

Distinct osteogenic activity of BMPs and their orthopaedic applications

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Bone is the only tissue that undergoes continual remodeling throughout life, and is one of the few organs that retain the potential for regeneration in adult life¹. The identification of growth factors that induce bone formation has stimulated an enormous interest in using bone-forming factors to improve bone regeneration. Although several BMPs (notably, BMP-2 and BMP-7) have been used as bone regeneration agents, it was not clear whether they were the most osteogenic BMPs. A thorough analysis of the osteogenic activity of all BMPs was hampered by the fact that recombinant BMP proteins are not available for all BMPs, and/or the available recombinant BMPs are not biologically active. To elucidate the osteogenic activity of all BMPs, we constructed recombinant adenoviruses that express human BMP-2 to 15, using our recently developed AdEasy system. We evaluated the *in vitro* effects of individual BMPs on osteogenic differentiation in mesenchymal progenitor cells C3H10T1/2 and C2C12 by measuring the osteogenic markers such as alkaline phosphatase (Figure 1) and osteocalcin, as well as matrix mineralization. Our *in vitro* findings strongly suggest that BMP-2, 6, and 9 (to a lesser extent, BMP-4 and 7) may be highly capable of stimulating mesenchymal stem cells to undergo osteoblastic differentiation.

We next examined the *in vivo* osteogenicity analysis of the 14 BMPs. We used an orthotopic ossification animal model, in which C2C12 cells were first transduced with AdBMPs and then injected into the quadriceps muscles of athymic nude mice by intramuscular injection (5×10^6 cell per injection). Orthotopic ossification was assessed by X-ray radiography and histological evaluation at three and five weeks after injection.

As illustrated in Figure 2A, ossification was readily detected on X-ray radiography from the animals injected with AdBMP-2, 6, and 9 (to a lesser extent, BMP-7) transduced C2C12 cells as early as at 3 weeks. Interestingly, we reproducibly failed to detect any signs of ossification in the animals injected with AdBMP-4-transduced C2C12 cells, and the discrepancy of *in vitro* vs. *in vivo* osteogenic activity of BMP-4 is not currently understood. We also examined the histology of the recovered injection sites. Overall, the histology correlated well with the findings from X-ray radiography. At 3-week time points, BMP-2, BMP-6, BMP-7, and BMP-9 demonstrated varying degrees of ossification (Figure 2B). For BMP-6 and BMP-9, histological examination at both time points revealed robust and highly mineralized woven bone with scattered osteoblast-rimming and occasional osteoclasts. BMP-7 was shown to induce much weaker ossification. The remaining samples had no evidence of ossification. BMP-2, 6, 7, and 9-induced osteogenesis was further confirmed by Masson's Trichrome staining. We also tested the osteoinductive activity of the 14 BMPs via direct intramuscular injection of AdBMPs. Recent studies suggest that skeletal muscles may harbor pluripotent mesenchymal stem cells, including osteoblast progenitors. At 3- and 5-week time points, we did not observe apparent ossification on X-ray radiography, while various degrees of cartilaginous and/or osteoid matrix formation were observed in BMP-2, 6, 7, and 9-injected animals when the 5-week injection sites were examined histologically. We have further confirmed the osteogenic activity of BMP-2, 6, and 9 in a rabbit spinal fusion model.

To gain insights into the molecular basis of BMP-mediated osteogenesis, we conducted an expression profile analysis of genes whose expression was regulated by osteogenic BMPs. We were particularly interested in elucidating the early signaling events upon BMP stimulation. Exponentially growing C2C12 cells were infected with three osteogenic BMP viruses (AdBM-2, 6, or 9), along with AdGFP (mock), AdBMP-3 (a negative BMP), and AdBMP-12 (a non-osteogenic BMP) for 30 hours. Using dChIP-based hierarchical clustering analysis, we found that the three osteogenic

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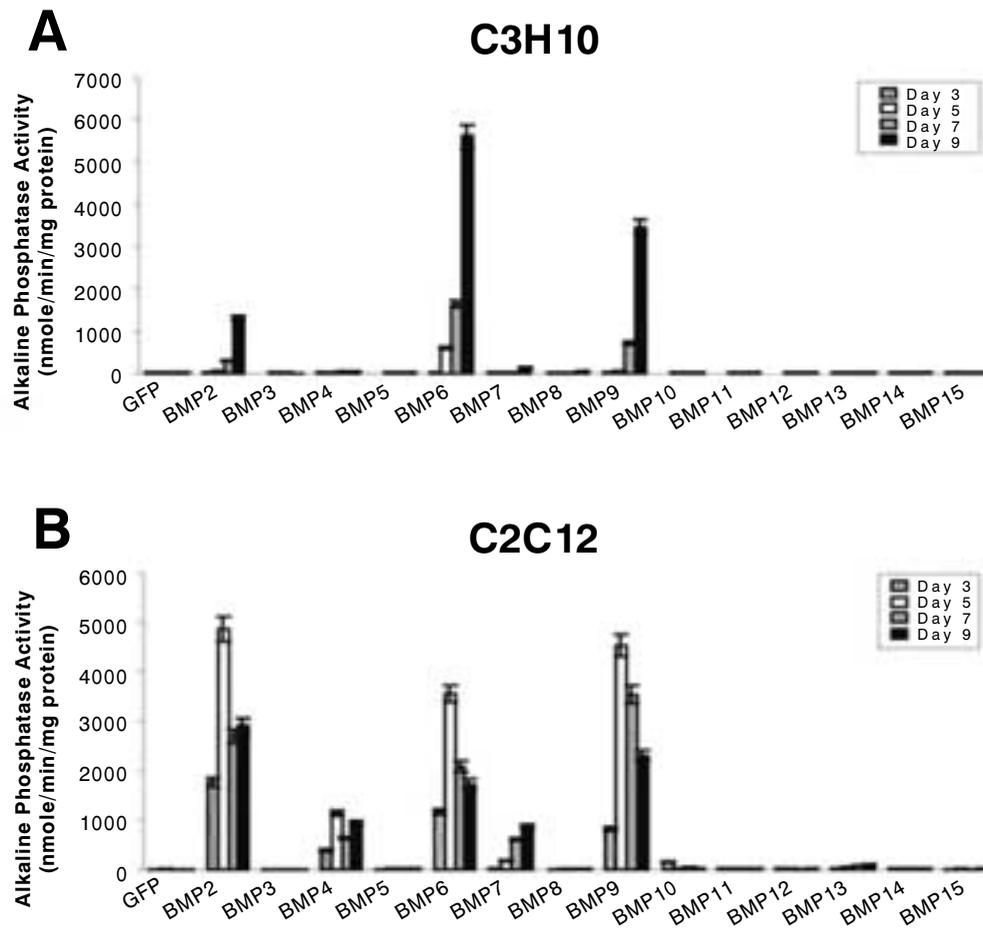


Figure 1. Effects of individual BMPs on the induction of ALP activity in C3H10T1/2 (A) and C2C12 (B) cells. Cells were infected with AdBMPs and a control AdGFP. Cells were lysed at indicated times for a colorimetric assay of ALP activity by using *p*-nitrophenyl phosphate as a substrate.

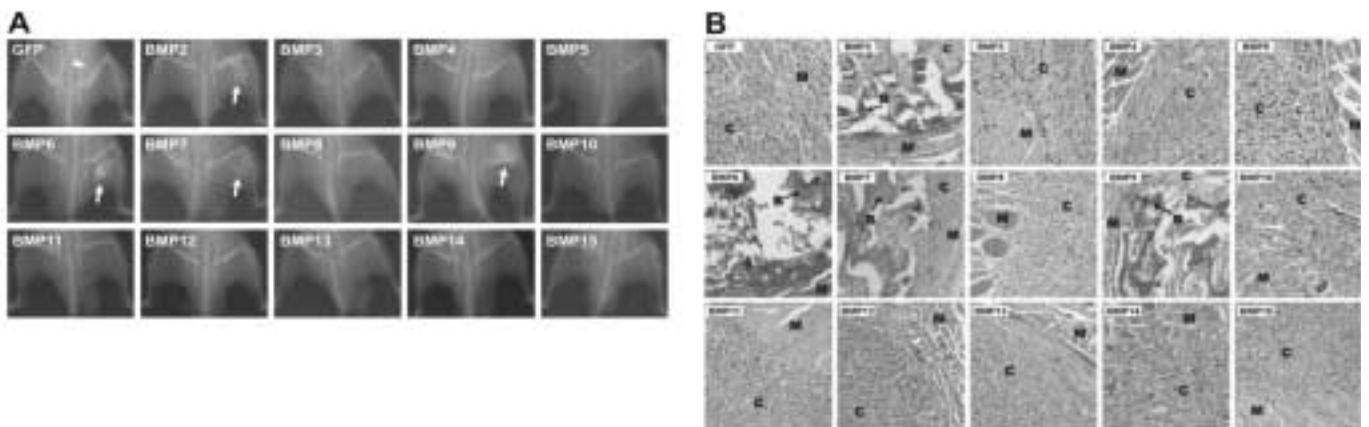


Figure 2. Orthotopic bone forming activity of BMPs in athymic mice. C2C12 cells were infected with AdBMPs or AdGFP, and injected into the right quadriceps of athymic mice. At 3 weeks, mice were sacrificed and subjected to X-ray radiography (A) and H & E stain (B). C, injected cells; M, muscle cells; B, bone.

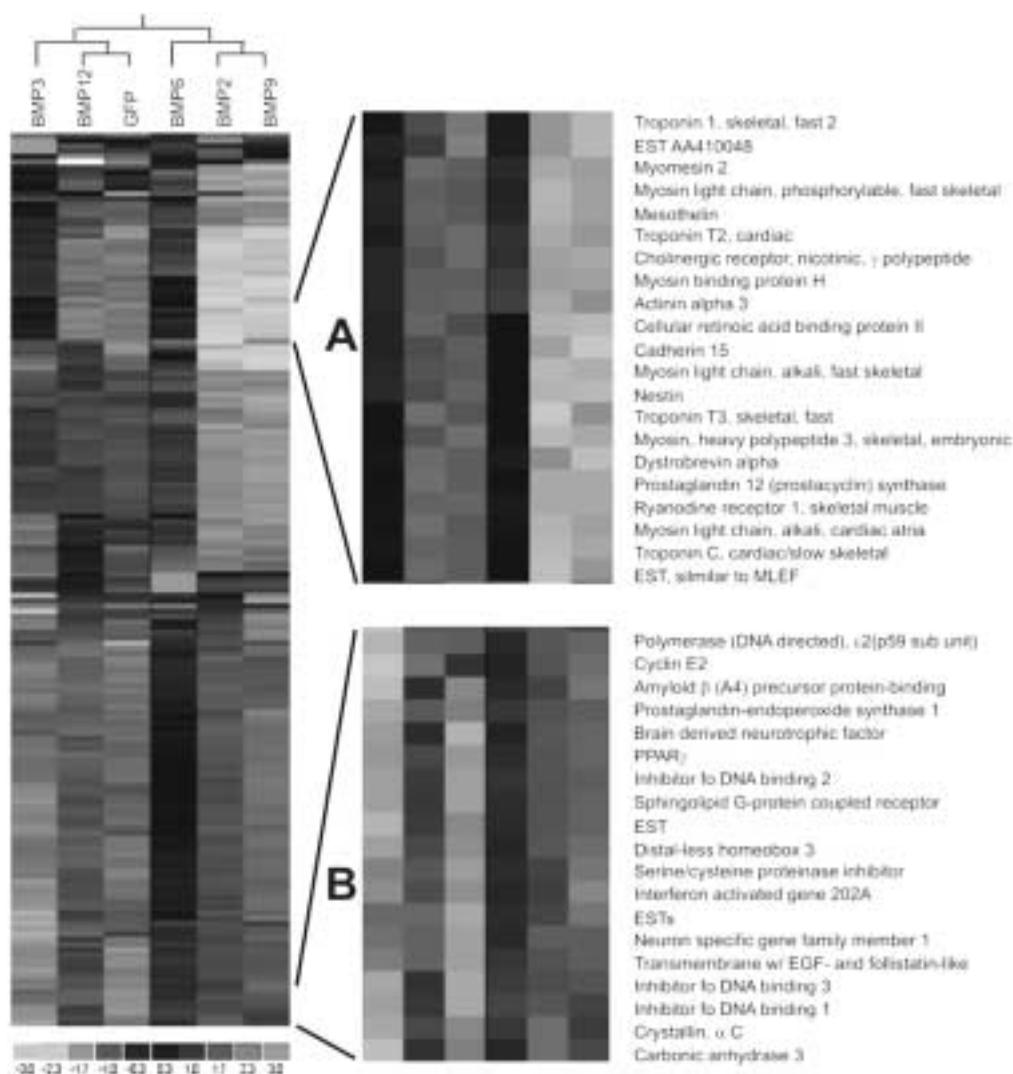


Figure 3. Molecular fingerprints of osteogenic BMPs. ~200 filtered genes regulated by osteogenic BMPs were used for hierarchical clustering analysis. Expression level matrix is shown in a log ratio representing normalized values from -3 (green) to +3 (red).

BMPs (i.e., BMP-2, 6, and 9) induced a similar overall expression pattern that was distinct from that of BMP-3 and 12, and the GFP control, suggesting that BMP-3 and 12 behaved differently from that of the osteogenic BMPs (Figure 3). Of the 3 osteogenic BMPs, BMP-2 induced a more closely related expression pattern to BMP-9 than to BMP-6. The similarities in expression pattern among osteogenic BMPs may underscore a fundamental mechanism behind bone formation. Based on the microarray analysis, we have demonstrated the important functional roles of several target genes in BMP-9-induced osteogenesis. Our ultimate goal is to formulate BMP cocktails that are efficacious for bone regeneration with great potential in orthopaedic applications.

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