

# Role of heat shock protein 27 in transforming growth factor- $\beta$ -stimulated vascular endothelial growth factor release in osteoblasts

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Received October 15, 2010; Accepted December 3, 2010

DOI: 10.3892/ijmm.2011.595

**Abstract.** We have previously reported that transforming growth factor- $\beta$  (TGF- $\beta$ ) stimulates heat shock protein 27 (HSP27) induction via p44/p42 mitogen-activated protein (MAP) kinase, p38 MAP kinase and stress-activated protein kinase/c-Jun N-terminal kinase in osteoblast-like MC3T3-E1 cells, and that the release of vascular endothelial growth factor (VEGF) is induced by TGF- $\beta$  in these cells. In the present study, we investigated the effect of HSP27 knock-down on the TGF- $\beta$ -stimulated VEGF release in these cells. Gene silencing using short interfering RNA against HSP27 (HSP27-siRNA) significantly suppressed the TGF- $\beta$ -induced VEGF release. Immunofluorescence microscopy also revealed that HSP27-siRNA suppressed the TGF- $\beta$ -stimulated VEGF induction as well as the reduction of HSP27 induction in these cells. However, the mRNA expression of VEGF stimulated by TGF- $\beta$  was not reduced even in cells transfected with HSP27-siRNA. These results strongly suggest that HSP27 induction is critical for TGF- $\beta$ -induced VEGF release in osteoblasts.

## Introduction

Heat shock proteins (HSPs) are expressed when the cells are exposed to biological stress such as heat stress and chemical stress (1). HSPs are classified into high-molecular-weight HSPs and low-molecular-weight HSPs based on the apparent molecular sizes. Low-molecular-weight HSPs with molecular masses from 10-30 kDa, such as heat shock protein 27 (HSP27),  $\alpha$ B-crystallin and HSP20, have a highly homologous amino acid sequence in their  $\alpha$ -crystallin domain (2,3).

It is well recognized that the high-molecular-weight HSPs act as chaperones that interact with other denatured proteins to facilitate normal cell functions. On the other hand, the functions of the low-molecular-weight HSPs are less known. Among the low-molecular-weight HSPs, HSP27 has been discovered as an inhibitor of actin polymerization. HSP27 becomes rapidly phosphorylated in response to various types of stress, as well as to exposure to cytokines and mitogens (4,5). Under unstimulated conditions, HSP27 exists in a high-molecular weight aggregated form. It is rapidly dissociated as a result of phosphorylation (6,7). It has been shown that HSP27 is implicated in cell survival, cell differentiation and tumor progression in addition to chaperoning functions. However, the exact roles of HSP27 and the implication of its phosphorylation have not yet been clarified.

Bone metabolism is mainly regulated by two functional cells, the osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively (8). The formation of bone structures and bone remodeling are reportedly the result of the following coupling process, bone resorption by the activated osteoclasts with subsequent deposition of new matrix by the osteoblasts. In previous studies (9-12), we have reported that various physiological regulators of the bone, such as endothelin-1, prostaglandin F<sub>2</sub> $\alpha$  (PGF<sub>2</sub> $\alpha$ ), PGD<sub>2</sub> and basic fibroblast growth factor stimulate the induction of HSP27 in osteoblast-like MC3T3-E1 cells. It has been shown that down-regulation of proliferation is accompanied by a transient increase of HSP27 mRNA expression in osteoblasts (13). However, the exact role of HSP27 in osteoblasts remains unclear.

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a multifunctional cytokine that regulates cell growth, differentiation and extracellular matrix production (14,15). In bone tissue, TGF- $\beta$  is produced by osteoblasts, and is abundantly stored in a latent form in bone matrix tissue (16). During the process of bone resorption by activated osteoclasts, generated active TGF- $\beta$  stimulates the recruitment and proliferation of osteoblasts responsible for new bone formation (16), indicating its essential role in the bone remodeling process. Although the intracellular signaling of TGF- $\beta$  is characterized by the Smad

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**Key words:** heat shock protein 27, transforming growth factor- $\beta$ , vascular endothelial growth factor, osteoblast

signaling pathway, it is recognized that Smad-independent signaling, such as that of the mitogen-activated protein (MAP) kinase superfamily, also plays a crucial role in the intracellular signal transduction mechanism of TGF- $\beta$  (14,15). In our previous studies in osteoblast-like MC3T3-E1 cells (17,18), we have shown that TGF- $\beta$  stimulates HSP27 induction through the activation of three major MAP kinases, the p44/p42 and p38 MAP kinases, and the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK).

It is well recognized that VEGF is an angiogenic growth factor displaying high specificity for vascular endothelial cells (19). As for bone metabolism, it has been shown that inactivation of VEGF causes complete suppression of blood vessel invasion concomitant with impaired trabecular bone formation and expansion of the hypertrophic chondrocyte zone in the mouse tibial epiphyseal growth plate (20). Furthermore, accumulating evidence indicates that among the bone cells, osteoblasts synthesize and secrete VEGF in response to various physiological agents (19,21-23). We have previously reported that TGF- $\beta$  stimulates VEGF synthesis and release via three major MAP kinases in osteoblast-like MC3T3-E1 cells (24,25), suggesting the relationship between HSP27 induction and VEGF release. Based on the assessment of their time course, the induction of HSP27 reaches its peak at 12 h after the stimulation of TGF- $\beta$  and decreases thereafter, while TGF- $\beta$ -induced VEGF release is sustained up to 48 h. These findings lead us to speculate that HSP27 induction plays a role in VEGF release in osteoblasts.

In the present study, we investigated how HSP27 is implicated in the TGF- $\beta$ -stimulated VEGF release from osteoblast-like MC3T3-E1 cells. We herein show that HSP27 induction is indispensable for the TGF- $\beta$ -induced VEGF release in these cells.

## Materials and methods

**Materials.** TGF- $\beta$  and the mouse VEGF enzyme-linked immunosorbent assay (ELISA) kit were purchased from R&D Systems, Inc. (Minneapolis, MN). Anti-HSP27 antibodies, anti-HSP70 antibodies, anti-HSP90 antibodies and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies for Western blot analysis were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The ECL Western blotting detection system was purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK). Control short interfering RNA (siRNA; Silencer Negative Control no. 1 siRNA) or HSP27-siRNAs (Silencer Predesigned siRNA, 159311 and 102070, presented as #1 and #2, respectively) were purchased from Ambion (Austin, TX). siLentFect was purchased from Bio-Rad (Hercules, CA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). The Omniscript Reverse Transcriptase kit was purchased from Qiagen (Hilden, Germany). Fast-start DNA Master SYBR-Green I was purchased from Roche Diagnostics (Mannheim, Germany). Other materials and chemicals were obtained from commercial sources.

**Cell culture.** Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (26) were maintained as previously described (27). Briefly, the cells were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) containing 10%

fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The cells were seeded into 35-mm (5x10<sup>4</sup>/dish) or 90-mm (25x10<sup>4</sup>/dish) diameter dishes in  $\alpha$ -MEM containing 10% FCS. After 5 days, the medium was exchanged for  $\alpha$ -MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

**siRNA transfection.** To knock down HSP27 in MC3T3-E1 cells, the cells were transfected with negative control siRNA or HSP27-siRNA utilizing siLentFect according to the manufacturer's protocol. In brief, the cells (1x10<sup>5</sup> cells) were seeded into 35-mm diameter dishes in  $\alpha$ -MEM containing 10% FCS and sub-cultured for 48 h. The cells were then incubated at 37°C with 50 nM siRNA-siLentFect complexes. After 24 h, the medium was exchanged to  $\alpha$ -MEM containing 0.3% FCS. They were then stimulated with 5 ng/ml TGF- $\beta$  in  $\alpha$ -MEM containing 0.3% FCS for the indicated periods.

**Western blot analysis.** The cells were washed twice with phosphate-buffered saline (PBS) and 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. The cytosolic fraction was collected as a supernatant after centrifugation at 12,500 x g for 10 min at 4°C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (28) in a 10% polyacrylamide gel. Western blot analysis was performed as described previously (29) by using anti-HSP27, anti-HSP70, anti-HSP90 and anti-GAPDH antibodies, and peroxidase-labeled antibodies raised in goat against rabbit IgG as secondary antibodies. The peroxidase activity on the PVDF membrane was visualized on X-ray film by means of the ECL Western blot detection system.

**Assay for VEGF.** The cultured cells transfected with siRNA were stimulated with 5 ng/ml of TGF- $\beta$  in 1 ml of  $\alpha$ -MEM containing 0.3% FCS for 48 h. The conditioned medium was collected at the end of the incubation, and the VEGF release was measured by the VEGF ELISA kit according to the manufacturer's instructions. The assay kit can detect the mouse VEGF in the range between 7.8 and 500 pg/ml. When the samples generated values >500 pg/ml, the samples were adequately diluted with a calibrator diluent provided with the kit, and then were re-assayed. The absorbance of the ELISA samples was measured at 450 nm with the EL340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT).

**Real-time RT-PCR.** Cultured cells transfected with siRNA were stimulated by 5 ng/ml TGF- $\beta$  for the indicated periods. Total RNA was isolated and transcribed into complementary DNA using the TRIzol reagent and the Omniscript Reverse Transcriptase kit, respectively. Real-time RT-PCR was performed using a LightCycler system (Roche Diagnostics) in capillaries and FastStart DNA Master SYBR-Green I provided with the kit. Sense and antisense primers were synthesized based on the report of Simpson *et al* (30) for mouse VEGF mRNA and GAPDH mRNA. The amplified products were evaluated by both a melting curve analysis and agarose electrophoresis. The VEGF mRNA levels were normalized to those of GAPDH mRNA.

**Immunofluorescence microscopy studies.** Immunofluorescence microscopy studies were performed as previously described (31). In brief, the live cells grown on coverslip-bottom dishes were first treated with or without siRNA of HSP27 for 48 h at 37°C, followed by stimulation with TGF- $\beta$  for 24 h. They were then fixed with 3% paraformaldehyde and exposed to 0.1% Triton X-100 for membrane permeabilization. After washing, fixed cells were exposed to anti-VEGF antibodies and anti-HSP27 antibodies, followed by exposure to Alexa Fluor 488® conjugated goat anti-rabbit IgG and Alexa Fluor 546® conjugated goat anti-mouse IgG antibodies. Finally, they were exposed to DAPI (Wako Inc. Tokyo, Japan) for 20 min and the cells were then examined by fluorescence microscopy, Bioevo (BZ-9000) (Keyence, Tokyo, Japan) according to the manufacturer's protocol.

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

## Results

**Effect of HSP27-siRNA on TGF- $\beta$ -stimulated HSP27 induction in MC3T3-E1 cells.** We first examined whether or not gene silencing using HSP27-siRNA suppresses TGF- $\beta$ -stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. According to our previous report showing that the maximum effect of TGF- $\beta$  on the accumulation of HSP27 occurs 12 h after stimulation (17), we performed the experiments under the condition of TGF- $\beta$  stimulation for 12 h. Both types of siRNAs successfully reduced the protein levels of HSP27 stimulated by TGF- $\beta$  (Fig. 1). On the contrary, HSP27-siRNA either with or without TGF- $\beta$  did not affect the protein levels of HSP70 or HSP90 in these cells (Fig. 1).

**Effect of HSP27-knock down on TGF- $\beta$ -stimulated VEGF release in MC3T3-E1 cells.** We have previously reported that TGF- $\beta$  induces the expression of HSP27 and subsequently stimulates VEGF release in osteoblast-like MC3T3-E1 cells (17,24). To clarify whether HSP27 induction is involved in TGF- $\beta$ -induced release of VEGF, we next investigated the effects of the HSP27 down-regulation on VEGF release stimulated by TGF- $\beta$  in MC3T3-E1 cells. In a previous study (24), we have demonstrated that the maximum effect of TGF- $\beta$  on the VEGF release occurs at 48 h. Thus, we examined the VEGF release stimulated by TGF- $\beta$  for 48 h in HSP27-knock down MC3T3-E1 cells. In comparison with the control cells, the TGF- $\beta$ -stimulated VEGF release was significantly diminished in HSP27-knock down cells (Fig. 2). Treatment with HSP27-siRNA #1 suppressed the TGF- $\beta$ -stimulated VEGF release by approximately 70% (Fig. 2). The treatment with HSP27-siRNA #2 also significantly suppressed the TGF- $\beta$ -stimulated VEGF release (data not shown).

**Effect of HSP27-knock down on TGF- $\beta$ -stimulated VEGF mRNA expression in MC3T3-E1 cells.** To clarify whether or not the suppression by HSP27 knock down of TGF- $\beta$ -

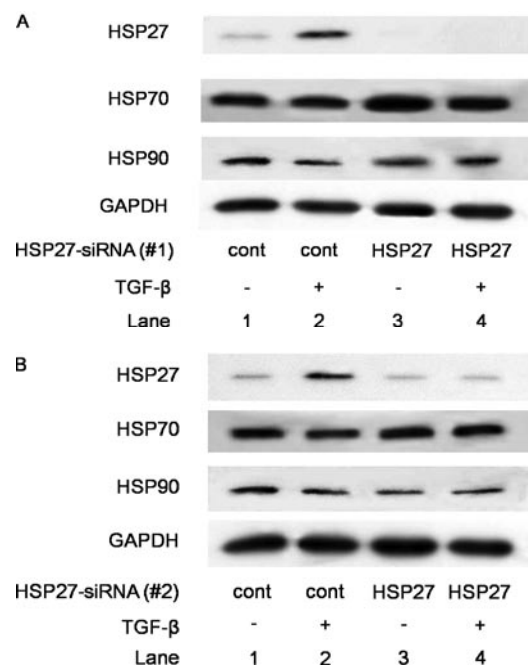


Figure 1. Effect of HSP27-siRNA on the TGF- $\beta$ -stimulated HSPs induction in MC3T3-E1 cells. Cultured cells were transfected with 50 nM control siRNA (cont) or 50 nM HSP27-siRNA using the siLentFect. (A) siRNA #1, ID 159311, (B) siRNA #2, ID 102070 (Ambion; HSP27). Forty-eight hours after transfection, the cells were stimulated by 5 ng/ml TGF- $\beta$  or vehicle for another 12 h. The extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against HSP27, HSP70, HSP90 or GAPDH.

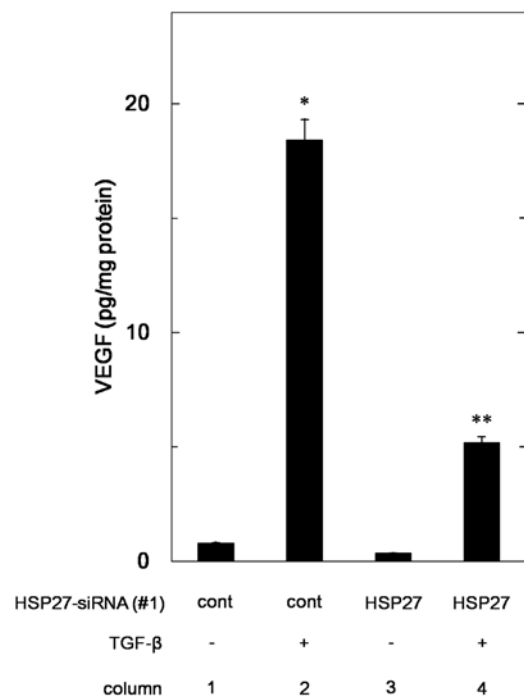


Figure 2. Effect of HSP27-siRNA on the TGF- $\beta$ -stimulated VEGF release in MC3T3-E1 cells. Cultured cells were transfected with 50 nM control siRNA (cont) or 50 nM HSP27-siRNA (siRNA #1, ID 159311, Ambion; HSP27) using the siLentFect. Forty-eight hours after transfection, the cells were stimulated by 5 ng/ml TGF- $\beta$  or vehicle for another 48 h. VEGF concentrations of the culture medium were determined by ELISA. Each value represents the mean  $\pm$  SEM of independent triplicate determinations. Similar results were obtained with two additional and different cell preparations. \* $P < 0.05$ , compared with the value of vehicle with control siRNA transfection (column 1). \*\* $P < 0.05$ , compared with the value of TGF- $\beta$  with control siRNA transfection (column 2).

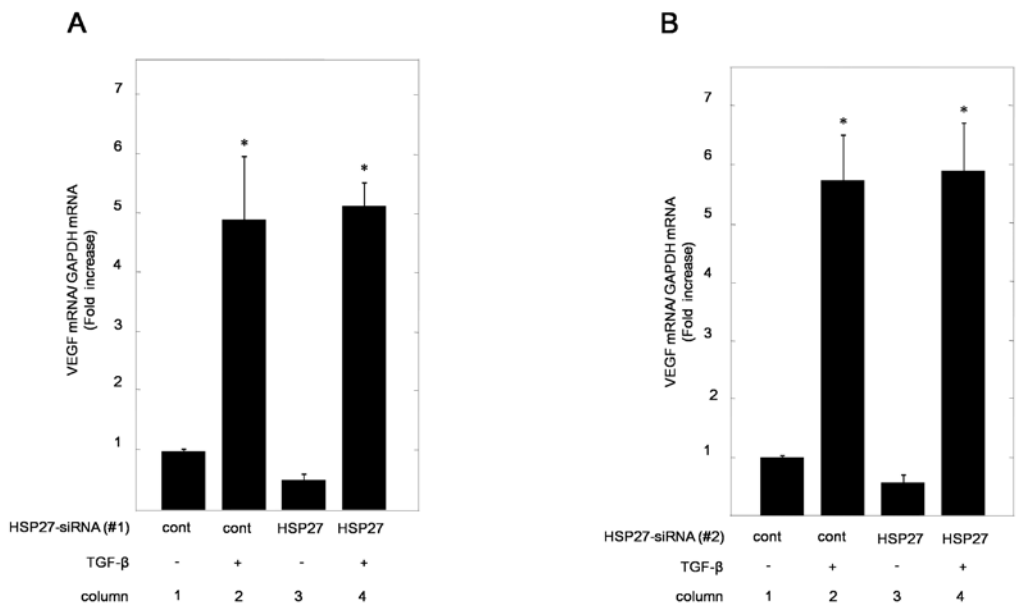


Figure 3. Effect of HSP27-siRNA on the TGF- $\beta$ -stimulated VEGF mRNA expression in MC3T3-E1 cells. Cultured cells were transfected with 50 nM control siRNA (cont) or 50 nM HSP27-siRNA. (A) siRNA #1, ID 159311, (B) siRNA #2, ID 102070 (Ambion; HSP27) using the siLentFect. Forty-eight hours after transfection, the cells were stimulated by 5 ng/ml TGF- $\beta$  or vehicle for another 12 h. Total RNA was isolated and transcribed into complementary DNA. The expression of VEGF mRNA and GAPDH mRNA were quantified by real-time RT-PCR. VEGF mRNA levels were normalized to those of GAPDH mRNA. Results were standardized for the value of vehicle with control siRNA transfection. Each value represents the mean  $\pm$  SEM of independent triplicate determinations. Similar results were obtained with two additional and different cell preparations. \* $P < 0.05$ , compared with the value of vehicle with control siRNA transfection (column 1).

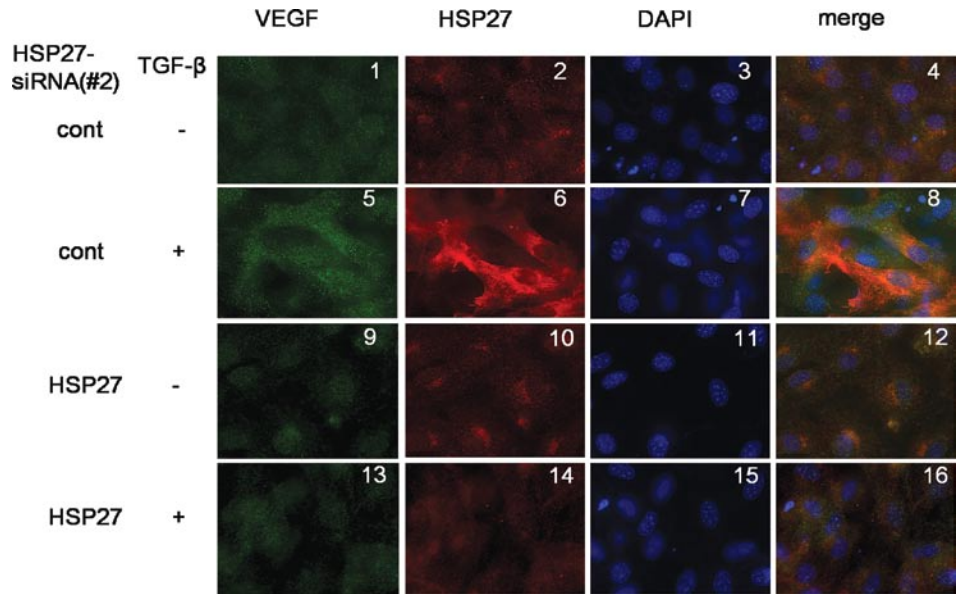


Figure 4. Immunofluorescence microscopy of HSP27 and VEGF proteins in MC3T3-E1 cells. The cells were first treated with or without siRNA of HSP27 (siRNA #2, ID 102070, Ambion; HSP27) for 48 h at 37°C, followed by stimulation with TGF- $\beta$  for 24 h. Fixed cells were exposed to anti-VEGF and anti-HSP27 antibodies (1:100 dilution) for 1 h, followed by exposure to Alexa Fluor 488<sup>®</sup> conjugated goat anti-rabbit IgG (green signal for VEGF) and Alexa Fluor 546<sup>®</sup> conjugated goat anti-mouse IgG antibodies (red signal for HSP27). After washing, the cells were exposed to DAPI (blue signal for the nuclear) for 20 min and then examined by fluorescence microscopy. Representative results from at least three independent experiments are shown.

stimulated VEGF release in osteoblast-like MC3T3-E1 cells is exerted at a point upstream of the transcriptional level, we next examined the effect of HSP27 down-regulation on the TGF- $\beta$ -stimulated VEGF mRNA expression. Treatment with either #1 or #2 HSP27-siRNA did not affect the TGF- $\beta$ -induced VEGF mRNA expression detected at 12 h after the stimulation (Fig. 3). Similar results were obtained at 24 h (data not shown).

*Effect of HSP27-knock down on TGF- $\beta$ -stimulated VEGF induction in MC3T3-E1 cells.* We further examined the effect of HSP27 down-regulation by HSP27-siRNA on VEGF induction stimulated by TGF- $\beta$  using immunofluorescence microscopy. As shown in Fig. 4, we confirmed that TGF- $\beta$  elicited both VEGF (green signal) and HSP27 (red signal) induction in the cytosol of MC3T3-E1 cells (Fig. 4, panel 5

in comparison with panel 1 and panel 6 in comparison with panel 2, respectively). VEGF and HSP27 induction closely correlated with each other (Fig. 4, panel 8 in comparison with panel 4). HSP27-siRNA (# 2) which alone had little effect on VEGF induction (Fig. 4, panel 9 in comparison with panel 1), clearly suppressed the TGF- $\beta$ -stimulated VEGF induction in these cells (Fig. 4, panel 13 and panel 16 in comparison with panel 5 and panel 8, respectively). We found that HSP27-siRNA markedly reduced the induction of HSP27 stimulated by TGF- $\beta$  (Fig. 4, panel 14 in comparison with panel 6).

## Discussion

In the present study, we have shown that HSP27-knock down by siRNA suppressed the VEGF release stimulated by TGF- $\beta$  in osteoblast-like MC3T3-E1 cells. Therefore, it is likely that HSP27 induction by TGF- $\beta$  is involved in the TGF- $\beta$ -induced VEGF synthesis or release in these cells. In previous studies (17,18,24,25), we have shown that TGF- $\beta$  stimulates both VEGF release and HSP27 induction commonly via three major MAP kinases in osteoblast-like MC3T3-E1 cells. In addition, the maximum effect of HSP27 induction was observed at 12 h after the TGF- $\beta$  stimulation whereas the VEGF release reached its peak at 48 h after stimulation of these cells (17,24). Thus the time course of HSP27 induction by TGF- $\beta$  seems to reach its peak earlier than that of VEGF release. Moreover, using immunofluorescence microscopy, we have shown that HSP27-knock down by siRNA concomitantly reduced TGF- $\beta$ -stimulated induction of both HSP27 and VEGF in the cytosol of MC3T3-E1 cells. These results strongly suggest that HSP27 induction by TGF- $\beta$  plays a crucial role in the synthesis or release of VEGF stimulated by TGF- $\beta$  in osteoblasts.

On the other hand, we have demonstrated that HSP27-siRNA has little effect on the VEGF mRNA expression induced by TGF- $\beta$  in MC3T3-E1 cells. Therefore, it seems unlikely that the suppressive effect of HSP27-siRNA on TGF- $\beta$ -induced VEGF release is exerted at the transcriptional level. Taking our findings into account as a whole, it is most likely that HSP27 induction plays a pivotal role in TGF- $\beta$ -induced VEGF release in osteoblasts, and that the effect of HSP27 is exerted at a post-transcriptional step. To the best of our knowledge, this is the first report indicating that HSP27 is involved in the intracellular signaling of TGF- $\beta$ .

In bone metabolism, osteoblast-secreted VEGF plays an important role in bone remodeling through the reconstruction of local microvasculature. VEGF is an important modulator that regulates vascularization and endochondral ossification during fracture healing (32,33). On the other hand, it is well recognized that TGF- $\beta$  synthesized by osteoblasts is stored in bone matrix as an inactive latent form, and takes part in the process of bone remodeling or fracture healing in its active form (16). In addition, it has been reported that TGF- $\beta$  and VEGF are actively involved in the recruitment and differentiation of mesenchymal stem cells in bone repair (34). It is probable that TGF- $\beta$ -induced VEGF release from osteoblasts is implicated in the process of bone remodeling, fracture healing or metabolic bone diseases. Thus, clarifying the mechanism of TGF- $\beta$ -stimulated VEGF release could provide a new therapeutic strategy for bone diseases or fracture treatment. Our present results, suggesting that the induction of

HSP27 is involved in the VEGF release stimulated by TGF- $\beta$ , might create a novel therapeutic aspect in inflammatory or metabolic osteopathy and/or fracture. However, the physiological and pathological significance of HSP27 induction in osteoblasts still remains unclear. Further investigations of the detailed role of HSP27 in the TGF- $\beta$ -stimulated VEGF release from osteoblasts are necessary to better understand bone metabolism and repair. In conclusion, our present results strongly suggest that HSP27 plays a pivotal role in TGF- $\beta$ -stimulated VEGF release at a post-transcriptional level in osteoblasts.

## Acknowledgements

We are very grateful to Yoko Kawamura for her skillful technical assistance. This investigation was supported in part by Grants-in-Aid for Scientific Research (16590873 and 16591482) from the Ministry of Education, Science, Sports and Culture of Japan, and by Research Grants for Longevity Sciences (21A-4, 21A-22) from the Ministry of Health, Labour and Welfare of Japan.

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