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Biochemical and Biophysical Research Communications 308 (2003) 636–645
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Adeno-associated virus-mediated bone morphogenetic protein-4 gene therapy for in vivo bone formation

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Received 16 July 2003

Abstract

Adeno-associated virus (AAV) is so far the most valuable vehicle for gene therapy because it has no association with immune response and human disease. The present study was conducted to investigate the feasibility of AAV-mediated BMP4 gene transfer for bone formation. In vitro study suggested that AAV-BMP4 vectors could transduce myoblast C2C12 cells and produce osteogenic BMP4. In vivo study demonstrated that new bone formation could be induced by direct injection of AAV-BMP4 into the skeletal muscle of immunocompetent rats. Histological analysis revealed that the newly formed bone was induced through endochondral mechanism. Immunohistochemical staining further demonstrated that AAV-BMP4 gene delivery could mediate long-term transduction, and the involvement of BMP4 expression was responsible for the endochondral ossification. This study is, to our knowledge, the first report in the field of AAV-based BMP gene transfer and should be promising for clinical orthopaedic applications.

Keywords: Adeno-associated virus; Gene therapy; Bone morphogenetic protein; Bone; Muscle

Bone regeneration for fracture repair or segmental bone defect healing may be the first major attempted procedure in orthopaedic surgery. Currently, the standard approaches including the autogenous and allogeneic bone graft have many disadvantages. Although a variety of animal studies with various individual or extracted bone morphogenetic proteins (BMPs) have yielded promising results in promoting fracture repair and bone healing [1], the inability to identify a suitable delivery system, the requirement of large doses and its half-life, and thus short-term bioavailability greatly limited their application into clinical trials. However, gene therapy could provide an alternative method for the delivery of BMP protein into tissues for either short-term or long-term expression. This novel approach also allows the targeted delivery of BMP to specific cells thus increasing the efficacy of protein transmission to specific target site. As a result, 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 [2].

So far, three kinds of vectors for BMP gene therapy have been applied to animal studies, which include plasmid [3,4], retrovirus [5,6], and adenovirus [7–9]. However, the gene delivery efficiency by plasmid vector is very poor and the targeted gene by this vector is only transiently expressed. Retrovirus can only infect dividing cells, and these viruses also insert themselves randomly into the host genome DNA, initiating an insertion mutation, activating a cell proto-oncogene, or disrupting a tumor suppressor gene. Therefore, retroviral gene transfer is usually applicable only in an ex vivo strategy. Although adenovirus vectors have been demonstrated to infect both dividing and nondividing cells with excellent efficiency, the application of adenovirus-based vectors is also limited by lack of persistent

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0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-291X(03)01429-3

expression and by the pronounced immune response in immunocompetent animals and humans [10].

Adeno-associated virus (AAV), however, is the only viral vector system that is based on a nonpathogenic and replication-defective virus [11,12]. AAV can mediate efficient and long-term gene transduction in a broad range of host tissues including both dividing and nondividing cells [13]. More importantly, nearly all of viral-coding sequences have been removed from recombinant AAV, so the expression of target gene delivered by AAV vectors does not activate severe cell-mediated immunogenicity or toxicity [14,15]. Since AAV can provide greater safety, efficiency, and longevity of all viral and nonviral vectors tested and can be directly applied to immunocompetent animals or humans, it is a valuable vector for gene therapy and may offer the best gene transfer in the musculoskeletal system.

So far, delivery of BMP gene by AAV vector has not been reported. Combining the merits of AAV and osteogenic BMP should confer another therapeutic advantage in promoting bone augmentation. In the present study, we investigated the feasibility of BMP4 gene transfer using this novel AAV vector and try to demonstrate for the first time whether AAV-BMP4 gene delivery could successfully induce in vivo bone formation in immunocompetent rats.

Materials and methods

Construction of adeno-associated virus vector carrying rat BMP4 gene. The recombinant AAV2 packaging plasmid, pAM/CAG-WPRE-BGH-polyA, was constructed by deleting all of the viral open reading frame (ORF) and introducing the chicken β-actin promoter and cytomegalovirus (CMV) enhancer, a multicloning site, woodchuck hepatitis B virus post-regulatory element (WPRE) enhancer, and the bovine growth hormone polyadenylation signal. The expression cassette was flanked by 145 bp inverted terminal repeats (ITRs), which contained palindromic sequences necessary in *cis* elements for replication of the viral genome. AAV vector plasmid, pAM/CAG-BMP4, was constructed by cloning a full-length rat BMP4 cDNA into pAM/CAG-WPRE-BGH-polyA at *Xho*I and *Eco*RI sites located between two ITRs (Fig. 1) using PCR amplification. In PCR subcloning, the 5′ end of rat BMP4 cDNA sequence was further modified by introducing a Kozak sequence to enhance BMP4 protein expression. The forward oligonucleotide primer for PCR amplification is 5′-CCGCTC GAGCCACCATGGCCATGATTCCTGGTAACCGAATGCTGAT GG-3′ (introduced Kozak sequence and modified ATG start codon is underlined). Reverse primer for PCR is 5′-GACTGGAATTCATTT CAGCGGCATCC GCACCCCTCCACC-3′. PCR was conducted with Platinum Pfx DNA Polymerase (Gibco-BRL, Gaithersburg, MD) according to the conditions recommended by the manufacturer. In addition, vector pAM/CAG-EGFP was constructed by inserting enhanced green fluorescent protein (EGFP) gene into the *Xho*I and *Eco*RI sites. All of plasmid DNA for virus packaging was purified by the MaxiPrep Plasmid Preparation Kit (Qiagen, Hilden, Germany).

AAV vectors were produced using a helper virus-free system [16,17], with some modifications. In general, trypsinized HEK 293 cells (ATCC, Manassas, VA) were plated at 4×10^6 cells in 150-mm culture dishes containing 20 ml Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Gaithersburg, MD), 1% penicillin/streptomycin, and 1% glutamine, and incubated at 37 °C under 5% CO_2 for 24–48 h. After cells reach 80% confluence, each plate of 293 cells was co-transfected with 11 μg pAM/CAG-BMP4 (or 11 μg pAM/CAG-EGFP) and 66 μg AAV helper plasmid pDG by means of the calcium phosphate co-precipitation method. After incubation at 37 °C for 10 h, growth medium was replaced with fresh medium and cells were further incubated for approximately 60 h. The transfected 293 cells were trypsinized, harvested by centrifugation, and re-suspended in 150 mM NaCl buffer containing 20 mM Tris at pH 8.0. After two cycles of freezing and thawing followed by centrifugation, supernatants containing AAV vectors were combined and purified by HiTrap Heparin column chromatography (Sigma, St. Louis, MO). Peak virus fractions were collected and dialyzed against PBS supplemented with 1 mM $MgSO_4$ and concentrated by 100K-MicroSep Centrifugal Concentrator (Life Technologies, Carlsbad, CA). The viral titer was quantified using an AAV Titration ELISA Kit (Progen Biotechnik GmbH, Heidelberg, Germany). The recombinant AAV-EGFP was also constructed and quantified by the same procedure.

Immunofluorescence analysis for BMP4 expression in myoblast C2C12 cells. For immunofluorescence analysis of BMP4 expression, mouse myoblast C2C12 cells (ATCC, Manassas, VA) were seeded at 1×10^5 onto a 6-well plate and cultured for 24 h in DMEM containing 10% FBS. Cells were then infected with AAV-BMP4 at an MOI (multiplicity of infection: viral particles/cell) of 10^6 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) in PBS–3% bovine serum albumin (BSA) overnight at 4 °C. Cells were then thoroughly washed with PBS and stained with fluorescein isothiocyanate (FITC) conjugated rabbit anti-goat IgG at 1:100 dilutions (Zymed Laboratories, San Francisco, CA) for 2 h in the dark, washed with PBS, and mounted for observation under fluorescence microscope.

Osteogenic activity assay in myoblast C2C12 cells. To demonstrate that BMP4 protein is osteoinductive, we investigated alkaline phosphatase (ALP) activity, an important osteoblastic differentiation marker, in myoblast C2C12 cell layers. Cells were cultured at 37 °C under 5% CO_2 in DMEM containing 10% FBS, 1% penicillin/streptomycin, and 1% glutamine. Cells were plated at 2×10^5 cells/well on 6-well plates and allowed to reach 70% confluence. They were infected with AAV-BMP4 at an MOI (viral particles/cell) of 5×10^5 and 0 (mock). Control cells were exposed to AAV-EGFP at an MOI of 5×10^5 (each group is composed of three wells). Twenty-four hours later, cells were rinsed with PBS and growth medium was replaced with fresh medium. Six days after infection, the phenotype changes of C2C12 cells were observed under conventional microscope. For ALP assay, 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 hydrolosis

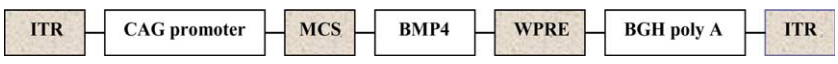

Fig. 1. Schematic diagram of AAV-BMP4 vector. ITR, inverted terminal repeat; CAG, chicken β-actin promoter and cytomegalovirus enhancer; MCS, multicloning site; BMP4, rat BMP4 cDNA; WPRE, woodchuck hepatitis B virus post-regulatory element; BGH polyA, bovine growth hormone polyadenylation signal.

method using an ALP Assay Kit (Upstate Biotechnology, Lake Placid, NY).

In vivo AAV-BMP4 gene transfer for new bone formation. All animal procedures were conducted in accordance with the Animal Ordinance (Chapter 340) in Hong Kong. Twelve male immunocompetent Sprague–Dawley (SD) rats aged between 5 and 7 weeks were used in this study. Animals were randomly assigned into three groups. Group I contained six animals, each of them injected with AAV-BMP4 (2×10^{12} viral particles, VP). Group II contained three animals, each of them injected with AAV-EGFP (2×10^{12} VP), and group III contained three animals injected with empty AAV (2×10^{12} VP). After the animals were adequately anesthetized with a mixture of ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), a 5-mm sterile incision was made in right hindlimbs. AAV-BMP4, AAV-EGFP or empty AAV vectors were injected directly into the muscle with a micro-syringe (Hamilton, Reno, NV). One interrupted 4-0 silk suture was used to close the incision. Animals were allowed ad libitum activity, food, and water after the injection, and were radiographically examined at 2, 3, 4, 5, and 8 weeks after operation.

For histological and immunohistochemical analyses, SD rats were sacrificed by administration of a fatally high dose of anesthetics at 1, 3, and 8 weeks post-injection. Muscles containing newly formed bone were dissected from the hindlimbs. The harvested ossified tissues were fixed in 10% formalin neutral buffer solution at pH 7.4 for 2 days and decalcified with decalcifying solution composed of 10% HCl and 0.1% EDTA for another 2 days. The specimens were then dehydrated through a series of graded ethanol, infiltrated, and embedded into paraffin wax. The tissues were cut into 10 μm sections and several of them were stained with standard hematoxylin and eosin. Immunohistochemical staining was conducted on other sections with a goat anti-rat BMP4 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000. Visualization of bound antibodies was performed using VECTASTAIN ABC *Elite* Kit and DAB Substrate Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. These sections were then counterstained with hematoxylin.

For analysis of AAV-mediated long-term transduction, muscles that had been injected with AAV-EGFP were harvested at 1 and 8 weeks post-injection and transversely oriented in cryomatrix embedding medium to be immediately frozen in liquid nitrogen. Ten micrometer sections were cut with a cryomicrotome at −20 °C, placed on gelatin-coated slides. Sections were fixed for 5 min with 4% paraformaldehyde/PBS, rinsed twice with PBS, mounted under coverslips, and observed under fluorescence microscope.

Results

Expression of BMP4 in myoblast C2C12 cells

To demonstrate the gene transfer of BMP4 by the AAV vector, we transduced mouse myoblast C2C12 cells with AAV-BMP4 at an MOI (multiplicity of infection: viral particles/cell) of 10^6 and 0 (mock). Twenty-four hours after infection, expression of BMP4 in the C2C12 cells was visualized by immunofluorescence staining using the polyclonal antibody against rat BMP4. Under fluorescence microscope, only those C2C12 cells infected with AAV-BMP4 developed positive staining, and the expressed BMP4 protein was mainly located in the cytoplasm. However, uninfected C2C12 cells showed negative immunofluorescence staining (Fig. 2).

Phenotype changes of myoblast C2C12 cells transduced with AAV-BMP4

Six days after infection of AAV-BMP4 or AAV-EGFP at an MOI of 5×10^5 and 0 (mock), the phenotype of C2C12 cells revealed an obvious change. The uninfected C2C12 cells or those transduced with AAV-EGFP significantly generated myogenic differentiation, as confirmed by the formation of numerous multinucleated myotubes on day 6 post-infection. However, C2C12 transduced with AAV-BMP4 inhibited the formation of myotubes and many of them remained as unfused mononuclear round-like or polygonal cells, suggestive of an osteoblast phenotype (Fig. 3).

Osteogenic induction activity in AAV-BMP4-infected C2C12 cells

To further confirm that the expressed BMP4 is osteoinductive, we measured alkaline phosphatase (ALP)

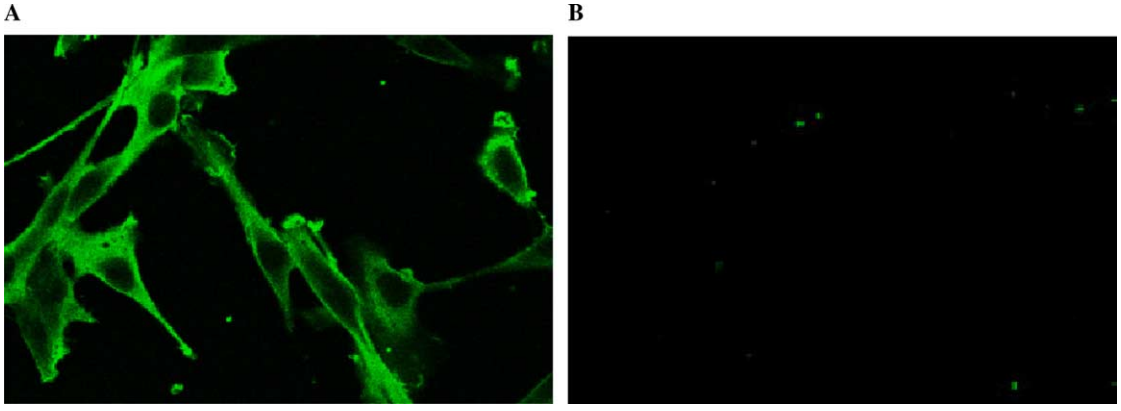

Fig. 2. Immunofluorescence analysis for BMP4 expression. Immunofluorescence of BMP4 protein was analyzed in C2C12 cells infected with AAV-BMP4 at an MOI of 10^6 (viral particles/cell) for 24 h. The expressed BMP4 protein is mainly located in the cytoplasm after immunostaining with BMP4 antibody. Uninfected cells showed negative staining. (A) AAV-BMP4-infected C2C12 cells under fluorescence microscope. (B) Uninfected C2C12 cells under fluorescence microscope. Original magnification 40×.

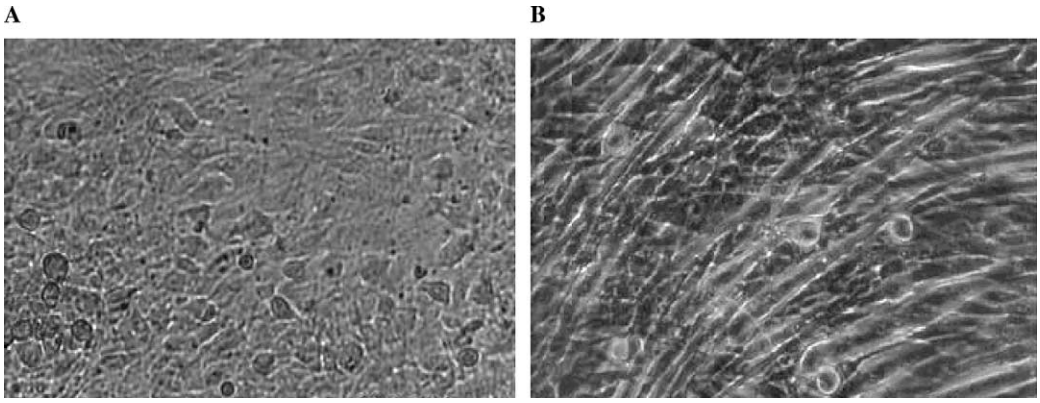

Fig. 3. Phenotype changes in C2C12 cells transduced with AAV-BMP4. Phenotype changes were observed under conventional microscope on day 6 after infection with AAV-BMP4 or AAV-EGFP at an MOI of 5×10^5 (viral particles/cell). C2C12 cells infected with AAV-EGFP generated a significant multinucleate myotube formation. In contrast, in C2C12 cells that had been infected with AAV-BMP4, many of them displayed an unfused mononuclear round-like or polygonal phenotype. (A) C2C12 cells infected with AAV-BMP4. (B) C2C12 cells infected with AAV-EGFP. Original magnification 20×.

activity, an important osteoblastic differentiation marker, in myoblast C2C12 cell layers on day 6 post-infection. Although there was an amount of cellular intrinsic ALP activity, C2C12 cells infected with AAV-BMP4 at an MOI of 5×10^5 (viral particles/cell) still showed an increase of ALP activity compared to those infected with the same dosage of AAV-EGFP or uninfected cells (Fig. 4).

Radiographic evidence for bone formation

To further demonstrate that AAV-BMP4 gene transfer could also induce in vivo osteogenic activity, we directly injected AAV-BMP4, AAV-EGFP, or empty AAV vectors in the hindlimb muscle of immunocompetent SD rats. X-ray films of animals were taken at 2, 3, 4, 5, and 8 weeks post-injection for evidence of new bone. Radiographic examination detected that visible ossification tissue had produced in all those rats receiving AAV-BMP4 as early as 3 weeks post-injection (Figs. 5A–E). In contrast, no radiographic evidence of bone formation was seen in rats injected with either AAV-EGFP (Fig. 5F) or empty AAV (data not shown) at all time intervals.

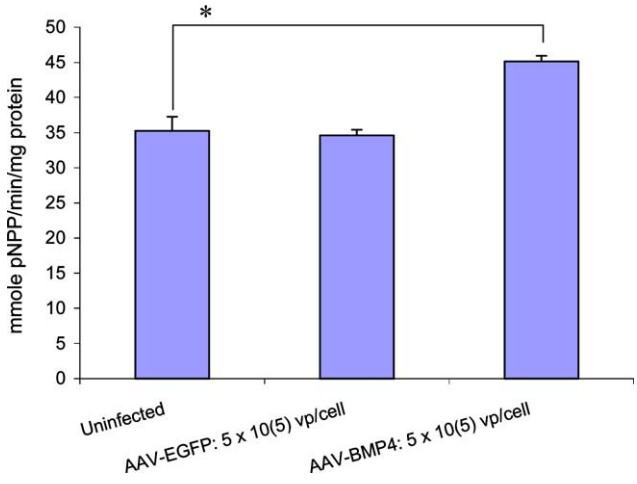

Fig. 4. Quantification of alkaline phosphatase activity in C2C12 cell lysates. Six days after infection of C2C12 cells with AAV-BMP4 or AAV-EGFP at an MOI (viral particles/cell) of 5×10^5, the ALP activity was measured from cell layers using the pNPP hydrolosis method. Values represent means ± SD ($n = 3$). Asterisk denotes statistically significantly differences from the uninfected cells ($p < 0.001$) as determined by two-tailed Student's test.

Histological evidences for endochondral ossification

To provide histological evidence for bone tissue, rats were sacrificed at 1, 3, and 8 weeks post-injection. Standard hematoxylin–eosin staining confirmed the bone structure only in rats injected with AAV-BMP4 vectors. One week after gene transfer, there was a significant accumulation of chondrocytes within the injected muscle tissues, which produced a cartilaginous matrix surrounded by some cells suggestive of undifferentiated mesenchymal cells (Figs. 6A and B). At 3 weeks post-injection, woven bone had formed characterized by the typical trabeculae structure, the irregular medullary cavity containing bone marrow-like cells and adipocyte-like cells. There were also many osteoblast-like cells, with some of them trapped within bone matrix as differentiated osteocyte-like cells. Some large multinucleate osteoclast-like cells also appeared which indicated the continuous remodeling of bone (Figs. 6C and D). Eight weeks later, woven bone became more mature, characterized by a well-defined and highly organized lamellar cortical bone matrix and an enlarged medullary cavity. Osteoblast- and osteoclast-like cells significantly reduced, and the mature osteocyte-like cells located in lacunae in the bone matrix were the predominant cell type at this stage (Figs. 6E and F). These observations

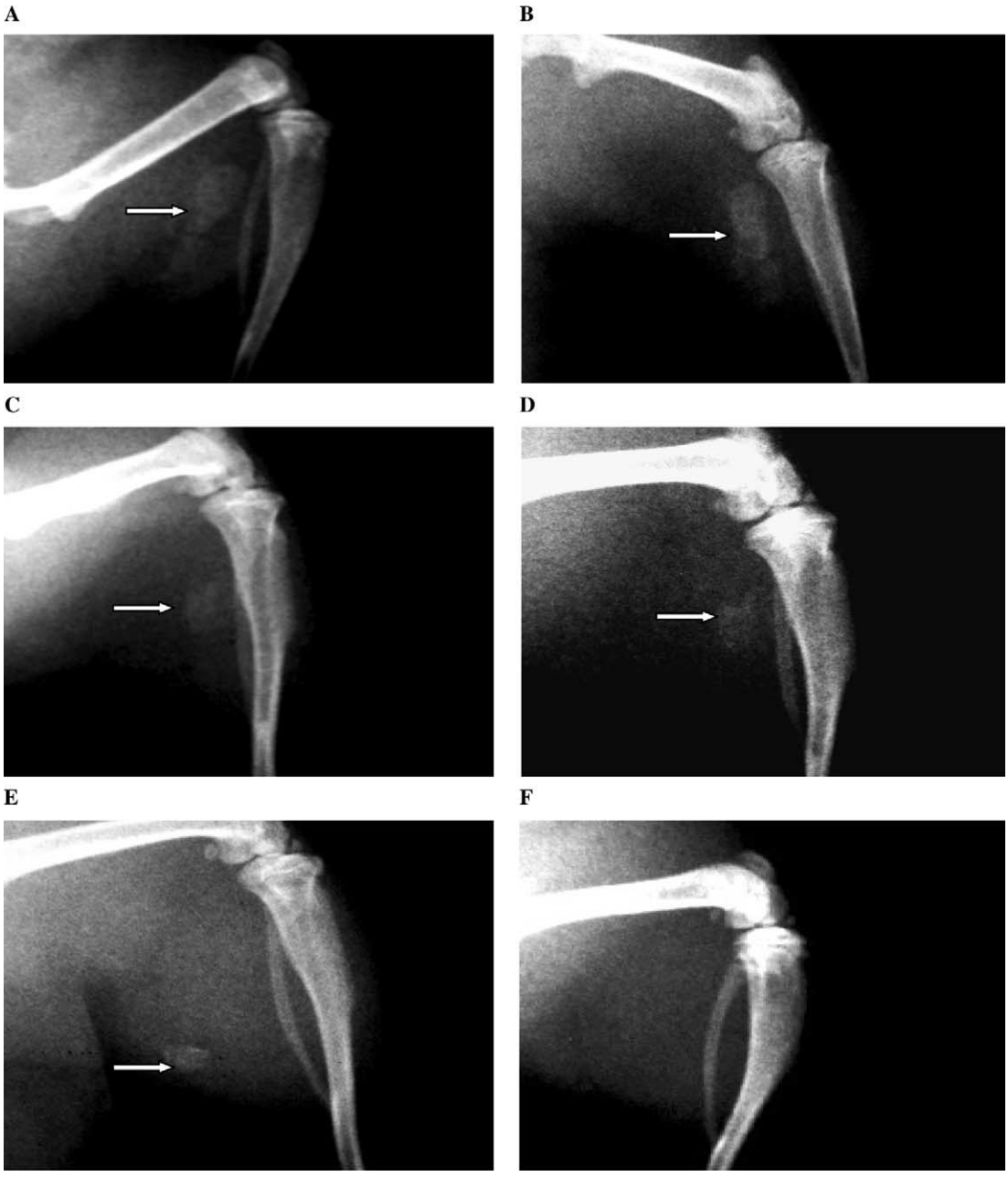

Fig. 5. Radiographic analysis of bone formation in immunocompetent SD rats. Significant bone formation could be detected by X-ray film in animals injected with AAV-BMP4 at a dosage of 2×10^{12} VP. No bone was formed in rats receiving AAV-EGFP or empty AAV. (A) Three weeks post-injection of AAV-BMP4; (B) 8 weeks post-injection of AAV-BMP4; (C) 3 weeks post-injection of AAV-BMP4; (D) 8 weeks post-injection of AAV-BMP4; (E) 8 weeks post-injection of AAV-BMP4; and (F) 8 weeks post-injection of AAV-EGFP. Arrows indicate the regional newly formed bone tissues.

demonstrated that AAV-BMP4 gene delivery had induced new bone formation via endochondral mechanism. In contrast, there were not any bone-related structures that had developed in rats receiving either AAV-EGFP or empty AAV (data not shown). It was also noticeable that there was no significant mononuclear cell infiltration, which indicated that no severe immunological responses had taken place. Throughout the whole experiment, all animals survived well with no apparent complications until they were sacrificed.

Immunohistochemical evidences for BMP4 expression

Immunostaining with BMP4 antibody in tissue sections indicated that BMP4 was only expressed in the whole bone-forming area and in closely adjacent muscle tissues at all time intervals. At 1 week post-injection, BMP4-positive staining was mainly detected in some chondrocytes within the injected muscles (Fig. 7A). Three weeks after transfer, only osteoblast-like cells invariably located on the margin of trabeculae structure

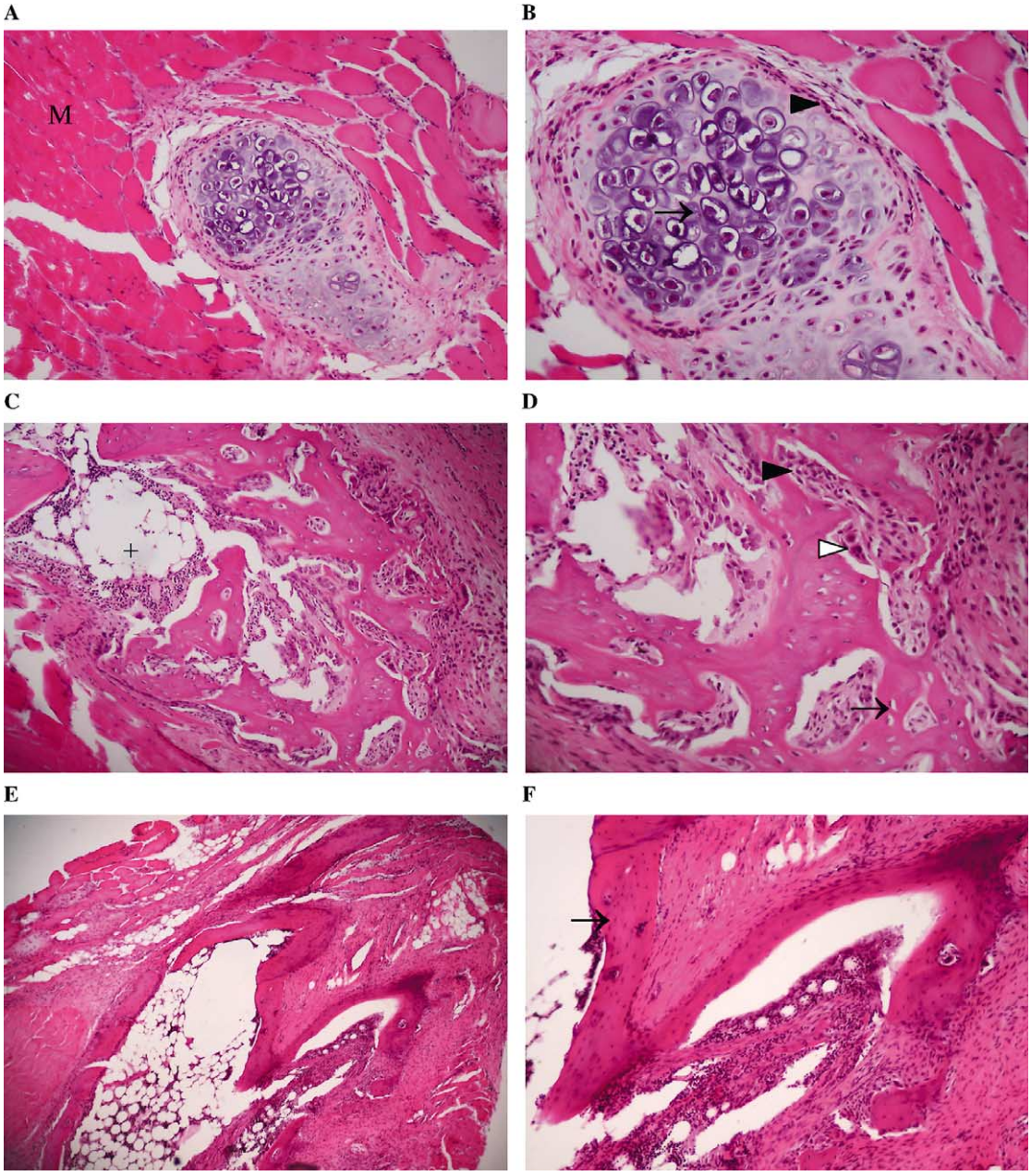

Fig. 6. Histological analysis of in vivo bone formation. At 1 week post-injection, there was a significant accumulation of immature chondrocytes (arrow) with an expanding cartilaginous matrix within skeletal muscle (M). Many cells surrounding the cartilaginous mass suggestive of undifferentiated mesenchymal cells (arrowhead) were also noticed. (A) Original magnification 10×. (B) Higher magnification 20×. At 3 weeks post-injection, woven bone was formed characterized by trabeculae structure, medullary cavity containing bone marrow-like cells and adipocyte-like cells (+). Note that other differentiated structures including osteoblast-like cells (solid arrowhead), multinucleated osteoclast-like cells (open arrowhead), and differentiated osteocyte-like cells (arrow) were also identified. (C) Original magnification 5×. (D) Higher magnification 10×. At 8 weeks post-injection, a highly organized lamellar cortical bone matrix with an enlarged medullary cavity was developed, which suggested that woven bone became mature. Note that more flattened osteocyte-like cells (arrow) were the major cell type within the bone tissue at this stage. (E) Original magnification 5×. (F) Higher magnification 10×.

displayed positive staining within woven bone area. No visible stains were developed in differentiated osteocyte-like cells in trabeculae (Fig. 7B). At 8 weeks post-injection, although the bone mass became more mature as characterized by high lamellar cortical bone matrix, the BMP4-positive staining could still be detected in many osteoblast-like cells lining the cortical matrix. In contrast, no BMP4 expression was detected in the highly differentiated osteocyte-like cells (Fig. 7C). In addition, there was also no positive staining detected in the distant muscles in AAV-BMP4-treated animals, or in injected muscles in AAV-EGFP- or empty AAV-treated animals at any time intervals (Fig. 7D). These observations suggested that AAV-BMP4 gene delivery could mediate

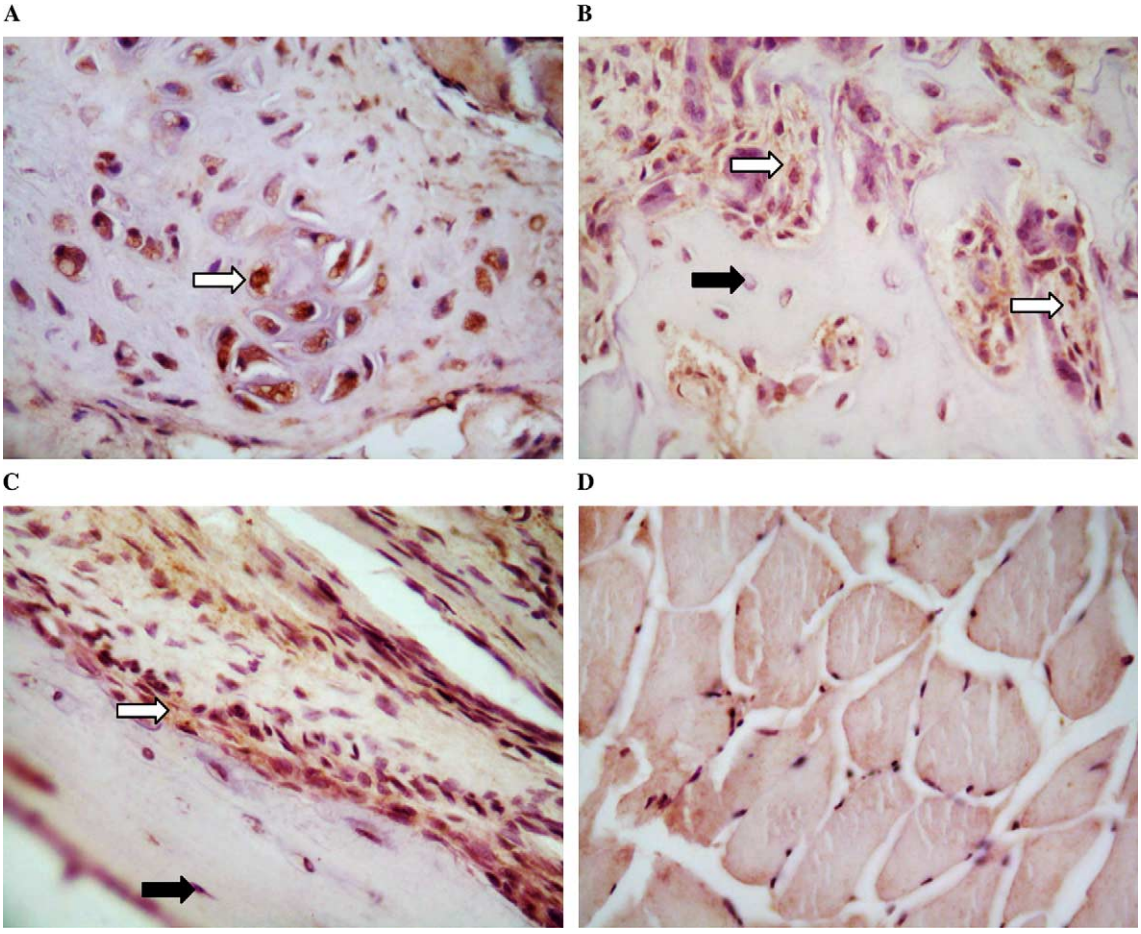

Fig. 7. Immunohistochemical analysis of in vivo BMP4 expression. At 1 week post-injection, BMP4 was mainly expressed in the cytoplasm of immature chondrocytes (arrow) within cartilaginous mass. (A) Original magnification 40×. At 3 weeks post-injection, positive staining was mainly detected in osteoblast-like cells (open arrow) located on the margin of trabeculae structure within bone mass. Note that no significant staining developed in differentiated osteocyte-like cells (solid arrow) in trabeculae. (B) Original magnification 40×. At 8 weeks post-injection, BMP4 was only expressed in osteoblast-like cells (open arrow) lining the highly organized lamellar cortical bone matrix. There was no positive staining developed in mature osteocyte-like cells (solid arrow) (C) Original magnification 40×. It was noticeable that no positive staining was detected in distant muscle tissues at all time intervals. (D) Original magnification 40×.

BMP4 expression, which plays an important role in the whole endochondral ossification. Moreover, since positive stains were still detected in closely adjacent muscles harvested at 8 weeks post-injection, these findings also suggested that AAV-BMP4 had mediated a long-term transduction in muscle tissues.

Fluorescence microscopy for AAV-mediated long-term transduction in muscle

At 1, 8 weeks post-injection, muscles that had been treated with AAV-EGFP were harvested for fluorescence microcopy. Based on gross visual assessment, there was still significant fluorescence intensity in the muscles harvested at 8 weeks post-injection. In contrast, no significant fluorescent signal was detected in the muscles injected with empty AAV (Fig. 8). These observations further confirmed that AAV-EGFP had mediated a long-term transduction (that is, at least 8 weeks after transfer) in skeletal muscles.

Discussion

Our present study demonstrated that AAV-mediated BMP4 gene delivery could successfully induce endochondral bone formation in SD rats. To the best of our knowledge, this is so far the first report in the field of AAV-based BMP gene transfer. Based on our results, we proposed that AAV-BMP4 gene therapy represents a novel and feasible approach to bone regeneration in immunocompetent animals, which could have a wide variety of preclinical and clinical applications.

In this study, mouse myoblast C2C12 cells were efficiently transduced with recombinant AAV-BMP4 vectors and produced BMP4 protein in vitro. This was

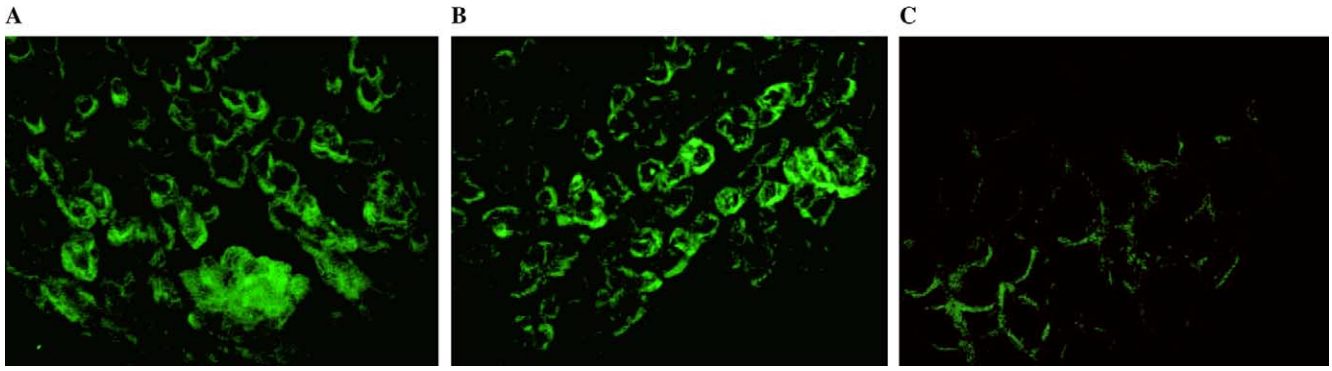

Fig. 8. Fluorescence analysis for AAV-EGFP-mediated transduction in muscle. Expression of EGFP was visualized in muscles injected with AAV-EGFP under fluorescence microscopy. (A) AAV-EGFP-infected muscles at 1 week post-injection. (B) AAV-EGFP-infected muscles at 8 weeks post-injection. (C) Empty AAV-infected muscles at 1 week post-injection. Original magnification 40×.

visualized by immunofluorescence analysis. Uninfected or AAV-EGFP-infected myoblast C2C12 cells generated multinucleated myotubes on day 6 post-injection. In contrast, cells treated with AAV-BMP4 remained as unfused mononuclear or polygonal phenotype, suggestive of an osteoblast phenotype [18]. Moreover, although there was an intrinsic ALP activity in untreated or AAV-EGFP-infected C2C12 cells, AAV-BMP4-transduced cells still demonstrated a significant increase of ALP activity. Since ALP is an important osteoblastic differentiation marker, therefore, in combination with phenotype changes, our in vitro study indicated that AAV-BMP4 gene delivery could not only inhibit myogenic differentiation of C2C12 cells, but also converted their differentiation pathway from a myoblast into an osteoblast.

To explore that AAV-mediated BMP4 gene delivery could also induce in vivo osteogenic activity, we performed direct intramuscular injection of AAV vectors in immunocompetent SD rats. Radiographic examination demonstrated that new bone tissues had developed as early as 3 weeks post-injection only in those rats receiving AAV-BMP4. Histological analysis revealed that newly formed bone tissues were induced via endochondral mechanism. Moreover, immunostaining with BMP4 antibody further indicated that BMP4 expression was mainly detected in chondrocytes and in osteoblast-like cells, but not in differentiated osteocyte-like cells. These results suggested that BMP4 was responsible for the bone-forming process. Meanwhile, positive BMP4 stains were also seen in those closely adjacent surrounding muscles in AAV-BMP4-injected animals, but not detected in any injected muscle samples harvested from AAV-EGFP- or empty AAV-treated animals. These findings demonstrated that BMP4 expression was only induced by gene delivery of AAV-BMP4 and that there was no intrinsic expression of BMP4 in muscle tissues.

Previous studies have documented that gene delivery by adenovirus vectors could only result in transient target gene expression, which usually persisted less than 3 weeks [19]. In contrast, our results suggested that BMP4 could be expressed for at least 8 weeks (the period tested in this study) after injection. However, since BMP-4 can elicit osteogenic differentiation of other cells that in turn express endogenous BMP4, we cannot conclude about such a cell whether it is a truly AAV-infected cell expressing exogenous transgene BMP4 or rather a host cell expressing endogenous BMP4 in response to exogenous transgene BMP-4 expression. To address this concern, we also observed expression of EGFP under fluorescence microscope in the muscles injected with AAV-EGFP. Since EGFP is a foreign protein expressed only in infected muscle cells, our observations demonstrated significant fluorescent signal even at 8 weeks post-injection. These findings supported the fact that AAV-based gene delivery could mediate a long-term transduction (e.g., at least 8 weeks post-injection in this study) for target gene. Moreover, since we also observed positive BMP4 staining in those closely adjacent muscle fibers surrounding bone mass even at 8 weeks post-injection, these results further confirmed that AAV-BMP4 gene delivery had also mediated a long-term transduction for new bone formation.

Recently, we also developed adenovirus-based BMP4 vectors and demonstrated that Ad-BMP4 gene delivery could exert osteogenic activity both in vitro and in vivo [9]. In that study, however, either the radiographic or the histological evidence of the newly formed bone mass was only presented in athymic nude (i.e., immunodeficient) rats, not in immunocompetent animals. Those results were consistent with a recent study in which bone formation by an adenoviral vector expressing BMP2 was only detected in nude rats but not in immunocompetent rats due to severe immunogenicity initiated by adenovirus [7]. In the present study, however, direct injection of AAV-BMP4 vectors in muscles had successfully induced new bone in immunocompetent animals without severe immune responses. These observations strongly demonstrated that AAV-based BMP vectors could provide better and safer gene transfer than its adenovirus counterpart.

In addition, our results also suggested that AAV-BMP4 gene therapy could induce bone formation only at the site of vector injection, which indicated that AAV-BMP4-mediated gene delivery would be clinically useful. The AAV-BMP vectors could be applied to fracture sites or segmental bone defects to stimulate osteoinduction, thus improving the rate and strength of bone healing. Addition of AAV-BMP4 to autologous bone grafts during spine stabilization may further improve their clinical efficacy by improving osteointegration of the graft and decreasing the amount of bone graft required. In addition, direct injection of BMP4 vectors into the spine may lead to long-term spine fusion, eliminating the requirement for an open spine surgery [20]. Furthermore, by utilizing the long-term transduction efficiency, AAV-BMP4 gene therapy may be particularly important and useful in the treatment of some chronic bone-wasting diseases (e.g., osteoporosis, osteopenia) that involve a long-term BMP expression.

Although our present results demonstrated the success of regional AAV-BMP4 gene therapy for bone reconstruction, many issues still need to be addressed before this novel approach can be further applied in clinical settings. For example, over a long period of time, whether the mass of the new bone will gradually increase due to the long-term expression of BMP4 should be considered. Recently, it has been reported that the use of a tetracycline-inducible promoter allows effective regulation of AAV-based therapeutic interleukin-10 (IL-10) gene expression after intramuscular injection into mice model of rheumatoid arthritis [21]. It seems that strictly regulated AAV-BMP gene therapy is very important for future clinical settings. Currently, our research group is investigating whether AAV vector, modified by addition of BMP promoter, could result in controlled BMP gene delivery. In addition, as to the source of "osteoprogenitors" in skeletal muscle, previous studies have indicated that muscle-derived stem cells (MDSCs), stimulated by BMP2 protein, could significantly undergo osteoblastic differentiation both in vitro and in vivo [22]. We therefore hypothesized that these MDSCs might play a pivotal role in this endochondral ossification in response to AAV-BMP4 gene transfer. Current work in our laboratory is also undertaken to further elucidate the role of MDSCs in this AAV-BMP4-mediated bone-forming process.

Gene therapy has received increasing attention during the past decade. There are numerous potential clinical applications for gene therapy approaches to enhance bone formation. In the present study, we have demonstrated that AAV-BMP4 gene therapy could induce new bone formation in immunocompetent animals. To our knowledge, this study is so far the first to establish the feasibility of AAV-based BMP gene transfer. Although many issues need to be addressed before AAV-BMP gene therapy can be further used for humans, this novel approach represents a promising and feasible technique for future clinical trials that require bone augmentation.

Acknowledgments

We thank Dr. Di Chen (Department of Medicine, University of Texas Health Science Center at San Antonio, TX 78284, USA) for kindly providing us pSK-BMP4 cloning plasmid containing full-length rat BMP4 cDNA. We are also grateful to the Laboratory Animal Unit, The University of Hong Kong for helping us with animal holding and maintenance. This study was supported by a University Research Grant (2000–2001) from The University of Hong Kong (Grant No. 10203525/07788/21000/323/01).

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