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Divergent Regulation by p44/p42 MAP Kinase and p38 MAP Kinase of Bone Morphogenetic Protein-4-Stimulated Osteocalcin Synthesis in Osteoblasts

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Abstract In the present study, we investigated whether the mitogen-activated protein (MAP) kinase superfamily is involved in the bone morphogenetic protein (BMP)-4-stimulated synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells. BMP-4 dose-dependently stimulated osteocalcin synthesis. BMP-4 markedly induced the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase, while having little effect on SAPK (stress-activated protein kinase)/JNK (c-*Jun* N terminal kinase) phosphorylation. SB203580 and PD169316, specific inhibitors of p38 MAP kinase, significantly reduced the osteocalcin synthesis stimulated by BMP-4. In contrast, PD98059 and U0126, inhibitors of upstream kinase of p44/p42 MAP kinase, markedly enhanced the BMP-4-stimulated osteocalcin synthesis. The BMP-4-induced phosphorylation of p44/p42 MAP kinase was suppressed by PD98059, which did not, however, affect the BMP-4-induced phosphorylation of p38 MAP kinase. Taken together, our results strongly suggest that p38 MAP kinase takes part in BMP-4-stimulated osteocalcin synthesis as a positive regulator in osteoblasts, whereas p44/p42 MAP kinase acts as a negative regulator in the synthesis. J. Cell. Biochem. 84: 583–589, 2002.

Key words: BMP; osteocalcin; MAP kinase; osteoblast

Bone morphogenetic proteins (BMPs) are multifunctional cytokines, which belong to the transforming growth factor-β (TGF-β) superfamily [Reddi, 1994; Kawabata et al., 1998]. BMPs were originally identified by their ability to form ectopic bone [Reddi, 1994]. It is well known that BMPs play an important role in the early development of vertebrates [Harland, 1994]. As for bone cells, BMP-2 and -4 are synthesized and secreted by osteoblasts [Centrella et al., 1994]. In osteoblasts [Yamaguchi et al., 1991; Centrella et al., 1994; Yamaguchi et al., 1996], it has been reported that BMPs stimulate alkaline phosphatase activity and the expression of osteocalcin, markers of a mature osteoblast phenotype [Stein and Lian, 1993]. It is well recognized that Smad proteins such as Smad1 and Smad5 act as intracellular mediators of BMPs similar to TGF-β [Heldin et al., 1997; Kawabata et al., 1998; Massague, 1998]. In addition, other signaling pathways such as the mitogen-activated protein (MAP) kinase superfamily have also been shown to be mediators of BMP signaling [Yamaguchi et al., 1999]. The MAP kinase superfamily plays crucial roles in the intracellular signaling of a variety of agonists [Widmann et al., 1999]. The three MAP kinases, p38 MAP kinase, p44/p42 MAP kinase, and SAPK (stress-activated protein kinase)/JNK (c-*Jun* N-terminal kinase) are known to be central elements used by mammalian cells to transduce the diverse messages [Widmann et al., 1999]. As for BMP-signaling in osteoblasts, p44/p42 MAP kinase reportedly takes part in BMP-2-stimulated gene expression of collagen type I in ROS 17/2.8 osteosarcoma cells [Palcy and Goltzman, 1999]. Recently, it has been shown that p44/p42 MAP kinase is involved in BMP-2-induced osteoblastic differentiation such as alkaline phosphatase activity of mesenchymal progenitor C3H10T1/2 cells [Lou et al., 2000]. However, the roles of the

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MAP kinase superfamily in BMP intracellular signaling in osteoblasts have not yet been precisely clarified.

Osteocalcin, an osteoblastic-specific phenotype marker, is a γ-carboxylated, calcium-binding protein synthesized and secreted only by mature osteoblasts [Ducy and Karsenty, 1996]. The level of initially expressed osteocalcin is low and then upregulated with time in cultured osteoblasts [Franchesi and Iyer, 1992]. It has previously been shown that osteocalcin-deficient mice develop a phenotype marked by increased bone formation [Ducy and Karsenty, 1996]. Thus, it is generally recognized that osteocalcin normally functions to limit without impairing bone resorption and mineralization. As for osteocalcin production, the expression of osteocalcin gene is reportedly stimulated in the constitutively active form of MEK (MAP kinase/extracellular signal-regulated kinase kinase)-transfected MC3T3-E1 cells [Xiao et al., 2000]. It is recognized that osteocalcin synthesis in osteoblasts is regulated by various extracellular stimuli. However, the exact mechanism behind osteocalcin production by agonists in osteoblasts has not yet been clarified.

In the present study, we investigated the possible involvement of the MAP kinase superfamily in BMP-4-stimulated osteocalcin synthesis in osteoblast-like MC3T3-E1 cells. We show that p38 MAP kinase is involved as a positive regulator in BMP-4-stimulated osteocalcin synthesis and that p44/p42 MAP kinase negatively regulates the synthesis by BMP-4 in these cells.

MATERIALS AND METHODS

Materials

BMP-4 was purchased from R&D Systems (Tokyo, Japan). The osteocalcin radioimmuassay (RIA) kit was obtained from Biomedical Technologies, Inc. (Stoughton, MA). PD98059, SB203580, and PD169316 were obtained from Calbiochem–Novabiochem (La Jolla, CA). U0126 was obtained from Promega (Madison, WI). Phospho-specific p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), phospho-specific p44/p42 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), p44/p42 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), phospho-specific SAPK/JNK antibodies (rabbit polyclonal IgG, affinity purified), and SAPK/JNK antibodies (rabbit polyclonal IgG, affinity purified) were purchased from New England BioLabs, Inc. (Beverly, MA). The ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. SB203580, PD169316, PD98059, and U0126 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for osteocalcin or Western blot analysis.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Sudo et al., 1983] were maintained as previously described [Kozawa et al., 1997]. In brief, the cells (5×10^4) were seeded into 35-mm-diameter dishes in 2 ml of α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS). After 5 days, the medium was exchanged for 2 ml of α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. When indicated, the cells were pretreated with SB203580, PD169316, PD98059, or U0126 for 60 min.

Assay for Osteocalcin

The cultured cells were stimulated by BMP-4 in 1 ml of α-MEM containing 0.3% FCS, and then incubated for the indicated periods. The conditioned medium was collected, and osteocalcin in the conditioned medium was then measured by an osteocalcin RIA kit.

Analysis by Western Blotting

The cultured cells were stimulated by BMP-4 in serum-free α-MEM for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/Cl, pH 6.8, 2% SDS, 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as the supernatant after centrifugation at 125,000*g* for 10 min at 4°C. SDS–PAGE was performed by the method of Laemmli [1970] in 10% polyacrylamide gel. Western blotting analysis was performed as previously described [Miwa et al., 1999] using phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK

antibodies or SAPK/JNK antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of an ECL Western blotting detection system. The densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA).

Determination of Radioactivity

The radioactivity of radioimmunoassay samples was determined using a Wallac 1480 WIZARD3″ automatic gamma counter (Turk, Finland).

Statistical Analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a $P < 0.05$ was considered significant. All data are presented as the mean ± SEM of triplicate determinations. Each experiment was repeated three times with similar results.

RESULTS

Effect of BMP-4 on Synthesis of Osteocalcin in MC3T3-E1 Cells

BMP-4 (70 ng/ml) time-dependently stimulated the synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells (Fig. 1A). The synthesis of osteocalcin stimulated by BMP-4 was significantly observed 36 h after the stimulation. The stimulatory effect of BMP-4 on osteocalcin synthesis was dose dependent in the range between 10 and 100 ng/ml (Fig. 1B).

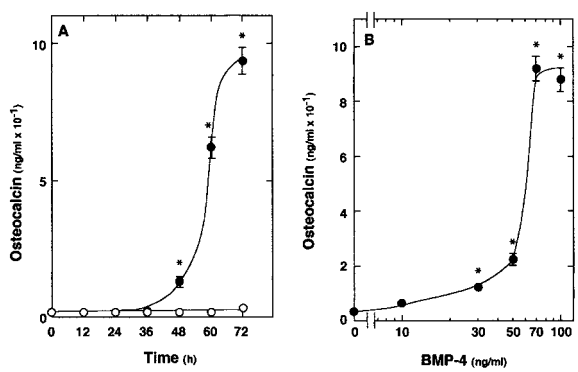

Fig. 1. Effect of BMP-4 on synthesis of osteocalcin in MC3T3-E1 cells. **A**: The cultured cells were stimulated by 70 ng/ml BMP-4 for the indicated periods. **B**: The cultured cells were stimulated by various doses of BMP-4 for 72 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$ vs. control value.

Effects of BMP-4 on Phosphorylation of p38 MAP Kinase, p44/42 MAP Kinase and SAPK/JNK in MC3T3-E1 Cells

We first examined the effects of BMP-4 on the phosphorylation of p38 MAP kinase, p44/p42 MAP kinase, and SAPK/JNK in MC3T3-E1 cells. BMP-4 significantly induced the phosphorylation of p38 MAP kinase and p44/p42 MAP kinase in a time-dependent manner (Fig. 2). On the contrary, it seems that BMP-4 had little effect on the phosphorylation of SAPK/JNK (Fig. 2).

Effects of SB203580, PD169316, PD98059, or U0126 on BMP-4-Induced Osteocalcin Synthesis in MC3T3-E1 Cells

To clarify whether p38 MAP kinase is involved in the BMP-4-stimulated synthesis of osteocalcin in MC3T3-E1 cells, we examined the effect on the synthesis of SB203580, a specific inhibitor of p38 MAP kinase [Cuenda et al., 1995]. SB203580, which alone did not affect the basal level of osteocalcin, significantly suppressed the osteocalcin synthesis stimulated by BMP-4 (Fig. 3A), and this inhibitory effect of SB203580 on the osteocalcin synthesis by BMP-4 was dose-dependent in the range

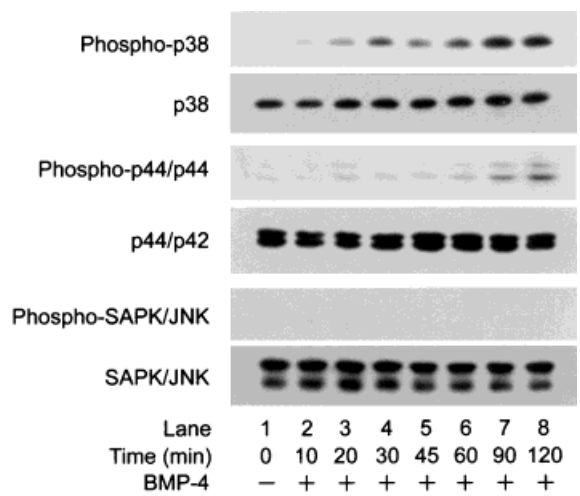

Fig. 2. Effects of BMP-4 on phosphorylation of p38 MAP kinase, p44/42 MAP kinase, and SAPK/JNK in MC3T3-E1 cells. The cultured cells were stimulated by 70 ng/ml BMP-4 for the indicated periods. Extracts of cells were subjected to SDS–PAGE against each antibodies. Similar results were obtained with two additional and different cell preparations.

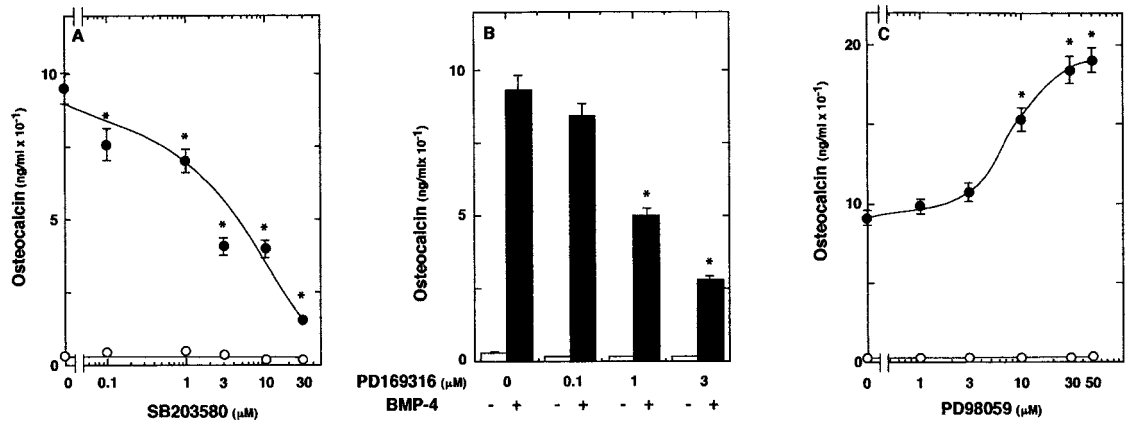

Fig. 3. Effects of SB203580, PD169316, and PD98059 on BMP-4-induced osteocalcin synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SB203580 (**A**), PD169316 (**B**), and PD98059 (**C**) for 60 min, and then stimulated by 70 ng/ml BMP-4 for 72 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$ vs. BMP-4 alone.

between 1 and 30 μM. The maximum effect of SB203580 was observed at 30 μM, a dose that caused about a 70% reduction in the BMP-4-effect. In addition, the effect of PD169316, another inhibitor of p38 MAP kinase [Kummer et al., 1997], on osteocalcin synthesis was examined. PD169316, which had little effect on the basal level of osteocalcin, reduced the BMP-4-stimulated osteocalcin synthesis as well as SB203580 (Fig. 3B).

We next investigated the involvement of p44/p42 MAP kinase in the osteocalcin synthesis by BMP-4 in MC3T3-E1 cells. PD98059, a specific inhibitor of upstream kinase that activates p44/p42 MAP kinase (MEK 1) [Alessi et al., 1995], which by itself did not affect the basal level of osteocalcin, markedly enhanced the BMP-4-induced osteocalcin synthesis (Fig. 3C). The maximum effect of PD98059 on osteocalcin synthesis was observed at 50 μM, a dose that caused about a 100% enhancement in the BMP-4-effect. The BMP-4-induced synthesis of osteocalcin was amplified by U0126, another inhibitor of upstream kinase that activates p44/p42 MAP kinase (MEK 1) [Favata et al., 1998], as well as PD98059 (control, 3.7 ± 0.3 ng/ml; 70 ng/ml BMP-4 alone, 90.0 ± 4.7 ng/ml; 0.3 μM U0126 alone, 5.7 ± 0.5 ng/ml; 70 ng/ml BMP-4 plus 0.3 μM U0126, 132.4 ± 6.0* ng/ml, as measured after 72 h stimulation, $^*P < 0.05$). In this study, we found that the viability of cells treated with 50 μM PD98059 or 30 μM U0126 for 73 h was more than 90%, as assessed by trypan blue staining.

Effects of PD98059 on BMP-4-Induced Phosphorylation of p44/p42 MAP Kinase and p38 MAP Kinase in MC3T3-E1 Cells

We found that PD98059 truly suppressed the BMP-4-induced phosphorylation of p44/p42 MAP kinase (Fig. 4A). According to the densitometric analysis, PD98059 caused about a 65% reduction in the BMP-4-effect (the mean density values in Fig. 4A; lane 1: 0.1 ± 0.01; lane 2: 57.8 ± 2.7; lane 3: 0.4 ± 0.02; lane 4: 19.9 ± 1*; $^*P < 0.05$). In addition, in order to investigate whether the BMP-4-activated p44/p42 MAP kinase affects p38 MAP kinase activation, we examined the effect of PD98059 on the p38 MAP kinase phosphorylation stimulated by BMP-4. PD98059 had little effect on the BMP-4-induced phosphorylation of p38 MAP kinase (the mean density values in Fig. 4B; lane 1: 7.0 ± 0.1; lane 2: 42.3 ± 2.8; lane 3: 7.3 ± 0.2; lane 4: 47.5 ± 3.2).

DISCUSSION

In the present study, we demonstrated that BMP-4 induced the phosphorylation of p38 MAP kinase and p44/p42 MAP kinase, but not the phosphorylation of SAPK/JNK in osteoblast-like MC3T3-E1 cells. It has been shown that BMP-2 increases p44/p42 MAP kinase activity in ROS 17/2.8 osteosarcoma cells [Palcy and Goltzman, 1999]. In addition, BMP-2 reportedly induces MAP kinase activity during osteoblastic differentiation in mesenchymal progenitor cells [Lou et al., 2000]. It is well

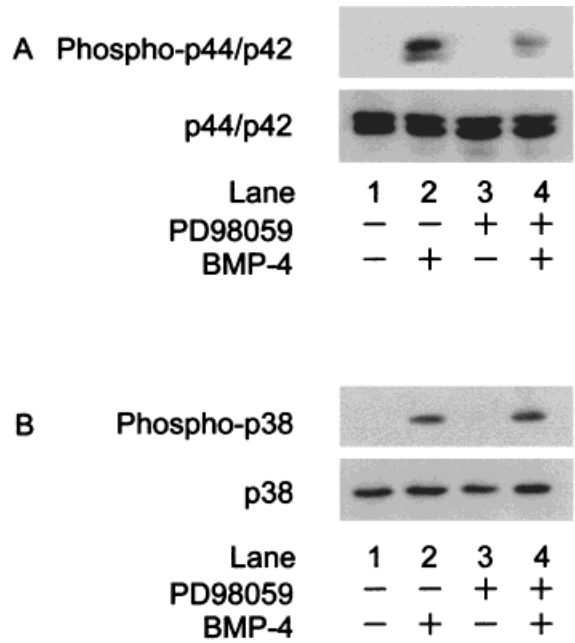

Fig. 4. Effects of PD98059 on BMP-4-induced phosphorylation of p44/p42 MAP kinase or p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM PD98059 for 60 min, and then stimulated by 70 ng/ml BMP-4 for 120 min. **A**: Extracts of cells were subjected to SDS–PAGE against phospho-specific p44/p42 MAP kinase antibodies or p44/p42 MAP kinase antibodies. **B**: Extracts of cells were subjected to SDS–PAGE against phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies.

known that MAP kinase is activated by phosphorylation on tyrosine and threonine by dual-specificity MAP kinase kinase [Raingeaud et al., 1995]. Based on these findings, it is probable that BMP-4 activates both p38 MAP kinase and p44/p42 MAP kinase, but not SAPK/JNK in osteoblast-like MC3T3-E1 cells.

It is well recognized that the MAP kinase superfamily including a three-kinase molecule plays crucial roles in the intracellular signaling of a variety of agonists [Widmann et al., 1999]. Recently, it has been shown that p44/p42 MAP kinase is involved in BMP-2-induced collagen type I gene expression in ROS 17/2.8 osteosarcoma cells [Palcy and Goltzman, 1999]. As for osteocalcin production, BMP-2 reportedly induces the expression of mRNA and the synthesis in rat osteoblast-like cells [Yamaguchi et al., 1991]. In this study, we measured osteocalcin in conditioned medium. Although the osteocalcin was secreted from osteoblast-like MC3T3-E1 cells, it is most likely that the changes in long-term secretion represent changes in synthesis rather than a specific secretory process. In the present study, we investigated the involvement of p44/p42 MAP kinase and/or p38 MAP kinase in the BMP-4-induced osteocalcin synthesis in MC3T3-E1 cells. First, PD98059, a specific inhibitor of MEK 1 [Alessi et al., 1995], upregulated the BMP-4-induced synthesis of osteocalcin. We confirmed that PD98059 truly suppressed the BMP-4-induced phosphorylation of p44/p42 MAP kinase. Thus, it seems that BMP-4-induced p44/p42 MAP kinase activation inhibits osteocalcin synthesis by BMP-4 itself. In addition, we showed that U0126, another inhibitor of MEK 1 [Favata et al., 1998], enhanced the osteocalcin synthesis stimulated by BMP-4. Therefore, taking our results into account, it is most likely that the BMP-4-stimulated synthesis of osteocalcin is negatively regulated via p44/p42 MAP kinase activated by BMP-4 itself in osteoblast-like MC3T3-E1 cells.

We next showed that the BMP-4-induced osteocalcin synthesis was significantly suppressed in a dose-dependent manner by SB20-3580, an inhibitor of p38 MAP kinase [Cuenda et al., 1995]. Therefore, our findings suggest that p38 MAP kinase is involved in BMP-4-induced osteocalcin synthesis in addition to p44/p42 MAP kinase in MC3T3-E1 cells. In addition, PD169316, another inhibitor of p38 MAP kinase [Kummer et al., 1997], reduced the osteocalcin synthesis stimulated by BMP-4. Thus, these results suggest that p38 MAP kinase is necessary for BMP-4-stimulated osteocalcin synthesis in osteoblast-like MC3T3-E1 cells. We next investigated whether p44/p42 MAP kinase affects the BMP-4-induced activation of p38 MAP kinase in these cells. We showed that PD98059 had little effect on the p38 MAP kinase phosphorylation by BMP-4. It seems unlikely that MEK 1 inhibitor enhances the BMP-4-induced synthesis of osteocalcin via amplifying the activation of p38 MAP kinase in osteoblast-like MC3T3-E1 cells. Taking these results into account, it is probable that the effect of p44/p42 MAP kinase is not exerted at a point upstream from p38 MAP kinase in BMP-4-stimulated osteocalcin synthesis. Further investigations would be necessary to clarify the exact mechanism of p44/p42 MAP kinase and p38 MAP kinase in the regulation of osteocalcin synthesis in osteoblasts.

It is well known that BMP-2 and -4 are synthesized and secreted by osteoblasts [Centrella et al., 1994]. In osteoblast-like MC3T3-E1 cells, it has been reported that

BMP-2 and -4 similarly stimulate alkaline phosphatase activity [Nakase et al., 1997]. It has also been shown that BMP-4 induces bone nodule formation more potently than BMP-2 in rat osteoblasts [Hughes et al., 1995]. On the contrary, BMP-2, but not BMP-4, reportedly stimulates chemotactic migration in human osteoblasts and human osteosarcoma cells [Llnd et al., 1996]. These findings led us to speculate on the qualitative differences between BMP-2 and -4 functional osteoblast biology. Further investigation is necessary to clarify the details.

In conclusion, our results strongly suggest that p38 MAP kinase takes part in BMP-4-stimulated osteocalcin synthesis as a positive regulator, and that p44/p42 MAP kinase acts as a negative regulator in osteoblasts.

REFERENCES

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. 1995. PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. J Biol Chem 270:27489–27494.

Centrella M, Horowitz MC, Wozney JM, McCarthy TL. 1994. Transforming growth factor-β gene family members and bone. Endocr Rev 15:27–39.

Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. 1995. SB203580 is a specific inhibitor of a MAP kinase homologue, which is stimulated by cellular stresses and interleukin-1. FEBS Lett 364:229–233.

Ducy P, Karsenty G. 1996. Osteocalcin. In: Bilezikian JP, Raisz LG, Rodan GA, editors. Principles of bone biology 1st. San Diego: Academic Press. pp 183–195.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273: 18625–18632.

Franchesi RT, Iyer BS. 1992. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J Bone Miner Res 7: 235–246.

Harland RM. 1994. The transforming growth factor β family and induction of the vertebrate mesoderm: Bone morphogenetic proteins are ventral induces. Proc Natl Acad Sci USA 91:10243–10246.

Heldin C-H, Miyazono K, ten Dijke P. 1997. TGF-β signalling from cell membrane to nucleus through SMAD proteins. Nature 390:465–471.

Hughes FJ, Collyer J, Stanfield M, Goodman SA. 1995. The effects of bone morphogenetic protein-2, -4, and -6 on differentiation of rat osteoblast cells in vitro. Endocrinology 136:2671–2677.

Kawabata M, Imamura T, Miyazono K. 1998. Signal transduction by bone morphogenetic proteins. Cyto Growth Factors Rev 9:49–61.

Kozawa O, Suzuki A, Tokuda H, Uematsu T. 1997. Prostaglandin $F_{2}\alpha$ stimulates interleukin-6 synthesis via activation of PKC in osteoblast-like cells. Am J Physiol 272:E208–E211.

Kummer JL, Rao PK, Heidenreich KA. 1997. Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. J Biol Chem 282: 20490–20494.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227: 680–685.

Llnd M, Eriksen EF, Bunger C. 1996. Bone morphogenetic protein-2, but not bone morphogenetic protein-4 and -6 stimulates chemotactic migration of human osteoblasts, human marrow osteoblasts, and U2-OS cells. Bone 18: 53–57.

Lou J, Tu Y, Li S, Manske PR. 2000. Involvement of ERK in BMP-2 induced osteoblastic differentiation of mesenchymal progenitor cell line C3H10T1/2. Biochem Biophys Res Commun 268:757–762.

Massague J. 1998. TGF-β signal transduction. Annu Rev Biochem 67:753–791.

Miwa M, Kozawa O, Tokuda H, Uematsu T. 1999. Mitogen-activated protein (MAP) kinases are involved in interleukin-1 (IL-1)-induced IL-6 synthesis in osteoblasts: Modulation not of p38 MAP kinase, but of p42/p44 MAP kinase by IL-1-activated protein kinase C. Endocrinology 140:5120–5125.

Nakase T, Takaoka K, Masuhara K, Shimizu K, Yoshikawa H, Ochi T. 1997. Interleukin-1β enhances and tumor necrosis factor-α inhibits bone morphogenetic protein-2-induced alkaline phosphatase activity in MC3T3-E1 osteoblastic cells. Bone 21:17–21.

Palcy S, Goltzman D. 1999. Protein kinase signalling pathways involved in the up-regulation of the rat α1(I) collagen gene by transforming growth factor β1 and bone morphogenetic protein 2 in osteoblastic cells. Biochem J 343:21–27.

Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ. 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation of tyrosine and threonine. J Biol Chem 270:7420–7426.

Reddi AH. 1994. Bone and cartilage differentiation. Curr Opin Genet Dev 4:737–744.

Stein GS, Lian JB. 1993. Molecular mechanisms mediating proliferation/differentiation interrelationship during progressive development of the osteoblastic phenotypes. Endocr Rev 14:424–442.

Sudo H, Kodama H, Amagai Y, Yamamoto S, Kasai S. 1983. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96:191–198.

Widmann C, Gibson S, Jarpe MB, Johnson GL. 1999. Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. Physiol Rev 79:143–180.

Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G, Franceschi RT. 2000. MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J Biol Chem 275:4453–4459.

Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, Wang EA, Kahn AJ, Suda T, Yoshiki S. 1991. Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation. J Cell Biol 113:681–687.

Yamaguchi A, Ishizuya T, Kitou N, Wada Y, Katagiri T, Wozney JM, Rosen V, Yoshiki S. 1996. Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST-2 and MC3T3-G2/PA6. Biochem Biophys Res Commun 220: 366–371.

Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H, Matsumoto K. 1999. XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptor to TAB1-TAK1 in the BMP signaling pathway. EMBO J 18:179–187.