Oncostatin M enhances osteoprotegerin synthesis but reduces macrophage colony-stimulating factor synthesis in bFGF-stimulated osteoblast-like cells

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Abstract. Bone remodeling is tightly controlled by various factors, including hormones, autacoids and cytokines. Among them, oncostatin M (OSM) is a multifunctional cytokine produced by osteal macrophages, which serves as an essential modulator of bone remodeling. Macrophage colony-stimulating factor (M-CSF) and osteoprotegerin are secreted by osteoblasts, and also have pivotal roles in the regulation of the bone remodeling process. The binding of basic fibroblast growth factor (bFGF), a key regulator of bone remodeling, to the corresponding receptor [fibroblast growth factor receptor (FGFR)] triggers the dimerization and activation of FGFRs, which causes the phosphorylation of FGFR substrates and subsequent activation of downstream effectors, including mitogen-activated protein kinases (MAPKs), via Grb2. bFGF can activate MAPKs, resulting in the synthesis of osteoprotegerin and vascular endothelial growth factor in osteoblast-like MC3T3-E1 cells. In the present study, the effects of OSM on bFGF-induced osteoblast activation were investigated in the synthesis of osteoprotegerin and M-CSF in osteoblasts. The release of osteoprotegerin and M-CSF were analyzed using ELISA. The mRNA expression levels of osteoprotegerin and M-CSF were analyzed using reverse transcription-quantitative PCR. Phosphorylation of p38 MAPK, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p44/p42 MAPK was assessed using western blotting. OSM enhanced bFGF-induced osteoprotegerin release and bFGF-stimulated mRNA expression of osteoprotegerin. By contrast, OSM suppressed the bFGF-induced release of M-CSF and bFGF-stimulated mRNA expression of M-CSF. SB203580, a p38 MAPK inhibitor, and SP600125, a SAPK/JNK inhibitor, suppressed the bFGF-stimulated M-CSF release, whereas PD98059, an upstream kinase inhibitor of p44/p42 MAPK, failed to suppress the M-CSF release stimulated by bFGF. Furthermore, OSM enhanced the bFGF-induced phosphorylation of p38 MAPK, but attenuated the bFGF-stimulated phosphorylation of SAPK/JNK. By contrast, OSM had little effect on the bFGF-induced phosphorylation of p44/p42 MAPK. SB203580 markedly reduced the amplification of bFGF-stimulated osteoprotegerin release enhanced by OSM. These results strongly suggested that OSM may possess divergent effects on bFGF-induced osteoblast activation, upregulation of p38 MAPK and downregulation of SAPK/JNK, leading to the amplification of osteoprotegerin synthesis and the attenuation of M-CSF synthesis.

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Introduction

Bone tissue homeostasis is maintained by osteoclast-mediated bone resorption and osteoblast-mediated bone formation (1,2). This continuous regenerative process is generally recognized as bone remodeling, an imbalance in which can cause metabolic bone diseases, such as osteoporosis or poor/inappropriate fracture healing (2). Bone remodeling is tightly controlled by various factors, including hormones, autacoids and cytokines (1,2). Because receptors for bone resorptive factors, such as parathyroid hormone and vitamin D, are not found on osteoclasts but on osteoblasts, osteoblasts serve a central role in the regulation of bone resorption (1,2). Macrophage colony-stimulating factor (M-CSF), which is released from osteoblasts, is a hematopoietic growth factor that promotes the proliferation and differentiation of osteoclast progenitor cells (3,4). M-CSF also stimulates the differentiation of osteoclast precursors into mature osteoclasts with bone resorptive activity and activates

bone resorption by cooperating with the receptor activator of nuclear factor-κB (RANK) ligand (RANKL) expressed on osteoblasts, which binds to RANK on the surface of osteoclast precursors (5,6). On the other hand, osteoprotegerin released from osteoblasts is a glycoprotein belonging to the tumor necrosis factor receptor family (7), which binds to RANKL as a decoy receptor and inhibits RANKL-RANK binding, thereby reducing bone resorption via the suppression of osteoclast differentiation (7,8). Thus, M-CSF and osteoprotegerin released from osteoblasts serve pivotal roles in the regulation of bone remodeling.

Osteal macrophages located on the surface of bone remodeling sites have been reported to play diverse roles in skeletal homeostasis (9). The removal of apoptotic osteoblasts by osteal macrophage phagocytosis generates specific proteins, such as transforming growth factor (TGF)-β, to promote the differentiation of progenitor cells into osteoblasts, leading to osteogenesis (9). Oncostatin M (OSM), which is secreted by osteal macrophages, is a member of the IL-6 family that shares gp130 as a common subunit of the IL-6 family receptor (10). OSM is expressed in several cell types, including osteoblast lineage cells, and has its effects through multiple receptors, such as OSM receptor (OSMR) and leukemia inhibitory factor receptor; the roles of both pathways have previously been explored in osteoblast regulation (11). Regarding bone metabolism, it has been reported that OSM stimulates the activation of osteoblasts and inhibits bone resorption (12). It has also been demonstrated that OSMR-deficient mice exhibit osteopetrosis with a reduced number of osteoblasts and osteoclasts (13). Additionally, the lack of OSM function reportedly leads to delayed bone fracture healing in mouse models (14). These findings suggest that OSM may act as an essential modulator of bone remodeling.

Basic fibroblast growth factor (bFGF), which is embedded in the bone matrix, is released from bone remodeling sites by the process of bone resorption and induces osteoblast lineage cells to promote osteogenesis (15,16). Thus, bFGF is considered to serve a role in the regulation of bone remodeling. Regarding the effects on osteoblast lineage cells, bFGF reportedly induces the expression of M-CSF mRNA, with an increase of M-CSF secretion from murine bone marrow stromal cells, precursors of osteoblasts (17). The binding of bFGF to the corresponding receptor, fibroblast growth factor receptor (FGFR), triggers the dimerization and activation of FGFRs, which causes the phosphorylation of FGFR substrates and subsequent activation of downstream effectors, including mitogen-activated protein kinases (MAPKs), via Grb2 (18). Notably, our previous study reported that bFGF elicits FGFR autophosphorylation due to dimerization in osteoblast-like MC3T3-E1 cells (19). In addition, our previous study demonstrated that bFGF stimulates the synthesis of osteoprotegerin via activation of p38 MAPK and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells (20). As for the intracellular signaling mechanism of bFGF, it has been indicated that bFGF activates p44/p42 MAPK, in addition to p38 MAPK and SAPK/JNK, resulting in the upregulation of vascular endothelial growth factor (VEGF) synthesis in these cells (21,22). These findings led to the hypothesis that osteoblast functions are finely tuned by MAPKs stimulated by bFGF. Furthermore, our recent study reported that OSM suppresses TGF-β-stimulated syntheses of M-CSF and VEGF in these cells (23). However, the details of the molecular mechanisms underlying how OSM affects osteoblast functions remain to be elucidated.

In the present study, the effects and the underlying mechanisms of OSM on the bFGF-induced synthesis of osteoprotegerin and M-CSF were investigated in osteoblast-like MC3T3-E1 cells.

Materials and methods

Cell culture. Clonal osteoblast-like MC3T3-E1 cells derived from newborn mouse calvariae (24) were donated by Dr Masayoshi Kumegawa (Graduate School of Dentistry, Department of Dentistry, Meikai University, Sakado, Japan) and maintained as previously described (20). Mouse MC3T3-E1 cells were cultured in α-minimum essential medium (α-MEM) (MilliporeSigma) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO_2/95% air. The cells were seeded onto 35-mm diameter dishes ($5x10^4$ cells/dish) for enzyme-linked immunosorbent assay (ELISA) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, or 90-mm diameter dishes ($2x10^5$ cells/dish) for western blot analysis in α-MEM supplemented with 10% FBS. After 5 days, the medium was replaced with α-MEM supplemented with 0.3% FBS. After 48 h, the cells were used for experiments. It is well known that the addition of ascorbate and β-glycerophosphate (BGP) practically induces the differentiation of MC3T3-E1 cells into osteoblasts (25). In the present study, α-MEM containing 50 mg/l ascorbate, but no BGP, was used, thus experiments were performed on a model of pre-osteoblasts.

ELISA. To assess osteoprotegerin, the cultured MC3T3-E1 cells were pretreated with 0, 3, 10, 30 or 50 ng/ml OSM for 60 min at 37˚C, and then stimulated with 30 ng/ml bFGF or vehicle (mast cell medium; 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.4) in 1 ml α-MEM supplemented with 0.3% FBS for 48 h at 37˚C. Pretreatment with 30 μM SB203580 or vehicle was performed for 60 min prior to pretreatment with OSM. To assess M-CSF, the cultured MC3T3-E1 cells were pretreated with 0, 3, 10, 30 or 50 ng/ml OSM, 50 μM PD98059, 10 μM SB203580, 3 μM SP600125 or vehicle (50 μl mast cell medium) for 60 min at 37˚C, and then stimulated with 30 ng/ml bFGF or vehicle (50 μl of mast cell medium) in 1 ml α-MEM supplemented with 0.3% FBS for 48 h at 37˚C. Recombinant mouse OSM, and ELISA kits for mouse osteoprotegerin (cat. no. MOP00) and M-CSF (cat. no. MMC00) were obtained from R&D Systems, Inc. Recombinant human bFGF was purchased from Gibco; Thermo Fisher Scientific, Inc. PD98059, SB203580 and SP600125 were purchased from Calbiochem; Merck KGaA. OSM and bFGF were dissolved in mast cell medium. PD98059, SB203580 and SP600125 were dissolved in dimethyl sulfoxide. The conditioned medium was then collected, and the concentrations of osteoprotegerin and M-CSF were measured using the mouse ELISA kits for osteoprotegerin or M-CSF, in accordance with the manufacturer's protocols.

RT-qPCR. The cultured MC3T3-E1 cells were pretreated with 50 ng/ml OSM or vehicle (50 μl of mast cell medium) for 60 min at 37˚C and were then stimulated with 30 ng/ml bFGF or vehicle (50 μl of mast cell medium) in α-MEM containing 0.3% FBS for 4 or 6 h at 37˚C. TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and Omniscript Reverse Transcriptase kit (Qiagen, Inc.) were used to isolate total RNA and transcribe it into cDNA, respectively. qPCR was performed using a LightCycler 2 Real-Time PCR system and software (version 3.5; Roche Diagnostics) with the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the manufacturer's protocol. Samples were subjected to thermocycling conditions as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 1 sec, annealing at 60˚C for 5 sec and elongation at 72˚C for 7 sec. Forward and reverse primers for mouse osteoprotegerin (primer set ID: MA026526) and M-CSF mRNA (primer set ID: MA171365) were obtained from Takara Bio Inc. The primer sequences were as follows: Osteoprotegerin, forward 5'-CAATGGCTGGCTTGGTTTCATAG-3', reverse 5'-CTGAACCAGACATGACAGCTGGA-3'; M-CSF, forward 5'-CATGTGGAGCAGCATGAGG-3' and reverse 5'-CAA TGTCTGAGGGTCTCGATGG-3'. Forward and reverse primers for mouse GAPDH mRNA were synthesized based on the report of Simpson *et al* (26). The primer sequences for GAPDH were as follows: Forward 5'-AACGACCCCTTC ATTGAC-3' and reverse 5'-TCCACGACATACTCAGCA C-3'. The amplified products were determined by melting curve analysis in accordance with the system protocol. The mRNA expression levels of osteoprotegerin and M-CSF were normalized to those of GAPDH using SPSS Statistics (version 22; IBM, Corp.), and the relative mRNA expression levels were analyzed using the $2^{-\Delta\Delta Cq}$ method (27), which was created automatically with the LightCycler software in each run (20,23).

Western blot analysis. The cultured MC3T3-E1 cells were pretreated with 0, 30, 50 or 70 ng/ml OSM for 60 min at 37˚C, and then stimulated with 30 ng/ml bFGF or vehicle (200 μl of mast cell medium) in 4 ml α-MEM containing 0.3% FBS for 10 or 20 min. The cells were then lysed, homogenized and sonicated (output 20W, 1 sec x20 cycles at 4˚C) in a lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The concentration of protein in the samples was assessed using a Pierce BCA protein kit (cat. no. 23225; Thermo Fisher Scientific, Inc.). Proteins (20 μg per lane) were separated by SDS-polyacrylamide electrophoresis, which was performed in accordance with the method of Laemmli using 10% polyacrylamide gels (28). The proteins were then transferred onto Immuno-Blot PVDF membranes (Bio-Rad Laboratories, Inc.), which were blocked with 5% fat-free dry milk in Tris-buffered saline-Tween (TBS-T: 20 mM Tris-HCl, pH 7.6; 137 mM NaCl; 0.1% Tween 20) for 1 h at room temperature before incubation with primary antibodies. The membrane was subsequently incubated at 4˚C overnight with primary antibodies (1:1,000) followed by incubation with the appropriate secondary antibodies (1:1,000) at room temperature for 1 h. Western blot analysis was performed as described previously (20) using primary antibodies against phosphorylated (p)-p38 MAPK (cat. no. 4511), p38 MAPK (cat. no. 9212), p-SAPK/JNK (cat. no. 4668), SAPK/JNK (cat. no. 9252), p-p44/p42 MAPK (cat. no. 9101), p44/p42 MAPK (cat. no. 9102) (all from Cell Signaling Technology Inc.) or GAPDH (cat. no. 60004-1-lg; Proteintech Group, Inc.). KPL horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (cat. no. 5220-0336; SeraCare Life Sciences, Inc.) or HRP-labeled anti-mouse IgG antibodies (cat. no. 7076; Cell Signaling Technology, Inc.) were used as a secondary antibody. The ECL western blot detection system was purchased from Cytiva. The primary and secondary antibodies were diluted in TBS-T with 5% fat-free dry milk to optimal concentrations. An X-ray film with the ECL western blot detection system was used to visualize peroxidase activity on the membrane, and different membranes were used for every single protein. A densitometric analysis was performed using a scanner and image analysis program (ImageJ version 1.48; National Institutes of Health). The background-subtracted signal intensity of each phosphorylation signal was normalized to the respective intensity of the total protein, and then plotted as the fold increase compared to that of control cells treated without stimulation (20).

Statistical analysis. All data were analyzed using Mini StatMate (version 2.01; ATMS Co., Ltd.). Data are presented as the mean ± SEM of at least triplicate determinations from independent cell preparations. The statistical significance of the data was analyzed using one-way or two-way analysis of variance, as appropriate, followed by the Bonferroni method for multiple comparisons between pairs. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of OSM on the bFGF-induced release of osteoprotegerin and M-CSF from MC3T3-E1 cells. To investigate the effect of OSM on osteoblasts, the present study examined the effect of OSM on bFGF-induced osteoprotegerin release from these cells. OSM, which by itself did not affect osteoprotegerin release, significantly enhanced the bFGF-stimulated osteoprotegerin release observed within a range of 3-50 ng/ml (Fig. 1A). The maximum effect of OSM observed at 30 ng/ml was ~320% amplification of the bFGF effect.

The present study next examined the effect of OSM on bFGF-induced M-CSF release from osteoblast-like MC3T3-E1 cells. OSM, which by itself did not affect M-CSF release, significantly suppressed the bFGF-stimulated M-CSF release within a range of 3-50 ng/ml (Fig. 1B). The maximum effect of OSM observed at 10 ng/ml was ~35% attenuation of the bFGF effect.

Effects of OSM on the mRNA expression levels of osteoprotegerin and M-CSF in MC3T3-E1 cells. To elucidate whether the OSM-induced increase in the release of osteoprotegerin stimulated by bFGF was mediated via transcriptional events, the present study examined the effect of OSM on the bFGF-induced mRNA expression of osteoprotegerin in osteoblast-like MC3T3-E1 cells. Although OSM by itself did not have any significant effect on the mRNA expression levels of osteoprotegerin, it significantly enhanced the bFGF-upregulated mRNA expression levels of osteoprotegerin when used at a dose of 50 ng/ml (Fig. 1C).

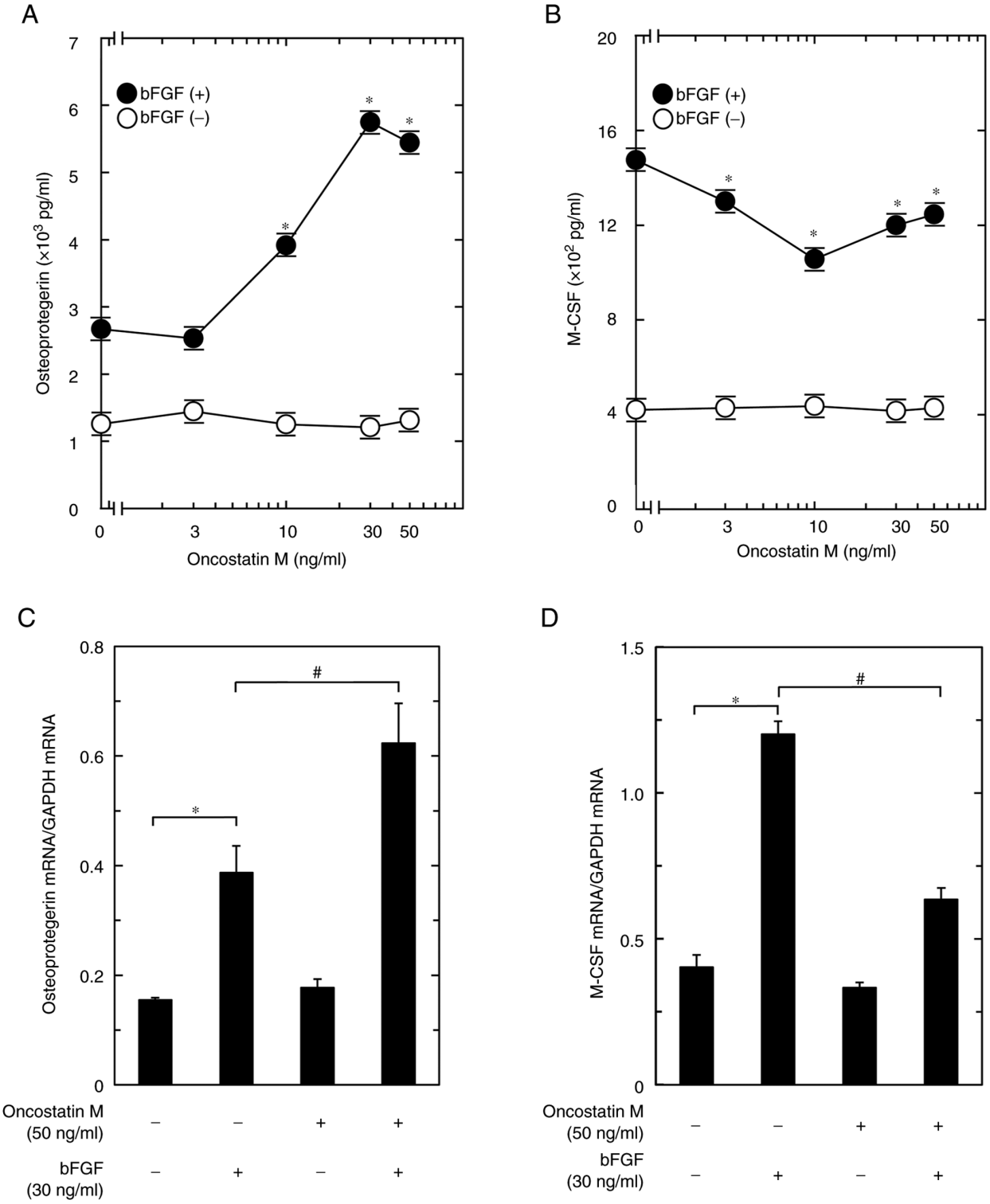

Figure 1. Effects of OSM on the bFGF-induced release and mRNA expression levels of osteoprotegerin and M-CSF in MC3T3-E1 cells. The cultured cells were pretreated with 0, 3, 10, 30 or 50 ng/ml OSM for 60 min, and subsequently stimulated with 30 ng/ml bFGF or vehicle for 48 h. The concentrations of (A) osteoprotegerin and (B) M-CSF in the culture medium were determined by enzyme-linked immunosorbent assay. Identical samples were analyzed. The cultured cells were pretreated with 50 ng/ml OSM or vehicle for 60 min, and subsequently stimulated with 30 ng/ml bFGF or vehicle for (C) 4 h or (D) 6 h. The total RNA was subsequently isolated and transcribed into cDNA. The mRNA expression levels of (C) osteoprotegerin and (D) M-CSF were quantified by reverse transcription-quantitative PCR. The mRNA expression levels of osteoprotegerin and M-CSF were normalized to those of GAPDH. Data are presented as the mean ± SEM of triplicate determinations from three independent cell preparations. *P<0.05 vs. control; #P<0.05 vs. bFGF alone. bFGF, basic fibroblast growth factor; M-CSF, macrophage colony-stimulating factor; OSM, oncostatin M.

To evaluate whether OSM suppressed the bFGF-stimulated M-CSF release through transcriptional events, the present study next examined the effect of OSM on the bFGF-induced mRNA expression of M-CSF in these cells. OSM alone did not have any significant effect on the mRNA expression levels of M-CSF; however, the bFGF-upregulated mRNA expression

levels of M-CSF were significantly suppressed by OSM at a concentration of 50 ng/ml (Fig. 1D).

Effects of PD98059, SB203580 or SP600125 on the bFGF-induced M-CSF release from MC3T3-E1 cells. To investigate the intracellular signaling mechanism underlying the bFGF-induced synthesis of M-CSF in MC3T3-E1 cells, the present study examined the effects of PD98059, an inhibitor of MEK1/2 which is the upstream kinase of p44/p42 MAPK (29), SB203580, a specific inhibitor of p38 MAPK (30), or SP600125, a specific inhibitor of SAPK/JNK (31), on the bFGF-induced M-CSF release from osteoblast-like MC3T3-E1 cells. PD98059 failed to suppress M-CSF release with or without bFGF. By contrast, SB203580 and SP600125, which by themselves had little effect on M-CSF release, significantly reduced the bFGF-stimulated M-CSF release (Fig. 2). Regarding the effects of PD98059, SB203580 and SP600125 on the activities of p44/p42 MAPK, p38 MAPK and SAPK/JNK, respectively, in these cells, we previously reported that the bFGF-induced phosphorylation of p44/p42 MAPK, p38 MAPK and SAPK/JNK was markedly suppressed by PD98059, SB203580 and SP600125, respectively (21,22). Thus, the present results suggested that M-CSF synthesis involves the bFGF-elicited activation of not p44/p42 MAPK, but of p38 MAPK and SAPK/JNK, in MC3T3-E1 cells.

Effects of OSM on the bFGF-induced phosphorylation of p38 MAPK, SAPK/JNK and p44/p42 MAPK in MC3T3-E1 cells. In order to investigate whether OSM modulates the activation of p38 MAPK, SAPK/JNK and p44/p42 MAPK, the present study next examined the effects of OSM on the phosphorylation levels induced by bFGF in osteoblast-like MC3T3-E1 cells. As previously reported (21,22), it was confirmed that bFGF significantly induced the phosphorylation of p38 MAPK, SAPK/JNK and p44/p42 MAPK (Fig. 3A-C). OSM, which alone hardly affected the levels of p-p38 MAPK, significantly enhanced the levels of bFGF-induced phosphorylation at 30, 50 and 70 ng/ml (Fig. 3A). By contrast, OSM, which by itself hardly affected the phosphorylation levels of SAPK/JNK, markedly dose-dependently suppressed the levels of phosphorylation stimulated by bFGF at 30, 50 and 70 ng/ml (Fig. 3B). Furthermore, OSM hardly affected the levels of p44/p42 MAPK phosphorylation when used at doses up to 70 ng/ml with or without bFGF stimulation (Fig. 3C).

Effect of SB203580 on the amplification by OSM of the bFGF-induced osteoprotegerin release from MC3T3-E1 cells. To elucidate whether the enhancing effect of OSM on the bFGF-stimulated osteoprotegerin release was truly mediated through p38 MAPK, the present study examined the effect of SB203580 (30) on the amplification by OSM of the osteoprotegerin release induced by bFGF in osteoblast-like MC3T3-E1 cells. As previously reported (20), SB203580, which alone had little effect on osteoprotegerin levels, significantly suppressed the bFGF-induced osteoprotegerin release (Fig. 4). SB203580 also markedly reduced the amplification of the bFGF-stimulated osteoprotegerin release caused by OSM (Fig. 4).

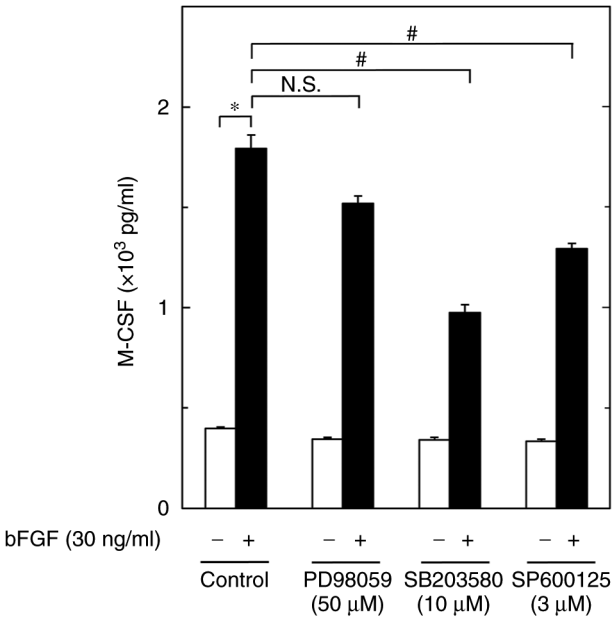

Figure 2. Effects of PD98059, SB203580 and SP600125 on the bFGF-induced release of M-CSF from MC3T3-E1 cells. The cultured cells were pretreated with 50 μM PD98059, 10 μM SB203580, 3 μM SP600125 or vehicle for 60 min, and subsequently stimulated with 30 ng/ml bFGF or vehicle for 48 h. M-CSF concentrations in the culture medium were determined using enzyme-linked immunosorbent assay. Data are presented as the mean ± SEM of quadruplicate determinations from four independent cell preparations. *P<0.05 vs. control; #P<0.05 vs. bFGF alone. bFGF, basic fibroblast growth factor; M-CSF, macrophage colony-stimulating factor; N.S., not significant.

Discussion

The present study used osteoblast-like MC3T3-E1 cells to explore the effects of OSM, a cytokine produced by osteal macrophages (9), on the bFGF-induced synthesis of osteoprotegerin and M-CSF, which are the inhibitory factor and the promoting factor of osteoclastogenesis, respectively (3,4,7). The results revealed that the bFGF-induced release of osteoprotegerin was clearly enhanced by OSM. In addition, the mRNA expression levels of osteoprotegerin stimulated by bFGF were amplified by OSM, indicating that the enhancing effect of OSM on the bFGF-induced osteoprotegerin release may be mediated through transcriptional events in these cells. Therefore, it is likely that OSM potentiates the synthesis of osteoprotegerin stimulated by bFGF in osteoblasts. The present study also demonstrated that the bFGF-stimulated M-CSF release was suppressed by OSM. In addition, the mRNA expression levels of M-CSF induced by bFGF were markedly reduced by OSM, suggesting that the suppressive effect of OSM on the bFGF-induced M-CSF release may be mediated through a reduction of transcriptional events in these cells. It is likely that OSM could diminish the synthesis of M-CSF stimulated by bFGF in osteoblasts. Thus, OSM seems to regulate bFGF-stimulated osteoblast functions, having diverse effects on the syntheses of osteoprotegerin and M-CSF, amplifying the former and suppressing the latter. To the best of our knowledge, this seems to be the first report that clearly presents the effects of OSM on bFGF-induced osteoblast activation. bFGF embedded in bone matrix is released by bone resorption in the process of bone remodeling and affects

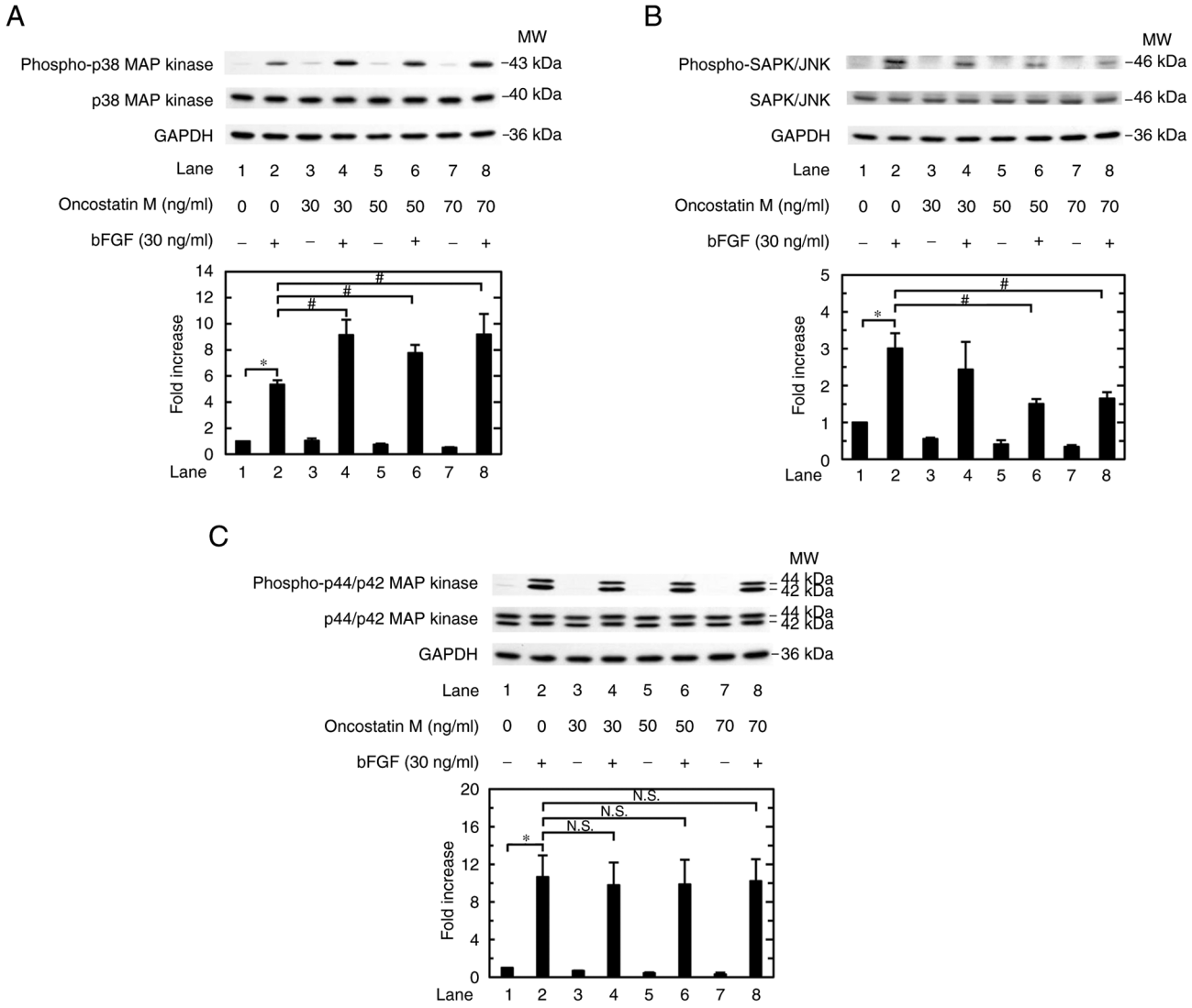

Figure 3. Effects of OSM on the bFGF-induced phosphorylation of p38 MAPK, SAPK/JNK and p44/p42 MAPK in MC3T3-E1 cells. The cultured cells were pretreated with 0, 30, 50 or 70 ng/ml OSM for 60 min, and then stimulated with 30 ng/ml bFGF or vehicle for (A) 10 or (B and C) 20 min. The cell extracts were then subjected to SDS-PAGE and western blot analysis with antibodies against (A) p-p38 MAPK, p38 MAPK and GAPDH; (B) p-SAPK/JNK, SAPK/JNK and GAPDH; or (C) p-p44/p42 MAPK, p44/p42 MAPK and GAPDH. The histograms show the semi-quantitative representations of the expression levels of (A) p-p38 MAPK after normalization to p38 MAPK, (B) p-SAPK/JNK after normalization to SAPK/JNK, and (C) p-p44/p42 MAPK after normalization to p44/p42 MAPK obtained from densitometric analysis. The levels were expressed as the fold increase with respect to the basal levels presented in lane 1. Data are presented as the mean ± SEM of (A) quadruplicate determinations from four independent cell preparations or (B and C) triplicate determinations from three independent cell preparations. *P<0.05 vs. control; $^{\#}$P<0.05 vs. bFGF alone. bFGF, basic fibroblast growth factor; MAPK, mitogen-activated protein kinase; MW, molecular weight N.S., not significant; p-, phosphorylated; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase.

osteoblast lineage cells to promote osteogenesis (15,16), and as such is considered a direct stimulator. OSM secreted by osteal macrophages translocated for bone remodeling, can stimulate the activation of osteoblasts and inhibit bone resorption (9), and as such is considered a modulator. Notably, the present study revealed that OSM by itself hardly affected the synthesis of osteoprotegerin or M-CSF in osteoblast-like MC3T3-E1 cells. Thus, the reason the present study performed pretreatment with OSM and stimulation with bFGF is that OSM itself cannot stimulate but instead modulates for the activation of osteoblast-like cells.

Regarding the intracellular signaling system underlying the effects of bFGF in osteoblasts, our previous study demonstrated that p38 MAPK and SAPK/JNK are involved in the bFGF-stimulated osteoprotegerin synthesis in osteoblast-like MC3T3-E1 cells (20). In addition, it has been reported that p44/p42 MAPK is activated by bFGF stimulation in these cells (21). The present study revealed that both SB203580 (30) and SP600125 (31) significantly reduced the release of M-CSF induced by bFGF, suggesting that activation of both p38 MAPK and SAPK/JNK are involved in bFGF-induced M-CSF synthesis as positive regulators in these cells. Furthermore, PD98059 (29) was shown to hardly affect bFGF-stimulated M-CSF release, thus it is unlikely that p44/p42 MAPK is involved in the M-CSF synthesis induced by bFGF in these cells. Therefore, it is likely that activation of p38 MAPK and SAPK/JNK, but not p44/p42 MAPK, is commonly involved in the syntheses of osteoprotegerin and M-CSF in osteoblast-like MC3T3-E1 cells.

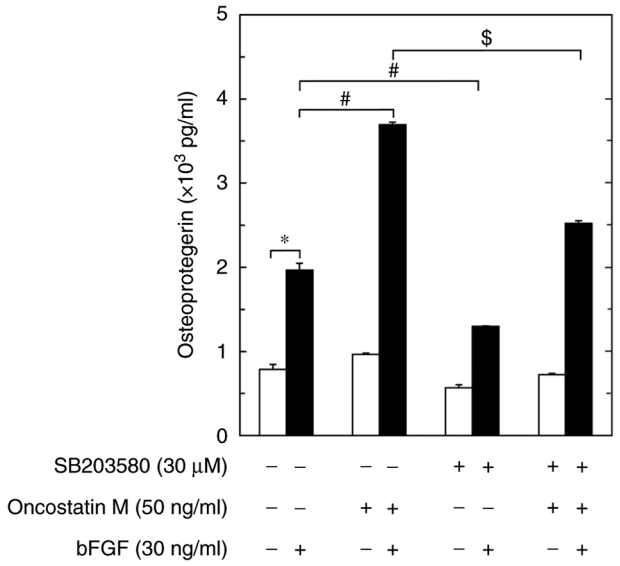

Figure 4. Effect of SB203580 on the amplification by OSM of the bFGF-induced osteoprotegerin release from MC3T3-E1 cells. The cultured cells were preincubated with 30 μM SB203580 or vehicle for 60 min, subsequently pretreated with 50 ng/ml OSM or vehicle for 60 min, and then stimulated with 30 ng/ml bFGF or vehicle for 48 h. Osteoprotegerin concentrations in the culture medium were determined by enzyme-linked immunosorbent assay. Data are presented as the mean ± SEM of triplicate determinations from three independent cell preparations. *P<0.05 vs. control; #P<0.05 vs. bFGF alone; $P<0.05 vs. OSM + bFGF. bFGF, basic fibroblast growth factor; OSM, oncostatin M.

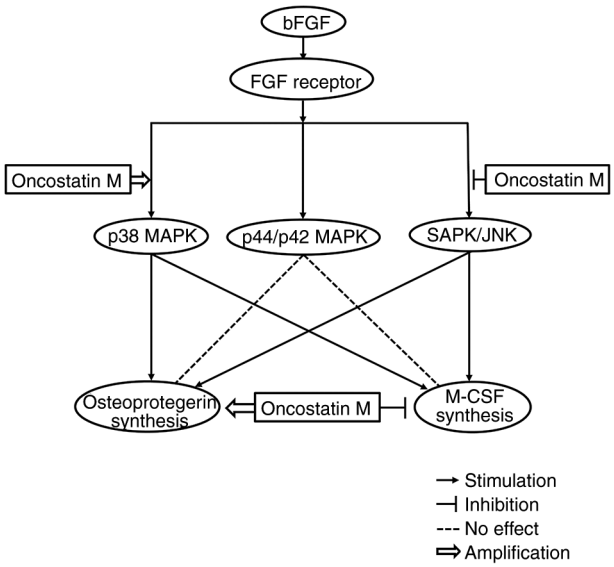

Figure 5. Schematic illustration of the mechanism underlying the bFGF-induced osteoprotegerin and M-CSF synthesis indicating how and where OSM affects osteoblast-like cells. bFGF, basic fibroblast growth factor; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase.

The present study demonstrated that OSM enhanced the bFGF-induced phosphorylation of p38 MAPK, indicating that OSM may upregulate activation of p38 MAPK induced by bFGF in MC3T3-E1 cells. By contrast, OSM did not enhance but suppressed the bFGF-induced phosphorylation of SAPK/JNK, suggesting that OSM may downregulate the activation of SAPK/JNK stimulated by bFGF in these cells. In addition, it was confirmed that bFGF-stimulated phosphorylation of p44/p42 MAPK was not affected by OSM, indicating that OSM is not able to affect the p44/p42 MAPK activation by bFGF in these cells. Thus, it is likely that the upregulation of p38 MAPK caused by OSM is involved in the enhancement of bFGF-induced osteoprotegerin synthesis in osteoblast-like MC3T3-E1 cells. The present study further examined the effect of SB203580 (30) on the OSM-induced enhancement of osteoprotegerin release stimulated by bFGF in these cells. The results demonstrated that SB203580 markedly reduced the amplification of bFGF-stimulated osteoprotegerin release caused by OSM in these cells. Therefore, it seems that OSM may amplify bFGF-induced osteoprotegerin synthesis, at least in part via the upregulation of p38 MAPK activation in osteoblasts. As SB203580 is a common selective inhibitor of p38α and p38β MAPK (32), it is not clear which subtype works in the amplification of bFGF-induced osteoprotegerin synthesis by OSM. However, it is recognized that p38α is the most highly expressed isoform of p38 MAPK in osteoblasts (33), suggesting that p38α is a promising candidate. In addition, OSM has been shown to downregulate the activation of SAPK/JNK stimulated by bFGF in osteoblasts. Taking into account this finding, it is possible that downregulating SAPK/JNK activation could result in the suppression of bFGF-stimulated M-CSF synthesis in osteoblasts. The schematic illustration of the mechanism underlying osteoprotegerin and M-CSF synthesis induced by bFGF, indicating where and how OSM affects this, is presented as Fig. 5.

In bone metabolism, osteoprotegerin is a pivotal regulator of osteoclastogenesis and osteoclastic bone resorption to competitively interrupt RANK and RANKL binding as the decoy receptor for RANKL (7,8). Thus, the enhancement of osteoblast-derived osteoprotegerin synthesis by OSM seems to suppress the accelerated bone resorption in metabolic bone diseases such as osteoporosis. In addition, M-CSF, which plays a pivotal role in osteoclastogenesis to promote the proliferation and differentiation of osteoclast progenitor cells (3,4), is recognized to activate osteoclastic bone resorption in cooperation with RANK and RANKL binding (5,6). It can be hypothesized that OSM-induced downregulation of M-CSF synthesis by osteoblasts would reduce both the number and activity of osteoclasts. It is probable that OSM is a potent functional modulator of osteoblasts to suppress osteoclastic bone resorption in bone remodeling. In addition, osteocytes, differentiated from osteoblasts, which are recognized as the most abundant cell type in bone, also produce the cytokines M-CSF, RANKL and osteoprotegerin (34). Taking this into account, the effects of OSM on the synthesis of osteoprotegerin and M-CSF by bFGF-stimulated osteocytes in addition to osteoblasts needs to be elucidated to clarify the detailed regulatory mechanism of bone remodeling. Regarding the effect of OSM on M-CSF synthesis, we recently reported that TGF-β-stimulated M-CSF synthesis is reduced by OSM via suppression of p44/p42 MAPK and SAPK/JNK in osteoblast-like cells (23). As aforementioned, it is probable that bFGF-elicited activation of p44/p42 MAPK is not affected by OSM in these cells. Moreover, SAPK/JNK is likely involved in the M-CSF synthesis induced by bFGF in these cells. Thus, the

mechanisms underlying M-CSF synthesis and the outcome of OSM effect on the synthesis are dependent on what stimulates the osteoblasts. Such a precisely regulated signaling mechanism of M-CSF synthesis and OSM effect might indicate the importance of both M-CSF synthesis and OSM action in the functions of osteoblasts in bone remodeling. Osteal macrophages may play a pivotal role as functional cells in regulating bone remodeling through OSM, which promotes bone formation. Thus, the present findings may provide new insights into the mechanism underlying physiological bone metabolism.

There are several limitations in the present study. First, the present study could not show that the effects of SB203580 were not detected in the suppressive effect of OSM on bFGF-induced M-CSF release. Furthermore, the effects of PD98059 and SP600125 on the release of osteoprotegerin and M-CSF stimulated by bFGF and OSM were not detected. However, these experiments may be unnecessary, because it is unlikely that p44/p42 MAPK is involved in the effects of OSM, or that SAPK/JNK would be involved in the down regulation by OSM of bFGF-stimulated M-CSF release. Furthermore, the findings were not confirmed in other cell lines. In addition, the spontaneous differentiation of MC3T3-E1 cells during culture could affect the levels of response to OSM. Further investigations, including those using primary cultured cells, or disease and development-related animal models, would be necessary to clarify the details. The effect of OSM via bFGF-stimulated osteoblasts on bone remodeling could also be strengthened if the conditioned medium from osteoblast-like cells treated with OSM and bFGF had a considerable impact on osteoclastogenesis.

In conclusion, the findings of the present study strongly suggested that OSM may possess diverse effects on bFGF-induced osteoblast activation via p38 MAPK and SAPK/JNK, leading to the amplification of osteoprotegerin synthesis and the attenuation of M-CSF synthesis. These results may provide novel insights for bone remodeling.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

OK and HT conceived and designed the experiments. TH, JT, KU and RMN performed the experiments. TH, RMN, HI, OK and HT analyzed the data. TH, OK and HT wrote the paper. All authors read and approved the final manuscript. OK and HT confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Kular J, Tickner J, Chim SM and Xu J: An overview of the regulation of bone remodeling at the cellular level. Clin Biochem 45: 863-873, 2012.
2. Kim JM, Lin C, Stavre Z, Greenblatt MB and Shim JH: Osteoblast-osteoclast communication and bone homeostasis. Cells 9: 2073, 2020.
3. Fuller K, Owens JM, Jagger CJ, Wilson A, Moss R and Chambers TJ: Macrophage colony-stimulating factor stimulates survival and chemotactic behavior in isolated osteoclasts. J Exp Med 178: 1733-1744, 1993.
4. Fixe P and Praloran V: M-CSF: Haematopoietic growth factor or inflammatory cytokine? Cytokine 10: 32-37, 1998.
5. Walsh MC, Kim N, Kadono Y, Rho J, Lee SY, Lorenzo J and Choi Y: Osteoimmunology: Interplay between the immune system and bone metabolism. Annu Rev Immunol 24: 33-63, 2006.
6. Amarasekara DS, Yun H, Kim S, Lee N, Kim H and Rho J: Regulation of osteoclast differentiation by cytokine networks. Immune Netw 18: e8, 2018.
7. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Lüthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, *et al*: Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell 89: 309-319, 1997.
8. Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F and Heymann D: The molecular triad OPG/RANK/RANKL: Involvement in the orchestration of pathophysiological bone remodeling. Cytokine Growth Factor Rev 15: 457-475, 2004.
9. Sinder BP, Pettit AR and McCauley LK: Macrophages: Their emerging roles in bone. J Bone Miner Res 30: 2140-2149, 2015.
10. Zarling JM, Shoyab M, Marquardt H, Hanson MB, Lioubin MN and Todaro GJ: Oncostatin M: A growth regulator produced by differentiated histiocytic lymphoma cells. Proc Natl Acad Sci USA 83: 9739-9743, 1986.
11. Sims NA and Quinn JMW: Osteoimmunology: Oncostatin M as a pleiotropic regulator of bone formation and resorption in health and disease. Bonekey Rep 3: 527, 2014.
12. Bellido T, Stahl N, Farruggella TJ, Borba V, Yancopoulos GD and Manolagas SC: Detection of receptors for interleukin-6, interleukin-11, leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor in bone marrow stromal/osteoblastic cells. J Clin Invest 97: 431-437, 1996.
13. Walker EC, McGregor NE, Poulton IJ, Solano M, Pompolo S, Fernandes TJ, Constable MJ, Nicholson GC, Zhang JG, Nicola NA, *et al*: Oncostatin M promotes bone formation independently of resorption when signaling through leukemia inhibitory factor receptor in mice. J Clin Invest 120: 582-592, 2010.
14. Guihard P, Boutet MA, Brounais-Le Royer B, Gamblin AL, Amiaud J, Renaud A, Berreur M, Rédini F, Heymann D, Layrolle P and Blanchard F: Oncostatin m, an inflammatory cytokine produced by macrophages, supports intramembranous bone healing in a mouse model of tibia injury. Am J Pathol 185: 765-775, 2015.

15. Aguirre JI, Leal ME, Rivera MF, Vanegas SM, Jorgensen M and Wronski TJ: Effects of basic fibroblast growth factor and a prostaglandin E2 receptor subtype 4 agonist on osteoblastogenesis and adipogenesis in aged ovariectomized rats. J Bone Miner Res 22: 877-888, 2007.
16. Charoenlarp P, Rajendran AK and Iseki S: Role of fibroblast growth factors in bone regeneration. Inflamm Regen 37:10, 2017.
17. Abboud SL and Pinzani M: Peptide growth factors stimulate macrophage colony-stimulating factor in murine stromal cells. Blood 78: 103-109, 1991.
18. Xie Y, Su N, Yang J, Tan Q, Huang S, Jin M, Ni Z, Zhang B, Zhang D, Luo F, *et al*: FGF/FGFR signaling in health and disease. Signal Transduct Target Ther 5: 181, 2020.
19. Suzuki A, Shinoda J, Kanda S, Oiso Y and Kozawa O: Basic fibroblast growth factor stimulates phosphatidylcholine-hydrolyzing phospholipase D in osteoblast-like cells. J Cell Biochem 63: 491-499, 1996.
20. Kuroyanagi G, Otsuka T, Yamamoto N, Matsushima-Nishiwaki R, Nakakami A, Mizutani J, Kozawa O and Tokuda H: Down-regulation by resveratrol of basic fibroblast growth factor-stimulated osteoprotegerin synthesis through suppression of Akt in osteoblasts. Int J Mol Sci 15: 17886-17900, 2014.
21. Tokuda H, Kozawa O and Uematsu T: Basic fibroblast growth factor stimulates vascular endothelial growth factor release in osteoblasts: Divergent regulation by p42/p44 mitogen-activated protein kinase and p38 mitogen-activated protein kinase. J Bone Miner Res 15: 2371-2379, 2000.
22. Tokuda H, Hirade K, Wang X, Oiso Y and Kozawa O: Involvement of SAPK/JNK in basic fibroblast growth factor-induced vascular endothelial growth factor release in osteoblasts. J Endocrinol 177: 101-107, 2003.
23. Doi T, Hioki T, Tachi J, Ueda K, Matsushima-Nishiwaki R, Iida H, Ogura S, Kozawa O and Tokuda H: Oncostatin M reduces the synthesis of macrophage-colony stimulating factor stimulated by TGF-β via suppression of p44/p42 MAP kinase and JNK in osteoblasts. Biomed Res 43: 41-51, 2022.
24. Sudo H, Kodama HA, Amagai Y, Yamamoto S and Kasai S: In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96: 191-198, 1983.
25. Zambelli A, Mongiardini E, Villegas SN, Carri NG, Boot-Handford RP and Wallis GA: Transcription factor XBP-1 is expressed during osteoblast differentiation and is transcriptionally regulated by parathyroid hormone (PTH). Cell Biol Int 29: 647-653, 2005.
26. Simpson DA, Feeney S, Boyle C and Stitt AW: Retinal VEGF mRNA measured by SYBR green I fluorescence: A versatile approach to quantitative PCR. Mol Vis 6: 178-183, 2000.
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
28. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
29. Alessi DR, Cuenda A, Cohen P, Dudley DT and Saltiel AR: PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 270: 27489-27494, 1995.
30. Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR and Lee JC: SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett 364: 229-233, 1995.
31. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, *et al*: SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA 98: 13681-13686, 2001.
32. Cuenda A and Rousseau S: p38 MAP-kinases pathway regulation, function and role in human diseases. Biochim Biophys Acta 1773: 1358-1375, 2007.
33. Rodríguez-Carballo E, Gámez B and Ventura F: p38 MAPK signaling in osteoblast differentiation. Front Cell Dev Biol 4: 40, 2016.
34. Han Y, You X, Xing W, Zhang Z and Zou W: Paracrine and endocrine actions of bone-the functions of secretory proteins from osteoblasts, osteocytes, and osteoclasts. Bone Res 6: 16, 2018.