

RESEARCH ARTICLE

Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery

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Efficacious bone regeneration could revolutionize the clinical management of bone and musculoskeletal disorders. Although several bone morphogenetic proteins (BMPs) (mostly BMP-2 and BMP-7) have been shown to induce bone formation, it is unclear whether the currently used BMPs represent the most osteogenic ones. Until recently, comprehensive analysis of osteogenic activity of all BMPs has been hampered by the fact that recombinant proteins are either not biologically active or not available for all BMPs. In this study, we used recombinant adenoviruses expressing the 14 types of BMPs (AdBMPs), and demonstrated that, in addition to currently used BMP-2 and BMP-7, BMP-6 and BMP-9 effectively induced orthotopic ossification when either AdBMP-transduced osteoblast

progenitors or the viral vectors were injected into the quadriceps of athymic mice. Radiographic and histological evaluation demonstrated that BMP-6 and BMP-9 induced the most robust and mature ossification at multiple time points. BMP-3, a negative regulator of bone formation, was shown to effectively inhibit orthotopic ossification induced by BMP-2, BMP-6, and BMP-7. However, BMP-3 exerted no inhibitory effect on BMP-9-induced bone formation, suggesting that BMP-9 may transduce osteogenic signaling differently. Our findings suggest that BMP-6 and BMP-9 may represent more effective osteogenic factors for bone regeneration. Gene Therapy (2004) 11, 1312–1320. doi:10.1038/sj.gt.3302298; Published online 22 July 2004

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Introduction

Bone regeneration is critical to the effective management of many bone and musculoskeletal disorders, such as fracture healing, spinal fusion, and osteoporosis, which are responsible for a large portion of healthcare expenditure in the developed countries—approximately \$14 billion is spent annually on treating osteoporotic fractures in the US alone.¹ Bone is a highly mineralized tissue and is one of the few organs that retains the potential for regeneration in adult life. Bone also undergoes continuous remodeling throughout life.^{2,3} Three major types of cells are present in bone tissues: bone-forming osteoblasts, bone-resorbing osteoclasts, and chondrocytes. Osteoblasts are derived from the mesenchymal stem cells, which also serve as precursor cells for myocytes, adipocytes, and chondrocytes. It has been known for almost half a century that demineralized bone

can induce *de novo* bone formation.⁴ The molecular identity of the bone-forming factors in demineralized bone was subsequently revealed to be bone morphogenetic proteins (BMPs).⁵ BMPs belong to the TGF β superfamily, and play an important role in embryonic development and bone formation.^{6,7} At least 15 types of BMPs have been identified in humans. BMP signal transduction begins via interaction with the heterodimeric complex of two transmembrane serine/threonine kinase receptors, BMPR type I and BMPR type II.^{8,9} The activated receptor kinases phosphorylate the transcription factors Smads 1, 5, and/or 8. The phosphorylated Smads then form a heterodimeric complex with Smad 4 in the nucleus and activate the expression of target genes in concert with other coactivators.^{10–12}

Although the molecular mechanisms underlying osteoblast differentiation remain to be defined, BMPs play an important role in regulating osteoblast differentiation and subsequent bone formation. Traditionally, various bone grafts have been used to promote osteogenesis in bone and musculoskeletal disorders. The identification of BMPs has generated great interest due to their potential use in bone regeneration.³ Several recombinant forms of BMPs, mostly rhBMP-2 and rhBMP-7 (a.k.a., OP-1), have been shown to induce bone formation *in vivo*,^{13–24} and both rhBMP-2 and rhBMP-7 have been

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tested in clinical trials.^{25–27} In addition to the direct application of recombinant BMP proteins, numerous reports have confirmed the ability of adenoviral or retroviral vector-mediated gene transfer of several BMPs to induce bone formation in animal models.^{18,20,21,23,28–44}

Although a plethora of studies have demonstrated the ability of several BMPs, mostly BMP-2 and BMP-7, to promote osteogenesis, it is unclear whether or not BMPs other than those currently being tested in clinical trials are more potent stimulators of new bone formation. Thus, it is important to conduct a comprehensive comparative analysis of the *in vivo* osteogenic activity of all BMPs. This line of investigation has been hampered by the fact that recombinant proteins are either not biologically active or not available for all BMPs. We have recently constructed a panel of recombinant adenoviral vectors that express the 14 types of human BMPs (BMP-2–BMP-15).⁴⁵ Recombinant adenoviral vectors are ideal for this line of investigation for following reasons.^{46–51} First, adenoviral vectors can transduce osteoblast progenitor cells with high efficiency. Second, the biologically active BMPs are continuously produced inside mammalian cells. Third, BMP-mediated bone osteoblast differentiation does not require long-term expression. Fourth, adenoviral vectors can be used in both *in vitro* and *in vivo* studies. In this study, we sought to carry out a comprehensive analysis of the distinct *in vivo* bone-forming activity of the 14 types of human BMPs. Using an orthotopic ossification animal model, we demonstrate that BMP-2, BMP-6, and BMP-9 (BMP-7 to a lesser extent) are the most potent osteoinductive BMPs. Our findings strongly suggest that, in addition to BMP-2 and BMP-7 that are currently used in clinical trails, BMP-6 and BMP-9 could represent

equally, if not more effective osteogenic factors for bone regeneration in a clinical setting.

Results

Distinct ability to induce an osteogenic marker, alkaline phosphatase (ALP), by 14 BMPs in the osteoblast progenitor C2C12 cells *in vitro*

In order to comprehensively analyze the distinct osteogenic activity of BMPs, we have recently constructed a panel of recombinant adenoviral vectors that express the 14 types of human BMPs, designated as AdBMPs.⁴⁵ As shown in Figure 1a, the level of transgene expression of the AdBMPs were in general comparable (ie, <2-fold difference among different BMPs), as demonstrated by RT-PCR analysis. These PCR products should be specifically derived from the adenoviral vector-mediated expression (rather than from the endogenous genes), as the 3'-end primer was derived from the SV40 poly-A cassette. Using these adenoviral vectors, we have recently demonstrated that BMPs displayed a distinct ability to induce osteoblast differentiation of mesenchymal progenitor cells *in vitro*.⁴⁵ In this study, we sought to determine the relative *in vivo* osteogenic activity of the 14 types of BMPs. We first tested the ability of individual AdBMPs to induce the earlier osteogenic marker alkaline phosphatase in the C2C12 line, which is myoblastic and can be trans-differentiated into osteoblast progenitors upon BMP stimulation. As shown in Figure 1b, ALP activity was remarkably induced by five of the 14 BMPs at four days after AdBMP infections. Among the five osteogenic BMPs, BMP-2, BMP-6, and BMP-9 induced the ALP activity to a much greater extent

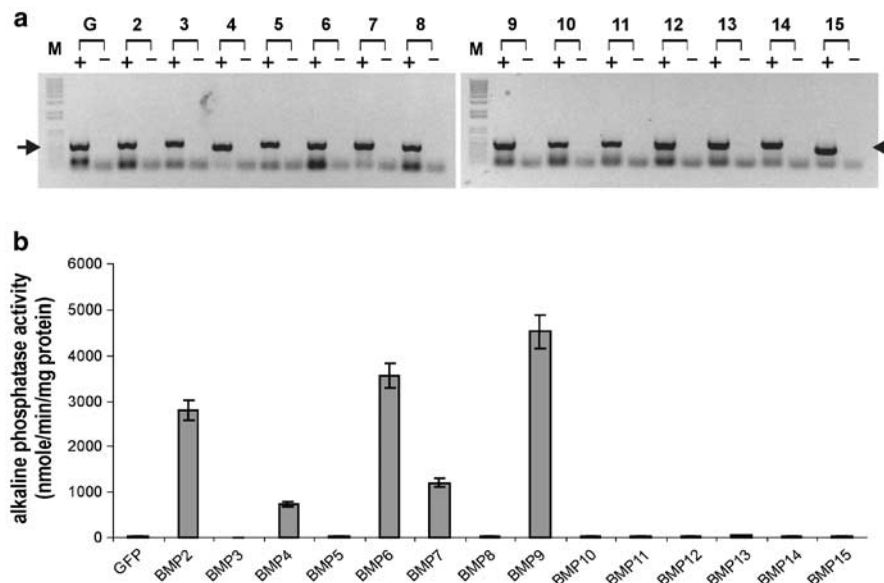


Figure 1 Induction of alkaline phosphatase activity by the 14 types of human BMPs in C2C12 cells. (a) Relative level of AdBMP-mediated transgene expression. C2C12 cells were infected with AdBMPs or AdGFP for 40 h. Total RNA was isolated and converted into cDNA products by reverse transcription, which were used for RT-PCR reactions using BMP-specific primers (5'-end) and a 3'-end primer derived from SV40 polyA. Resultant products ranged from 500 to 600 bps (indicated by arrows). '+', PCR products from +RT reactions of the original cDNA synthesis; '-', PCR products from -RT reactions of the original cDNA synthesis; 'M', 1-Kb Plus ladder from Invitrogen; 'G', AdGFP; '2–15', AdBMP-2 to AdBMP-15. (b) Subconfluent C2C12 cells were infected with AdBMPs and the control AdGFP. At 4 days after infection, cells were lysed for colorimetric assays of alkaline phosphatase activity using p-nitrophenyl phosphate as a substrate. Representative results from at least three independent experiments are shown. See Materials and methods for details.

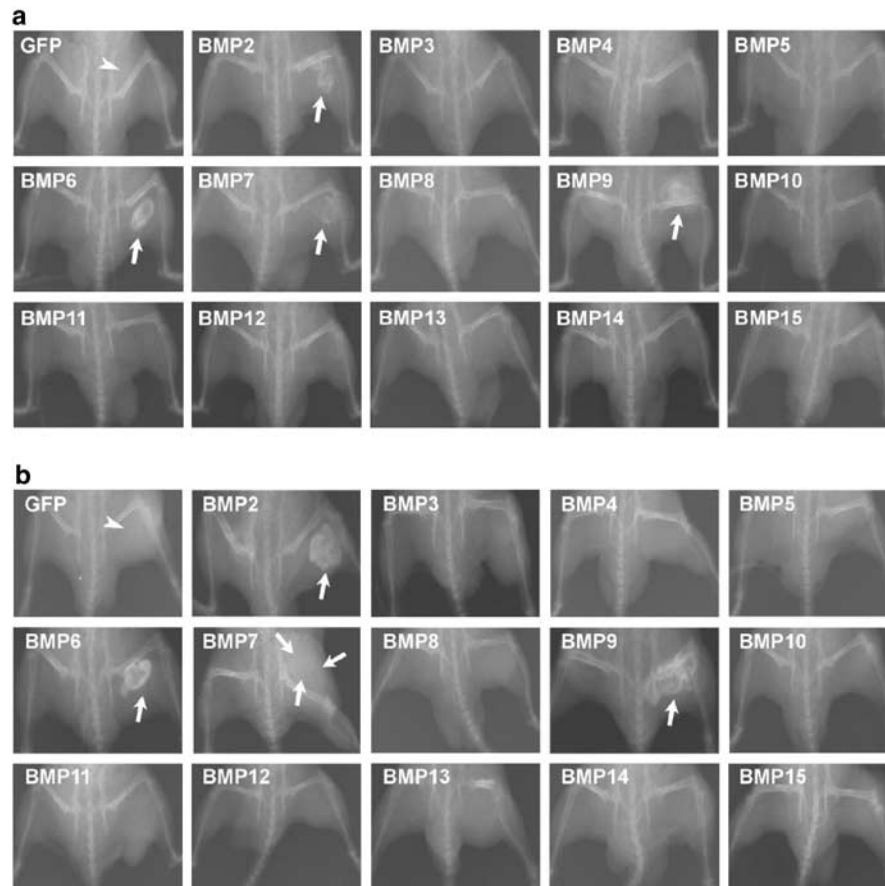


Figure 2 Orthotopic bone formation induced by AdBMP-transduced C2C12 in athymic nude mice. Exponentially growing C2C12 cells were infected with AdBMPs or the control AdGFP for 15 h. Approximately 5×10^6 of the infected cells were injected into the right quadriceps of athymic nude mice (a triangle indicated in the GFP group as an example). At 3 weeks (a) and 5 weeks (b) after injections, mice were killed and subjected to X-ray radiography. Each experimental group contained four mice, and representative radiographies from three batches of experiments were shown.

(approx. 169-, 215-, and 273-fold over the GFP control, respectively), while BMP-4 and BMP-7 increased ALP activity by 44- and 73-fold, respectively. These findings are consistent with our previous studies.⁴⁵ It should be pointed out that several BMPs (eg, BMP-10, and BMP-13) also induced a 2–3-fold increase of ALP activity over the basal level under the same assay conditions.

Orthotopic bone formation effectively induced by several but not all BMPs in athymic mice

We next sought to test the *in vivo* osteogenic effect of the 14 BMPs. In order to effectively assess the osteogenic ability of the BMPs, we used an orthotopic ossification animal model, in which C2C12 cells were first transduced with AdBMPs and subsequently implanted into the quadriceps muscles of athymic nude mice by intramuscular injection. Orthotopic ossification was assessed by X-ray radiography and histological evaluation at 3 and 5 weeks after injections. As illustrated in Figure 2, ossification was readily detected on X-ray radiographies from the animals injected with AdBMP-2, 6, 7, and 9-transduced C2C12 cells at 3 weeks (Figure 2a) and 5 weeks (Figure 2b). For BMP-6 and BMP-9, histologic examination at both time points (Figure 3a and b) revealed robust and highly mineralized woven bone with scattered osteoblast-rimming and occasional

osteoclasts. Osteoid was also present. In addition, BMP-6 showed bone marrow elements with a range of hematopoietic cells. At the 3-week time point, BMP-2 was characterized by well-calcified foci without bone formation; however, at the 5-week time point, these foci had developed into mature bone. BMP-7, on the other hand, was shown to induce much weaker ossification. Interestingly, we failed to detect any signs of ossification in the animals injected with AdBMP-4-transduced C2C12 cells, which is surprising because we have demonstrated that BMP-4 is capable of inducing ALP activity *in vitro* (Figure 1b).⁴⁵ These results were reproducible in two additional rounds of animal studies using different batches of AdBMP-4 preparations, which were consistently shown to induce ALP activity in C2C12 cells *in vitro* (data not shown). Further, our RT-PCR analysis demonstrated that the expression level of BMP-4 was comparable with that of other BMPs, especially BMP-2, BMP-6, and BMP-9 under the same assay condition (Figure 1a). Currently, we are still searching for any satisfactory explanations for this discrepancy between BMP-4's *in vitro* versus *in vivo* osteogenic activity. Nevertheless, all of the above findings were reproducible in three batches of independent experiments. The remaining samples had no evidence of ossification at 5 weeks. The injection site in these sections demonstrated exuberant granulation tissue and reparative changes.

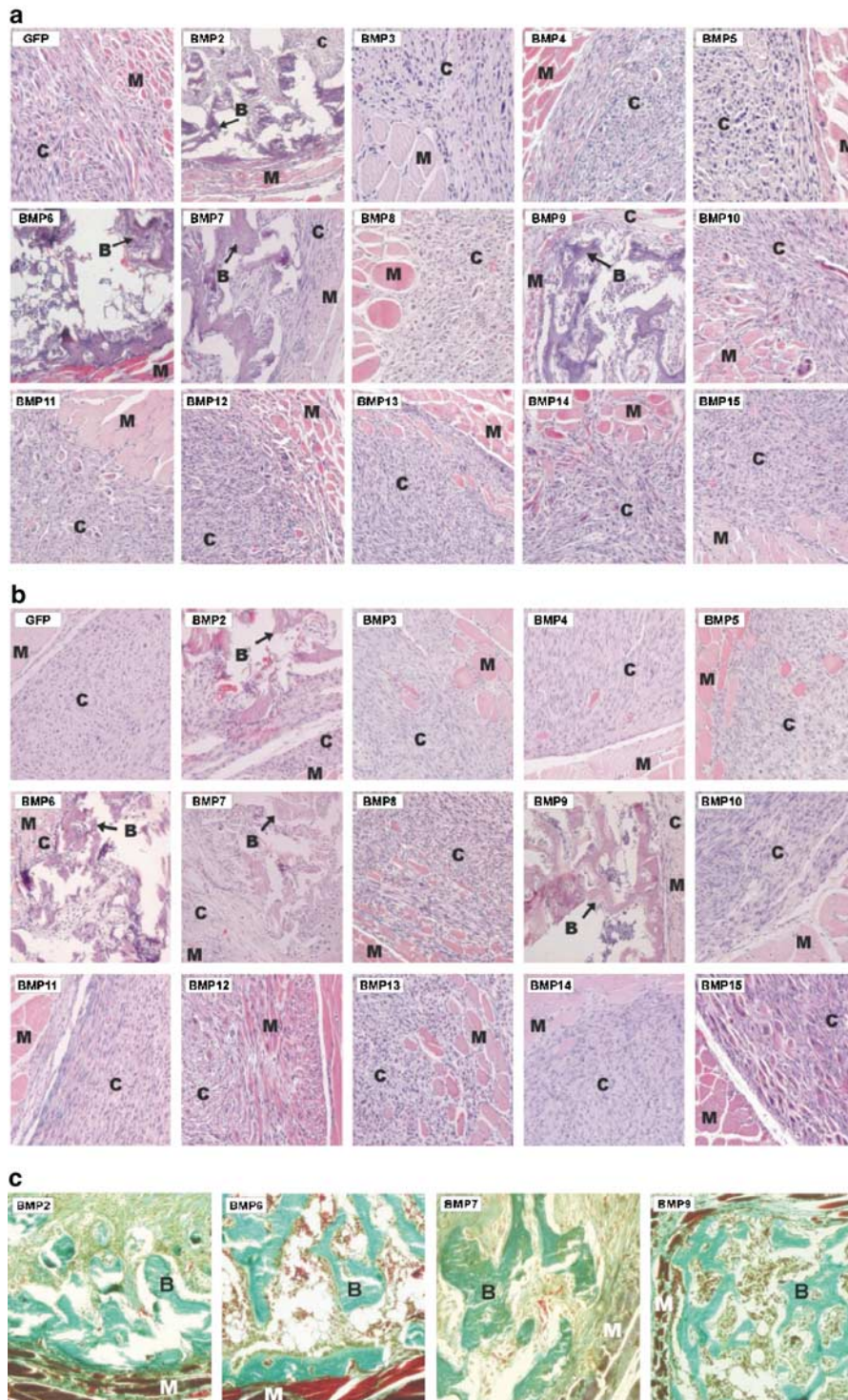


Figure 3 Histological evaluation of AdBMP-induced orthotopic ossification. (a) H & E staining of the AdBMP-transduced C2C12 injection sites at 3 weeks. (b) H & E staining of the AdBMP-transduced C2C12 injection sites at 5 weeks. (c) Masson's Trichrome staining of the AdBMP-transduced C2C12 injection sites at 3 weeks. Muscle fibers and cytoplasm were stained red and collagen of osteoid matrix were stained blue. B, osteoid matrix (indicated by arrows); C, injected C2C12 cells; and M, muscle cells. Magnification, $\times 200$.

Histology of BMP-induced bone formation

We next examined the histology of the recovered injection sites. Overall, the histology correlated well with the findings from X-ray radiography. At the 3-week time point, BMP-2, BMP-6, BMP-7, and BMP-9 demonstrated varying degrees of ossification. BMP-2 and BMP-7 were

the least developed with small foci of woven bone (Figure 3a). Both BMP-6 and BMP-9, however, had multiple foci of immature woven trabecular bone. In addition, BMP-9 demonstrated focal cartilaginous differentiation. The bone in BMP-6 and BMP-9-treated animals formed a shell-like rim around a proliferating mass of

spindle-shaped C2C12 cells. The 5-week samples demonstrated increased maturation with more mature osteoid matrix and trabecular bone-like structures with accentuation of the shell-like rim, especially in BMP-6 and BMP-9-treated animals. There was less extensive ossification in the BMP-2 sections. Bone marrow elements were present in the BMP-6 sections and chondrocytes and cartilaginous matrix were increased in the BMP-9 sections (Figure 3b). Interestingly, the injected C2C12 cells formed a desmoid-like cell mass in nonosteogenic BMP injections and the GFP control. Even in the animals injected with BMP-2, BMP-6, BMP-7, and BMP-9-transduced C2C12 cells, such cell mass was still visible, and multiple ossification centers were observed at the periphery of the cell mass. BMP-2-, BMP-6-, BMP-7-, and BMP-9-induced osteogenesis was further confirmed by Masson's Trichrome staining (Figure 3c).

Antagonistic effect of BMP-3 on BMP-induced bone formation

We sought to investigate how the osteogenic BMPs were affected by BMP-3, a known negative regulator of bone formation, as BMP-3 knockout animals exhibited an increase in bone density.⁵² When C2C12 cells transduced by AdBMP-3 and one of the four osteogenic AdBMPs

were coinjected intramuscularly for 3 weeks, BMP-2 and BMP-6-induced ossification was completely blocked by BMP-3, and most of the BMP-7-induced ossification was inhibited by BMP-3 (Figure 4a). However, BMP-3 coinjection did not exert any effect on BMP-9-induced calcification (Figure 4a), strongly suggesting that BMP-9 may exert its osteogenic activity via a distinct signaling mechanism. Similar results were obtained for the 5-week groups (data not shown). The histological findings were consistent with those from X-ray radiographic results (Figure 4b). The three samples without ossification demonstrated C2C12 cell proliferation with entrapped skeletal muscle while the BMP-3+BMP-9 sections had multiple foci of woven trabecular bone similar to BMP-9 injection alone.

Bone formation induced by direct intramuscular injection of AdBMPs

Recent studies suggest that skeletal muscles may harbor pluripotent mesenchymal stem cells, including osteoblast progenitors.^{34,44,53} We next tested the osteoinductive activity of the 14 BMPs via direct intramuscular injection of AdBMPs. At the 3 and 5-week time points, we did not observe apparent ossification on X-ray radiography (data not shown). However, when the 5-week injection sites

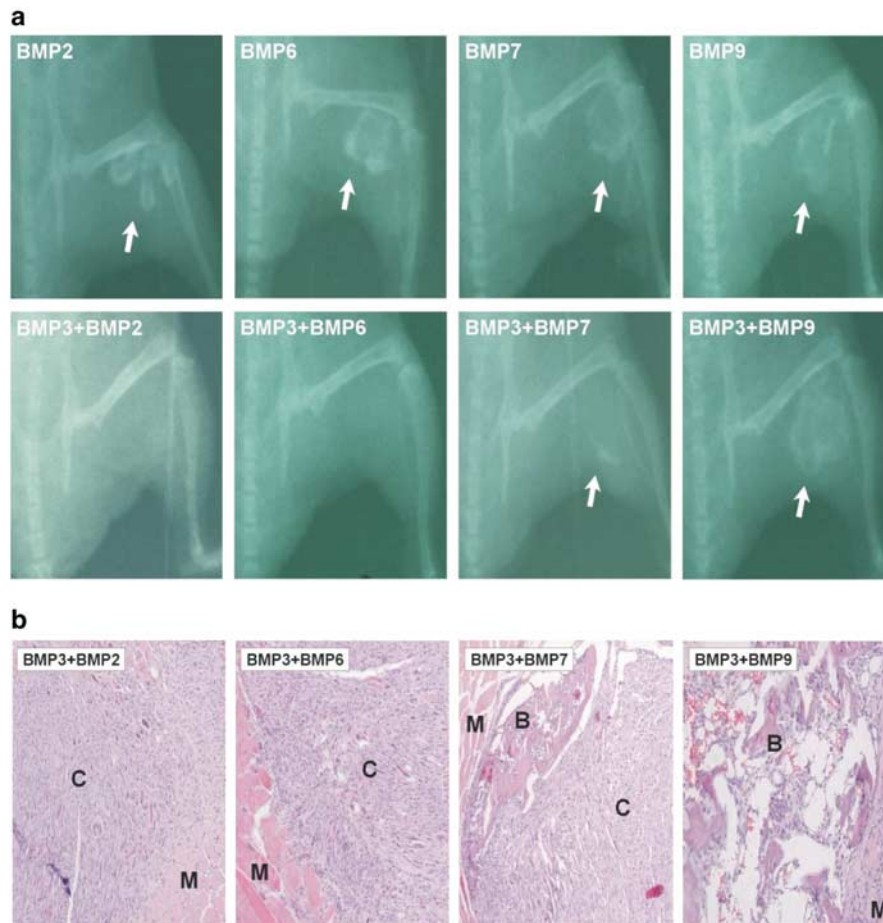


Figure 4 BMP-3-mediated inhibition of bone formation induced by BMP-2, BMP-6, and BMP-7, but not by BMP-9. (a) Osteogenic AdBMPs (ie, BMP-2, -6, -7, and -9)-transduced C2C12 cells were either injected alone (top row) or coinjected with AdBMP-3 (bottom row) into the right quadriceps of athymic mice. Animals were killed at 3 weeks and subjected to X-ray radiography. Ossification sites were indicated by arrows. Each experimental group contained four mice, and representative radiographies from three batches of experiments are shown. (b) Histological evaluation of the BMP-3 coinjection sites. B, osteoid matrix; C, injected C2C12 cells; and M, muscle cells. Magnification, $\times 150$.

were examined histologically, various degrees of cartilaginous and/or osteoid matrix formation were observed in BMP-2, BMP-6, BMP-7, and BMP-9-injected animals (Figure 5). Samples derived from BMP-2 and, to a lesser extent, BMP-7 injection sites contained more cartilage-like chondrocyte-containing structure, while osteoid matrix and mature lamellar bone were present with evidence of bone marrow colonization and remodeling in BMP-6 and BMP-9-injected animals. Unlike in the experiments with C2C12 injections, direct intramuscular injections with AdBMPs (ie, the above-mentioned four osteogenic BMPs) induced more diffuse ossification. This may also explain why the calcification (ie, by BMP-9) was not readily detected by X-ray radiography. These findings also suggest that orthotopic osteogenesis induced by direct intramuscular injection with osteogenic AdBMPs may be less efficient than that induced by introduction of AdBMP-transduced osteoblast progenitor cells, implying that osteoblast progenitor cell-based gene therapy may be a more efficacious approach to bone regeneration, although it is possible that the reduced bone formation was resulted from the potentially less-efficient gene transfer associated with direct intramuscular injections than that with AdBMP-transduced C2C12 cells.

Discussion

Successful bone regeneration mediated by biofactors could revolutionize the clinical management of musculoskeletal disorders, including fracture healing and

spinal fusion.⁵⁴ Several biological factors, such as TGF β , BMP, FGF, PDGF, IGF, and LMP-1, have been investigated for their potential use in bone regeneration and skeletal repair.^{55–69} BMPs have been shown to be the most promising, and clinical trials with recombinant BMP-2 and -7 are ongoing.^{54,70–73} These BMPs have shown varying degrees of success in the clinical setting and further study on their mechanisms of action and optimal formulations is required to optimize effectiveness of this strategy for promoting osteogenesis.

To the best of our knowledge, this reported study represents the first of its kind to evaluate the *in vivo* osteogenic activity of BMPs in a comprehensive fashion. The observation that BMP-2 exhibited osteogenic activity in our study is consistent with early data from human clinical trials.^{25,27} While BMP-7 exhibited apparent osteogenic activity, its ability to induce ossification was significantly less robust than that of BMP-2, BMP-6, and BMP-9. These findings mirror the moderate success of the rhBMP-7 (ie, OP-1) in a recent clinical trial.²⁶

It is intriguing, however, that BMP-6 and BMP-9 emerged as the most potent inducers of orthotopic ossification *in vivo*. Although considerable genetic and developmental studies have been conducted to elucidate the biological functions of BMP-6, its osteogenic activity has not been investigated to any significant degree in animal studies or clinical trials. BMP-6-deficient mice are largely unremarkable, with the exception of a defect in the sternum.⁷⁴ Its expression during embryogenesis is closely coupled with BMP-2, and the lack of noticeable defects in BMP-6-deficient mice may be due to functional compensation by BMP-2.⁷⁴ Nevertheless, our findings corroborate well with a recent study in which BMP-6 was shown to induce the most rapid tissue calcification when compared with BMP-2 or BMP-4 in an athymic nude rat model,⁷⁵ although for reasons to be determined AdBMP-4 reproducibly failed to induce orthotopic bone formation in this study.

BMP-9 is one of the least studied members of the BMP family. Originally identified from fetal mouse liver cDNA libraries, BMP-9 (a.k.a., GDF-2) is highly expressed in the developing mouse liver, and recombinant human BMP-9 (rhBMP-9) stimulates hepatocyte proliferation.⁷⁶ BMP-9 has also been shown to be a potent synergistic factor for hematopoietic progenitor cell generation and colony formation,⁷⁷ and may also play a role in the induction and maintenance of the neuronal cholinergic phenotype in the central nervous system.⁷⁸ In addition, it has recently been shown that BMP-9 exhibits an apparent osteoinductive effect in rat models.^{21,23,79} However, the mechanisms underlying BMP-9-mediated osteogenic signaling remain to be defined. It is a very intriguing finding that BMP-9-mediated bone formation was not inhibited by BMP-3 in our studies. This result strongly suggests that BMP-9 may transduce a distinct osteogenic signaling pathway that is significantly different from that of BMP-2, BMP-6, and BMP-7. Through an expression profiling analysis, we have recently identified a group of downstream targets that may play an important role in the osteogenic BMP signaling pathway mediated by BMP-2, BMP-6, and BMP-9.⁸⁰ Interestingly, while BMP-2, BMP-6, and BMP-9 induced a very similar overall gene expression pattern, the clustering analysis revealed that BMP-2 and BMP-9 exhibited a more similarly related expression pattern.⁸⁰

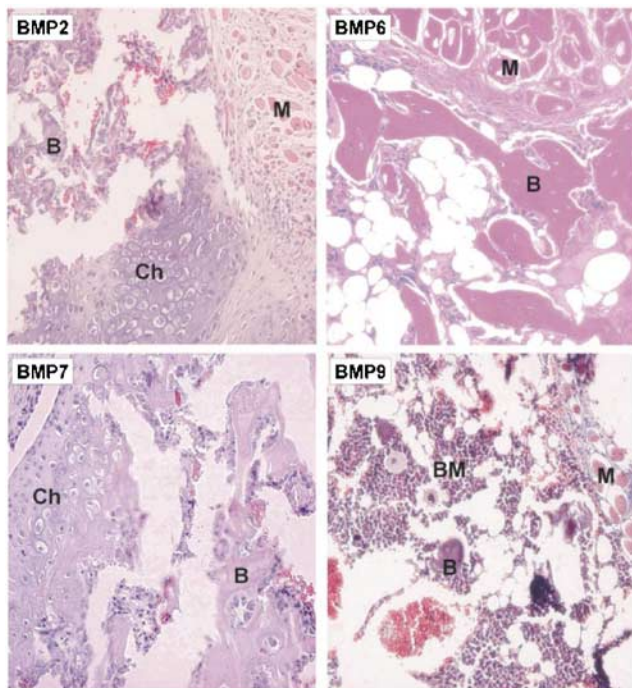


Figure 5 Orthotopic bone formation induced by direct intramuscular injection of AdBMPs. Approximately 10^9 PFU of AdBMPs or AdGFP were directly injected into the quadriceps of athymic mice. Animals were killed at 5 weeks after injection and subjected to X-ray radiography (not shown). Each experimental group had four mice. Representative results from two independent experiments are shown. BMP-6-treated sample was decalcified. B, osteoid matrix; BM, bone marrow cells; Ch, chondrocytes; and M, muscle cells. Magnification, $\times 200$.

In conclusion, we have demonstrated the relative osteogenic ability of 14 BMPs and identified BMP-6 and BMP-9 (in addition to the currently used BMP-2 and BMP-7) as the most potent BMPs to induce orthotopic bone formation *in vivo*. Our results also suggest that the stem/progenitor cell-based *ex vivo* gene therapy may represent a more effective approach to bone regeneration. Future studies will focus on elucidating the major signaling differences among BMPs so that maximal synergy in bone formation can be achieved by combining BMPs that act through overlapping or converging signaling pathways. This line of investigation would help to elucidate the molecular mechanisms underlying bone formation and lead to the development of more efficacious approaches towards bone regeneration.

Materials and methods

Cell culture and chemicals

HEK 293 and C2C12 cell lines were obtained from the ATCC (Manassas, VA, USA), and were maintained in complete DMEM supplemented with 10% fetal calf serum (FCS, Mediatech, Herndon, VA, USA), 100 U of penicillin, and 100 μ g of streptomycin at 37°C in 5% CO₂. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

Recombinant adenoviral vectors expressing BMPs

The cDNA clones for human BMP-2, -3 (a.k.a., osteogenin), -4, -5, -6, -8 (a.k.a., OP-2), -9 (a.k.a., GDF-2), -10, -12 (a.k.a., GDF-7 or CDMP-3), and -13 (a.k.a., GDF6 or CDMP2) were kindly provided by the Genetics Institute (Cambridge, MA, USA). The coding sequences for BMP-7 (a.k.a., OP-1), -11 (a.k.a., GDF-11), -14 (a.k.a., GDF-5 or CDMP-1), and -15 (a.k.a., GDF-9) were PCR amplified from a human osteosarcoma cDNA library. The coding regions of the above BMPs were subcloned into pAdTrack-CMV, resulting in pAdTrack-BMPs; recombinant adenoviruses expressing BMPs (ie, AdBMPs) were subsequently generated as previously described.^{45,81} For a control, we used an analogous adenovirus expressing only GFP (ie, AdGFP), as previously described.⁸¹ Details on vector constructions are available upon request.

RNA purification and reverse transcriptase-PCR analysis

The RT-PCR analysis was carried out as previously described.⁸⁰ Specifically, C2C12 cells were seeded in 25 cm² cell culture flasks, and infected with an optimal and compatible titer of AdBMPs or AdGFP. At 40 h after infection, total RNA was isolated using RNAgent Total RNA Isolation kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Total RNA (10 μ g) was used to generate cDNA templates for reverse transcriptase-PCR. The first-strand cDNA synthesis was performed using a hexamer (Promega, Madison, WI, USA) and Superscript II reverse transcriptase (Invitrogen). The first-strand cDNA products were further diluted 50-fold and used as PCR templates. Expression level was determined by touchdown PCR analysis using respective pairs of oligonucleotides to amplify the 3'-end of each BMP gene and the 5'-end of SV40 poly A region. Touchdown PCR was performed by using the following program: 94°C \times 2 min for one cycle,

12 cycles at 92°C \times 20 s, 68°C \times 30 s, and 70°C \times 45 s with a decrease of one degree per cycle, and 35 cycles at 92°C \times 20 s, 55°C \times 30 s, and 70°C \times 45 s. The amplified products (ranging from 500–600 bps) were resolved on 1% agarose gels, and visualized under UV light after ethidium bromide staining.

Determination of alkaline phosphatase activity

Exponentially growing C2C12 cells were seeded in 48-well cell culture plates, and infected with AdBMPs or AdGFP (multiplicities of infection, or MOIs = 50–200). The induction of alkaline phosphatase activity was assessed at 4 days after infection. Alkaline phosphatase activity was determined by using *p*-nitrophenyl phosphate (Sigma-Aldrich) as a substrate. Absorbance at 405 nm was recorded at 1, 2, and 3 min after the cell lysate was mixed with the substrate. Each assay condition was carried out in triplicate and normalized with the concentrations of total cellular proteins. Enzyme activity was expressed as nanomoles of *p*-nitrophenol produced per minute per mg of total cellular proteins.

Orthotopic bone formation in athymic nude mice

The use of animals was approved by the Institutional Animal Care and Use Committee. Young athymic nude mice (male, 5–6 months, Frederick Cancer Research Center) were used in this study. Each experimental group had four animals. For the injection with adenovirus-transduced C2C12 cells, subconfluent C2C12 cells were infected with AdBMPs or AdGFP at preoptimized titers (MOIs ~ 50–100). At 15 h after infection, cells were collected and resuspended in PBS at an approximate density of 1×10^8 cells/ml. In total, 50 μ l of the cell suspension (approx. 5×10^6 cells) was used for the intramuscular injection of right quadriceps. For cell mixing experiments, approximately 2.5×10^6 cells of AdBMP-3- and AdBMP-infected cells were combined prior to intramuscular injections. For direct intramuscular injections, approximately 10^9 PFU of AdBMPs or AdGFP were briefly dialyzed against PBS to remove CsCl, and suspended in a final volume of 50 μ l for direct injections into the right quadriceps. Injected animals resumed activities immediately without any restraints on food and drink. At 3 and 5 weeks after injections, animals were killed and subjected to X-ray radiography. The injected sites were harvested for histological evaluation. Representative results from three independent batches of experiments are shown.

H & E staining and Masson's Trichrome staining

After X-ray radiography, the injected thighs were recovered, fixed in 10% formalin overnight, and embedded in paraffin. Serial sections at 12 μ m of the embedded specimens were carried out, and mounted onto treated slides. The sections were stained with hematoxylin and eosin (H & E) and Masson's Trichrome. Some samples were subjected to calcification prior to H & E staining.

Note added in proof

While this report was under review, Li LZ *et al* coincidentally analyzed the osteogenic activity of BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9 (*Gene Therapy* 2003;

10:1735–1743). Their findings also suggest that BMP-9 is one of the most osteogenic BMPs in rat models of bone formation.

Acknowledgements

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