**Endocrine,** *vol. 9, no. 3, 273–280, December 1998* 0969–711X/98/8:273–280/$10.00 

# Treatment of Resting Zone Chondrocytes with Bone Morphogenetic Protein-2 Induces Maturation into a Phenotype Characteristic of Growth Zone Chondrocytes by Downregulating Responsiveness to 24,25$(OH)_2D_3$ and Upregulating Responsiveness to 1,25-$(OH)_2D_3$

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**To determine if bone morphogenetic protein-2 (BMP-2) can induce the endochondral maturation of resting zone (RC) chondrocytes, confluent fourth-passage cultures of these cells were pretreated for 24, 36, 48, 72, or 120 h with recombinant human BMP-2. At the end of pretreatment, the media were replaced with new media containing $10^{-10}$–$10^{-8}$ *M* 1,25-$(OH)_2D_3$ or $10^{-9}$–$10^{-7}$ *M* 24,25-$(OH_2)D_3$, and the cells incubated for an additional 24 h. This second treatment was chosen, because prior studies had shown that the more mature growth zone (GC) chondrocytes and RC cells respond to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in distinctly different ways with respect to the parameters examined. The effect of BMP-2 pretreatment on cell maturation was assessed by measuring alkaline phosphatase specific activity (ALPase). In addition, changes in matrix protein production were assessed by measuring collagen synthesis, as well as [$^{35}$S]-sulfate incorporation into proteoglycans. When RC cells were pretreated for 72 or 120 h with BMP-2, treatment with 1,25-$(OH)_2D_3$ caused a dose-dependent increase in ALPase specific activity and collagen synthesis, with no effect on proteoglycan sulfation. RC cells pretreated with 1,25-$(OH)_2D_3$ responded like RC cells that had not received any pretreatment. RC cells normally respond to 24,25-$(OH)_2D_3$; however, RC cultures pretreated for 72 or 120 h with BMP-2 lost their responsiveness to 24,25-$(OH)_2D_3$. These results indicate that BMP-2 directly regulates the differentiation and maturation of RC chondrocytes into GC chondrocytes. These observations support the hypothesis that BMP-2 plays a significant role in regulating chondrocyte maturation during endochondral ossification.**

**Key Words:** Chondrocytes; differentiation; maturation; growth plate cartilage; BMP-2; 1,25-$(OH)_2D_3$; 24,25-$(OH)_2D_3$

## Introduction

The process of endochondral bone formation consists of a developmental cascade of chondrocyte maturation states that culminates in extracellular matrix mineralization before osteogenesis can occur. The process is required for normal growth and development of long bones and for certain kinds of bone repair. During the chondrogenic phase of the process, chondrocytes are responsible for the synthesis, maintenance, and maturation of a calcifiable extracellular matrix that is composed mainly of proteoglycan and collagen *(1,2)*.

Bone morphogenetic proteins (BMPs) are the only known factors capable of inducing this process in an ectopic site *(3–5)*. They are believed to initiate the expression of the endochondral cartilage cell phenotype by inducing the commitment of pluripotent mesenchymal and chondroprogenitor cells. Several studies have shown that purified and recombinant BMPs, including recombinant human BMP-2, can cause these cells to express a cartilage cell phenotype in vivo *(6–11)*. In vitro, BMP-2 has been shown to promote expression of a chondrogenic phenotype by a multipotent mesenchymal cell line *(12)*.

Received August 17, 1998; Revised October 6, 1998; Accepted October 6, 1998.

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BMPs can also enhance endochondral development by direct action on chondrocytes in the growth plate *(13–15)*. BMP-2 has been shown to inhibit collagenase-3 mRNA production during human fetal cartilage growth and development *(16)*. We have shown that BMP-2 affects proliferation and differentiation of rat costochondral growth plate chondrocytes *(17)*. The effects are dose-dependent, but they also depend on the maturation state of the responding cell population. Resting zone cells exhibit greater sensitivity to BMP-2 than do cells derived from the prehypertrophic and upper hypertrophic zones (growth zone) of the growth plate. BMP-2 upregulates BMP-2 production in both populations of cells. BMP-2 also causes an increase in BMP-4 mRNA levels in resting zone cells, whereas it causes a decrease in growth zone cells.

This differential expression of BMPs by growth plate cells in postfetal development is also seen in fetal tissues *(18)*. In the developing limb bud, there is a differential distribution of BMP-2 and BMP-13 transcripts *(5,19)*. Similarly, Chang et al. *(20)* have shown that two other members of the transforming growth factor-β (TGF-β) superfamily are also differentially expressed in limb bud development. These observations support the hypothesis that BMPs are produced locally and act on the cells in a paracrine/autocrine manner, suggesting that they function to promote the transition of growth plate chondrocytes from one maturation state to another.

The effects of BMP-2 on mesenchymal cell response also depend on the duration of exposure. Puleo *(21)* has shown that the C3H1OT1/2 cell line exhibits an initial proliferative response to BMP-2 followed by increased alkaline phosphatase activity, osteocalcin production, and mineralized matrix dependent on the length of exposure to BMP-2. In contrast, BMP-2 inhibited proliferation of bone marrow stromal cells, but elicited a similar effect on markers of osteoblastic differentiation that became greater as exposure to the factor was prolonged. Similarly, BMP-2 modulates differentiation of fetal rat calvarial cells in a time- and dose-dependent manner *(22)*, suggesting that BMP-2 enhances expression of other BMP genes during the process in much the same way as noted in the growth plate. These results also suggest that BMP-2 may cause cells to become sensitive to other regulatory agents. BMP-2 has been shown to cause increased synthesis of insulin-like growth factors 1 and 2 by skeletal cells in a dose- and time-dependent manner *(23)* and to enhance the response of osteoblast-like 2T9 cells to 1,25-$(OH)_2D_3$ *(24)*. Whether this is true for chondrocytes as well is not known.

Recent attention has focused on the transition of cells from the proliferative zone to the zone of prehypertrophy, since it is at this juncture that cells in the growth plate become demonstrably endochondral rather than hyaline in their phenotypic expression. *In situ* hybridization and immunolocalization studies can distinguish between these cell maturation states on the basis of tissue morphology. However, in cell culture, both the hyaline-like resting zone cells and the postproliferative growth zone cells undergo proliferation before they re-express their in vivo phenotype.

To understand the transition from the resting zone to the zone of prehypertrophy, we have established a chondrocyte model in which these two cell types are cultured separately after discarding the intervening proliferative cell zone *(25–28)*. Characterization of these two distinct populations has shown that they produce matrix vesicles with different compositions, including activity of alkaline phosphatase *(29,30)* and matrix metalloproteinases *(31)*; their basal Ca ion flux *(32,33)* and membrane fluidity *(34)* are different; phospholipid composition of their membranes is different *(26)*, as is their basal production of prostaglandin $E_2$ *(35)* and 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ *(36)*. Also, they exhibit marked differences in their response to vitamin D metabolites. Whereas resting zone cells respond primarily to 24,25-$(OH)_2D_3$, growth zone cells respond primarily to 1,25-$(OH)_2D_3$ *(37,38)*.

Even though resting zone chondrocytes will form nodules in long-term culture and exhibit increased alkaline phosphatase as a function of time, these cells do not mineralize their matrix, nor do they synthesize type X collagen, even after 28 d postconfluence, indicating that their hyaline chondrocyte phenotype is retained *(39,40)*. However, if these cells are treated for 36 h or more with 24,25-$(OH)_2D_3$, they will exhibit a growth zone phenotype and become responsive to 1,25-$(OH)_2D_3$ *(37)*. rhTGF-β1 also promotes this shift in phenotypic expression, but the effect requires 72 h of exposure *(41)*. In contrast, resting zone cells exposed to 1,25-$(OH)_2D_3$ for up to 5 d do not become responsive to 1,25-$(OH)_2D_3$ *(37,41)*. These observations suggest that acquisition of a 1,25-$(OH)_2D_3$-responsive phenotype is a hallmark of the shift from the resting zone maturation state to the growth zone maturation state.

Once the phenotypic shift occurs, at least with respect to TGF-β1, it does not appear to be reversible, since the cells lose responsiveness to 24,25-$(OH)_2D_3$ as they acquire responsiveness to 1,25-$(OH)_2D_3$. Because of the sensitivity of resting zone cells to BMP-2 *(17)*, we hypothesized that this growth factor may also play an important role in promoting the phenotypic transition of these cells. To test this hypothesis, we assessed whether resting zone cells acquired responsiveness to 1,25-$(OH)_2D_3$ following exposure to BMP-2, if the effect was dose- or time-dependent, and if the cells retained responsiveness to 24,25-$(OH)_2D_3$ after acquiring responsiveness to 1,25-$(OH)_2D_3$.

## Results

### *Alkaline Phosphatase*

BMP-2 induced resting zone cells to become responsive to 1,25-$(OH)_2D_3$, but the effect was dependent on BMP-2 concentration and time of exposure. In resting zone chondrocyte cultures treated with BMP-2 alone for 24 h (Fig. 1A), alkaline phosphatase specific activity was increased,

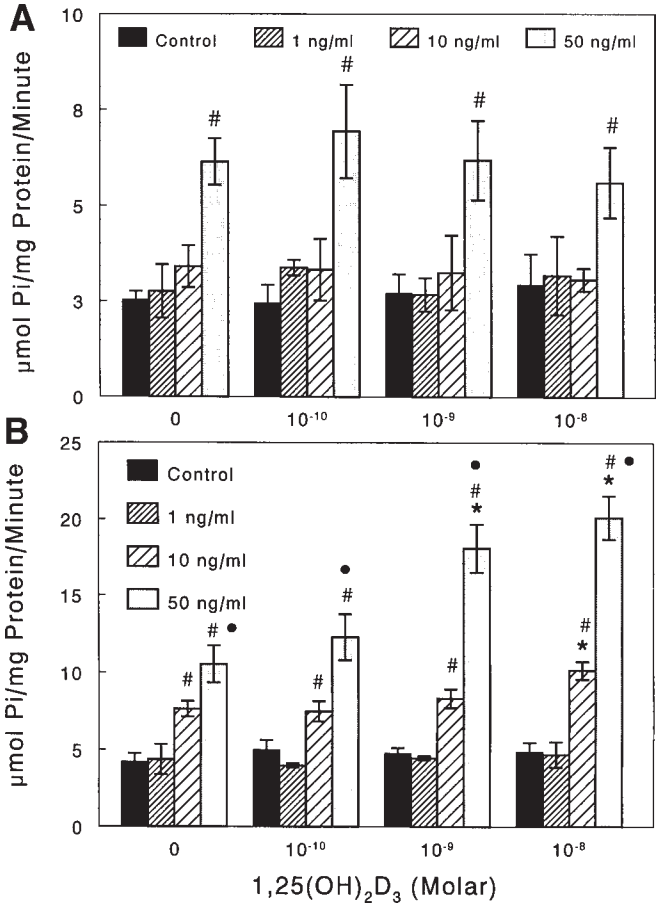


**Fig. 1.** Effect of BMP-2 pretreatment for 24 h **(A)** or 72 **(B)** on alkaline phosphatase specific activity in cell layer lysates of resting zone chondrocytes following challenge with 1,25-$(OH)_2D_3$. Cultures were treated for 24 or 72 h with 1, 10, or 50 ng/mL of BMP-2 and then treated with $10^{-10}$–$10^{-8}$M 1,25-$(OH)_2D_3$ for an additional 24 h. At harvest, alkaline phosphatase specific activity in the cell layer was measured as described in the Materials and Methods section. The data are from one of three replicate experiments yielding similar results. Each data point is the mean ± SEM of six cultures. $^{\#}p < 0.05$, treatment with BMP-2 vs cultures not treated with BMP-2; $^{*}p < 0.05$, treatment with 1,25-$(OH)_2D_3$ vs cultures not treated with 1,25-$(OH)_2D_3$; $\bullet p < 0.05$, 50 ng/mL BMP-2 vs 10 ng/mL BMP-2.

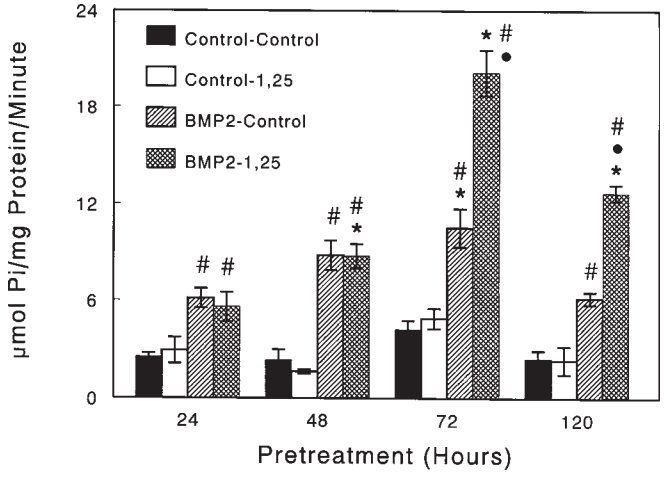


**Fig. 2.** Effect of BMP-2 pretreatment time on alkaline phosphatase specific activity of resting zone chondrocytes following challenge with 1,25-$(OH)_2D_3$. Cultures were pretreated with 50 ng/mL BMP-2 for 24, 48, 72, or 120 h and then treated with $10^{-8}$ *M* 1,25-$(OH)_2D_3$ for an additional 24 h. At harvest, alkaline phosphatase specific activity in the cell layer was measured as described in the Materials and Methods section. The data are from one of three replicate experiments yielding similar results. Each data point is the mean ± SEM for six cultures. $^{\#}p < 0.05$ treatment with BMP-2 vs cultures not treated with BMP-2; $^{*}p < 0.05$ vs 24 h; $\bullet p < 0.05$ vs cells pretreated with BMP-2, but not subsequently treated with 1,25-$(OH)_2D_3$.

but only at the highest concentration of growth factor. As noted previously *(37,41)*, alkaline phosphatase was unresponsive to 1,25-$(OH)_2D_3$ treatment alone. In addition, treatment of the cultures with BMP-2 for 24 h had no effect on the cells' responsiveness to $10^{-10}$–$10^{-8}$ *M* 1,25-$(OH)_2D_3$.

At 72 h (Fig. 1B), cultures treated with either 10 or 50 ng/mL BMP-2 had elevated alkaline phosphatase specific activity, and the effect was dose-dependent. The level of alkaline phosphatase specific activity in cultures treated with 50 ng/mL BMP-2 for 72 h was further augmented by $10^{-9}$*M* 1,25-$(OH)_2D_3$, whereas cultures treated with 10 or 50 ng/mL BMP-2 followed by $10^{-8}$ *M* 1,25-$(OH)_2D_3$ also showed increased enzyme specific activity.

The effect of BMP-2 on alkaline phosphatase specific activity was time-dependent (Fig. 2). Alkaline phosphatase specific activity was increased over controls after 24, 48, 72, and 120 h of exposure to BMP-2. Addition of BMP-2 for 24 or 48 h did not induce responsiveness to 1,25-$(OH)_2D_3$. However, responsiveness to 1,25-$(OH)_2D_3$ was seen after 72 and 120 h of treatment with BMP-2. Maximal stimulation of alkaline phosphatase in 1,25-$(OH)_2D_3$-challenged cultures was observed in cells pretreated for 72 h with BMP-2.

Treatment with BMP-2 resulted in a loss of cellular response to 24,25-$(OH)_2D_3$ (Fig. 3). In cultures pretreated with control media followed by 24,25-$(OH)_2D_3$, resting zone cells had increased alkaline phosphatase specific activity at all times examined, but similar treatment with 1,25-$(OH)_2D_3$ had no effect. In cultures pretreated with BMP-2 for 24 or 48 h, 24,25-$(OH)_2D_3$ caused no further increase in enzyme activity over that seen in cultures treated with 24,25-$(OH)_2D_3$ alone, whereas 1,25-$(OH)_2D_3$ had an effect that was comparable to that seen with 24,25-$(OH)_2D_3$. In cultures treated for 72 or 120 h with BMP-2, however, addition of 1,25-$(OH)_2D_3$ caused an increase in alkaline phosphatase, whereas enzyme activity in the cultures challenged with 24,25-$(OH)_2D_3$ was comparable to that of the untreated controls.

### *Collagen Production*

Collagen synthesis was inhibited by $10^{-9}$–$10^{-8}$ *M* 1,25-$(OH)_2D_3$ in control cultures, and by $10^{-8}$ *M* 1,25-$(OH)_2D_3$ in BMP-2-pretreated cultures at 24 h (Fig. 4A). However,

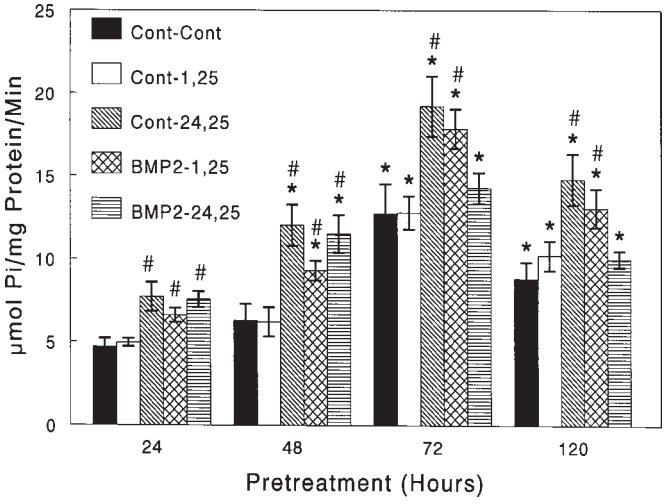


**Fig. 3.** Effect of BMP-2 pretreatment time on 24,25-$(OH)_2D_3$-dependent alkaline phosphatase specific activity of resting zone chondrocytes. Cultures were pretreated with 50 ng/mL BMP-2 for 24, 48, 72, or 120 h and then treated with $10^{-8}$ *M* 1,25-$(OH)_2D_3$ or $10^{-7}$ *M* 24,25-$(OH)_2D_3$ for an additional 24 h. At harvest, alkaline phosphatase specific activity in the cell layer was measured as described in the Materials and Methods section. The data are from one of three replicate experiments yielding similar results. Each data point is the mean ± SEM for six cultures. $^{\#}p < 0.05$, treatment vs control; $^{*}p < 0.05$ vs 24 h.

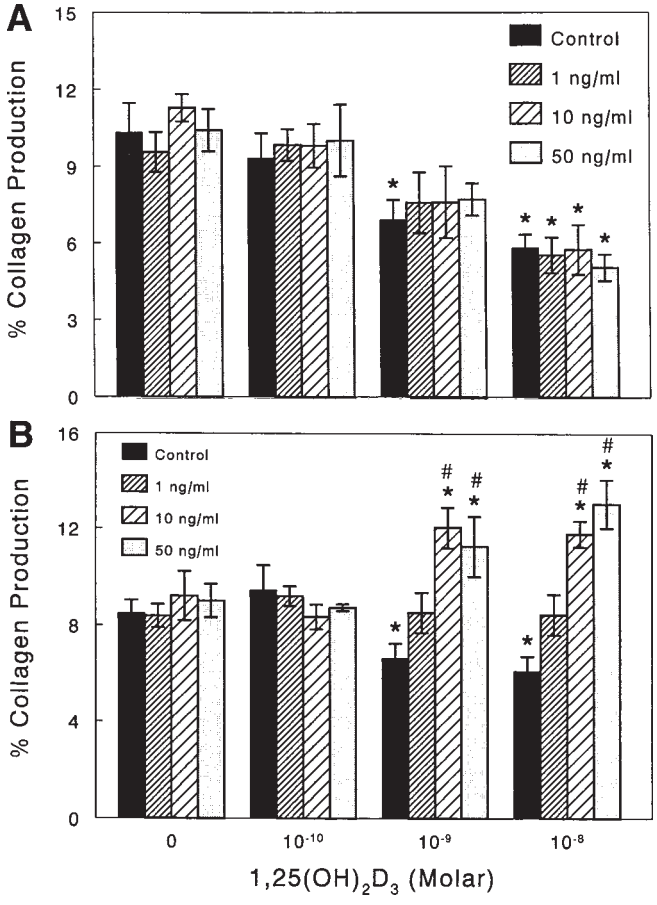


**Fig. 4.** Effect of BMP-2 pretreatment for 24 **(A)** or 72 **(B)** h on percent collagen production by resting zone chondrocytes following challenge with 1,25-$(OH)_2D_3$. Cultures were pretreated with BMP-2 (1, 10, or 50 ng/mL) for 24 or 72 h and then treated with $10^{-10}$–$10^{-8}$ *M* 1,25-$(OH)_2D_3$ for an additional 24 h. At harvest, % collagen production was measured as described in the Materials and Methods section. The data are from one of three replicate experiments yielding similar results. Each data point is the mean ± SEM for six cultures. $^{\#}p < 0.05$, + BMP-2 vs –BMP-2; $^{*}p < 0.05$, +1,25-$(OH)_2D_3$ vs -1,25-$(OH)_2D_3$.

this decrease was independent of BMP-2 concentration. In cultures treated with control media for 72 h, $10^{-9}$ and $10^{-8}$ *M* 1,25-$(OH)_2D_3$ inhibited collagen production (Fig. 4B). In contrast, when cultures were pretreated with 10 or 50 ng/mL BMP-2, there was a dose-dependent increase in collagen synthesis after challenge with $10^{-8}$ *M* 1,25-$(OH)_2D_3$. The stimulatory effect of BMP-2 on cell response to 1,25-$(OH)_2D_3$ was also noted after 120 h of BMP-2 treatment (Fig. 5).

### *Proteoglycan Sulfation*

Proteoglycan sulfation by resting zone cells was unaffected by treatment with BMP-2, or by challenge with 1,25-$(OH)_2D_3$, whether or not the cells were treated with BMP-2 for up to 120 h (Fig. 6).

## Discussion

The results of this study demonstrate that BMP-2 can induce resting zone cells to acquire a 1,25-$(OH)_2D_3$ responsive phenotype characteristic of more mature growth zone chondrocytes. This effect is dose- and time-dependent and is specific to the parameter being examined. Responsiveness of alkaline phosphatase to 1,25-$(OH)_2D_3$ required at least 72 h of exposure to the growth factor. Although cells pretreated with BMP-2 for 24 h appeared to become responsive to 1,25-$(OH)_2D_3$, the effect was comparable to that seen in cultures pretreated with BMP-2 alone, indicating that 1,25-$(OH)_2D_3$ did not elicit a further effect until 72 h. Responsiveness of collagen production to 1,25-$(OH)_2D_3$ also required at least 72 h of exposure. This suggests that BMP-2 initiates a cascade of responses leading to upregulation of receptors to this vitamin D metabolite.

The effects of BMP-2 were similar to those noted previously in response to TGF-β1, suggesting that similar mechanisms may be involved. Whereas 24,25-$(OH)_2D_3$ induced the phenotypic shift by 36 h *(37)*, both BMP-2 and TGF-β1 *(41)* required 72 h to elicit a comparable response. The requirement for long exposures to differentiating factors has been noted by others *(42–44)*, supporting the hypothesis that a cascade of events is involved. BMP-2 exerts its effects on cells via specific receptors activating a kinase cascade leading to new gene expression, sharing certain common mediators with other members of the TGF-β superfamily *(45,46)*. We have shown that TGF-β1 stimulates PKCα activity in resting zone chondrocytes via a mechanism that is independent of phospholipase C and tyrosine kinase, but requires new gene expression and protein synthesis *(47)*. Activation of PKC can also lead to new

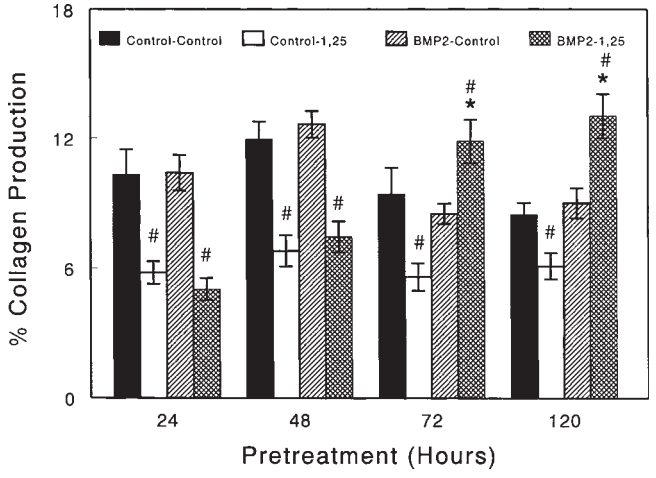


**Fig. 5.** Effect of BMP-2 pretreatment time on percent collagen production by resting zone chondrocytes following challenge with 1,25-$(OH)_2D_3$. Cultures were pretreated with 50 ng/mL BMP-2 for 24, 48, 72, or 120 h and then treated with $10^{-8}$ *M* 1,25-$(OH)_2D_3$ for an additional 24 h. At harvest, % collagen production was measured as described in the Materials and Methods section. The data are from one of three replicate experiments yielding similar results. Each data point is the mean ± SEM for six cultures. $^{\#}p < 0.05$, treatment vs control; $^{*}p < 0.05$; vs 24 h.

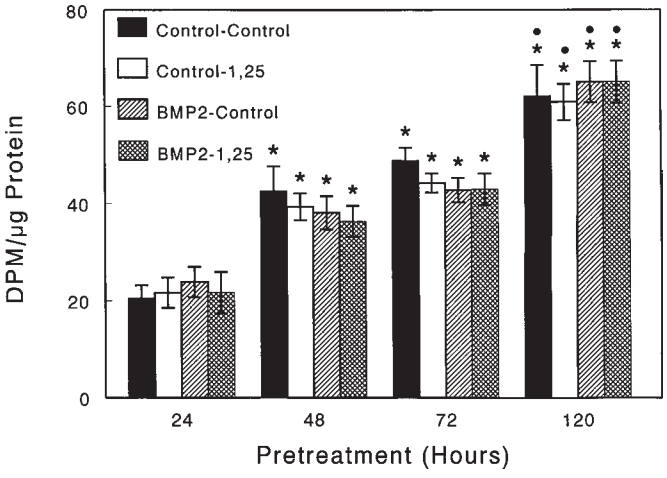


**Fig. 6.** Effect of BMP-2 pretreatment time on [$^{35}$S]-sulfate incorporation by resting zone chondrocytes following challenge with 1,25-$(OH)_2D_3$. Cultures were pretreated with 50 ng/mL BMP-2 for 24, 48, 72, or 120 h and then treated with $10^{-8}$ *M* 1,25-$(OH)_2D_3$ for an additional 24 h. At harvest, [$^{35}$S]-sulfate incorporation was measured as described in the Materials and Methods section. The data are from one of three replicate experiments yielding similar results. Each data point is the mean ± SEM for six cultures. $^{*}p < 0.05$ vs 24 h; $^{\bullet}p < 0.05$ vs 48 h.

gene expression *(48–50)*. Thus, it is likely that TGF-β1 and BMP-2 initiate a comparable series of reactions, at least with respect to the parameters measured here, potentially including crosstalk *(46,51)*.

At least some of the pathways modulated by TGF-β1 and BMP-2 may be in common with those activated by 24,25-$(OH)_2D_3$. This vitamin $D_3$ metabolite has a rapid, direct effect on plasma membrane PKCα in resting zone cells *(52)*. In addition, 24,25-$(OH)_2D_3$ stimulates PKCα activity by activating gene expression and protein synthesis *(53)*. Given the importance of regulating this transition during endochondral bone formation in vivo, it is not surprising that some redundancy may exist.

It is also possible that 24,25-$(OH)_2D_3$ produced by the resting zone chondrocytes plays a role. TGF-β1 upregulates 24-hydroxylase in resting zone cells, resulting in increased production of 24,25-$(OH)_2D_3$ within 1 h *(54)*. Increased local production of this vitamin D metabolite could contribute to the phenotypic shift. It is unknown if BMP-2 elicits a similar change in local production of 24,25-$(OH)_2D_3$.

Concomitant with the increase in responsiveness to 1,25-$(OH)_2D_3$ there was a reduction in responsiveness to 24,25-$(OH)_2D_3$, further supporting the presence of a phenotypic shift in maturation state. In the absence of BMP-2, resting zone chondrocytes continue to express increased alkaline phosphatase activity as a function of time, and they retain their ability to respond to 24,25-$(OH)_2D_3$, but not 1,25-$(OH)_2D_3$. When treated with BMP-2 for 48 h, they continue to exhibit a 24,25-$(OH)_2D_3$ responsive phenotype. However, once the maturation shift occurs, these cells no longer respond to 24,25-$(OH)_2D_3$.

Our data suggest that the transition event is definitive in this regard. The transition involves multiple phenotypic markers. Not only is alkaline phosphatase affected, but collagen production is affected as well. At 5 d, collagen synthesis was still inhibited in control cultures challenged with $10^{-9}$–$10^{-8}$ *M* 1,25-$(OH)_2D_3$, a phenotypic response of the resting zone cell *(28)*. However, BMP-2 treatment for 72 h induced a dose-dependent increase in collagen in response to 1,25-$(OH)_2D_3$. These data are consistent with the resting zone cultures acquiring a growth zone-like phenotype, since 1,25-$(OH)_2D_3$ stimulates collagen synthesis in growth zone chondrocyte cultures.

Unlike the results obtained for collagen synthesis, proteoglycan production was not affected by BMP-2 pretreatment, suggesting that this marker of chondrocyte differentiation may either require additional culture time with BMP-2 or additional factors are involved in the acquisition of this phenotypic marker. TGF-β1 pretreatment also had no effect on sulfate incorporation following challenge by 1,25-$(OH)_2D_3$ *(41)*, indicating that it, too, does not modulate this aspect of the cell. In contrast, following pretreatment with 24,25-$(OH)_2D_3$ for 36 h, resting zone cells respond to 1,25-$(OH)_2D_3$ with a dose-dependent increase in [$^{35}$S]-sulfate incorporation typical of the growth zone cell phenotype *(37)*. Thus, although crosstalk among differentiating factors may exist, they are not interchangeable. Moreover, the effects of BMP-2 appear to be more limited than those of 24,25-$(OH)_2D_3$, suggesting that it regulates a subset of cells or that

its action is restricted to discrete components of the endochondral developmental cascade.

We did not observe an effect of BMP-2 alone on proteoglycan sulfation in this study. However, studies examining the effects of BMP-2 *(55)* and BMP-7 *(56)* on articular chondrocytes did demonstrate increased proteoglycan production. The difference in our findings may be owing to the source of the cells, articular vs costochondral resting zone cartilage. Embryonic mouse limb bud cultures also exhibit an increase in proteoglycan in response to BMP-2 *(57)*. Whether this is specific to chondroblastic embryonic cells rather than to postfetal chondrocytes is not yet known. In addition, we used much lower concentrations of growth factor in our cultures than have been reported by others. The concentrations used were sufficient to elicit a phenotypic shift, as well as other physiologically relevant effects on the cells *(17)*, although they may not have been sufficient to have activated the proteoglycan synthetic pathway. Under the same experimental protocols as used in the present study, 24,25-$(OH)_2D_3$ increased proteoglycan sulfation in resting zone cells, but after a 36-h exposure, the cells become responsive to 1,25-$(OH)_2D_3$ instead *(37)*. Thus, it is unlikely that the chondrocytes were already maximally incorporating [$^{35}$S]-sulfate under pretreatment conditions and were consequently unable to exhibit a further increase when challenged with 1,25-$(OH)_2D_3$.

In summary, this study demonstrates that resting zone chondrocytes in culture can be induced to differentiate into the more mature phenotype characteristic of growth zone chondrocytes, showing that BMP-2 plays a significant role in growth plate cartilage development. The exact mechanism utilized by BMP-2 in this process is not clear, although it is likely to be mediated by its effects on kinase cascades and vitamin $D_3$ metabolism. Resolution of these issues is presently under way.

## Materials and Methods

### *Chondrocyte Cultures*

The culture system used in this study has been described previously *(25)*. Chondrocytes were isolated from the resting zone of the costochondral cartilage of 125 *g* Sprague-Dawley rats. Cells were released from the matrix by enzymatic digestion and then cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 50 μg/mL vitamin C, and 1% penicillin–streptomycin–fungizone in an atmosphere of 5% $CO_2$ and 100% humidity at 37°C. Culture media were replaced after the first 24 h and then at 72-h intervals. Fourth-passage cells were used for the experiments, since previous studies have demonstrated that these cells retain their differential phenotype, including response to BMP-2 and the vitamin D metabolites at this passage (*see Experimental Design*).

At confluence, media were replaced with fresh media containing vehicle alone or 1, 10, or 50 ng/mL recombinant human BMP-2 (Genetics Institute, Andover, MA) in PBS. At appropriate time-points (24, 48, 72, or 120 h), these media were replaced with fresh media containing vehicle alone or $10^{-10}$ *M*–$10^{-8}$ *M* 1α,25-$(OH)_2D_3$ for an additional 24 h. In some experiments, $10^{-7}$ *M* 24R,25-$(OH)_2D_3$ was used. Both metabolites were a gift of Milan Uskokovic (Hoffman-LaRoche, Nutley, NJ). For those cells pretreated with 50 ng/mL BMP-2 for 120 h, fresh media containing the appropriate concentration of BMP-2 were added at 72 h.

### *Experimental Design*

In this culture model, resting zone cells respond to 24,25-$(OH)_2D_3$ with dose-dependent decreases in proliferation, dose-dependent increases in alkaline phosphatase specific activity, and proteoglycan sulfation, but no change in collagen synthesis *(26,25,28,37)*. 24,25-$(OH)_2D_3$ also inhibits [$^3$H]-thymidine incorporation by growth zone cells, but it has no effect on alkaline phosphatase, collagen production, or sulfate incorporation. 1,25-$(OH)_2D_3$ also inhibits [$^3$H]-thymidine incorporation by both cell types, but its effects on alkaline phosphatase, collagen, and proteoglycan are cell maturation-dependent. Growth zone cells exhibit increased alkaline phosphatase and proteoglycan sulfation, but 1,25-$(OH)_2D_3$ has no effect on these parameters in resting zone cells. In contrast, 1,25-$(OH)_2D_3$ inhibits collagen production by resting zone chondrocytes. When resting zone cells are exposed for 36 or more hours to 24,25-$(OH)_2D_3$ *(37)* or for 72 h to TGF-β1 *(41)*, they respond to 1,25-$(OH)_2D_3$ in a manner typical of a growth zone cell; treatment of resting zone cells with 1,25-$(OH)_2D_3$ for up to 5 d does not exert a similar transition in phenotype. Therefore, we used these markers in our present study.

### *Alkaline Phosphatase Specific Activity*

Specific activity of alkaline phosphatase (orthophosphoric monoester phosphohydrolase alkaline [EC 3.1.3.1]) was measured in cell layer lysates as a function of *para*-nitrophenol release from *para*-nitrophenylphosphate at pH 10.2, as previously described *(25,58,59)*.

### *Collagen and Noncollagen Protein Synthesis*

Incorporation of L-[$^3$H]-proline (New England Nuclear, Boston, MA) into collagenase-digestible protein (CDP) and noncollagenase-digestible protein (NCP) in the cell layer was used to estimate matrix protein synthesis by resting zone cells as previously described *(28,37,60)*. Data were expressed as dpm and were calculated with respect to protein content. Percent collagen synthesis was calculated after multiplying the labeled proline in NCP by 5.4 to correct for its relative abundance in collagen *(61)*. This assay did not take into account any degradation that may have occurred.

### *[$^{35}$S]-Sulfate Incorporation*

Proteoglycan synthesis was assessed by measuring [$^{35}$S]-sulfate incorporation in the cell layer according to the method of O'Keefe et al. *(62)*, as described by us previously *(37,63)*.

### *Statistical Analysis*

The data presented in the figures are from one experiment that was repeated three or more times with comparable results. For any given experiment, each data point represents the mean ± SEM for six individual cultures. The data were analyzed by analysis of variance, and statistical significance determined by comparing each data point to the control (containing ethanol vehicle) using Bonferroni's modification of the *t*-test.

## Acknowledgments

The authors acknowledge the contributions of Yuhong Liu, Zhi Chang, Roland Campos, and Jayme Solis to the technical aspects of this work, and to Sandra Messier for assistance with manuscript preparation. The studies were supported through grants from the National Institute of Dental Research (DE-08603 and DE-05937), the Center for the Enhancement of the Biology/Biomaterials Interface, and OsteoBiologics, Inc., San Antonio, TX.

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