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In vivo new bone formation by direct transfer of adenoviral-mediated bone morphogenetic protein-4 gene

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Abstract

Previous studies have demonstrated that bone morphogenetic protein-4 (BMP4) could participate in vivo endochondral ossification and is one of the main local contributing factors in the early stage of fracture healing. To investigate the effectiveness of BMP4 gene transfer, we constructed an adenoviral vector, Ad-BMP4, and evaluated its osteoinduction activity both in vitro and in vivo. In vitro study suggested that this vector could efficiently transduce mouse myoblast C2C12 cells and produce osteogenic BMP4 protein, as confirmed by immunofluorescence analysis and alkaline phosphatase activity assay. For in vivo study, Ad-BMP4 was directly injected into the hind limb muscles of male athymic nude rats. Visible new bone formation under X-ray films could be detected as early as three weeks post-injection. The bone tissue was further analyzed by histological staining and revealed a typical remodeled bone structure. In conclusion, this study is the first to establish the feasibility of adenovirus-based BMP4 gene therapy for bone regeneration.

Keywords: Bone morphogenetic protein-4; Gene therapy; Adenovirus

Treatment of fracture nonunion and segmental bone defect is the commonly attempted procedure in orthopaedic surgery, in which bone augmentation is usually required. However, the currently used approaches including autogenous bone and allogenic bone graft are limited by their disadvantages such as their association with lack of volume, donor site morbidity, potential for antigenicity, and disease transmission. However, bone morphogenetic proteins (BMPs), members of the transforming growth factor β (TGF-β), have been shown to exert osteogenic activity in a variety of animal studies [1]. Previous investigators have demonstrated that recombinant BMP2, BMP4, BMP7, and BMP9 proteins all have potentials to stimulate osteoinductive cascade [2]. Nevertheless, the applications of BMPs have also been limited by the requirement of large doses, their short half-life, and thus short-term bioavailability, and inability to identify a suitable delivery system as well. However, gene therapy could be a better strategy than direct administration of a therapeutic BMP protein to maximally stimulate osteogenesis in animals as well as in humans [3]. Because gene therapy has the advantage of being able to express BMPs in target cells continuously for either short or long time, and this novel approach could be conducted using a less invasive fashion (e.g., direct injection), it makes delivery of BMPs safe and practicable [4]. Furthermore, gene therapy also allows the delivery of BMPs to specific cells, thus increasing the efficacy of this protein at specific target site [5].

BMP4 is one of the structurally related BMPs belonging to TGF-β superfamily. Mature BMP4 is a dimer that binds to a multimeric transmembrane receptor with serine/threonine kinase activity [6]. Although BMP4 has important roles as a signaling molecule in embryonic development [7], the major practical application of BMP4 is maybe for its capacity of stimulating bone

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repair. It has been reported that BMP4 could participate in vivo endochondral ossification and is one of the main local contributing factors in callus formation in the early phase of fracture healing [8].

In the present study, we constructed an adenovirus vector containing rat BMP4 cDNA and investigated the osteoinductivity of Ad-BMP4 both in vitro and in vivo. Our results clearly demonstrated that Ad-BMP4 gene therapy could successfully induce new bone formation in athymic nude rats, an apparent therapeutic benefit for bone augmentation in future clinical settings.

Materials and methods

Construction of adenovirus BMP4 vector. Recombinant adenovirus BMP4 vector was constructed by a novel in vitro ligation approach developed by Mizuguchi and Kay [9] using Adeno-X Expression System (Clontech, Laboratories, Palo Alto, CA, USA). Briefly, rat BMP4 cDNA was inserted into the *Nhe*I and *Not*I sites of pShuttle plasmid, then the recombinant pShuttle-BMP4 was digested by I-*Ceu*I and PI-*Sce*I, and further ligated to the adenoviral genome DNA. The resultant DNA was transformed into DH5α *Escherichia coli* and positive colonies were screened by PCR and endonuclease analysis to identify the correct recombinant clones. This recombinant adenoviral DNA was then transfected into HEK 293 cells (ATCC, Manassas, VA, USA) for 10 days until full cytopathic effect (CPE) was evident. The cells were harvested and had undergone three cycles of freezing and thawing. The cell lysates were then collected after centrifugation. This cell lysate stock containing primary recombinant adenovirus was further applied to a series of consecutive propagation procedure for in vitro packaging. Finally, the propagated viral particles were purified by Viraprep Kit (Virapur, Carlsbad, CA, USA) and quantified based on the evaluation of absorbance of disrupted virions at 260 nm. In addition, the recombinant adenovirus containing the bacterial β-galactosidase gene (Ad-LacZ) was also constructed by the same procedure.

In vitro transduction of Ad-LacZ in mouse myoblast C2C12 cells. To demonstrate the transduction efficiency by adenovirus, mouse myoblast C2C12 cells (ATCC, Manassas, VA, USA) were seeded at 3×10^5 onto a 6-well plate in growth medium (DMEM containing 10% FBS, 1% penicillin/streptomycin, and 1% glutamine) and allowed to reach 80% confluence. The cells were infected with Ad-LacZ at an MOI (multiplicity of infection: viral particles/cell) of 10^5, 10^4, and 0 (mock) for 24 h. The cells were then washed with PBS, fixed with 3.7% formaldehyde in PBS for 10 min, and stained for 2 h at 37 °C in a solution composed of 4 mm potassium ferricyanide, 2 mm magnesium chloride, and 1 mg/ml X-gal in PBS.

Immunofluorescence analysis for BMP4 expression in myoblast C2C12 cells. For immunofluorescence analysis of cellular BMP4 expression, myoblast C2C12 cells were seeded at 1×10^5 onto a 6-well plate and cultured for 24 h in growth medium. Cells were then infected with Ad-BMP4 at an MOI (viral particles/cell) of 10^4 and 0 (mock) for 24 h. Then, C2C12 cells were washed with PBS, fixed with 3.7% formaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and then incubated with a goat polyclonal antibody against rat BMP4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS-3% bovine serum albumin (BSA) overnight at 4 °C. Cells were then thoroughly washed with PBS and stained with fluorescein isothiocyanate conjugated rabbit anti-goat IgG (Zymed Laboratories, San Francisco, CA, USA) at 1:100 dilutions for 2 h in the dark, washed with PBS, and mounted for observation under fluorescence microscopy.

Osteogenic activity assay in myoblast C2C12 cells infected with Ad-BMP4. To investigate the osteogenic activity of Ad-BMP4, we evaluated alkaline phosphatase (ALP) activity in myoblast C2C12 cell layers. C2C12 cells were seeded on 6-well plates and cultured in growth medium at 37 °C under 5% CO_2. When cells reached 70% confluence, the cells were infected with Ad-BMP4 at an MOI (viral particles/cell) of 10^4 and 0 (mock). Control cells were exposed to Ad-LacZ at an MOI of 10^4 (each group is composed of 3 wells). Twenty-four hours later, cells were rinsed with PBS and growth medium was replaced with fresh medium. Six days after infection, the cell layers were rinsed with PBS and lysed in a buffer containing 50 mm Tris–HCl and 0.5% NP-40 at pH 7.5. The cellular ALP activity was determined by the pNPP hydrolysis method using an ALP Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA).

Radiographic and histological evidences for Ad-BMP4 mediated in vivo new bone formation in athymic nude rats. Nine male athymic nude rats (Harlan, Indianapolis, IN, USA) aging between 5 and 7 weeks were used in this study and all animal procedures were in accordance with Animal Ordinance, made by Department of Health, Hong Kong Regional Office. Animals were randomly assigned to two groups with Ad-BMP4 treated five animals forming group I and Ad-LacZ treated four animals forming group II. After adequately anesthetized with a mixture of ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), a 5 mm incision was made on the left hind limb that was prepared in a sterile fashion. Then, each animal in group I was directly injected with Ad-BMP4 at a dosage of 4×10^{10} viral particles (VP) into the musculature using a micro-syringe (Hamilton, Reno, NV, USA). Each animal in group II was injected with same dosage of Ad-LacZ as a control. Incision was routinely closed with an interrupted 4-0 silk suture. Animals were allowed ad libitum activity, food, and water after the injection.

At 2, 3, and 5 weeks post-injection, all animals were examined by X-ray film for evidence of new bone formation. At 5 weeks after operation, rats were killed by administration of a fatally high dose of anesthetics and the newly formed bone at the injection site was evaluated by radiographic and histological analyses. Left hind limbs were harvested so that the posterior musculature could then be dissected from the rest of hind limb and the muscle samples were stored at −20 °C until ready for histological analysis. The harvested ossified tissues were fixed in 10% formalin neutral buffer solution at pH 7.4 for 2 days and then decalcified with decalcifying solution composed of 10% HCl and 0.1% ethylenediaminetetraacetic acid for another 2 days. The specimens were then dehydrated through a series of graded ethanol, followed by infiltration and embedding into paraffin wax. The tissues were cut into 10 μm sections and stained with hematoxylin and eosin.

Results

X-gal staining in myoblast C2C12 cells infected with Ad-LacZ

After infection with Ad-LacZ for 24 h, cultured myoblast C2C12 cells were stained for expression of bacterial β-galactosidase gene with X-gal. Based on visual assessment of the percentage of X-gal stained cells, the expression of β-galactosidase was detected only in those C2C12 cells infected by Ad-LacZ. The transduction efficiency increased with increasing MOI, with over 80% transduction rate at an MOI (viral particles/cell) of 10^5. About 30% of C2C12 cells infected at an MOI of 10^4 was stained positive (Figs. 1A and B). However, the cells without infection showed negative for staining (data not shown).

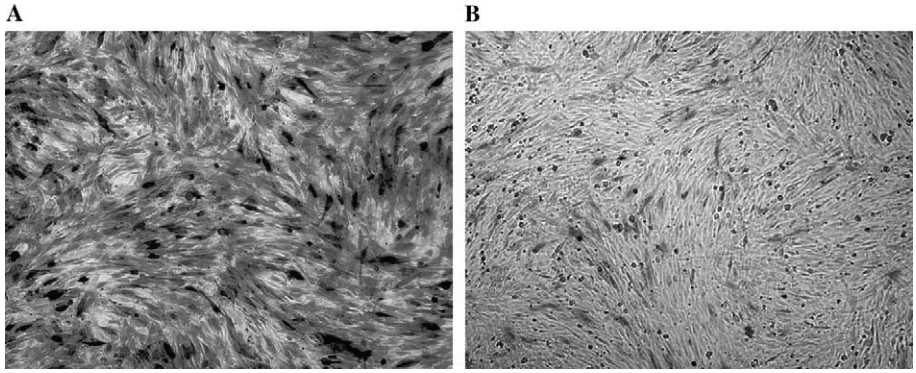

Fig. 1. X-gal staining in C2C12 cells. Cultured myoblast C2C12 cells were transduced with Ad-LacZ vector at different MOIs (viral particles/cell). Cells were fixed and stained for β-galactosidase expression 24 h after infection. Transduced cells showed blue staining. (A) C2C12 cells infected with Ad-LacZ at an MOI of 10^5. (B) C2C12 cells infected with Ad-LacZ at an MOI of 10^4. Original magnification 20×.

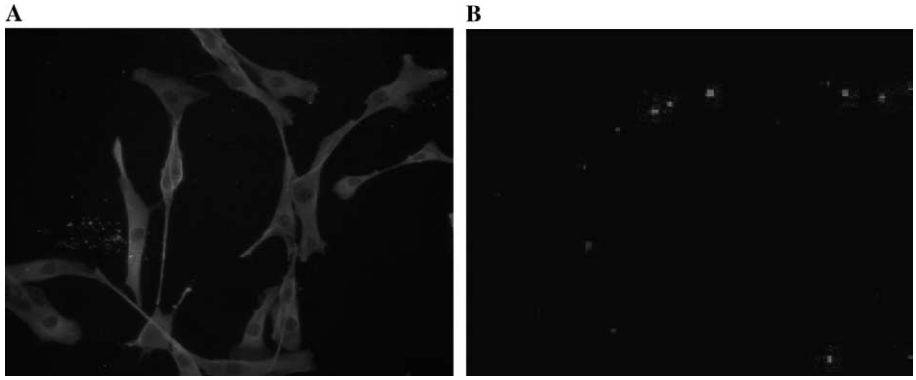

Fig. 2. Immunofluorescence analysis for BMP4 expression in C2C12 cells. Immunofluorescence of BMP4 protein was analyzed in C2C12 cells infected with Ad-BMP4 at an MOI (viral particles/cell) of 10^4 for 24 h. The expressed BMP4 protein is mainly located in the cytoplasm after immunostaining with BMP4 antibody. Those uninfected cells showed negative staining. (A) Ad-BMP4 infected C2C12 cells under fluorescence microscopy. (B) Uninfected C2C12 cells under fluorescence microscopy. Original magnification 40×.

Expression of BMP4 in myoblast C2C12 cells infected with Ad-BMP4

To demonstrate efficient gene transfer of BMP4 by the adenovirus vector, we transduced myoblast C2C12 cells with Ad-BMP4 at an MOI (viral particles/cell) of 10^4 and 0 (mock). Twenty-four hours after infection, the expression of BMP4 in the C2C12 cells was visualized by immunofluorescence staining using the polyclonal antibody against rat BMP4. Under fluorescence microscopy, only those C2C12 cells infected with Ad-BMP4 showed positive BMP4 staining, the expressed BMP4 protein was mainly located in the cytoplasm. However, those uninfected C2C12 cells showed negative immunofluorescence staining (Figs. 2A and B).

Osteogenic induction activity in Ad-BMP4 infected C2C12 cells

To demonstrate the osteoinductive activity of the expressed BMP4, we measured alkaline phosphatase (ALP) activity in mouse myoblast C2C12 cell layers on day 6 after infection. C2C12 cells infected with

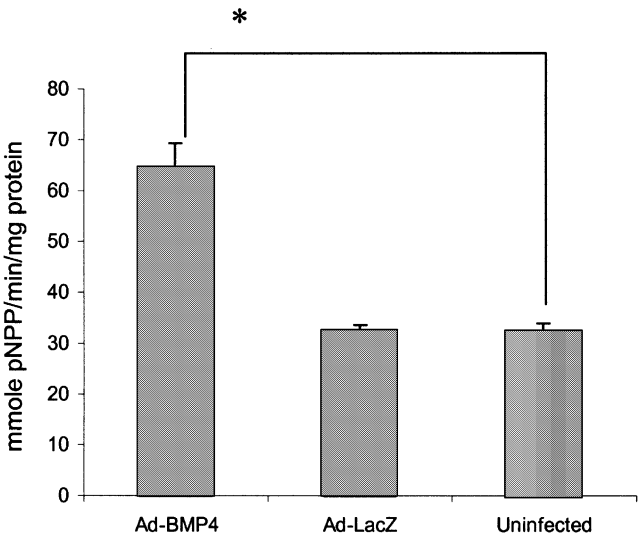

Fig. 3. Quantification of alkaline phosphatase activity (ALP) in myoblast C2C12 cell lysates. Six days after infection of C2C12 cells with Ad-BMP4 or Ad-LacZ at an MOI (viral particles/cell) of 10^4, the ALP activity was measured from cell layers using pNPP hydrolosis method. Values represent means ± SD ($n = 3$). Asterisk denotes statistically significantly different from that of the uninfected cells ($p < 0.001$) as determined by two-tailed Student's test.

Ad-BMP4 at an MOI (viral particles/cell) of 10^4 showed a significant increase of ALP activity, as compared to those infected with same dosage of Ad-LacZ or uninfected cells (Fig. 3), which indicated that after treatment with Ad-BMP4, those transduced myoblast C2C12 cells underwent an inhibition of myogenic differentiation and converted their differentiation pathway from myoblast into that of osteoblast cells.

Radiographic and histological evidences for Ad-BMP4 mediated in vivo new bone formation in nude rats

To demonstrate the capacity of Ad-BMP4 mediated in vivo new bone formation, nine male athymic nude rats were intramuscularly injected with Ad-BMP4 and Ad-LacZ, respectively. Under X-ray films, visible new bone could be detected in all those rats injected with Ad-BMP4 as early as three weeks post-injection. The bone intensity increased at 5 weeks post-injection (Figs. 4A and B). In contrast, no radiographic evidence for bone formation could be seen in those rats injected with Ad-LacZ (data not shown). Five weeks after operation, all rats were killed and had undergone histological analysis. Hematoxylin–eosin staining of specimens revealed remodeled bone tissue only in rats receiving Ad-BMP4. Woven bone was formed in the sections, with a well-defined cortical rim and trabecular structure. The medullary cavity in which hematopoietic cells and adipocytes were filled was also visible. There were also some other differentiated structures including osteoblasts, osteoclasts, and osteocytes, which indicated a typical bone remodeling process. In addition, adjacent myofibers showed somewhat fatty degeneration (Figs. 5A and B). In contrast, no bone tissue structure was found in rats receiving Ad-LacZ injection (data not shown). There were no detectable immunological and inflammatory responses in any rats, and all rats survived until the scheduled date of killing with no apparent complications.

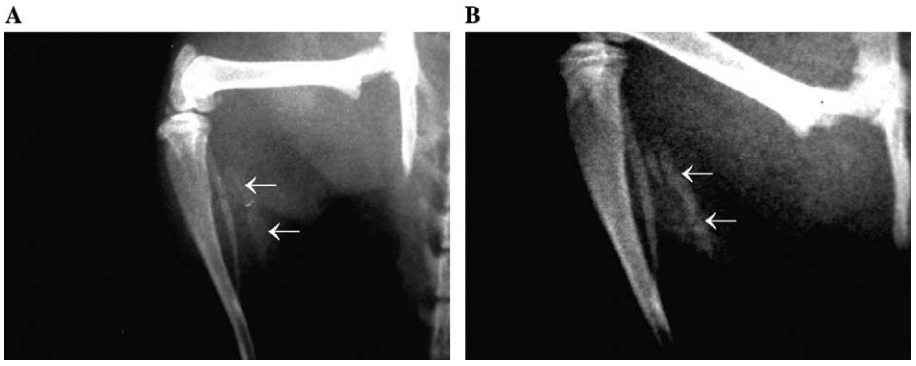

Fig. 4. Radiographic analysis of bone formation in athymic nude rats. Visible radiopaque area indicating new bone formation could be detected by X-ray film in animals injected with Ad-BMP4. (A) Three weeks post-injection of Ad-BMP4. (B) Five weeks post-injection of Ad-BMP4. Arrows indicate the newly formed bone tissue.

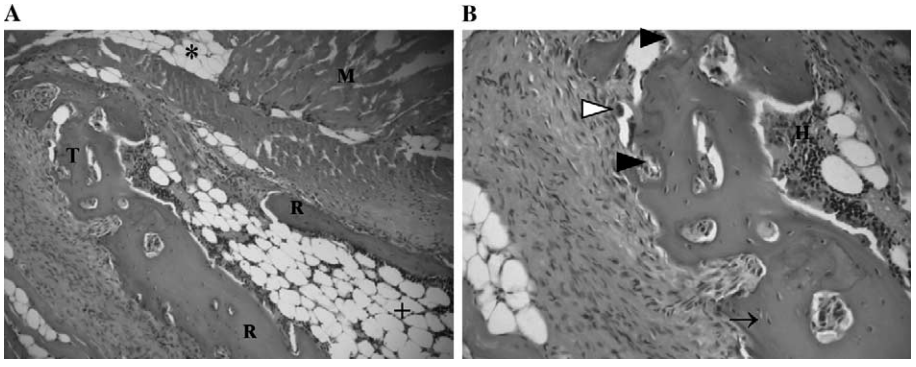

Fig. 5. Histological analysis of in vivo bone formation. Samples were harvested at 5 weeks post-injeciton and undergone hematoxylin–eosin staining. Woven bone was formed within the muscles (M), characterized by a well-define cortical rim (R), trabeculae (T), medullary cavity containing hematopoietic cells (H), and adipocytes (+). Note that other differentiated structures including osteoblasts (solid arrowhead) lining the trabeculae, large multinucleate osteoclasts (open arrowhead), and osteocytes (arrow) trapped in the lacunae were also identified. Fatty degeneration (asterisk) was shown in those adjacent muscles. (A) Original magnification 5×. (B) Higher magnification 10×.

Discussion

Our results clearly demonstrated the feasibility of adenoviral BMP4 gene therapy for both in vitro and in vivo new bone formation. Mouse myoblast C2C12 cells transduced with this recombinant Ad-BMP4 vector could produce osteogenic BMP4 protein, which could increase alkaline phosphatase activity in C2C12 cells. Therefore, our in vitro study indicated that the expressed BMP4 protein could not only inhibit the myogenic differentiation of those transduced C2C12 cells, but also converted their phenotype from myoblast into osteoblast. The mechanism by which BMP4 acts on C2C12 cells is still unknown; however, it has been concluded that BMP2 could inhibit myotube formation of C2C12 cells by suppressing myogenin mRNA and inducing Id-1 mRNA expression [10]. In addition, BMP2 could also specifically convert the differentiation pathway of C2C12 myoblasts into that of osteoblast lineage cells by suppressing the transcriptional activity of the myogenic factor including MyoD and myogenin [11]. Since the sequence of mature region of BMP4 shares 86% identity with that of BMP2 [12] and BMP4 seems to possess similar functions in stimulating a variety of bone cell markers in vitro [13] therefore it is possible that the expressed BMP4 protein could also act on C2C12 cells by the similar mechanism. More importantly, after intramuscular injection of Ad-BMP4 into athymic rats, radiographic examination demonstrated that significant new bone formation could be induced as early as 3 weeks. The newly formed bone was further analyzed by hematoxylin–eosin staining, in which the typical characteristics of bone remodeling structure, such as cortical rim, trabeculae, medullary cavity, osteoblasts, osteoclasts, and osteocytes could be obviously detected. To our knowledge, this is the first report that new bone could be induced by adenovirus mediated BMP4 gene delivery, which may have much potential benefits for bone augmentation.

Previous investigators have demonstrated the potential of BMP4 gene therapy for bone regeneration using plasmid and retrovirus vectors. Fang and colleagues [14] were the first to document the osteoinductivity of plasmid mediated BMP4 gene transfer for segmental bone defect healing in rats. By means of electroporation, BMP4 gene by plasmid vector could also be transferred in vivo and induce new bone in mice [15]. Recently, a retroviral vector carrying BMP4 cDNA was also developed to produce secretion of high levels of BMP4 protein. Rat marrow stromal cells transduced with this MFG-based BMP4 vector could induce in vivo ectopic bone formation and promote critical-sized skull defect healing in mice [16,17]. However, application of plasmid vector as gene therapy vehicle was mainly limited by low transfection efficiency and only transient target gene expression. Retrovirus vector however, requires only dividing cells for nuclear entry [18] and these viruses also insert themselves randomly into the host DNA, which could result into insertion mutation, activation of a cell proto-oncogene or disruption of a tumor suppressor gene. Therefore, retroviral gene transfer usually is applicable only in an ex vivo gene delivery strategy.

In contrast, adenovirus has the advantage of being able to infect both dividing and nondividing cells with excellent efficiency [19] and adenoviral vectors have been widely investigated with use of both in vivo and ex vivo gene delivery strategies in musculoskeletal system [3]. Adenovirus-based BMP2 gene therapy have been demonstrated to have great potential for induction of new bone in a variety of animal studies [20,21]. In addition, Ad-BMP7 and Ad-BMP9 have also been shown to have osteoinductive activity in vivo [22,23]. Nevertheless, since adenoviral vector contains viral gene and expression of viral gene itself could initiate immune responses [24] therefore current work is under investigation in order to delete more viral gene and attenuate this immunogenicity. For example, the E1-deleted adenoviral vector was observed to express low level of viral antigens [25]. Second generation vectors, with deletion of E1, E2B, and E3, has been demonstrated to prolong transgene expression and reduce immunogenicity [26], therefore offering significant promise in some settings. In addition, since it has been found that $CD4^+$ and $CD8^+$ T-cells play an important role in the initiation of immunity, therefore, to overcome T-cell responses, several methods have been investigated, such as inhibiting the CD28/B7 pathway [27], administering an anti-CD4 antibody [28], and immunosuppression [29,30]. Recently, Okubo [31] reported that, under transient immunosuppression by the use of cyclophosphamide, Ad-BMP2 could successfully induce new bone formation in immunocompetent rats without any inflammatory responses. In general, all of these improvements make it practicable and feasible for adenovirus vector as gene therapy vehicles in humans.

Furthermore, our result also strongly suggested that direct transfer of Ad-BMP4 into skeletal muscle scaffold could represent a novel approach to bone augmentation. Previous studies have documented that muscle is a useful target for gene delivery approach because of its large mass, vascularity, and accessibility. Since muscle fibers are nondividing, effective gene delivery could potentially result in long-lived protein production. Post-translational modification of proteins in muscle cells allows these proteins to be secreted with full potency and bioavailability [32]. In the present study, after being directly injected into skeletal muscle, Ad-BMP4 vectors could efficiently transduce muscle fibers, which express and secrete osteogenic BMP4 protein. Then, through a series of osteoinductive cascades, new bone could be finally induced in the muscle tissue. Therefore, our studies provided evidence that skeletal muscle can

successfully function as a valuable bone substitute after being injected with Ad-BMP4.

There are numerous potential clinical applications for gene therapy approaches to enhance bone formation. In the present study, we are the first to demonstrate that adenoviral mediated BMP4 gene therapy could successfully induce new bone formation both in vitro and in vivo. Although there are many issues that need to be addressed before Ad-BMP4 can be further applied in humans, our result is still much more promising for bone augmentation in future clinical settings, including treatment of fracture nonunion, segmental bone defect, spinal fusion, or other orthopaedic disorders.

Acknowledgments

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