BONE MORPHOGENETIC PROTEINS 2, 6 AND 9 EXHIBIT POTENT OSTEOGENIC ACTIVITY IN OSTEOBLASTIC PROGENITOR CELLS

*Cheng, H; *Jiang, W; *Haydon, R; *Breyer, B; *Zhou, L; **Feng, T; *Vanichakarn, P; *Szatkowski, J; *Park, J Y; *Phillips, F; and +*He, T C
+* University of Chicago, Chicago, IL

**Chongqing University of Medical Sciences, Chongqing, People’s Republic of China

Listing for additional author affiliation

Introduction It has been known for nearly half a century that demineralized bone can induce de novo bone formation in various animal models. The molecular identity of the bone-forming factors responsible for this observation was subsequently revealed to be bone morphogenetic proteins (BMPs). Several recombinant versions of BMPs, most notably rhBMP-2 and rhBMP-7 (OP-1), have been shown to induce bone formation in vivo, and both have been tested in clinical trials. Initial data from these trials suggest that BMP-induced bone formation may be comparable to autogenous bone graft in the treatment of tibial non-unions and interbody spine fusions, and rhBMP-2 recently received FDA approval for use in spine fusions. When delivered by adenoviral or retroviral vectors, BMP2, 7, and 9 have each been shown to induce bone formation in various animal models.

To date, however, no comprehensive evaluation of the relative osteoinductive activity of each BMP has been performed, either as solitary factors or in combination. Therefore, it remains unclear whether other BMPs may be significantly more efficacious at inducing new bone formation than those currently being tested in animal studies and in clinical trials. In this study, we sought to determine the distinct osteogenic activities of the 14 types of human BMPs and their potential synergistic effect on osteoblastic differentiation in osteogenic precursor cells. These studies should expand our current knowledge of bone biology and provide important insight into which BMP(s) may be the most potent agents for bone regeneration.

Methods To elucidate the osteogenic activity of individual BMPs, we constructed a series of recombinant adenoviruses that express human BMPs 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15. BMP cDNA was amplified from a human cDNA library. We did not include BMP1 in this study because of its established inability to induce bone formation.

We used these adenoviruses to infect two mesenchymal progenitor lines (C2C12 and C3H10T1/2). The ability to promote osteoblastic differentiation by different BMPs was assessed by measuring the induction of alkaline phosphatase activity using both colorimetric and histochemical methods. Further differentiation into osteoblasts was evaluated by measuring the induction of osteocalcin, as well as matrix mineralization, at later time points.

To test the ability of BMP3 to inhibit BMP-mediated osteogenesis, we co-expressed BMP3 and other osteogenic BMPs (i.e., BMP2, 4, 6, 7, and 9) in subconfluent C2C12 cells. ALP activity was measured at 5 and 7 days after infection. Similarly, we also carried out a pilot study to determine the possible synergistic effect among BMPs that could not induce BMP-induced bone formation.

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**Essential Results** When alkaline phosphatase activity was measured by a colorimetric enzymatic assay, only BMP2, 6, and 9 showed a significant increase in C3H10T1/2 cells. Of these three BMPs, BMP6 induced ALP activity as early as 5 days after infection (i.e., >13-fold increase), continuing to increase at 9 days post infection (i.e., >292-fold increase). Similarly, at 9 days after infection BMP2 and BMP9 were shown to induce approx. 67-fold and 180-fold increases in ALP activity, respectively. In C2C12 cells, BMP2, BMP6 and BMP9 again induced the largest increase, in ALP activity, although BMP4 and BMP7 also resulted in detectable increases. The results of histochemical analyses of ALP activity were completely consistent with the colorimetric data.

We also measured the expression of osteocalcin, a marker of late osteoblastic differentiation, in response to the exogenous expression of each BMP. In C3H10T1/2 and C2C12 cells, BMP2, 6, and 9 induced significant expression of osteocalcin at 10 days after infection, while BMP 4 and 7 also resisted in appreciable levels of osteocalcin expression. Next, we sought to evaluate the ability of the BMPs to induce matrix mineralization, a functional assay of terminally differentiated osteoblasts. At 21 days after infection in C3H10T1/2 cells, mineralized nodules were readily detected in the cells infected with AdBMP2, AdBMP6, and AdBMP9, while some mineralization was detectable in the AdBMP4 and AdBMP7-infected cells.

To assess the inhibitory activity of BMP3 on other osteogenic BMPs, we co-expressed BMP3 and other osteogenic BMPs 2, 4, 6, 7, and 9 in subconfluent C2C12 cells. At 3 days and 5 days after infection, the ALP activity normally induced by all five BMPs was significantly inhibited. These findings are consistent with the notion that BMP3 may antagonize BMP-induced bone formation.

In order to determine the possible synergistic effect among BMPs in promoting osteoblastic differentiation, we infected C2C12 cells with pairs of AdBMPs. ALP activity was measured at 5 days after infection. Interestingly, when we analyzed the co-expression of a panel of BMPs that individually exhibit no or low osteogenic activity, several pairs of BMPs exerted strong synergy in inducing ALP activity. Specifically, the combinations of BMP5+BMP10, BMP5+BMP12, BMP5+BMP13, BMP7+BMP10, BMP7+BMP12, and BMP7+BMP13 were shown to exhibit significant synergy in their ability to induce alkaline phosphatase. Similar results (to a lesser extent) were obtained in C3H10T1/2 cells.

Discussion Using two model cell lines for osteogenesis studies, we demonstrated that BMP2, BMP6, and BMP9 exhibited the greatest ability to induce both early and late osteogenic markers, as well as matrix mineralization. Consistent with a recent gene disruption study, BMP3 exhibits no osteogenic activity and effectively antagonizes the osteogenic activity of BMP2, BMP4, BMP6, BMP7, and BMP9. Finally, we observed strong synergy between specific BMPs that could not induce differentiation alone, such as BMP5+10, BMP5+12, BMP5+13, BMP7+10, or BMP7+13. To the best of our knowledge, this line of investigation is the first of its kind to evaluate the osteogenic activity of most, if not all, human BMPs in a comprehensive fashion.

While it is not entirely surprising that BMP2 possesses strong osteoinductive abilities, it is intriguing that BMP6 and BMP9 emerge as two of the most potent inducers of osteogenic differentiation. Both of these BMPs are relatively uncharacterized when compared to BMP2, 4 and 7, although both have been tested in at least one in vivo study, and stimulate robust bone formation. These data provide important in vivo correlates to the findings reported here, and suggest that our in vitro findings will be born out in more extensive, comparative animal models of bone formation.

In addition, the potential use of combinations of BMPs has not been exploited previously. Our findings suggest that such combinations, which may eventually include more than just two BMPs, can significantly increase the osteoinductive activity of BMPs far above that of any single BMP. Additional investigations into the synergy among BMPs, as well as between BMPs and other classes of osteogenic factors, such as Cbfa1, will likely lead to much more effective strategies of bone regeneration. Ultimately, these approaches will lead to novel treatments for a variety of orthopaedic disorders.