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Smad3 Differently Affects Osteoblast Differentiation in a Manner Dependent upon its Differentiation Stage.

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Running title: Smad3 and osteoblast differentiation

Abstract

Smad3, a critical component of the TGF- β signaling pathways, plays an important role in the regulation of bone formation. However, how Smad3 affects osteoblast at the different differentiation stage remains still unknown. In the present study, we examined the effects of Smad3 on osteoblast phenotype by employing mouse bone marrow ST-2 cells and mouse osteoblastic MC3T3-E1 cells at the different differentiation stage. Smad3 overexpression significantly inhibited bone morphogenetic protein-2 (BMP-2)-induced ALP activity in ST-2 cells, indicating that Smad3 suppresses the commitment of pluripotent mesenchymal cells into osteoblastic cells. Smad3 increased the levels of COL1 and ALP mRNA at 7 day cultures in MC3T3-E1 cells, and its effects on COL1 were decreased as the culture periods progress, although its effects on ALP were sustained during 21 day cultures. Smad3 overexpression enhanced the level of Runx2 and OCN mRNA at 14 day and 21 day cultures. Smad3 increased the levels of MGP and NPP-1 mRNA, although the extent of increase in MGP and NPP-1 was reduced and enhanced during the progression of culture period,

respectively. Smad3 did not affect the level of ANK mRNA. On the other hand, Smad3 enhanced the level of MEPE mRNA at 14 and 21 day cultures, although Smad3 decreased it at 7 day cultures. In conclusion, Smad3 inhibits the osteoblastic commitment of ST-2 cells, while promotes the early stage of differentiation and maturation of osteoblastic committed MC3T3-E1 cells. Also, Smad3 enhanced the expression of mineralization-related genes at the maturation phase of MC3T3-E1 cells.

Keywords; Smad3; osteoblast, TGF- β , mineralization

Introduction

Transforming growth factor- β (TGF- β) is produced by osteoblasts and appears to regulate bone metabolism in various ways, including skeletal development and bone remodeling [1]. TGF- β modulates the proliferation, differentiation and production of bone matrix proteins of osteoblasts. TGF- β also induces bone formation when locally administered into bone tissues in rats [2]. The Smad family proteins are critical components of the TGF- β signaling pathways [3]. TGF- β exerts growth inhibitory and transcriptional response through the two receptor-regulated Smads, Smad2 and Smad3. 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. As for osteoblasts, Li *et al.* [4] reported that overexpression of Smad2 suppressed Runx2 and osteocalcin mRNA expression in primary rat calvaria cells and ROS17/2.8 cells. Moreover, TGF- β stimulated the α v-integrin subunit expression and p57 kip2 proteolysis via Smad signaling in osteoblastic cells [5]. Our study revealed that Smad3 inhibited

the proliferation and enhanced the levels of bone matrix proteins, such as type I collagen (Col1), osteopontin (Opn), and matrix Gla protein (MGP) in a manner similar to TGF- β in MC3T3-E1 cells [6]. On the other hand, unlike TGF- β , Smad3 enhanced alkaline phosphatase (ALP) activity and mineralization of MC3T3-E1 cells in that study. Indeed, Borton *et al.* [7] reported that mice with targeted deletion of Smad3 were osteopenic, compared with wild type littermates, because of a lower rate of bone formation. These findings suggested that Smad3 plays an important role in the regulation of bone formation.

The increased synthesis of COLI in MC3T3-E1 cells was a common effect of both TGF- β and Smad3. However, Smad3 greatly increased ALP activity as well as mineralization, and TGF- β inhibited them. For its reason, TGF- β might inhibit ALP activity and mineralization of osteoblasts through a pathway other than the Smad3 pathway. JNK as well as c-Jun and JunB represses Smad3-mediated transcriptional activity [8]. Moreover, our previous 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 [9]. Alternatively, Smad3 might differently affect osteoblast dependent upon its differentiation stage. However, how Smad3 affects osteoblast at different differentiation stage is still unknown.

In the present study, we therefore examined the effects of Smad3 on osteoblast phenotype by employing mouse bone marrow ST-2 cells, a pluripotent mesenchymal cell-line, which can differentiate into osteoblastic cells with bone morphogenetic protein-2 (BMP-2) treatment, as well as mouse osteoblastic MC3T3-E1 cells, which differentiate into the mineralization stage during 21 day cultures.

Materials and Methods

Materials

MC3T3-E1 cells were kindly provided by Dr. H. Kodama (Ozu Dental College, Japan). Human recombinant BMP-2 was kindly provided by Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan). Myc-tagged Smad3 was kindly provided by Dr JJ Jebrun (McGill University). Anti- β -actin antibody was from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Cell culture

Mouse bone marrow ST-2 and osteoblastic MC3T3-E1 cells were cultured in α -MEM (containing 50 μ g/ml ascorbic acid) with 10% FBS and 1% Penicillin-Streptomycin (Gibco BRL, Rockville, MD, USA). The medium was changed twice a week.

Constructs and transient or stable transfection

Myc-Smad3 and empty vector (pcDNA3.1+) (each 3 μ g) were transfected into ST-2 or MC3T3-E1 cells with Lipofectamine (Invitrogen). Six h later, the cells were fed with fresh α -MEM containing 10% FBS. Forty-eight h later, the transiently transfected cells were used for experiments. For experiments with stably transfected cells, after incubation in α -MEM containing 10% FBS for 48 h, the cells were passaged and 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, at least three independent clones for each stable transfection were

characterized. Empty vector (V)-transfected cells were used as control.

Assay of ALP activity

Confluent cells in 24-well plates were rinsed three times with phosphate-buffered saline, and 600 μ l of distilled water was added to each well. ALP activity was assayed at 37 ° C, as previously described [6]. 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.

RNA extraction and semi-quantitative RT-PCR

Total RNA was prepared from cells using the acid guanidinium-thiocyanate-phenol-chloroform extraction method. Reverse transcription of 5 μ g of cultured cell total RNA was carried out for 50 min at 42 ° C and then 15 min at 70 ° C, using the Super Script First-Strand

Synthesis system for RT-PCR (Gibco), which contained RT buffer, oligo (dT)₁₂₋₁₈, 5x first-strand solution, 10 mM dNTP, 0.1 M dithiothreitol (DTT), Super Script II (RT enzyme) and RNaseH (RNase inhibitor). PCR using primers to unique sequences in each cDNA was carried out in a volume of 10 μ l reaction mixture for PCR (as supplied by TaKaRa, Otsu, Japan), supplemented with 2.5 units of TaKaRa TaqTM, 1.5 mM of each dNTP (Takara) and PCR buffer (10x) (100 mM Tris-HCl (pH 8.3), 500 mM KCl and 15 mM MgCl₂); 25 ng of each primer and 1 μ l of template (from a 50 μ l RT reaction) were used. Thermal cycling conditions and primer sequences are described below. (1) Initial denaturation at 96 ° C for 2 min. (2) Cycling for cDNA-specific number of cycles: 96 ° C for 1 min, cDNA-specific annealing temperature for 2 min, and 72 ° C for 2 min. (3) Final extension at 72 ° C for 5 min. Primer sequences, annealing temperature and cycle numbers were as follows: Col1; 5'-GCAATCGGGATCAGTACGAA-3' and 5'-CTTTCACGCCTTTGAAGCCA-3'(61 ° C; 37 cycles); MGP; 5'-GAATCTCACGAAAGCAT-3' and 5'-ACCCGCAGAAGGAAGGA-3' (49 ° C;

20 cycles); OCN, 5'-CTCTGTCTCTCTGACCTCACAG-3' and
5'-GGAGCTGCTGTGACATCCA-3' (53 ° C; 28 cycles); Runx2,
5'-CAGGAAGACTGCAAGAAGGCTCTGG-3' and
5'-ACACGGTGTCCTGCGCTGAAGA-3' (62 ° C; 25 cycles); ALP;
5'-GCCCTCTCCAAGACATATA-3' and 5'-CCATGATCACGTCGATATCC-3'
(51 ° C; 28 cycles), ANK 5'-CTAGCAGGGTTTGTGGGAGAA-3' and
5'-TTTATGAAGCAGGGGCGTGAA-3' (56° C; 23 cycles); NPP-1,
5'-GCCAGGATCAGATGTGGAGATTG-3' and
5'-TAACCGAGCAGCAGGTCCATAC (63 ° C; 32 cycles); MEPE,
5'-ACTATCCACAAGTGGCCTCG-3' and 5'-CTGTTGGCTTGCTCAGTTCC-3'
(57 ° C; 31 cycles), and GAPDH; 5'-ATCCCA TCACCATCTTCCAGGAG-3' and
5'-CCTGCTTCA CCACCTTCTTGATG-3' (47 ° C; 24 cycles). For
semi-quantitative RT-PCR, the number of cycles was chosen so that
amplification remained well within the linear range, as assessed by densitometry
(NIH Image J, version 1.08i, public domain program). An equal volume from
each PCR was analyzed by 6% non-denaturing PAGE and ethidium bromide

stained PCR products were evaluated. Marker gene expression was normalized to GAPDH expression in each sample.

Statistics

Data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using ANOVA.

Results

Smad3 inhibits the differentiation of pluripotent mesenchymal cells into osteoblastic cells

We employed mouse mesenchymal ST-2 cells as pluripotent mesenchymal cells, because ST-2 cells differentiate into osteoblastic cells with the addition of BMP-2, as previously described [10, 11]. We examined the effects of Smad3 on ALP activity induced by BMP-2 in ST-2 cells. As shown in Fig. 1, BMP-2 significantly enhanced ALP activity in ST-2 cells. Transient overexpression of Smad3 significantly inhibited BMP-induced ALP activity in these cells. These

findings indicated that Smad3 suppresses the commitment of pluripotent mesenchymal cells into osteoblastic cells.

Effects of Smad3 on the levels of COLI and ALP mRNA in 21 day cultures of MC3T3-E1 cells

We previously revealed that Smad3 increased COLI and ALP activity in MC3T3-E1 cells [6]. Therefore, we examined the effects of Smad3 on the levels of COLI and ALP mRNA in 21 day cultures of MC3T3-E1 cells to explore whether Smad3 would differently affect osteoblastic cells in a manner dependent upon the differentiation stage. We employed stably Smad3-overexpressed MC3T3-E1 cells [6]. During 21 day cultures, the levels of Smad3 protein did not change (data not shown). As shown in Fig. 2, Smad3 increased the levels of COLI and ALP mRNA at 7 day cultures in MC3T3-E1 cells, and Smad effects on COL1 were decreased as the culture periods progress. The induction of Smad3 on ALP mRNA was similar during 21 day cultures.

Effects of Smad3 on the levels of Runx2 and OCN mRNA in 21 day cultures of MC3T3-E1 cells

Next, we examined the effects of Smad3 on the levels of Runx2 and OCN mRNA in 21 day cultures of MC3T3-E1 cells. We previously reported that Smad3 decrease the level of Runx2 and OCN mRNA at confluent MC3T3-E1 cells corresponding to 1 week culture in the present study [6]. On the other hand, Smad3 overexpression enhanced the level of Runx2 and OCN mRNA at 14 day and 21 day cultures (Fig. 3).

Effects of Smad3 on the levels of mineralization-related genes in 21 day cultures of MC3T3-E1 cells

Osteoblastic mineralization is controlled by several mineralization inhibitors as well as ALP and OCN, and our previous study revealed that Smad3 enhances mineralization by using Alizarin Red and von Kossa stain [6]. We therefore examined the effects of Smad3 overexpression on several putative mineralization inhibitors, such as ANK, NPP-1, MGP and MEPE in 21 day

cultures of MC3T3-E1 cells by using semi-quantitative RT-PCR. As shown in Fig. 4, Smad3 increased the levels of MGP and NPP-1 mRNA, although the extent of increase in MGP and NPP-1 was reduced and enhanced during the progression of culture period, respectively. Smad3 overexpression did not affect the level of ANK mRNA. On the other hand, Smad3 enhanced the level of MEPE mRNA at 14 and 21 day cultures, although Smad3 decreased it at 7 day cultures.

Discussion

Recent several studies revealed that TGF- β inhibits osteoblast differentiation, in contrast to BMP-2 [1]. We previously confirmed this evidence in MC3T3-E1 cells. TGF- β inhibited ALP activity and mineralization in these cells. However, the roles of Smad3, a crucial TGF- β signaling molecule, in the commitment of undifferentiated mesenchymal stem cells into osteoblastic cells as well as osteoblast differentiation still remained unclear in the present. As for the commitment of undifferentiated mesenchymal cells into osteoblastic cells, Maeda et al [12] reported that endogenous TGF- β signaling suppresses

maturation of osteoblastic mesenchymal cells by employing the TGF- β type I receptor kinase inhibitor SB431542. In that study, SB431542 promoted osteoblastic differentiation of mouse C2C12 cells induced by BMP-4 and the effects of endogenous TGF- β was more potent in well-differentiated osteoblasts. The present study revealed that Smad3 significantly inhibits ALP activity induced by BMP-2 in mouse mesenchymal ST-2 cells, indicating that Smad3 suppresses the commitment of mesenchymal cells into osteoblastic cells induced by BMP-2.

As for the differentiation of osteoblastic cells, we previously demonstrated that Smad3 enhanced ALP activity as well as mineralization, in contrast to TGF- β in confluent MC3T3-E1 cells [6]. Recently, the Smad-independent actions of TGF- β were elaborated [1, 8, 13]. As the explanation for the discrepant effects of Smad3 and TGF- β on ALP activity and mineralization, we previously suggested that TGF- β -responsive ERK1/2 and JNK cascades negatively regulate Smad3-induced transcriptional activity as well as ALP activity and mineralization [9]. On the other hand, numerous hormones and growth factors, including TGF- β , differently affect osteoblast dependent upon the transformed state or the

differentiation stage of cells. For example, TGF- β promotes the production of COLI in ROS17/2.8 and MC3T3-E1 cells. However, TGF- β stimulates and inhibits ALP activity in ROS 17/2.8 and MC3T3-E1 cells, respectively [13, 14].

These findings raised the possibility that Smad3 differently might affect osteoblast phenotype including ALP activity, which might partly explain the discrepant effects of Smad3 and TGF- β on ALP activity and mineralization. In the present study, the enhancement of COLI decreased with the progression of osteoblast differentiation stage and these effects were much less at the mineralization stage. Since the expression of Smad3 in these cells was sustained during the long-term culture, these changes of COLI did not seem to be the reduction of Smad3 expression. These findings suggest that Smad3 differently affects COLI in a manner dependent upon the differentiation stage in osteoblasts. During the long term culture of wild type MC3T3-E1 cells, we have previously shown that in MC3T3-E1 cells ALP activity increased with time of culture (up to 21 days). The levels of COL1 and OPN expression were highest at days 7 and 14 of culture, respectively, whereas the expression of OCN mRNA, a

terminal differentiation marker, was highest at day 21 of culture. Smad3 might exert bone anabolic effects mainly at osteoblastic cells, which express ALP and COL1 well, but not OCN and not mineralized yet.

Several studies revealed that TGF- β suppresses the expression of Runx2 and OCN [1, 6]. Moreover, Alliston et al [15] reported that Smad3 decreased Runx2 and OCN expression in MC3T3-E1 cells, and we confirmed their evidence previously [6]. The role of OCN in bone formation remains unclear. OCN is highly expressed at the late stage during osteoblastic differentiation[16]. Ducy et al. reported that OCN-deficient mice exhibited increased bone formation and OCN might normally function to limit bone formation [17]. In our previous study, Smad3 overexpression suppressed OCN expression and OCN significantly antagonized Smad3-mediated ALP activity and mineralization [6]. These findings suggest that reduced OCN expression would be partly related to Smad3-stimulated ALP activity and mineralization. In the present study, Smad3 inhibited the levels of Runx2 and OCN mRNA at 7 day cultures, then Smad3 rather enhanced the levels of them at 21 day cultures (mineralization stage).

These findings suggest that Smad3 play some role in the control of mineralization partly through Runx2 and OCN.

Our previous study revealed that Smad3 promotes mineralization in MC3T3-E1 cells [6]. In that study, we showed the enhancement of mineralization by Smad3 overexpression employing Alizarin-Red and von Kossa staining in the long-term culture of the stable cell lines, which were similar conditions with those in the present study. However, its mechanism remains ambiguous, although the increased ALP activity and COL1 synthesis as well as reduced OCN by Smad3 partly explain it. In 21 day culture study by using MC3T3-E1 cells, mineralization assessment by employing Alizarin-Red and von Kossa staining is difficult, because mineralization occurs only at 21 day cultures, but not 7 or 14 day cultures. Bone mineralization is a tightly controlled process, the initial stages of which begin in chondrocyte- and osteoblast-derived matrix vesicles [18].

Inorganic pyrophosphate (PPi) antagonizes the ability of Pi to crystallize with calcium to form hydroxyapatite and thereby suppresses hydroxyapatite crystal propagation [19-22]. Mineralization occurs only in bone because of the exclusive

coexpression in osteoblasts of COLI and tissue non-specific ALP [23]. For normal mineral deposition to proceed, a tight balance is required between the levels of extracellular Pi and PPI. Nucleotide pyrophosphatase phosphodiesterase 1 (NPP1), which generates PPI from nucleoside triphosphates [24] and the multiple-pass transmembrane protein ANK, which mediates intracellular to extracellular channeling of PPI [25]. NPP1 serves as a physiological inhibitor of calcification, at least in part by generating PPI [24, 26]. MGP is a putative inhibitors of mineralization [20]. MEPE is expressed in bone, and MEPE displays a temporal maturation-dependent expression in osteoblast cultures, and is highly expressed within osteocytes that are imbedded within bone [27, 28]. MEPE plays an inhibitory role in bone mineralization. We therefore examined the effects of Smad3 overexpression on these molecules, which regulate by suppressing bone mineralization. In the present study, Smad3 increased the levels of ANK and NPP-1 mRNA as well as MGP mRNA in MC3T3-E1 cells. During 21 day cultures, the effects of Smad3 on MGP mRNA were decreased, although the effects of Smad3 on NPP-1 mRNA was increased.

On the other hand, the effects of Smad3 on MEPE mRNA were analogous to its effects on Runx2 and OCN mRNA. Since Smad3 may induce mineralization partly through ALP activity, those factors, especially OCN, MEPE and NPP-1, might play some role to regulate Smad-3-induced mineralization by antagonizing excessive mineralization.

In the present study, overexpression of Smad3 without addition of exogenous TGF- β ligands may produce abundant non-phosphorylated Smad3 in the cells, and endogenous TGF- β ligands may not induce phosphorylation of all the exogenously induced Smad3. We therefore cannot rule out the artificial effects of accumulated non-phosphorylated Smad3 in the present study.

In conclusion, Smad3 inhibits the osteoblastic commitment of ST-2 cells, while promotes the early stage of differentiation and maturation of osteoblastic committed MC3T3-E1 cells. Also, Smad3 enhanced the expression of mineralization-related genes at the maturation phase of MC3T3-E1 cells.

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Figure Legends

Fig. **1** Effects of Smad3 overexpression on ALP activity induced by BMP-2 in ST2 cells. Empty vector (V)- or Smad3 (S)-transiently transfected cells were treated without or with BMP-2 (100 ng/ml) in serum-free medium for 48 h, and the ALP assay was performed as described in Materials and methods. Each value is the mean $\pm$ SEM (nmol/min/mg of protein). *, $p < 0.01$, compared with the Smad3-transfected group.

Fig. **2** Effects of Smad3 overexpression on the levels of Col1 and ALP mRNA in MC3T3-E1 cells. Empty vector (V)- or Smad3 (S)-transfected MC3T3-E1 cells were cultured for 7, 14 and 21 days. RNA extraction and semi-quantitative RT-PCR analyses were performed as described in Materials and methods.

Fig. **3** Effects of Smad3 overexpression on the levels of Runx2 and OCN mRNA in MC3T3-E1 cells. Empty vector (V)- or Smad3 (S)-transfected MC3T3-E1 cells were cultured for 7, 14 and 21 days. RNA extraction and semi-quantitative

RT-PCR analyses were performed as described in Materials and methods.

Fig. 4 Effects of Smad3 overexpression on the levels of MGP, ANK, NPP-1 and MEPE mRNA in MC3T3-E1 cells. Empty vector (V)- or Smad3 (S)-transfected MC3T3-E1 cells were cultured for 7, 14 and 21 days. RNA extraction and semi-quantitative RT-PCR analyses were performed as described in Materials and methods.

Figure 1

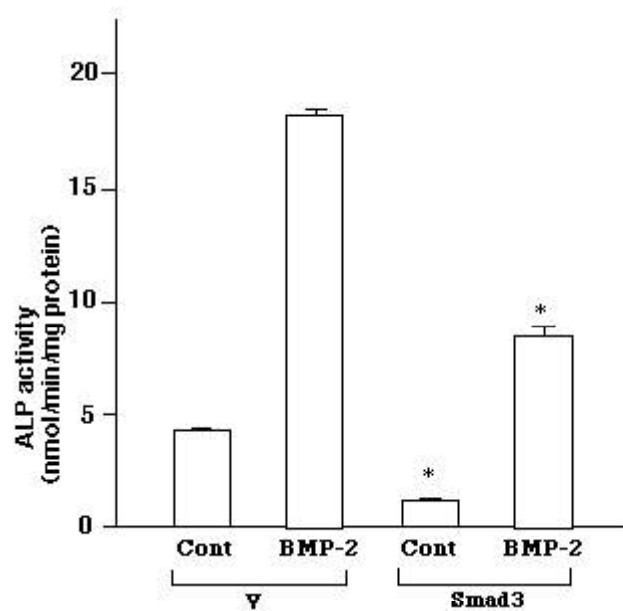
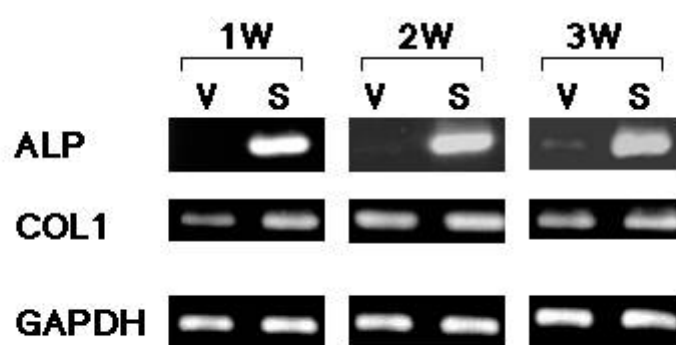


Figure 2



	2W		3W	
	V	S	V	S
Runx2				
OCN				
GAPDH				

	1W		2W		3W	
	V	S	V	S	V	S
ANK	+	+	+	+	+	+
NPP-1	+	+	+	+	+	++
MGP	+	+	+	+	+	+
MEPE	+	+	+	+	+	+
GAPDH	+	+	+	+	+	+