Activations of ERK1/2 and JNK by Transforming Growth Factor \( \beta \) Negatively Regulate Smad3-induced Alkaline Phosphatase Activity and Mineralization in Mouse Osteoblastic Cells

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Transforming growth factor (TGF-\( \beta \)) inhibits alkaline phosphatase (ALP) activity and mineralization in mouse osteoblastic MC3T3-E1 cells, whereas local administration of TGF-\( \beta \) stimulates bone formation \textit{in vivo}. We recently demonstrated that Smad3, a TGF-\( \beta \) signaling molecule, promotes ALP activity and mineralization in MC3T3-E1 cells. Moreover, the target disruption of Smad3 in mice is reported to cause a decrease in bone mineral density. These findings indicate that Smad3 plays an important role in the regulation of bone formation. However, why the effects of TGF-\( \beta \) and Smad3 on ALP activity and mineralization are different remains unknown. The purpose of the present study is to clarify the role of mitogen-activated protein kinase (MAPK) in TGF-\( \beta \) and Smad3 pathways in osteoblast. TGF-\( \beta \) activated extracellular signal-regulated kinases/p42/p44 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK) in mouse osteoblastic MC3T3-E1 cells. The expression of dominant negative type Smad3, Smad3\( \Delta \)C, affected neither TGF-\( \beta \)-activated MAPKs nor TGF-\( \beta \)-inhibited ALP activity. Specific inhibitors of ERK1/2 activation (PD98059 and U0126), as well as JNK inhibitors (curcumin and dicumarol) antagonized the inhibitory effects of TGF-\( \beta \) on ALP activity and mineralization, whereas the specific inhibitor of p38 MAPK (SB203580) did not affect them. PD98059 and curcumin enhanced Smad3-induced ALP activity and mineralization, whereas SB203580 inhibited them. In the luciferase reporter assay using 3TP-lux with the specific Smad3-responsive element, PD98059, and curcumin enhanced TGF-\( \beta \)- and Smad3-induced transcriptional activity in MC3T3-E1 cells. On the other hand, TGF-\( \beta \)-inhibited production of type I collagen was antagonized by curcumin but not by PD98059. The present study indicated that TGF-\( \beta \)-responsive ERK1/2 and JNK cascades negatively regulate Smad3-induced transcriptional activity as well as ALP activity and mineralization in osteoblasts.

Transforming growth factor \( \beta \) (TGF-\( \beta \))\(^*\) is most abundant in bone matrix compared with other tissues (1). TGF-\( \beta \) is stored in an inactive form, released from the bone matrix, and activated in the bone microenvironment (2). It is produced by osteoblasts and appears to regulate bone metabolism in various ways, including skeletal development and bone remodeling (3). Several reports demonstrated that TGF-\( \beta \) induced bone formation when it was locally administered into bone tissues in rat (4–7). However, it is disputable whether TGF-\( \beta \) would possess bone anabolic effects \textit{in vitro} (8–10), and the mechanism by which TGF-\( \beta \) stimulates bone formation \textit{in vivo} remains unknown.

The Smad family proteins are critical components of the TGF-\( \beta \) signaling pathways (11). TGF-\( \beta \) exerts growth inhibitory and transcriptional response through the two receptor-regulated Smads, Smad2 and Smad3 (11). Receptor-mediated phosphorylation of Smad2 or Smad3 induces their association with the common partner Smad4, followed by translocation into the nucleus where these complexes activate transcription of specific genes (12). As for osteoblasts, Li et al. (13) reported that overexpression of Smad2 suppressed Runx2(cbfα1) and osteocalcin mRNA expression in primary rat calvaria cells and ROS17/2.8 cells. Moreover, TGF-\( \beta \) stimulated the \( \beta \)-integrin subunit expression and \( \beta 5\)\(^{\alpha 2}\) proteolysis via Smad signaling in osteoblastic cells (14). Although Alliston et al. (15) reported that Smad3 decreased Runx2 and osteocalcin gene expressions in MC3T3-E1 cells, our recent study revealed that Smad3 inhibited the proliferation and enhanced the levels of bone matrix proteins, such as type I collagen, osteopontin, and matrix Gla protein in a manner similar to TGF-\( \beta \) in these cells (16). On the other hand, unlike TGF-\( \beta \)-, Smad3 enhanced ALP activity and mineralization of MC3T3-E1 cells in that study. Our findings suggested that Smad3 plays an important role in the regulation of bone formation. Indeed, Horton et al. (17) recently reported that mice with targeted deletion of Smad3 were osteopenic, compared with wild type littermates, because of a lower rate of bone formation. The increased synthesis of type I collagen was common effect of TGF-\( \beta \) and Smad3 on osteoblasts. However, Smad3 greatly increased ALP activity and mineralization, whereas TGF-\( \beta \) inhibited them in these cells. The reason for the discrepant effects of TGF-\( \beta \) and Smad3 on ALP activity and mineralization remained unknown in our previous study (16). We therefore hypothesized that TGF-\( \beta \) might inhibit ALP activity and mineralization of osteoblasts through some pathways other than the Smad3 pathway. Alternatively, it is also possible that some kinds of TGF-\( \beta \)-responsive intracellular signalings that are independent of Smad3 pathway might alter the activity of Smad3 signaling.

There are actually three distinct MAPKs that have been identified in mammalian cells, referred to as extracellular signal-regulated kinases/p42/p44 MAPK (ERK1/2), p38 MAPK (P38), and c-Jun N-terminal kinases (JNK)/stress-activated protein kinases (18). These MAPKs are all proline-directed,
serine-threonine kinases that are activated on threonine and tyrosine residues in response to a wide variety of extracellular stimuli. TGF-β also stimulates ERK1/2, P38, and JNK in a variety of cell lines (18). Numerous reports suggested that MAPK pathways cross-talk with Smad pathway and modulate the transcriptional regulation of the target genes (19–25). Our aim of this study is to clarify the role of MAPKs in TGF-β and Smad3 pathways in osteoblastic cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**MC3T3-E1 cells were kindly provided by Dr. H. Kodama (Ohu Dental College, Ohu, Japan). Human recombinant TGF-β1, mouse anti-c-Myc antibody, and mouse anti-β-actin monoclonal antibody were purchased from Sigma. Anti-Smad3 antibody was purchased from Zymed Laboratories (San Francisco, CA). PD98059, U0126, SB203580, and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma. 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT)/β-D-galactosidase activity was measured as described (26).

**Cell Culture—**MC3T3-E1 cells were cultured in α-MEM (containing 50 μg/ml ascorbic acid) supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen). The medium was changed twice a week. For the mineralization assay, the cells were cultured in α-MEM (containing 50 μg/ml ascorbic acid) supplemented with 10% FBS, 1% penicillin/streptomycin and 10 mM β-glycerophosphate for 14 days after reaching confluency.

**Construct and Transient or Stable Transfection—**Myc-tagged Smad3 was prepared as described previously (26). Smad3 DNA was derived from rat. A mutant form of Myc-tagged Smad3 (Smad3ΔC), in which the MH2 domain corresponding to amino acid residues 278–425 was removed, was kindly provided by Dr. Y. Chen. Myc-Smad3, Myc-Smad3ΔC, and empty vector (pcDNA3.1+) (each 3 μg) were transfected into MC3T3-E1 cells with LipofectAMINE (Invitrogen). 6 h later, the cells were fed with fresh α-MEM containing 10% FBS. 48 h later, the cells were employed as transiently transfected ones for the experiments. For a stable transfection, after incubation in α-MEM containing 10% FBS for 48 h, the cells were passaged, and the clones were selected in α-MEM supplemented with G418 (0.3 mg/ml) (Invitrogen) and 10% FBS. To rule out the possibility of clonal variation, we characterized at least three independent clones for each stable transfection. Empty vector (V)-transfected cells were used as the control.

**Luciferase Assay—**MC3T3-E1 cells were seeded at a density of 2 × 10⁵/6-well plate. 24 h later, the cells were transfected with 3 μg of the reporter plasmid (p3TP-Lux), the pCH110 plasmid expressing β-galactosidase (1 μg), and 3 μg of V-Smad3ΔC. 48 h later, the cells were stimulated with 5 ng/ml TGF-β. Then, 24 h later, the cells were harvested, and the luciferase activity was measured as described under “Experimental Procedures.” The values of relative luciferase activity represent the means ± S.E., *p < 0.01, compared with the TGF-β-unstimulated group. C, confluent cells were fed with fresh serum-free medium with or without 2.5 ng/ml TGF-β for 48 h. The ALP activity was measured as described under “Experimental Procedures.” Each bar is expressed as the mean ± S.E. (μmol/min/mg protein) of four determinations. *, p < 0.01, compared with the TGF-β-unstimulated group.

**Protein Extraction and Western Analysis—**The cells were lysed with radiomimunoprecipitation buffer with 0.5 mM phenylmethylsulfonyl fluoride, complete protease inhibitor mixture, 1% Triton X-100, and 1 mM sodium orthovanadate. The cell lysates were centrifuged at 12,000 g for 20 min at 4 °C, and the supernatants were stored at −80 °C. Protein quantitation was performed with BCA protein assay reagent (Pierce). 20 μg of protein was denatured in SDS sample buffer and separated on 10% polyacrylamide-SDS gel. The protein was transferred in 25 mM Tris, 192 mM glycine, and 20% methanol to polyvinylidene difluoride. The blots were blocked with Tris-buffered saline (20 mM Tris-HCl, pH 7.5, and 157 mM NaCl) plus 0.1% Tween 20 containing 3%...
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The hybridization probes were the 2.8-kb fragment of the Cb ya gene of V-/-Smad3+/+ cells, treated with TGF-β (2.5 ng/ml) in the presence or absence of PD98059, U0126, SB203580, curcumin, or dicumarol (each 10 μM) for 48 h after pretreatment with these inhibitors for 1 h, respectively, and then ALP stain (A) and measurement of ALP activity (B) were performed. Each bar is expressed as the mean ± S.E. (n mol/min/mg protein). *, p < 0.01, compared with TGF-β-untreated group.

**Fig. 4. Effects of MAPK inhibitors on TGF-β-inhibited ALP activity.** Confluent MC3T3-E1 cells were treated with TGF-β (2.5 ng/ml) in the presence or absence of PD98059, U0126, SB203580, curcumin, or dicumarol (each 10 μM) for 48 h after pretreatment with these inhibitors for 1 h, respectively, and then ALP stain (A) and measurement of ALP activity (B) were performed. Each bar is expressed as the mean ± S.E. (n mol/min/mg protein). *, p < 0.01, compared with TGF-β-untreated group.

**Map for ALP Activity**—Confluent cells in 24-well plates were rinsed three times with phosphate-buffered saline, and 600 μl of distilled water was added to each well. ALP activity was assayed at 37 °C by a method modified from that of Lowry et al. (28). In brief, the assay mixtures contained 0.1 M 2-amino-2-methyl-1-propanol (Sigma), 1 mM MgCl₂, 8 mM p-nitrophenyl phosphate disodium, and cell homogenates. After 3 min of incubation, the reaction was stopped with 0.1 N NaOH, and the absorbance was read at 405 nm. A standard curve was prepared with p-nitrophenol (Sigma). Each value was normalized with the value in protein content. ALP staining was performed as described previously by Harlow and Lane (29). In brief, cultured cells were rinsed in phosphate-buffered saline, fixed in 100% methanol, and then overlaid with 1.5 ml of 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Sigma) plus 0.3 mg/ml nitro blue tetrazolium chloride (Invitrogen) in 0.1 M Tris-HCl, pH 9.5, 0.01 N NaOH, 0.05 M MgCl₂, followed by incubation at room temperature for 2 h in the dark.

**Assay of Mineralization**—The mineralization of MC3T3-E1 cells was determined in 6- and 12-well plates using von Kossa staining and Alizarin Red staining, respectively. After 3 min of incubation, the reaction was stopped with 0.1 N NaOH, and the absorbance was read at 405 nm. A standard curve was prepared with p-nitrophenol (Sigma). Each value was normalized with the value in protein content. ALP staining was performed as described previously by Harlow and Lane (29). In brief, cultured cells were rinsed in phosphate-buffered saline, fixed in 100% methanol, and then overlaid with 1.5 ml of 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Invitrogen) plus 0.3 mg/ml nitro blue tetrazolium chloride (Invitrogen) in 0.1 M Tris-HCl, pH 9.5, 0.01 N NaOH, 0.05 M MgCl₂, followed by incubation at room temperature for 2 h in the dark.

**Statistics**—The data are expressed as the means ± S.E. The statistical analysis was performed using an unpaired t test or analysis of variance.

**Fig. 3. Activation of MAPKs by TGF-β in V-/Smad3ΔC-transfected MC3T3-E1 cells.** MC3T3-E1 cells expressing V- or myc-Smad3ΔC were cultured for 48 h after transfection and then stimulated with 2.5 ng/ml TGF-β in serum free α-MEM. Ten min later, protein extraction and Western blot were performed.
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RESULTS

TGF-β-activated MAPKs in MC3T3-E1 Cells—Previous study indicated that TGF-β stimulates MAPKs, such as ERK1/2, P38, and JNK, in several kinds of cells (18). We therefore investigated whether TGF-β would phosphorylate these MAPKs in MC3T3-E1 cells with Western blot using anti-phospho-ERK1/2, phospho-P38, and phospho-JNK antibodies. TGF-β (2.5 ng/ml) promoted the phosphorylation of ERK1/2, P38, and JNK within 10 min, whereas it did not alter the levels of total ERK, P38, and JNK (Fig. 1). These results indicated that TGF-β activates these MAPKs. Second, we investigated whether specific inhibitors of MAPKs would inhibit TGF-β-induced activations of MAPKs. 10 μM PD98059 and 10 μM U0126, specific inhibitors of the phosphorylation of ERK1/2, antagonized TGF-β-induced phosphorylation of ERK, whereas 10 μM SB203580, a P38-specific inhibitor, antagonized TGF-β-induced phosphorylation of P38. Moreover, curcumin and dicumarol, JNK-specific inhibitors, antagonized TGF-β-induced phosphorylation of JNK (data not shown). These inhibitors did not affect the basal levels of total ERK1/2, P38, and JNK. These specific inhibitors did not affect the phosphorylation of other MAPKs; for example, PD98059 could not inhibit TGF-β-induced phosphorylation of P38 and JNK (data not shown). These results indicated that the inhibitors of MAPKs employed in the present study specifically antagonized TGF-β-induced activation of ERK1/2, P38, and JNK in MC3T3-E1 cells.

TGF-β-induced Activations of MAPKs Were Independent of Smad3 Signaling Pathway—We investigated whether TGF-β-induced activations of MAPKs would be independent of Smad3 signaling pathway. The MH2-region of Smad3 is indispensable for protein-protein interaction and the transcriptional regulation of the target genes (11, 12). In several studies, C-terminally truncated Smad3 was used to inactivate endogenous Smad3 in a dominant negative manner (20). We therefore used the Smad3ΔC, which lacks the MH2-region. We confirmed that the Myc signal was detected in Myc-Smad3ΔC-transfected MC3T3-E1 cells but not in V-transfected cells (Fig. 2A). To investigate whether Smad3ΔC has a dominant negative effect on TGF-β-induced transcriptional activity, we employed luciferase assay using 3TP-Lux containing the promoter of plasminogen inhibitor 1 with a Smad3-specific responsive element. Although TGF-β promoted luciferase activity in V-transfected MC3T3-E1 cells, Smad3ΔC suppressed TGF-β-induced luciferase activity (Fig. 2B). These findings suggested that Smad3ΔC exhibited dominant negative effects on TGF-β-Smad3 signaling in MC3T3-E1 cells. We examined whether activations of MAPKs by TGF-β would be dependent or independent of Smad3. TGF-β increased the phosphorylation of ERK1/2, P38, and JNK, and Smad3ΔC did not affect the phosphorylation of these MAPKs by TGF-β in MC3T3-E1 cells (Fig. 3). These results indicated that TGF-β-induced activations of ERK1/2, P38, and JNK were independent of TGF-β-Smad3 signaling pathway.

Requirement of ERK1/2 and JNK in the Inhibitory Effects of TGF-β on ALP Activity and Mineralization—Although TGF-β inhibits ALP activity and mineralization in MC3T3-E1 cells in our study and previous studies (4–7, 16), our recent study revealed that Smad3 promoted them in MC3T3-E1 cells (16). These findings raised the hypothesis that TGF-β inhibits ALP activity and mineralization of osteoblasts through a pathway other than Smad3 signaling in MC3T3-E1 cells. As shown in Fig. 2C, Smad3ΔC did not affect ALP activity inhibited by TGF-β, suggesting that TGF-β inhibited ALP activity through pathways other than Smad3. We therefore investigated the effects of MAPK inhibitors on TGF-β-inhibited ALP activity and mineralization. PD98059 and U0126 (each 10 μM) rescued the reduction of ALP activity and mineralization by TGF-β (Figs. 4 and 5). Curcumin and dicumarol (each 10 μM) also rescued them (Figs. 4 and 5). On the other hand, 10 μM SB203580 did not affect the inhibitory effects of TGF-β (Figs. 4 and 5). These results indicated that TGF-β inhibited ALP activity and mineralization through ERK1/2 and JNK pathways in osteoblasts.

Inhibitors of ERK1/2 and JNK Enhanced Smad3-induced ALP Activity and Mineralization—To test the hypothesis that MAPKs activated by TGF-β negatively regulates Smad3-induced ALP activity and mineralization, we investigated the effects of MAPK inhibitors on Smad3-induced ALP activity and mineralization by using stably Smad3-overexpressed MC3T3-E1 cells. Smad3 overexpression promoted ALP activity and mineralization in MC3T3-E1 cells (Figs. 6 and 7), as described in our previous study (16). PD98059, U0126, curcumin, and dicumarol enhanced Smad3-induced ALP activity and mineralization, whereas SB203580 suppressed Smad3-induced ALP activity and mineralization (Figs. 6 and 7), indicating that inhibitors of ERK1/2 and JNK augmented Smad3-induced ALP activity and mineralization in osteoblasts. ERK1/2 and JNK

FIG. 5. Effects of MAPK inhibitors on TGF-β-induced mineralization. Confluent MC3T3-E1 cells were treated with TGF-β (2.5 ng/ml) in the presence or absence of PD98059, U0126, SB203580, curcumin, or dicumarol (each 10 μM) in α-MEM (containing 50 μg/ml ascorbic acid) supplemented with 10% FBS, 1% penicillin/streptomycin, and 10 mM β-glycerophosphate for 14 days. The mineralized matrix was stained with the von Kossa method (A) or with Alizarin Red (B). C, Alizarin Red stain was quantitated as described under “Experimental Procedures.” Each value is expressed as a ratio of the control value. Each bar is expressed as the mean ± S.E. *p < 0.01, compared with the TGF-β-un-treated group.
pathways might negatively regulate Smad3-induced ALP activity and mineralization in osteoblasts.

Inhibitors of ERK1/2 and JNK Enhanced Transcriptional Activity of Smad3—To investigate whether MAPKs activated by TGF-β would negatively regulate the transcriptional activity of Smad3, we employed luciferase assay using 3TP-lux. Without MAPK inhibitors, TGF-β/H9252 promoted transcriptional activity at a level of about three times that of the basal line in MC3T3-E1 cells (Fig. 8A). However, PD98059 and curcumin significantly enhanced TGF-β/H9252-induced transcriptional activity more effectively than that without these MAPK inhibitors (Fig. 8A). Moreover, Smad3-induced transcriptional activity was also significantly increased by PD98059 and curcumin, compared with that without these inhibitors (Fig. 8B). These results indicated that ERK and JNK pathways negatively regulated transcriptional activity of the TGF-β/Smad3 signaling pathway in osteoblasts.

Inhibitor of ERK1/2 but Not JNK Enhanced TGF-β-induced Expression of COLI—Although the effects of TGF-β and Smad3 on ALP activity and mineralization were contrary, both TGF-β and Smad3 promoted the expression of COLI in MC3T3-E1 cells (16). We investigated the effects of PD98059 and curcumin on TGF-β-induced COLI mRNA expression in MC3T3-E1 cells. PD98059, but not curcumin, enhanced TGF-β-induced COLI mRNA expression (Fig. 9). These findings suggested that inhibition of ERK, but not JNK, enhanced TGF-β-induced COLI expression in osteoblasts.

DISCUSSION

In the present study, the inhibition of the Smad3 signaling pathway by the expression of dominant negative type Smad3, Smad3C, did not affect TGF-β-induced activations of MAPKs, ERK1/2, P38, and JNK. These findings indicated that activations of these MAPKs by TGF-β were independent of Smad3 signaling pathways. Indeed, Engel et al. (20) reported that JNK was rapidly and transiently activated by TGF-β receptor type I in Rho-GTPase-dependent and Smad-independent manner in mink lung epithelial (Mv1Lu) cells and a breast carcinoma cell line (MDA-MB-468). These findings suggested that the coincident activation of the Smad and JNK/AP-1 pathways is necessary for full transcriptional activation in response to TGF-β. Moreover, TGF-β induced proliferation in colon carcinoma cells by Ras-dependent but Smad-independent down-regulation of p21
cip1 (32). On the other hand, Lai and Cheng (33) reported that TGF-β activated Ras/MAPK/AP-1 signaling and that the stimulation of AP-1 by TGF-β was dependent on Smad signaling in human osteoblastic cells. In addition, several studies suggested the cross-talk of Ras-ERK/MAPKs and TGF-β-Smad pathway (19, 22–25). Taken into account with our findings, TGF-β-Smad signaling, MAPK pathways, and AP-1 might...
cross-talk in a complex manner, although there are TGF-β-responsive and Smad3-independent MAPK pathways in mouse osteoblastic cells.

Several studies and our previous study indicated that TGF-β suppressed ALP activity and mineralization in osteoblasts including MC3T3-E1 cells (8, 9, 34). In contrast, Smad3 promoted them in MC3T3-E1 cells (16). In the present study, inactivation of ERK1/2 and JNK with their specific inhibitors antagonized the inhibitory effects of TGF-β on ALP activity and mineralization in MC3T3-E1 cells (Figs. 4 and 5). These findings suggested that the activation of ERK1/2 and JNK contributes to the inhibitory regulation of ALP activity and mineralization by TGF-β. In the present study, the specific inhibitors of ERK1/2 and JNK enhanced the transcriptional activity (Fig. 8), as well as ALP activity and mineralization (Figs. 6 and 7) induced by Smad3. Taken together, these findings suggested that ERK1/2 and JNK signaling pathways negatively regulate Smad3 signaling pathway, resulting in the suppression of Smad3-induced ALP activity and mineralization in osteoblasts. In support of this speculation, several studies have shown that JNK, as well as c-Jun and JunB, represses Smad3-mediated transcriptional activity in the human hepatoma cell line (HepG2), mouse fibroblasts, and keratinocytes (35, 36). Moreover, several studies indicated that oncogene Ras or epidermal growth factor-induced Ras repressed TGF-β/Smad signaling in cancer cells or cell lines other than osteoblasts (19, 22–25). The present study indicated that TGF-β activates ERK1/2 as well as JNK and inhibits ALP activity in a manner independent of Smad3 in MC3T3-E1 cells. These findings therefore suggested that TGF-β-responsive ERK1/2 and JNK cascades negatively regulate Smad3-induced transcriptional activity as well as ALP activity and mineralization in osteoblasts. The negative signal of TGF-

**FIG. 7. Effects of MAPK inhibitors on Smad3-induced mineralization.** Confluent V-/Smad3-transfected MC3T3-E1 cells were treated with PD98059, U0126, SB203580, curcumin, or dicumarol (each 10 μM) in α-MEM (containing 50 μg/ml ascorbic acid) supplemented with 10% FBS, 1% penicillin/streptomycin, and 10 mM β-glycerophosphate for 14 days. The mineralized matrix was stained with the von Kossa method (A) or with Alizarin Red (B). C, Alizarin Red stain was quantitated as described under “Experimental Procedures.” Each value is expressed as a ratio of untreated V values. Each bar is expressed as the mean ± S.E. *p < 0.01, compared with the inhibitor-untreated group.
collagen and H9251 cells. Therefore, H9251 expressing ALP activity. Takeuchi et al. TGF-

ever, we cannot rule out the possibility that some other mechanism ALP activity and mineralization in MC3T3-E1 cells. How-

vised and inhibits ALP activity in ROS 17/2.8 and MC3T3-E1 cells, respectively (10, 38). Although the effects of Smad3 on

ROS 17/2.8 cells are unknown, the intracellular signals that modulate the effects of TGF-β and Smad3 might be different, depending on the cell lines, species, and how the cells have been transformed.

The present study could not clarify the exact molecular mechanism by which TGF-β negatively regulates Smad3-induced transcriptional activity as well as ALP activity and mineralization through ERK1/2 and JNK. Several studies revealed the mechanisms by which RAS/ERK1/2 or JNK cascades negatively regulate TGF-β signaling pathway. Kretzschmar et al. (22) reported that oncogenic Ras inhibited the TGF-β-induced nuclear accumulation of Smad2 as well as Smad3 and Smad-dependent transcription by phosphorylation of Smad2 and Smad3 via ERK1/2, whereas Saha et al. (24) reported that oncogenic Ras repressed TGF-β signaling by ERK1/2-dependent down-regulation of Smad4. The AP-1 family protein c-Jun, which is a substrate for JNK, directly suppressed Smad/DNA interaction (36). It is also possible that the target step in which TGF-β-responsive ERK1/2 and JNK negatively regulate Smad3 signaling pathway might be the recruitment of Smad3 to intracellular membranes that contains TGF-β-receptor type I by Smad anchor for receptor activation (39, 40), phosphorylation/activation of a motif SSxxS in the C terminus of Smad3 by serine/threonine kinase activity of TGF-β-receptor type I (41), the association of Smad3 and the common partner, Smad4, the translocation of the Smad3-Smad4 complex into the nucleus, and its DNA binding or interaction with other transcriptional regulators. In our preliminary study, both ERK1/2 and JNK inhibitors did not promote the TGF-β-responsive nuclear localization of Smad3 (data not shown). These findings suggested that the TGF-β-responsive ERK1/2 and JNK cascade might not affect the nuclear translocation of Smad3 in MC3T3-E1 cells. Furthermore, there might be the autoinduction system that TGF-β up-regulates the production of TGF-β itself (42). It is possible that TGF-β increases the production of Smad3 and that ERK1/2 and JNK negatively regulate Smad3 expression by the TGF-β-responsive autoinduction system. Further studies are in progress in our laboratory. Smad3 enhanced ALP activity and mineralization in MC3T3-E1 cells (16), suggesting that Smad3 is involved in the transcriptional mechanism leading to bone formation. In support of this, Borton et al. (17) recently reported that mice with targeted deletion of Smad3 were osteopenic compared with wild type littermates, because of a lower rate of bone formation. In the present study, inhibitors of ERK1/2 and JNK rescued TGF-β-inhibited ALP activity and mineralization in MC3T3-E1 cells. Moreover, these inhibitors enhanced Smad3-induced ALP activity and mineralization in these cells. The negative effects of TGF-β on ALP activity and mineralization in osteoblasts negatively influence bone formation. If in vivo ERK1/2 inhibitor and/or JNK inhibitor

\[ \text{Relative luciferase activity} = \frac{\text{Relative luciferase activity of treated cells}}{\text{Relative luciferase activity of control cells}} \times 100 \]

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antagonize the negative effects of TGF-β on bone formation and enhance the positive effects of TGF-β-responsive Smad3 on bone formation, the combination of TGF-β and inhibitors of ERK1/2 and/or JNK may be a novel therapeutic strategy for bone disease or fracture healing. Type I collagen is the abundant protein in bone matrix and plays an important role in bone formation, mineralization, and maintenance of bone strength (37). As shown in Fig. 9, TGF-β-induced expression of COLI was enhanced by the ERK1/2 inhibitor but not by the JNK inhibitor. We therefore speculated that the combination of TGF-β with ERK1/2 inhibitors might be better than with JNK inhibitor for inducing bone anabolic action.

In conclusion, the present study indicated that TGF-β-activated ERK1/2 and JNK cascades negatively regulated the transcriptional activity as well as ALP activity and mineralization induced by Smad3 in mouse osteoblastic cells. We propose that Smad3 is an important molecule in the regulation of bone formation and that the local combined administration TGF-β with inhibitors of ERK1/2 might be a novel therapeutic strategy for the stimulation of bone formation.

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Activations of ERK1/2 and JNK by Transforming Growth Factor β Negatively Regulate Smad3-induced Alkaline Phosphatase Activity and Mineralization in Mouse Osteoblastic Cells

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