin specimen. Defects treated with Ad-BMP-7 demonstrated evidence of extensive periodontal bone regeneration with complete bridging. The open arrows show islands of ectopic bone that were seen in all of the Ad-BMP-7 specimens lateral to the carrier material. **Panels b, d,** and **f** reveal at high magnification (×20). Note minimal evidence of cementogenesis and insertion of fibers to the denuded tooth root surfaces in Ad-GFP and Ad-noggin treated specimens while the Ad-BMP-7-treated site shows evidence of a thin layer of cementum-like material and evidence of a fibrous attachment to the tooth root surface (H & E staining; $n$ = 4 to 7 animals/group).

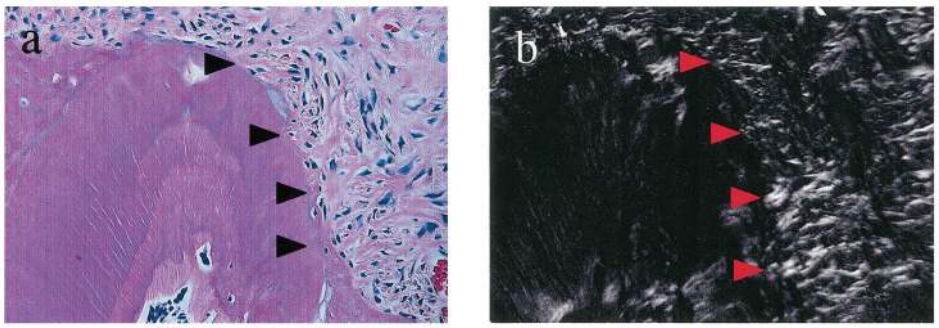


**Figure 7.**

BMP-7 gene transfer stimulates cementogenesis and fibrous attachment in vivo. Ex vivo gene transfer of SDFs transduced by Ad-BMP-7 were transplanted to periodontal alveolar defects. A thin layer of new cementum (black arrowheads) can be seen over the denuded root surface 35 days following surgery **(a). Panel b** reveals polarized light microscopy revealing the presence of fibers consistent with nascent Sharpey's fibers emanating from the immature cemental surface (red arrowheads). (H&E staining and polarized light; 40× original magnification).

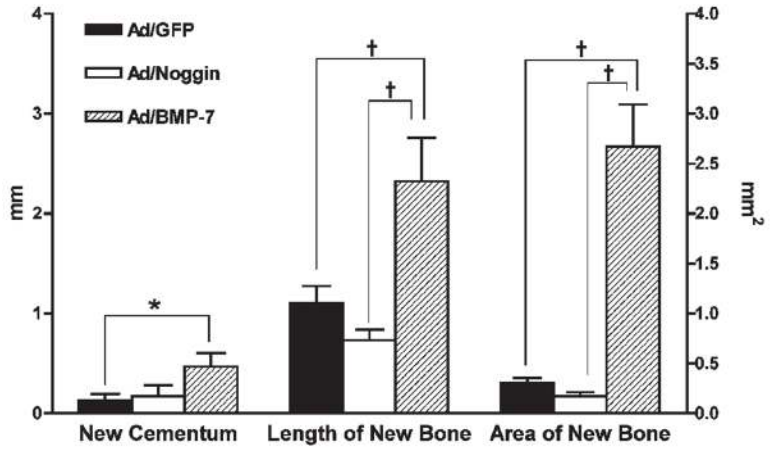


**Figure 8.**

BMP-7 gene delivery stimulates osteogenesis and cementogenesis. Histomorphometric analysis was performed to determine: 1) length of new cementum on the distal root of the first molar in mm; 2) length of bridging supporting bone across the osseous lesion (mesially-distally) in mm; and 3) the total area of new bone regenerated in $mm^2$. Note that for all categories, Ad-BMP-7 gene transfer ($n = 6$) resulted in statistically significant improvements above control (Ad-GFP; $n = 7$) treatment alone ($^*P < 0.01$) or both Ad-GFP and Ad-noggin ($n = 4$) treatment ($^{\dagger}P < 0.01$).