Original Paper

Heat Shock Protein 70 Negatively Regulates TGF-β-Stimulated VEGF Synthesis via p38 MAP Kinase in Osteoblasts

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Key Words

Heat shock protein 70 • TGF-β • VEGF • Mitogen-activated protein kinase • Osteoblast

Abstract

Background/Aims: We previously demonstrated that transforming growth factor-β (TGF-β) stimulates the synthesis of vascular endothelial growth factor (VEGF) through the activation of p38 mitogen-activated protein (MAP) kinase in osteoblast-like MC3T3-E1 cells. Heat shock protein70 (HSP70) is a ubiquitously expressed molecular chaperone. In the present study, we investigated the involvement of HSP70 in the TGF-β-stimulated VEGF synthesis and the underlying mechanism in these cells. ***Methods:*** Culture MC3T3-E1 cells were stimulated by TGF-β. Released VEGF was measured using an ELISA assay. VEGF mRNA level was quantified by RT-PCR. Phosphorylation of each protein kinase was analyzed by Western blotting. ***Results:*** VER-155008 and YM-08, both of HSP70 inhibitors, significantly amplified the TGF-β-stimulated VEGF release. In addition, the expression level of VEGF mRNA induced by TGF-β was enhanced by VER-155008. These inhibitors markedly strengthened the TGF-β-induced phosphorylation of p38 MAP kinase. The TGF-β-induced phosphorylation of p38 MAP kinase was amplified in HSP70-knockdown cells. SB203580, an inhibitor of p38 MAP kinase, significantly suppressed the amplification by these inhibitors of the TGF-β-induced VEGF release. ***Conclusion:*** These results strongly suggest that HSP70 acts as a negative regulator in the TGF-β-stimulated VEGF synthesis in osteoblasts, and that the inhibitory effect of HSP70 is exerted at a point upstream of p38 MAP kinase.

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Introduction

Heat shock proteins (HSPs), which have been originally identified as stress-responsive proteins possessed cytoprotective function, are induced in the cells exposed to biological stresses, including heat, hypoxia, oxidative and toxic stresses [1]. HSPs are generally established to function as molecular chaperones that protect unfolded proteins from aggregation. Up to date, a large number of HSPs have been discovered, and they were termed according to the corresponding molecular weight, such as HSP27, HSP40, HSP60, HSP70, HSP90 and HSP110 [2-5]. The HSP family have been recently classified into seven groups including HSPA (HSP70), HSPC (HSP90) and HSPH (HSP110) [6]. Among the members of HSPs, HSP70 (HSPA) ubiquitously exists in unstressed cells and functions as an ATP-dependent molecular chaperone for the assistance of folding newly synthesized proteins, and the translocation of proteins into the different cellular compartments [7-10]. Accumulating evidence indicates potent effects of HSP70 toward various diseases, such as cancer, infection and autoimmune diseases [4, 11]. Since the increase of HSP70 protein expression is reportedly related to poor outcome [12], it is currently considered that inhibition of HSP70 could become one of therapeutic targets against these diseases.

Bone metabolism is tightly coordinated by two types of functional cells, osteoblasts and osteoclasts [13]. The former cells are responsible for bone formation, whereas the latter are for bone resorption. In the adult skeletal system, bone tissue is continuously regenerated by the sequential process of resorption of old bone and formation of new bone, and bone mass is moderately maintained, so called bone remodeling [14]. Disorder of this remodeling process is considered to cause metabolic bone diseases such as osteoporosis or fracture healing distress. Multiple humoral factors including cytokines, hormones and growth factors take a part, and play a crucial role in bone remodeling process [15]. In addition, the microvasculature is essential for bone metabolism owing to blood circulation, which supplies precursor cells, nutrients, bone modulating factors needed for the adequate bone remodeling [16]. Therefore, it is currently recognized that the strict control of activity in capillary endothelial cells as well as osteoblasts and osteoclasts is important to regulate the proper bone metabolism. Vascular endothelial growth factor (VEGF), which is produced and secreted by various cells including osteoblasts, is a potent mitogen for vascular endothelial cells and acts as an angiogenic factor [17-20]. It has been shown that various growth factors including transforming growth factor-β (TGF-β) stimulate the VEGF synthesis in osteoblasts [21]. However, the exact mechanism of VEGF production and the secretion in osteoblasts has not yet been fully elucidated.

TGF-β, a member of the TGF-β superfamily including bone morphogenic proteins (BMPs) and activin, stimulates the osteoblastic bone formation in an autocrine or paracrine manner [22]. During the bone remodeling, TGF-β is released from bone matrix through the osteoclastic bone resorption, and subsequently promotes the recruitment of osteoblast progenitor cells and the stimulation of their proliferation, directing osteoblasts toward bone formation [21, 23]. Thus, TGF-β is now established as one of the most important factors in the process of bone remodeling [21]. Regarding the intracellular signaling system of TGF-β, it is firmly established that TGF-β signal is mainly mediated through the Smad-dependent pathway, including Smad2 and Smad3 [24, 25]. In addition, accumulating evidence indicates that the Smad-independent pathway such as the mitogen-activated protein (MAP) kinase superfamily including p38 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) plays an important role in the TGF-β signaling [26]. In osteoblasts, p38 MAP kinase reportedly regulates the expression of Runx2 gene induced by TGF-β in addition to the Smad-dependent pathway [27]. We have previously demonstrated that TGF-β stimulates VEGF synthesis at least in part though the p38 MAP kinase activation in osteoblast-like MC3T3-E1 cells [28]. Nowadays, p38 MAP kinase is well recognized to be a crucial pathway in the TGF-β signaling in osteoblasts [29]. However, the exact mechanism behind TGF-β-induced VEGF synthesis in osteoblasts remains to be clarified.

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Regarding the effect of HSP70 on bone tissue, it has been reported that extracellular HSP70 increases alkaline phosphatase activity and promotes mineralization in human mesenchymal stem cells [30]. However, the precise roles of HSP70 in osteoblasts remain to be elucidated. In the present study, therefore, we investigated whether HSP70 is implicated in the TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells, and the mechanism.

Materials and Methods

Materials

TGF-β and the mouse VEGF enzyme-linked immunosorbent assay (ELISA) kit were purchased from R&D Systems, Inc. (Minneapolis, MN). VER-155008 and YM-08, which are HSP70 inhibitors, were obtained from Sigma-Aldrich Co. LLC (St. Louis, MO). Phospho-specific Smad2 antibodies, phospho-specific Smad3 antibodies, phospho-specific p38 MAP kinase antibodies and p38 MAP kinase antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Enhanced Chemiluminescence (ECL) Western blotting detection system was purchased from GE Healthcare Life Sciences (Chalfont, UK). Acrylamide monomer, Tris (hydroxymethyl) aminomethane, sodium dodecyl sulfate (SDS), dithiothreitol and glycerol were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Control short interfering RNA (siRNA; Silencer Negative Control siRNA) and HSP70-siRNA (Silencer Select Pre-designed siRNA, s201488) were purchased from Sigma-Aldrich Co. Ambion (Austin, TX). Other materials and chemicals were obtained from commercial sources. VER-155008 and YM-08 was dissolved in dimethyl sulfoxide, and the maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect either the assay for VEGF or detection of the protein level using Western blotting.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells that have been derived from newborn mouse calvaria [31] were maintained as previously described [32]. In brief, the MC3T3-E1 cells were cultured in α-minimum essential medium (α-MEM; Sigma-Aldrich Co.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35- or 90-mm diameter dishes (5×10^4 cells/dish and 2×10^5 cells/dish, respectively) in α-MEM containing 10% FBS. Five days later, the medium was exchanged for α-MEM containing 0.3% FBS, and then the cells were used for experiments after 48 h.

Assay for VEGF

The MC3T3-E1 cells were pretreated with various doses of VER-155008 and YM-08 for 60 min, and then stimulated by various doses of TGF-β in 1 ml of α-MEM containing 0.3% FBS for the indicated periods. The conditioned medium was collected at the end of incubation, and the VEGF concentration was measured using mouse VEGF ELISA kit according to the manufacturer's instructions.

Real-time RT-PCR

The MC3T3-E1 cells were pretreated with 30 μM of VER-155008 or vehicle for 60 min, and then stimulated by 5 ng/ml of TGF-β or vehicle in α-MEM containing 0.3% FBS for 6 h. Total RNA was isolated and transcribed into complementary DNA using Trizol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and Omniscript Reverse Transcriptase kit (Qiagen Inc., Valencia, CA), respectively. Real-time RT-PCR was performed using a LightCycler system (version 3.5; Roche Diagnostics, Basel, Switzerland) in capillaries and Fast Start DNA Master SYBR Green I provided with the kit (Roche Diagnostics). Sense and antisense primers for mouse VEGF mRNA and GAPDH mRNA were purchased from Sigma-Aldrich Co. The amplified products were determined using a melting curve analysis. The VEGF mRNA level was normalized to that of GAPDH mRNA.

siRNA transfection

To knockdown HSP70 in osteoblast-like MC3T3-E1 cells, the cells were transfected with a HSP70-siRNA or negative control siRNA utilizing siLentFect according to the manufacturer's instruction. The cell

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sub-cultured in α-MEM containing 10% FBS for 48 h were incubated at 37°C with 30 nM siRNA-siLentFect complexes for 24 h. The medium was exchanged to α-MEM containing 0.3% FBS, and then the cells were used for the experiments.

Western blot analysis

The MC3T3-E1 cells were pretreated with various doses of VER-155008 or YM-08 for 60 min, and then stimulated by 5 ng/ml of TGF-β or vehicle in α-MEM containing 0.3% FBS for the indicated periods. The cells were then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli in 10% polyacrylamide gels [33]. The proteins were fractionated and transferred onto an Immun-Blot polyvinylidine fluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Hercules, CA). The membranes 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 2 h before incubation with primary antibodies. A Western blot analysis was performed as previously described [34] using antibodies against phospho-Smad2, phospho-Smad3, GAPDH, phospho-p38 MAP kinase or p38 MAP kinase as primary antibodies in 5% milk in TBS-T overnight at 4°C. Goat-anti rabbit IgG horseradish peroxidase-labeled antibodies (KPL, Inc., Gaithersburg, MD) were used as secondary antibodies in 5% milk in TBS-T for 1 h at room temperature. The peroxidase activity on the PVDF sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

Determination of densitometric analysis

The densitometric analysis was performed using a scanner and image analysis software package (imageJ version 1.48; National Institutes of Health, Bethesda, MD). The phosphorylated protein levels were calculated as follows: the background-subtracted signal intensity of each phosphorylation signal was respectively normalized to the total protein signal, and plotted as the fold increase in comparison with that of the control cells treated without stimulation.

Statistical analysis

The data were analyzed by an ANOVA followed by Bonferroni method for multiple comparisons between pairs, and a *p* value of <0.05 was considered to be statistically significant. All data are presented as the mean ± SEM of triplicate determinations from three independent cell preparations.

Results

Effects of VER-155008 or YM-08 on the TGF-β-stimulated VEGF release in osteoblast-like MC3T3-E1 cells

We have previously demonstrated that TGF-β stimulates VEGF synthesis in osteoblast-like MC3T3-E1 cells [28]. To clarify the involvement of HSP70 in the TGF-β-induced VEGF synthesis in MC3T3-E1 cells, in the present study, we first examined the effect of VER-155008, an inhibitor of HSP70 [35], on the TGF-β-stimulated VEGF release. VER-155008, which alone had little effect on the VEGF release, significantly enhanced the TGF-β-stimulated VEGF release time-dependently in comparison to TGF-β alone (Fig. 1). In addition, we examined the effect of YM-08, another inhibitor of HSP70 [36], on the TGF-β-stimulated VEGF release in these cells. YM-08 as well as VER-155008 markedly strengthened the TGF-β-stimulated VEGF release (Fig. 1).

The amplifying effect of VER-155008 on the TGF-β-induced VEGF release was dose-dependent over the range 10 and 30 μM (Fig. 2A). The enhancing effect of VER-155008 (30 μM) on the VEGF release caused an approximately 180% increase in the TGF-β-effect. YM-08 in addition to VER-155008 dose-dependently enhanced the VEGF release by TGF-β over the range 30 and 100 μM (Fig. 2B). YM-08 (100 μM) led to an about 80% increase in the TGF-β-effect.

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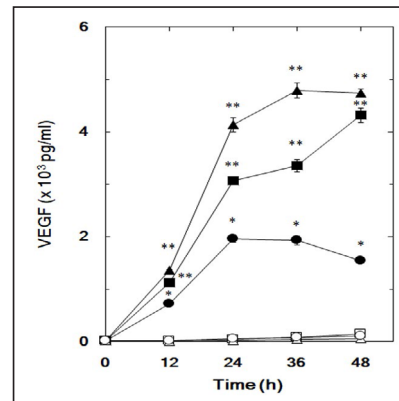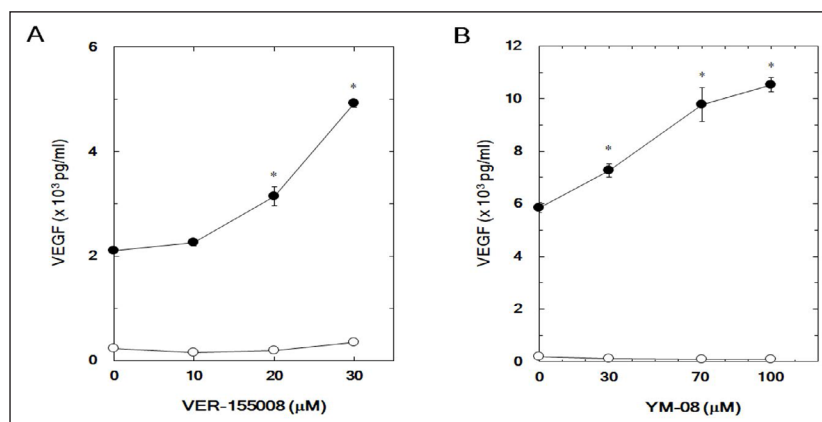www.karger.com/cpb

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Fig. 1. Time-dependent effects of VER-155008 or YM-08 on the TGF-β-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. The cultured cells were pretreated with 30 μM of VER-155008 (■,□), 100 μM of YM-08 (▲,△) or vehicle (●,○) for 60 min, and then stimulated by 3 ng/ml of TGF-β (●,■,▲) or vehicle (○,□,△) for the indicated periods. The VEGF concentrations in the conditioned medium were determined by ELISA. Each value represents the mean ± SEM of triplicate determinations from three independent cell preparations. *p<0.05 compared to the value of control. **p<0.05, compared to the value of TGF-β alone.
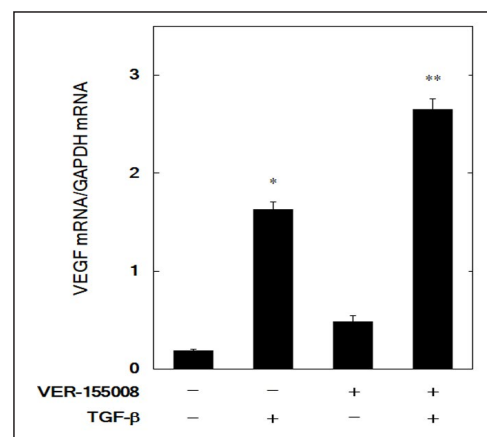

Fig. 2. Dose-dependent effects of VER-155008 or YM-08 on the TGF-β-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. (A) The cultured cells were pretreated with various doses of VER-155008 for 60 min, and then stimulated by 3 ng/ml of TGF-β (●) or vehicle (○) for 48 h. (B) The cultured cells were pretreated with various doses of YM-08 for 60 min, and then stimulated by 5 ng/ml of TGF-β (●) or vehicle (○) for 48 h. The VEGF concentrations in the conditioned medium were determined by ELISA. Each value represents the mean ± SEM of triplicate determinations from three independent cell preparations. *p<0.05, compared to the value of TGF-β alone.

Fig. 3. Effect of VER-155008 on the TGF-β-induced expression of VEGF mRNA in osteoblast-like MC3T3-E1 cells. The cultured cells were pretreated with 30 μM of VER-155008 or vehicle for 60 min, and then stimulated by 5 ng/ml of TGF-β or vehicle for 6 h. The respective total RNA was then isolated and quantified using real-time RT-PCR. Each value represents the mean ± SEM of triplicate determinations from three independent cell preparations. *p<0.05, compared to the value of control. **p<0.05, compared to the value of TGF-β alone.

Effect of VER-155008 on the TGF-β-induced expression of VEGF mRNA in osteoblast-like MC3T3-E1 cells

In order to elucidate whether the enhancement by HSP70 inhibitors of the TGF-β-stimulated VEGF release is mediated via transcriptional events or not in osteoblast-like MC3T3-E1 cells, we examined the effect of VER-155008 on the TGF-β-induced VEGF mRNA expression by real-time RT-PCR. VER-155008 at 30 μM, which by itself had a little effect on

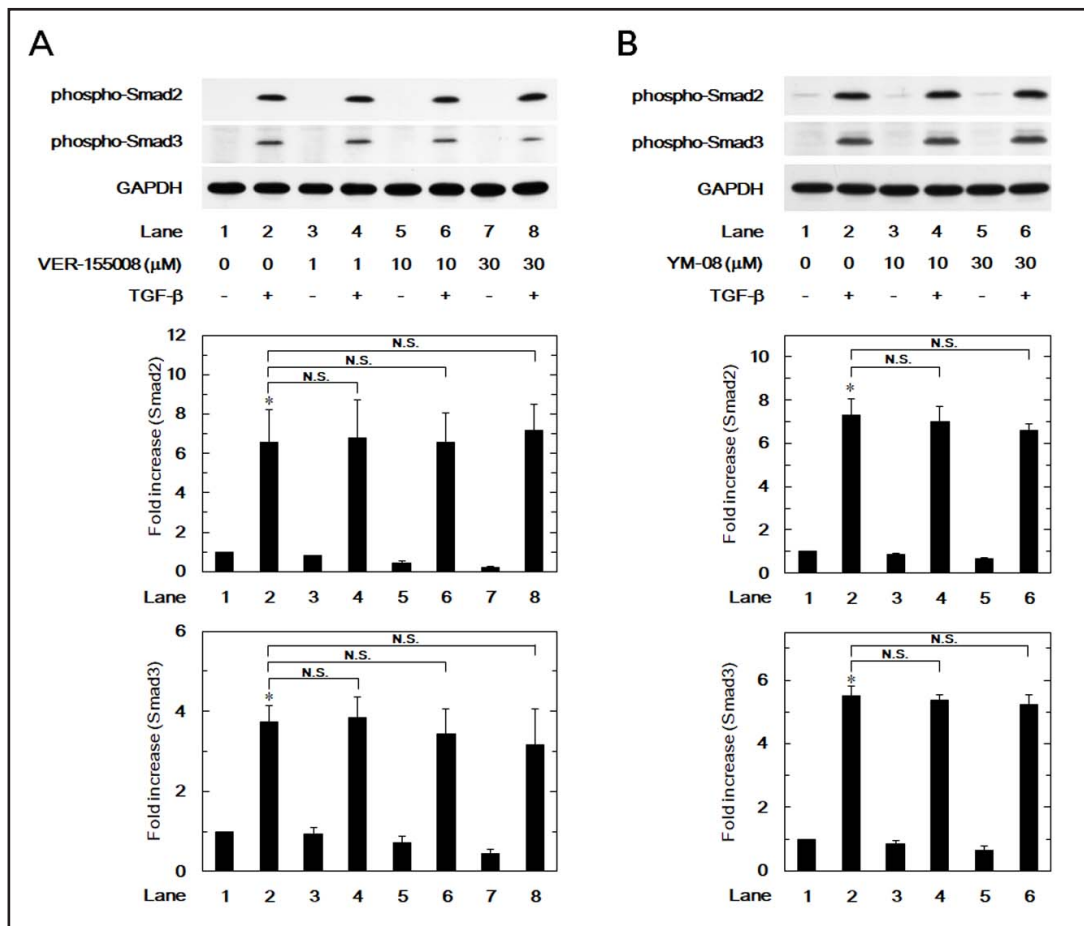# KARGER

Fig. 4. Effects of VER-155008 or YM-08 on the TGF-β stimulated phosphorylation of Smad2 or Smad3 in osteoblast-like MC3T3-E1 cells. The cultured cells were pretreated with various doses of VER-155008 (A) or YM-08 (B) for 60 min, and then stimulated by 5 ng/ml of TGF-β or vehicle for 60 min. The cell extracts were then subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific Smad2, phospho-specific Smad3 or GAPDH. The histogram shows quantitative representation of the levels of TGF-β-stimulated phosphorylation obtained from a laser densitometric analysis of three independent cell experiments. The levels were expressed as the fold increase to the basal levels presented as lane 1. Each value represents the mean ± SEM of triplicate determinations. *$p<0.05$, compared to the value of control. N.S. designates no significant differences between the indicated pairs.

the VEGF mRNA levels, significantly amplified the TGF-β-stimulated VEGF mRNA expression levels (Fig. 3).

Effects of VER-155008 or YM-08 on the TGF-β-stimulated phosphorylation of Smad2 or Smad3 in osteoblast-like MC3T3-E1 cells

As for the intracellular signaling system, it is firmly established that TGF-β-effects are mediated through the Smad-dependent pathway, and that Smad2 and Smad3 are receptor-regulated Smads for TGF-β [24, 25]. Thus, we next examined the effects of HSP70 inhibitors on the TGF-β-induced phosphorylation of Smad2 or Smad3 in osteoblast-like MC3T3-E1 cells. However, VER-155008 failed to affect the phosphorylation of Smad2 or Smad3 induced by TGF-β (Fig. 4A). As well as VER-155008, YM-08 did not exert any effect on the TGF-β-induced phosphorylation of Smad2 or Smad3 (Fig. 4B).
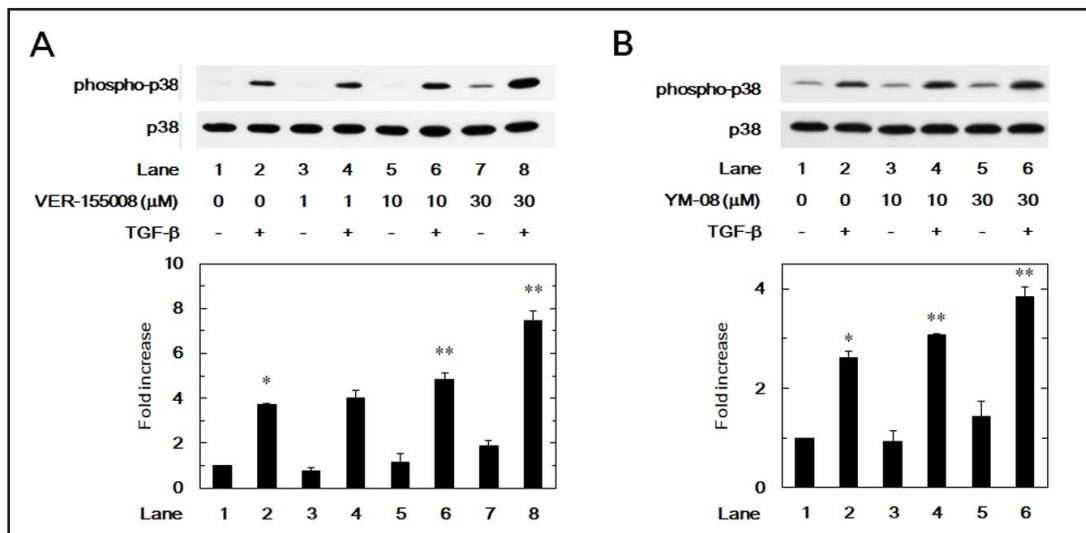
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Fig. 5. Effects of VER-155008 or YM-08 on the TGF-β stimulated phosphorylation of p38 MAP kinase in osteoblast-like MC3T3-E1 cells. The cultured cells were pretreated with various doses of VER-155008 (A) or YM-08 (B) for 60 min, and then stimulated by 5 ng/ml of TGF-β or vehicle for 120 min. The cell extracts were then subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representation of the levels of TGF-β-stimulated phosphorylation obtained from a laser densitometric analysis of three independent cell experiments. The levels were expressed as the fold increase to the basal levels presented as lane 1. Each value represents the mean ± SEM of triplicate determinations. $^*p<0.05$, compared to the value of control. $^{**}p<0.05$, compared to the value of TGF-β alone.

Fig. 6. Effect of HSP70 siRNA on the TGF-β-induced phosphorylation of p38 MAP kinase in osteoblast-like MC3T3-E1 cells. The cultured cells were transfected with 30 nM negative control siRNA (Neg) or 30 nM HSP70-siRNA. Twenty-four hours after transfection, the cells were stimulated by 5 ng/ml TGF-β or vehicle for 120 min. The cell extracts were then subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific p38 MAP kinase or GAPDH. The histogram shows the quantitative representations of the levels of phosphorylated p38 MAP kinase after normalization with respect to GAPDH obtained from laser densitometric analysis of three independent cell experiments. The levels were expressed as the fold increase to the basal levels presented as lane 1. Each value represents the mean ± SEM of triplicate determinations. $^*p<0.05$ compared to the value without TGF-β stimulation in the negative control siRNA transfected cells. $^{**}p<0.05$ compared to the value of TGF-β stimulation in the negative control siRNA transfected cells.
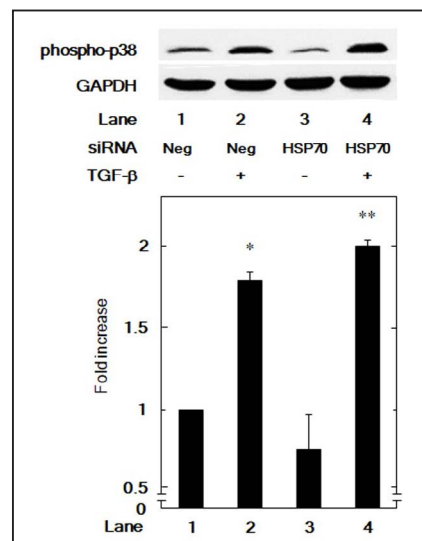
Effects of VER-155008 or YM-08 on the TGF-β-stimulated phosphorylation of p38 MAP kinase in osteoblast-like MC3T3-E1 cells

In our previous study [28], we have demonstrated that TGF-β stimulates VEGF synthesis at least in part via p38 MAP kinase activation in osteoblast-like MC3T3-E1 cells. Therefore, we additionally examined the effects of HSP70 inhibitors on the TGF-β-stimulated phosphorylation of p38 MAP kinase in these cells. The phosphorylation of p38 MAP kinase induced by TGF-β was significantly amplified by VER-155008 in a dose-dependent manner

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Fig. 7. Effect of SB203580 on the amplification by VER-155008 of the TGF-β-induced VEGF release in osteoblast-like MC3T3-E1 cells. The cultured cells were pretreated with 10 μM of SB203580 or vehicle for 60 min, subsequently preincubated with 30 μM of VER-155008 or vehicle for 60 min, and then stimulated by 3 ng/ml of TGF-β or vehicle for 48 h. The VEGF concentrations of the conditioned mediums were determined by ELISA. Each value represents the mean ± SEM of triplicate determinations from three independent cell preparations. *p<0.05 compared to the value of control. **p<0.05 compared to the value of TGF-β alone. ***p<0.05 compared to the value of TGF-β stimulation with the preincubation of VER-155008.
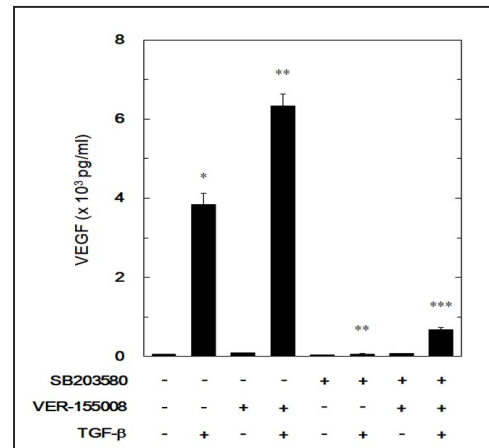

Fig. 8. Effect of SB203580 on the amplification by YM-08 of the TGF-β-induced VEGF release in osteoblast-like MC3T3-E1 cells. The cultured cells were pretreated with 10 M of SB203580 or vehicle for 60 min, subsequently preincubated with 100 μM of YM-08 or vehicle for 60 min, and then stimulated by 3 ng/ml of TGF-β or vehicle for 48 h. The VEGF concentrations of the conditioned mediums were determined by ELISA. Each value represents the mean ± SEM of triplicate determinations from three independent cell preparations. *p<0.05 compared to the value of control. **p<0.05 compared to the value of TGF-β alone. ***p<0.05 compared to the value of TGF-β stimulation with the preincubation of YM-08.
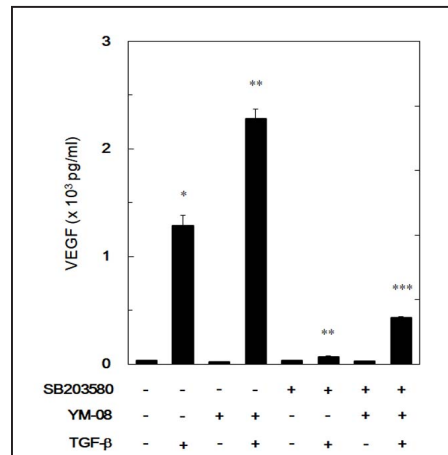
over the range 1 and 30 μM (Fig. 5A). YM-08 also remarkably enhanced the TGF-β-induced phosphorylation of p38 MAP kinase in a dose-dependent manner over the range 10 and 30 μM (Fig. 5B).

Effect of HSP70-siRNA on the TGF-β-induced phosphorylation of p38 MAP kinase in osteoblast-like MC3T3-E1 cells

In order to further clarify the involvement of HSP70 in the TGF-β-induced phosphorylation of p38 MAP kinase in osteoblast-like MC3T3-E1 cells, we next examined the effect of HSP70-siRNA on the TGF-β-induced phosphorylation of p38 MAP kinase. The level of TGF-β-induced phosphorylation of p38 MAP kinase in the HSP70 knockdown MC3T3-E1 cells was markedly up-regulated in comparison with that in the control cells (Fig. 6).

Effect of SB203580 on the amplification by HSP70 inhibitors of TGF-β-stimulated VEGF release in osteoblast-like MC3T3-E1 cells

Furthermore, we examined the effect of SB203580, an inhibitor of p38 MAP kinase [37], on the amplification of TGF-β-stimulated VEGF release by VER-155008 or YM-08 in osteoblast-like MC3T3-E1 cells. We confirmed here that the TGF-β-induced VEGF release was reduced by SB203580 as previously reported [28] (Fig. 7 and Fig. 8). SB203580 markedly suppressed the amplification by VER-155008, which caused an approximately 90% decrease in the effect of TGF-β (Fig. 7). In addition, the VEGF release up-regulated by YM-08 was also reduced by SB203580, which caused an approximately 80% decrease (Fig. 8). Based these findings, it is most likely that p38 MAP kinase pathway is implicated in the amplification of TGF-β-stimulated VEGF synthesis by HSP70 inhibitors.

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Discussion

In the present study, we demonstrated that HSP70 inhibitor, VER-155008 [35], markedly potentiated the TGF-β-stimulated VEGF release in osteoblast-like MC3T3-E1 cells, and that VER-155008 augmented the VEGF mRNA expression levels induced by TGF-β. Thus, it is probable that the amplification by HSP70 inhibitors of TGF-β-stimulated VEGF release is mediated through a gene transcriptional event in MC3T3-E1 cells. Additionally, we showed that another inhibitor of HSP70, YM-08 [36], as well as VER-155008 significantly enhanced the TGF-β-stimulated VEGF release in these cells. Based on these findings, it is most likely that HSP70 acts as a negative regulator in the TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. As far as we know, this is probable the first report demonstrating the relationship between HSP70 and TGF-β-stimulated VEGF synthesis in osteoblasts.

We next investigated the mechanism underlying the enhancement by HSP70 inhibitors of the TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. It is currently recognized that TGF-β exerts various biological effects through both the Smad-dependent pathway and the Smad-independent pathway including the MAP kinase superfamily [24, 25, 38]. Regarding the intracellular signaling in TGF-β-induced VEGF synthesis in osteoblasts, we have recently reported that the Smad-dependent pathway mediates the TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells [39]. On the other hand, we have previously shown that TGF-β stimulates the synthesis of VEGF at least in part through p38 MAP kinase in these cells [28]. Based on our findings, therefore, we investigated whether HSP70 affects the TGF-β-stimulated activation of Smad2/3 and p38 MAP kinase in osteoblast-like MC3T3-E1 cells. However, HSP70 inhibitors, VER-155008 or YM-08, hardly affected the phosphorylation of Smad2 or Smad3 induced by TGF-β. On the other hand, we clearly showed that both VER-155008 and YM-08 significantly augmented the TGF-β-stimulated phosphorylation of p38 MAP kinase. These results suggest that the enhancement by HSP70 inhibitors of TGF-β-stimulated VEGF synthesis is resulting from the upregulation of p38 MAP kinase activation but not Smad2/3 activation in osteoblast-like MC3T3-E1 cells. We additionally examined the effect of HSP70-siRNA on the TGF-β-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. We demonstrated that the level of phosphorylated p38 MAP kinase induced by TGF-β in the HSP70 knockdown cells was remarkably amplified compared with that in the control cells. Based on these findings, it is probable that HSP70 limits the activation of p38 MAP kinase induced by TGF-β in MC3T3-E1 cells. Furthermore, SB203580 [37] significantly reduced the amplification by VER-155008 or YM-08 of the TGF-β-stimulated VEGF release, suggesting that the inhibition of p38 MAP kinase truly suppresses the enhancement by HSP70 inhibitors of TGF-β-stimulated VEGF synthesis in these cells. As for the TGF-β-induced VEGF synthesis in MC3T3-E1 cells, we have previously shown that Rac, a small GTP-binding protein, limits the VEGF synthesis via the inhibition of p38 MAP kinase [39]. However, we found that HSP70 was not coimmunoprecipitated with Rac in these cells (data not shown), suggesting that HSP70 regulates p38 MAP kinase independently of Rac in MC3T3-E1 cells. Taking these findings into account as a whole, it is most likely that HSP70 negatively regulates the TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells, and the suppressive effect of HSP70 is exerted at a point upstream of p38 MAP kinase. The potential mechanism of HSP70 in the TGF-β-stimulated VEGF synthesis in osteoblasts shown here is summarized as Fig. 9.

Regarding the involvement of HSP70 in the TGF-β signaling, it has been reported that HSP70 prevents the activation of SAPK/JNK and p38 MAP kinase in lymphoid cells, which associates with holding an upstream component of MAP kinase in an inactivate form [40]. In addition, the induction of HSP70 reportedly inhibits the TGF-β-induced epithelial-mesenchymal transition through the suppression of Smad2 activation in renal proximal tubular epithelial cells [41]. It is possible that HSP70 affects either Smad-dependent pathway or Smad-independent pathway of TGF-β signaling depending upon the cell species. On the other hand, it is well known that HSP70, conjuncted with the E3 ubiquitin ligase C-terminal Hsp70-interacting protein, promotes the ubiquitination and subsequent proteasomal

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Fig. 9. Diagram of the potential mechanism of HSP70 on the TGF-β-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. TGF-β, transforming growth factor-β; MAPK, mitogen-activated protein kinase; HSP70, heat shock protein 70; VEGF, vascular endothelial growth factor.
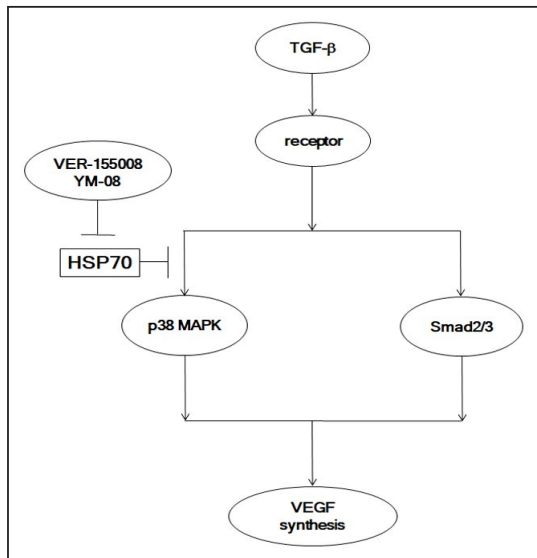
degradation [10, 42]. It has been shown that the induction of HSP70 inhibits the TGF-β signaling through its direct binding to TGF-β type I receptor, resulting in the ubiquitination and degradation of the complex in embryonic kidney cells [43]. Based on our findings here showing that the suppressive effect of HSP70 on the TGF-β-induced VEGF synthesis is mediated via inhibition of p38 MAP kinase without affecting the Smad2/3 activation, it is probable that the effect of HSP70 is exerted at the point between TGF-β receptors and p38 MAP kinase in osteoblast-like MC3T3-E1 cells. Regarding the relationship between TGF-β and the p44/p42 MAP kinase pathway, it has been shown that p44/p42 MAP kinase as well as p38 MAP kinase mediates the suppressive effect of TGF-β on the expression of osteocalcin, a determining factor in bone formation, in osteoblastic cells including MC3T3-E1 cells [44]. In addition, TGF-β2 reportedly enhances the proliferation of MC3T3-E1 cells via p44/p42 MAP kinase, and accelerates cranial suture closer resulting from osteoprogenitor cell proliferation mediated by p44/p42 MAP kinase [45]. Thus, these findings make us to speculate that the TGF-β-induced activation of p44/p42 MAP kinase plays an important role in osteoblast proliferation and bone formation. Indeed, we have previously demonstrated that p44/p42 MAP kinase in addition to p38 MAP kinase participates in TGF-β-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells [28]. Therefore, it is possible that HSP70 affects the TGF-β-stimulated VEGF synthesis via upregulation of p44/p42 MAP kinase in MC3T3-E1 cells.

Although accumulating evidence indicates that HSP70 has antagonistic roles in the TGF-β-signaling, the mechanism of HSP70 seems to be quite different in each type of cells. Recently, it has been reported that various stress conditions including electromagnetic wave irradiation, intermittent stretching and chemicals such as cadmium modulate osteoblast functions through the activation of p38 MAP kinase [46-48]. We have previously shown that HSP70 exists in resting state of osteoblast-like MC3T3-E1 cells [49]. Therefore, our present findings showing that inhibitory effect of HSP70 on p38 MAP kinase activity could present an important role of HSP70 in the protection of osteoblasts from various environmental stresses. In bone tissue, TGF-β, which is stored in the matrix and is released during bone resorption by osteoclasts, is generally recognized to promote bone remodeling process [21]. On the other hand, it is widely accepted that VEGF plays an important role as an angiogenic factor in the bone remodeling and the repair [19]. It has been reported that microRNA-10a plays suppressive roles in the differentiation of osteoblast-like MC3T3-E1 cells and pro-angiogenic activity including VEGF synthesis of umbilical vein endothelial cells, representing a novel potential therapeutic target for bone regeneration-related diseases [50]. Thus, the modulation of angiogenesis through regulating VEGF levels is probably a candidate of therapeutic strategy for metabolic bone diseases. Taking our present findings into account,

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it is possible that HSP70 inhibitor, in addition to the anti-cancer chemotherapeutics [51], could be utilized as a potent bone remodeling agent through up-regulating VEGF synthesis in osteoblasts, proposing a therapeutic tool for the acceleration of fracture healing and the remedy for bone metabolic diseases such as osteoporosis. Further investigations would be required to clarify the precise roles of HSP70 in osteoblasts and the detailed mechanism.

Conclusion

Our results strongly suggest that HSP70 functions as a negative regulator in the TGF-β-stimulated VEGF synthesis in osteoblasts, and that the suppressive effect of HSP70 is exerted via regulation of p38 MAP kinase.

Disclosure Statement

All authors declare that we have no Disclosure Statement in connection with this paper.

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References

1 Kiang JG, Tsokos GC: Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. Pharmacol Ther 1998;80:183-201.

2 Bukau B, Horwich AL: The Hsp70 and Hsp60 chaperone machines. Cell 1998;92:351-366.

3 Easton DP, Kaneko Y, Subjeck JR: The hsp110 and Grp170 stress proteins: newly recognized relatives of the Hsp70s. Cell Stress Chaperones 2000;5:276-290.

4 Calderwood SK, Khaleque MA, Sawyer DB, Ciocca DR: Heat shock proteins in cancer: chaperones of tumorigenesis. Trends Biochem Sci 2006;31:164-172.

5 Jee H: Size dependent classification of heat shock proteins: a mini-review. J Exerc Rehabil 2016;31:255-259.

6 Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, Bruford EA, Cheetham ME, Chen B, Hightower LE: Guidelines for the nomenclature of the human heat shock proteins. Cell Stress Chaperones 2009;14:105-111.

7 Beckmann RP, Mizzen LE, Welch WJ: Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. Science 1990;248:850-854.

8 Shi Y, Thomas JO: The transport of proteins into the nucleus requires the 70-kDa heat shock protein or its cytosolic cognate. Mol Cell Biol 1992;12:2186-2192.

9 Hartl FU, Bracher A, Hayer-Hartl M: Molecular chaperones in protein folding and proteostasis. Nature 2011;20:324-332.

10 Radons J: The human HSP70 family of chaperones: where do we stand? Cell Stress Chaperones 2016;21:379-404.

11 Qu B, Jia Y, Liu Y, Wang H, Ren G, Wang H: The detection and role of heat shock protein 70 in various nondisease conditions and disease conditions: a literature review. Cell Stress Chaperones 2015;20:885-892.

1144

12 Sherman MY, Gabai VL: Hsp70 in cancer: back to the future. Oncogene 2015;6:4153-4156.

13 Kular J, Tickner J, Chim SM: An overview of the regulation of bone remodeling at the cellular level. Clin Biochem 2012:45;863-873.

14 Sims NA, Gooi JH: Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption. Semin. Cell Dev Biol 2008;19:444-451.

15 Parfitt AM: Targeted and nontargeted bone remodeling: relationship to basic multicellular unit origination and progression. Bone 2002;30:5-7.

16 Brandi ML, Collin-Osdoby P: Vascular biology and the skeleton. J Bone Miner Res 2006;21:183-192.

17 Clarkin CE, Gerstenfeld LC: VEGF and bone cell signalling: an essential vessel for communication? Cell Biochem Funct 2013;31:1-11.

18 Ferrara N: Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004;25:581-611.

19 Hu K, Olsen BR: The roles of vascular endothelial growth factor in bone repair and regeneration. Bone 2016;91:30-38.

20 Zelzer E, Olsen BR: Multiple roles of vascular endothelial growth factor (VEGF) in skeletal development, growth, and repair. Curr Top Dev Biol 2005;65:169-187.

21 Janssens K, Dijke P, Janssens S, Hul WV: Transforming Growth Factor-β1 to the Bone. Endocr Rev 2005;26:743-774.

22 Guo X, Wang XF: Signaling cross-talk between TGF-β/BMP and other pathways. Cell Res 2009;19:71-88.

23 Tang Y, Wu X, Lei W, Pang L, Wan C, Shi Z, Zhao L, Nagy TR, Peng X, Hu J, Feng X, Van Hul W, Wan M, Cao X: TGF-β1-induced migration of bone mesenchymal stem cells couples bone resorption with formation. Nat Med 2009;15:757-765.

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

25 Hata A, Chen YG: TGF-β Signaling from Receptors to Smads. Cold Spring Harb Perspect Biol 2016;8 DOI: 10.1101/cshperspect.a022061.

26 Moustakas A, Heldin CH: Non-Smad TGF-β signals. J Cell Sci 2005;118:3573-3584.

27 Lee KS, Hong SH, Bae SC: Both the Smad and p38 MAPK pathways play a crucial role in Runx2 expression following induction by transforming growth factor-β and bone morphogenetic protein. Oncogene 2002;21:7156-7163.

28 Tokuda H, Hatakeyama D, Akamatsu S, Tanabe K, Yoshida M, Shibata T, Kozawa O: Involvement of MAP kinases in TGF-β-stimulated vascular endothelial growth factor synthesis in osteoblasts. Arch Biochem Biophys 2003;415:117-125.

29 Rodríguez-Carballo E, Gámez B, Ventura F: p38 MAPK signaling in osteoblast differentiation. Front Cell Dev Biol 2016;4:40.

30 Chen E, Xue D, Zhang Wei, Lin F, Pan Z: Extracellular heat shock protein 70 promotes osteogenesis of human mesenchymal stem cells through activation of the ERK signaling pathway. FEBS Lett 2015;21:4088-4096.

31 Sudo H, Kodama H, Amagai Y, Yamamoto S, Kasai S: *In vivo* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 1982;96:191-198.

32 Kozawa O, Tokuda H, Miwa M, Kotoyori J, Oiso Y: Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E$_2$ in osteoblast-like cells. Exp Cell Res 1992;198:130-134.

33 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4 Nature 1970;227:680-685.

34 Kato K, Ito H, Hasegawa K, Inaguma Y, Kozawa O, Asano T: Modulation of the stress-induced synthesis of hsp27 and αB-crystallin by cyclic AMP in C6 rat glioma cells. J Neurochem 1996;66:946-950.

35 Schlecht R, Scholz SR, Dahmen H, Wegener A, Sirrenberg C, Musil D, Bomke J, Eggenweiler HM, Mayer MP, Bukau B: Functional analysis of Hsp70 inhibitors. PLoS One 2013;8:e78443.

36 Miyata Y, Li X, Lee HF, Jinwal UK, Srinivasan SR, Seguin SP, Young ZT, Brodsky JL, Dickey CA, Sun D, Gestwicki JE: Synthesis and initial evaluation of YM-08, a blood-brain barrier permeable derivative of the heat shock protein 70 (Hsp70) inhibitor MKT-077, which reduces tau levels. ACS Chem Neurosci 2013;4:930-939.

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

38   Zhang YE: Non-Smad pathways in TGF-β signaling. Cell Res 2009;19:128-139.

39   Yamamoto N, Otsuka T, Kondo A, Matsushima-Nishiwaki R, Kuroyanagi G, Kozawa O, Tokuda H: Rac limits TGF-β-induced VEGF synthesis in osteoblasts. Mol Cell Endocrinol 2015;15:35-41.

40   Gabai VL, Meriin AB, Mosser DD, Caron AW, Rits S, Shifrin VI, Sherman MY: Hsp70 prevents activation of stress kinases. A novel pathway of cellular thermotolerance. J Biol Chem 1997;272:18033-18037.

41   Li Y, Kang X, Wang Q: HSP70 decreases receptor-dependent phosphorylation of Smad2 and blocks TGF-β-induced epithelial-mesenchymal transition. J Genet Genomics 2011;38:111-116.

42   Luo W, Zhong J, Chang R, Hu H, Pandey A, Semenza GL: Hsp70 and CHIP selectively mediate ubiquitination and degradation of hypoxia-inducible factor (HIF)-1α but Not HIF-α. J Biol Chem 2010;285:3651-3663.

43   Yun CH, Yoon SY, Nguyen TT, Cho HY, Kim TH, Kim ST, Kim BC, Hong YS, Kim SJ, Lee HJ: Geldanamycin inhibits TGF-β signaling through induction of Hsp70   Arch Biochem Biophys 2010;495:8-13.

44   Lai CF, Cheng SL: Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor-β in normal human osteoblastic cells. J Biol Chem 2002;277:15514-15522.

45   Lee SW, Choi KY, Cho JY, Jung SH, Song KB, Park EK, Choi JY, Shin HI, Kim SY, Woo KM, Baek JH, Nam SH, Kim YJ, Kim HJ, Ryoo HM: TGF-β2 stimulates cranial suture closure through activation of the Erk-MAPK pathway. J Cell Biochem 2006;98:981-991.

46   Yumoto H, Hirao K, Tominaga T, Bando N, Takahashi K, Matsuo T: Electromagnetic wave irradiation promotes osteoblastic cell proliferation and up-regulates growth factors via activation of the ERK1/2 and p38 MAPK pathways. Cell Physiol Biochem 2015;35:601-615.

47   Xiao WL, Zhang DZ, Fan CH, Yu BJ: Intermittent Stretching and Osteogenic Differentiation of Bone Marrow Derived Mesenchymal Stem Cells via the p38MAPK-Osterix Signaling Pathway. Cell Physiol Biochem 2015;36:1015-1025.

48   Hu KH, Li WX, Sun MY, Zhang SB, Fan CX, Wu Q, Zhu W, Xu X: Cadmium Induced Apoptosis in MG63 Cells by Increasing ROS, Activation of p38 MAPK and Inhibition of ERK 1/2 Pathways. Cell Physiol Biochem 2015;36:642-654.

49   Hatakeyama D, Kozawa O, Niwa M, Matsuno H, Ito H, Kato K, Tatematsu N, Shibata T, Uematsu T: Upregulation by retinoic acid of transforming growth factor-β-stimulated heat shock protein 27 induction in osteoblasts: involvement of mitogen-activated protein kinases. Biochim Biophys Acta 2002;1589:15-30.

50   Li J, Zhang Y, Zhao Q, Wang J, He X: MicroRNA-10a Influences Osteoblast Differentiation and Angiogenesis by Regulating β-Catenin Expression. Cell Physiol Biochem 2015;37:2194-2208.

51   Massey AJ, Williamson DS, Browne H, Murray JB, Dokurno P, Shaw T, Macias AT, Daniels Z, Geoffroy S, Dopson M, Lavan P, Matassova N, Francis GL, Graham CJ, Parsons R, Wang Y, Padfield A, Comer M, Drysdale MJ, Wood M: A novel, small molecule inhibitor of Hsc70/Hsp70 potentiates Hsp90 inhibitor induced apoptosis in HCT116 colon carcinoma cells. Cancer Chemother. Pharmacol 2009;66:535-545.

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