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Involvement of p70 S6 Kinase in Bone Morphogenetic Protein Signaling: Vascular Endothelial Growth Factor Synthesis by Bone Morphogenetic Protein-4 in Osteoblasts

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Abstract In the present study, we investigated the effect of bone morphogenetic protein (BMP)-4 on the synthesis of vascular endothelial growth factor (VEGF) in osteoblast-like MC3T3-E1 cells. BMP-4 significantly stimulated VEGF synthesis time-dependently up to 48 h. The stimulatory effect was dose-dependent in the range between 1 and 100 ng/ml. BMP-4 time-dependently phosphorylated p70 S6 kinase. Rapamycin, an inhibitor of p70 S6 kinase, suppressed the BMP-4-stimulated VEGF synthesis as well as the phosphorylation of p70 S6 kinase. The VEGF synthesis by BMP-4 was suppressed by wortmannin and LY294002, inhibitors of phosphatidylinositol 3-kinase. Both wortmannin and LY294002 inhibited the BMP-4-stimulated phosphorylation of p70 S6 kinase. BMP-4 did not affect the phosphorylation of Akt/protein kinase B. Taken together, our results strongly suggest that p70 S6 kinase takes part in BMP-4-stimulated VEGF synthesis as a positive regulator in osteoblasts and that phosphatidylinositol 3-kinase acts at a point upstream from p70 S6 kinase. J. Cell. Biochem. 81:430–436, 2001.

Key words: BMP; VEGF; S6 kinase; PI-3-kinase; osteoblasts

Bone metabolism is known to be regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [Nijweide et al., 1986]. The formation of bone structures and bone remodeling results from coupling bone resorption by activated osteoblasts with subsequent deposition of new matrix by osteoblasts. During bone remodeling, capillary endothelial cells provides the microvasculature. It is currently recognized that the activity of osteoblasts, osteoclasts and vascular endothelial cells are closely coordinated [Erlebacher et al., 1995].

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that induces endothelial cell proliferation, angiogenesis and capillary permeability [Neufeld et al., 1999]. VEGF is known to be produced and secreted from many cell types. As for osteoblasts, it has been reported that prostaglandin (PG) E_2, PGE_1 and insulin-like growth factor I stimulate synthesis of VEGF in osteoblasts [Harada et al., 1994; Goad et al., 1996]. It is currently recognized that VEGF secreted from osteoblasts play important roles in bone remodeling. However, the exact mechanism underlying VEGF production in osteoblasts has not yet been clarified.

Bone morphogenetic proteins (BMPs) that have been originally identified by their ability to form ectopic bone, are multifunctional cytokines, which belong to the transforming growth factor-β (TGF-β) superfamily [Reddi, 1994; Kawabata et al., 1998]. It is known that BMPs play a crucial role in the early development of vertebrates [Harland, 1994]. As for osteoblasts, it has been shown that BMP-2 and BMP-4 are synthesized by osteoblasts [Centrella et al., 1994]. In addition, BMPs reportedly stimulate alkaline phosphatase activity and the expression of osteocalcin, markers of mature osteoblast phenotype [Yamaguchi et al., 1991, 1996]. It is well recognized that Smad proteins such as Smad1 and Smad5 act as mediators of BMPs similar to TGF-β [Reddi, 1994; Heldin et al., 1997; Massague, 1998]. In addition, other signaling pathways such as the mitogen-activated protein kinase superfamily have also been

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shown to be mediators of BMP signaling [Yamaguchi et al., 1995]. However, the exact mechanisms behind BMP intracellular signaling have not yet been precisely clarified.

p70 S6 kinase is a mitogen-activated serine/threonine kinase that is recognized to be required for cell proliferation and G1 cell cycle progression [Pullen and Thomas, 1997]. It has been shown that phosphatidylinositol 3-kinase and Akt/protein kinase B act as upstream regulators of p70 S6 kinase [Pullen and Thomas, 1997]. As for osteoblasts, it has been reported that fluoroaluminate induces an increase in p70 S6 kinase phosphorylation [Susa et al., 1997]. In the present study, we investigated the effect of BMP-4 on VEGF synthesis and the involvement of p70 S6 kinase on the signaling of BMP-4-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. Herein, we show that BMP-4 stimulates VEGF synthesis in these cells and that p70 S6 kinase is involved in the BMP-4-induced VEGF synthesis.

MATERIALS AND METHODS

Materials

BMP-4 and mouse VEGF enzyme immunoassay (EIA) kit were purchased from R&D Systems (Tokyo, Japan). Rapamycin was obtained from Calbiochem-Novabiochem (La Jolla, CA). Wortmannin and LY294002 were purchased from Biomol Research laboratories, Inc. (Plymouth, PA). Phospho-specific p70 S6 (Thr389) kinase antibodies (rabbit polyclonal IgG, affinity purified), p70 S6 kinase antibodies (rabbit polyclonal IgG, affinity purified), phospho-specific Akt (Thr308) antibodies (rabbit polyclonal IgG, affinity purified), Akt 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. Rapamycin, wortmannin and LY294002 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for VEGF nor 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.

Assay for VEGF

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 VEGF in the medium was then measured by a VEGF EIA kit. When indicated, the cells were pretreated with rapamycin, wortmannin or LY294002 for 20 min.

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% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as the supernatant after centrifugation at $125{,}000 \times g$ for 10 min at 4°C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli [Laemmli, 1970] in 10% polyacrylamide gel. Western blotting analysis was performed as previously described [Miwa et al., 1999] using phospho-specific p70 S6 kinase antibodies, p70 S6 kinase antibodies, phospho-specific Akt antibodies or Akt 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 ECL Western blotting detection system. When indicated, the cells were pretreated with rapamycin, wortmannin or LY294002 for 20 min.

Determination

The absorbance of EIA samples was measured at 450 nm with SLT-Labinstruments EAR 340 AT. Absorbance was correlated with concentration through a standard curve.

Statistical Analysis

The data were analyzed by ANOVA followed by Bonferroni method for multiple comparisons

between pairs, and a $P < 0.05$ was considered significant. All data are presented as the mean ± SD of triplicate determinations.

RESULTS

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

BMP-4 (30 ng/ml) significantly stimulated the synthesis of VEGF in MC3T3-E1 cells (Fig. 1A). The stimulatory effect of BMP-4 on VEGF synthesis was time-dependent and significant after 24 h from the stimulation up to 48 h. The effect of BMP-4 on VEGF synthesis was dose-dependent in the range between 1 and 100 ng/ml (Fig. 1B). The maximum effect was observed at 100 ng/ml.

Effect of BMP-4 on p70 S6 Kinase Phosphorylation in MC3T3-E1 Cells

In order to clarify whether BMP-4 activates p70 S6 kinase in MC3T3-E1 cells, we next examined the effect of BMP-4 on the phosphorylation of p70 S6 kinase. p70 S6 kinase was time-dependently phosphorylated by BMP-4 (Fig. 2). The maximum effect on phosphorylation of p70 S6 kinase was observed at 3 h after the stimulation of BMP-4.

Effect of Rapamycin on BMP-4-Induced VEGF Synthesis in MC3T3-E1 Cells

To investigate whether p70 S6 kinase is involved in the BMP-4-induced VEGF synthesis in MC3T3-E1 cells, the effect of rapamycin, a specific inhibitor of p70 S6 kinase [Price et al., 1992; Kuo et al., 1992], on the synthesis of VEGF by BMP-4 was examined. Rapamycin, which alone had little effect on VEGF synthesis, significantly suppressed the BMP-4-induced VEGF synthesis (Fig. 3A). The inhibitory effect of rapamycin on the BMP-4-induced VEGF synthesis was dose-dependent between 1 and 30 ng/ml, and the maximum effect of rapamycin was observed at 10 ng/ml, a dose that caused a 75% reduction in the BMP-4-effect.

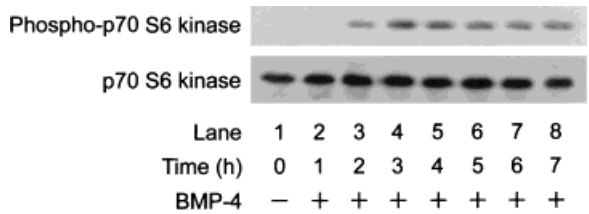

Fig. 2. Effect of BMP-4 on p70 S6 kinase phosphorylation in MC3T3-E1 cells. The cultured cells were stimulated by 30 ng/ml BMP-4 for the indicated periods. Extracts of cells were subjected to SDS-PAGE against phospho-specific p70 S6 kinase antibodies or p70 S6 kinase antibodies.

Effect of Rapamycin on BMP-4-Stimulated Phosphorylation of p70 S6 Kinase in MC3T3-E1 Cells

We next examined the effect of rapamycin on the BMP-4-stimulated phosphorylation of p70 S6 kinase. Rapamycin actually inhibited the

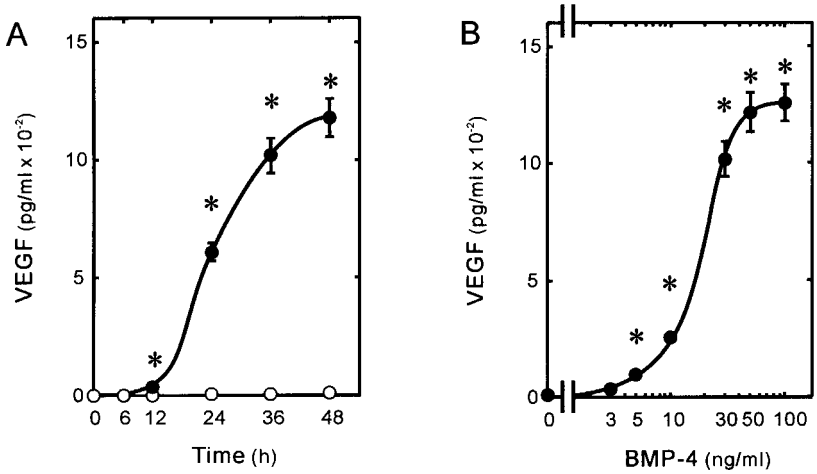

Fig. 1. Effect of BMP-4 on synthesis of VEGF in MC3T3-E1 cells. (**A**) The cultured cells were stimulated by 30 ng/ml BMP-4 (solid circle) or vehicle (open circle) for the indicated periods. (**B**) The cultured cells were stimulated by various doses of BMP-4 for 36 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$ vs. control value.

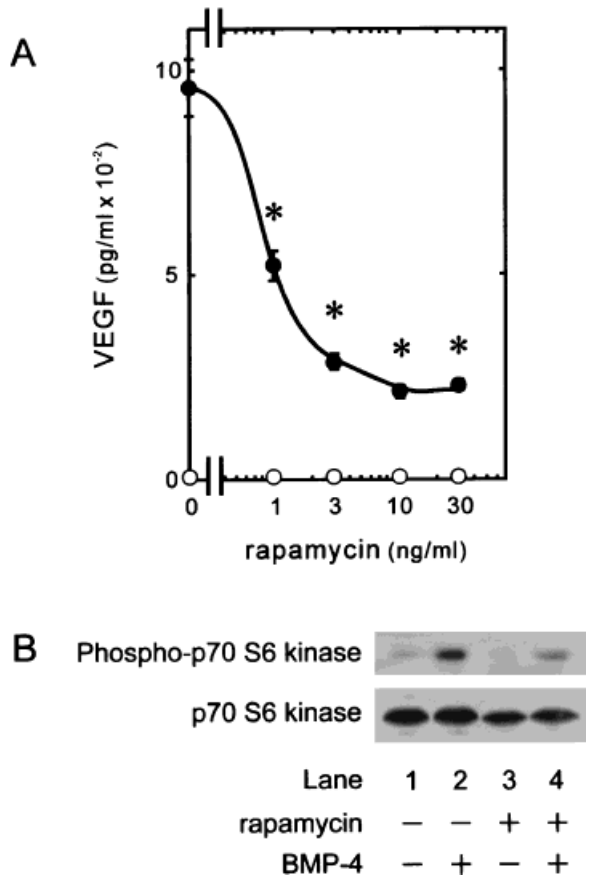

Fig. 3. Effect of rapamycin on BMP-4-induced VEGF synthesis and phosphorylation of p70 S6 kinase in MC3T3-E1 cells. (**A**) The cultured cells were pretreated with various doses of rapamycin for 20 min, and then stimulated by 30 ng/ml BMP-4 for 36 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$ vs. control value. (**B**) The cultured cells were pretreated with 30 ng/ml rapamycin for 20 min, and then stimulated by 30 ng/ml BMP-4 for 3 h. Extracts of cells were subjected to SDS-PAGE against phospho-specific p70 S6 kinase antibodies or p70 S6 kinase antibodies.

phosphorylation of p70 S6 kinase stimulated by BMP-4 (Fig. 3B).

Effects of Wortmannin or LY294002 on BMP-4-Induced VEGF Synthesis in MC3T3-E1 Cells

It has been shown that p70 S6 kinase acts at a point downstream from phosphatidylinositol 3-kinase [Pullen and Thomas, 1997]. Thus, to clarify whether p70 S6 kinase is involved in the BMP-4-induced VEGF synthesis in MC3T3-E1 cells, the effect of wortmannin, an inhibitor of phosphatidylinositol 3-kinase [Arcaro and Wymann, 1993], on the synthesis of VEGF by BMP-4 was examined. Wortmannin, which alone did not affect the basal levels of VEGF, significantly suppressed the BMP-4-induced VEGF synthesis (Fig. 4A). The inhibitory effect of wortmannin was dose-dependent between 1 and 100 nM, and the maximum effect of wortmannin was observed at 100 nM, a dose that caused a 67% reduction in the effect of BMP-4. In addition, the BMP-4-induced synthesis of VEGF was markedly reduced by LY294002, a specific inhibitor of phosphatidylinositol 3-kinase [Vlahos et al., 1994], which by itself had little effect on VEGF synthesis (Fig. 4B). LY294002 (10 μM) almost completely suppressed the VEGF synthesis by BMP-4.

Effects of Wortmannin or LY294002 on BMP-4-Stimulated Phosphorylation of p70 S6 Kinase in MC3T3-E1 Cells

In order to further clarify whether phosphatidylinositol 3-kinase acts at a point upstream from p70 S6 kinase in MC3T3-E1 cells, we next examined the effect of wortmannin or LY294002 on the phosphorylation of p70 S6 kinase. The BMP-4-stimulated phosphorylation of p70 S6 kinase was markedly reduced by wortmannin or LY294002 (Fig. 4C).

Effect of BMP-4 on Akt/Protein Kinase B Phosphorylation in MC3T3-E1 Cells

It has been shown that Akt/protein kinase B acts between phosphatidylinositol 3-kinase and p70 S6 kinase in several cell types [Pullen and Thomas, 1997]. To investigate whether BMP-4 activates Akt/protein kinase B in MC3T3-E1 cells, we next examined the effect of BMP-4 on the phosphorylation of Akt/protein kinase B. However, BMP-4 hardly affected the phosphorylation of Akt/protein kinase B (data not shown).

DISCUSSION

In the present study, we showed that BMP-4 stimulated VEGF secretion in osteoblast-like MC3T3-E1 cells. It has been reported that osteoblasts synthesize VEGF in response to PGE_2, PGE_1 and insulin-like growth factor I [Harada et al., 1994; Goad et al., 1996]. We here measured VEGF secreted from MC3T3-E1 cells and showed that BMP-4 did not affect the secretion of VEGF until 12 h after the stimulation and thereafter the values of VEGF were significantly increased by BMP-4 in MC3T3-E1 cells. Thus, it is probable that values in long-term

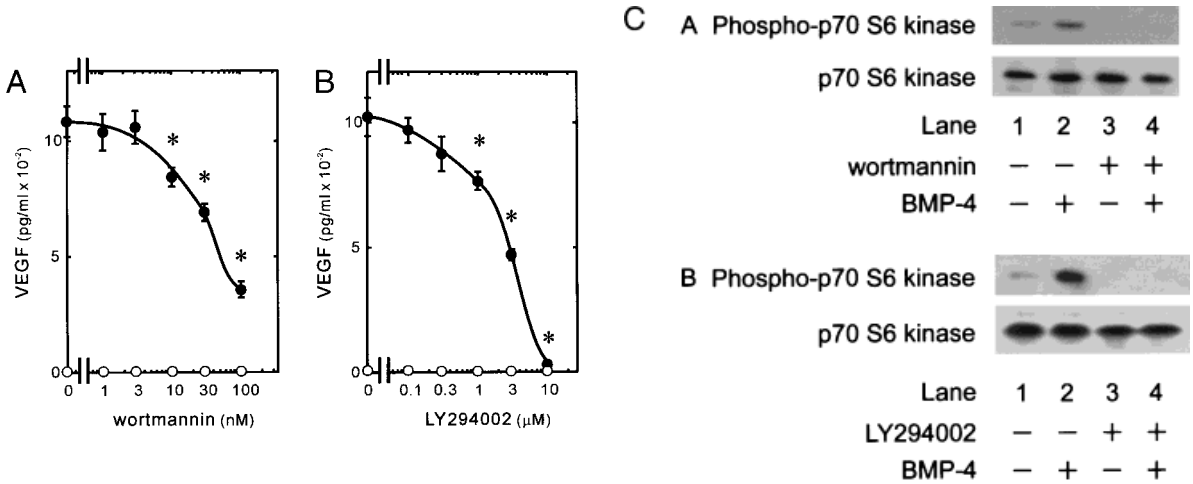

Fig. 4. Effects of wortmannin and LY294002 on BMP-4-induced VEGF synthesis and BMP-4-stimulated phosphorylation of p70 S6 kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of wortmannin (**A**) or LY294002 (**B**) for 20 min, and then stimulated by 30 ng/ml BMP-4 for 36 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$ vs. control value. (**C**) The cultured cells were pretreated with 100 nM wortmannin or 10 μM LY294002 for 20 min, and then stimulated by 30 ng/ml BMP-4 for 3 h. Extracts of cells were subjected to SDS-PAGE against phospho-specific p70 S6 kinase antibodies or p70 S6 kinase antibodies.

secretion of VEGF stimulated by BMP-4 reflect the values in the synthesis.

We showed here that BMP-4 stimulated the phosphorylation of p70 S6 kinase in osteoblast-like MC3T3-E1 cells, using phospho-specific p70 S6 kinase (Thr389) antibodies. It is well recognized that the activity of p70 S6 kinase is regulated by multiple phosphorylation events [Pullen and Thomas, 1997]. Among the phosphorylation, it has been reported that phosphorylation at Thr389 most clearly correlates with p70 S6 kinase activity [Pullen and Thomas, 1997]. Therefore, our results suggest that BMP-4 activates p70 S6 kinase in osteoblast-like MC3T3-E1 cells. We next demonstrated that rapamycin inhibited the BMP-4-induced synthesis of VEGF. Rapamycin is structurally related to immunosuppressant FK506 [Pullen and Thomas, 1997]. It has been shown that rapamycin inhibits the phosphorylation and activation of p70 S6 kinase stimulated by interleukin-2 or insulin [Price et al., 1992; Kuo et al., 1992]. Currently, it is well recognized that rapamycin selectively inhibits the phosphorylation and activation of p70 S6 kinase [Pullen and Thomas, 1997]. Thus, rapamycin is a useful tool for investigating the involvement of p70 S6 kinase. We found that rapamycin actually inhibited the BMP-4-induced phosphorylation of p70 S6 kinase. Taking our findings into account, it is most likely that p70 S6 kinase is required for the VEGF synthesis stimulated by BMP-4 in osteoblast-like MC3T3-E1 cells. To the best of our knowledge, this is probably the first report showing the stimulatory effect of BMP-4 on the activation of p70 S6 kinase resulting in VEGF synthesis.

We next showed that the BMP-4-induced VEGF synthesis was significantly inhibited by wortmannin, an inhibitor of phosphatidylinositol 3-kinase [Arcaro and Wymann, 1993]. Therefore, our findings seem that phosphatidylinositol 3-kinase is involved in the BMP-4-induced VEGF synthesis in MC3T3-E1 cells. In addition, LY294002 [Vlahos et al., 1994] dose-dependently suppressed the VEGF synthesis stimulated by BMP-4. Thus, our results suggest that phosphatidylinositol 3-kinase is necessary for the BMP-4-stimulated VEGF synthesis in MC3T3-E1 cells. It has been reported that phosphatidylinositol 3-kinase acts at a point upstream from p70 S6 kinase in several cell types [Pullen and Thomas, 1997]. In addition, we demonstrated that wortmannin and LY294002 suppressed the phosphorylation of p70 S6 kinase induced by BMP-4. Based on these findings, it is most likely that BMP-4 activates p70 S6 kinase through phosphatidylinositol 3-kinase, resulting in the VEGF synthesis in osteoblast-like MC3T3-E1 cells. However, the amounts of rapamycin, wortmannin or LY294002 needed to suppress the VEGF synthesis induced by BMP-4 were pharmacological doses. Thus, the effects of these pharmacologi-

cal inhibitors may be indirect. Further investigations would be necessary to clarify the details.

Akt/protein kinase B that is activated by phospholipid binding and phosphorylation of both Thr308 and Ser473 is a serine/threonine kinase and the cellular homologue of the product of the retroviral oncogene v-akt [Bellacasa et al., 1991; Alessi et al., 1996]. Recent evidence indicates that phosphatidylinositol 3-kinase mediates the activation of Akt/protein kinase B by several growth factors [Alessi et al., 1996] and that Akt/protein kinase B acts at a point upstream from p70 S6 kinase in several cell types [Pullen and Thomas, 1997]. In this study, BMP-4 had little effect on Akt/protein kinase B phosphorylation using phospho-specific Akt (Thr308) antibodies. Based on these findings, it seems unlikely that BMP-4 activates Akt/protein kinase B and that Akt/protein kinase B is involved in the BMP-4-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells.

VEGF is a highly specific mitogen for vascular endothelial cells [Neufeld et al., 1999]. Therefore, our results lead us to speculate that BMP-4 modulates endothelial cell function via VEGF, which is produced and secreted from osteoblasts by BMP-4 itself. Taking these findings as a whole into account, it is probable that osteoblasts and endothelial cells are closely related and these two cell populations regulate each other in bone remodeling. Further investigations would be required to clarify the details.

In conclusion, our results strongly suggest that p70 S6 kinase participates in BMP-4-stimulated VEGF synthesis as a positive regulator in osteoblasts and that phosphatidylinositol 3-kinase acts at a point upstream from p70 S6 kinase.

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