Smad2 Overexpression Enhances Smad4 Gene Expression and Suppresses CBFA1 Gene Expression in Osteoblastic Osteosarcoma ROS17/2.8 Cells and Primary Rat Calvaria Cells*

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Jinghong Li‡, Kunikazu Tsujii‡, Toshihisa Komori‡, Kohei Miyazono‡, Jeffrey L. Wrana‡, Yoshiaki Ito**#, Akira Nifuji‡, and Masaki Noda‡ ‡‡

From the ‡Department of Molecular Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, 3-10 Kanda-Surugadai 2-Chome, Chiyoda-ku, Tokyo 101, Japan, the §Department of Medicine III, Osaka University Medical School, 2-2 Yamada-oka Suita, Osaka 565, Japan, the ¶Department of Biochemistry, Cancer Institute, 1-37-1 Kamiikebukuro, Toshima-ku, Tokyo 170, Japan, the §Program in Developmental Biology, Division of Gastroenterology, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada, and the **Laboratory of Cell Regulation, Department of Viral Oncology, Institute for Virus Research, Kyoto University, Sakyoku, Kyoto 606, Japan

Mothers against decapentaplegic-related proteins (SMads) are essential intracellular components for the signal transduction of transforming growth factor-β (TGF-β) family members. Smad1 mediates bone morphogenetic protein (BMP) signals, whereas Smad2 functions downstream of TGF-β. TGF-β is expressed in osteoblastic cells and acts as an autocrine and/or paracrine factor in regulation of osteoblastic functions. In this study, we examined the levels and functions of Smad2 in osteoblastic cells. Smad2 mRNA expression was hardly detectable by Northern blot analysis in an osteoblast-like cell line, ROS17/2.8, as well as in primary rat calvaria (PRC) cells. Overexpression of Smad2 enhanced endogenous Smad4 gene expression in both ROS17/2.8 and PRC cells, while Smad3 levels were not altered. Smad2 overexpression suppressed osteocalcin mRNA expression in ROS17/2.8 cells. Furthermore, Smad2 overexpression also suppressed transcriptional activity of the 1-kilobase pair osteocalcin gene promoter, which was linked to chloramphenicol acetyltransferase reporter gene in both ROS and PRC cells. Since core binding factor A1 (CBFA1) is involved in osteocalcin gene expression, we further examined CBFA1 expression in the Smad2-overexpressing ROS17/2.8 and PRC cells. The levels of CBFA1 mRNA were suppressed by the overexpression of Smad2 by about 50% in both ROS17/2.8 and PRC cells. TGF-β treatment enhanced Smad4 mRNA expression in PRC cells, and this TGF-β effect was blocked by the cotreatment with BMP, indicating that TGF-β signaling pathway is interfered by BMP. These data indicate that Smad2 regulates Smad4 specifically and that CBFA1 gene is one of the downstream targets of Smad2.

The TGF-β1 superfamily is a large family of multifunctional ligands that regulate cellular growth and differentiation. Among them, TGF-β signals through distinct heteromeric receptor complexes including type I and type II serine/threonine kinase type receptors. Activation of the receptor complex initiates upon the binding of the ligand to type II receptor, which then recruits and phosphorylates the GS domain of type I receptor to activate it (1–5). The activated type I receptor then propagates the signal to downstream targets including Smads (4, 5).

Smads act as well conserved components in TGF-β family signal transduction pathway and have been identified in a variety of species including fruit fly and humans. Smad1 and Smad2 are rapidly and specifically phosphorylated by BMP2 and TGF-β, respectively (4–7), and translocated to nuclei to be involved in regulation of gene expression. Smads are highly conserved across species and share conserved amino- and carboxyl-terminal regions termed MH1 and MH2 domains, respectively (8, 9). The main active domains of the Smad proteins appear to be located in the carboxyl-terminal MH2 region. The activities of the MH2 domain are masked by the presence of amino-terminal MH1 domain, whereas they are unmasked upon the removal of the inhibition by MH1 domain upon activation, possibly by phosphorylation (9).

The gene for Smad2 has been mapped to the site closely linked to Smad4 (also called deleted in pancreatic carcinoma 4, DPC4) on chromosome 18q21 (10), a region deleted in some of the human cancers, for instance pancreatic carcinoma, colorectal carcinoma, and ovary or lung carcinoma (10–14). Missense mutations of Smad2 or Smad4/DPC4 gene lead to either loss of protein and/or loss of TGF-β-regulated responses (10, 15).

TGF-β is most abundantly stored in bone matrix in the body. It is produced by osteoblasts and appears to regulate bone metabolism in various ways, including skeletal development and bone remodeling (16). It modulates the expression of several markers of the osteoblastic phenotype. Although TGF-β promotes extracellular matrix production, it inhibits some features of fully differentiated osteoblastic phenotype, such as osteocalcin expression (17). Osteocalcin, a bone-specific calcium-binding protein, is a major non-collagenous component of the bone matrix and acts as a suppressor of bone formation as chloramphenicol acetyltransferase; CBF, core binding factor; DPC4/Smad4, deleted in pancreatic carcinoma 4; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase(s); OC, osteocalcin; pBS, pBluescript SK+; PEBP, polyomavirus enhancer-binding protein; PRC, primary rat calvaria; Smad, mothers against decapentaplegic.
shown in knock-out mice (18). It is expressed during differentiation of normal rat osteoblasts (19–21) and is produced constitutively in a rat osteosarcoma cell line, ROS17/2.8 (22). Osteocalcin expression is down-regulated by TGF-β1 treatment in both normal osteoblasts and osteosarcoma cells (17, 23).

PEBP2/EBF (core binding factor) was originally identified as a polyomavirus enhancer-binding protein 2 (24) and later as a core binding factor (25). The two names refer to an identical molecule. CBF is a complex of two different subunits, A and B. CBFA directly binds to DNA, while CBFB does not interact directly with DNA, but it associates with A subunit to increase DNA binding affinity of the A subunit (25, 26). CBFA contains a 128-residue domain, homologous to the Drosophila pair-rule gene, runt. CBFA1 is involved in regulation of T cell gene expression (25), and CBFA2 is homologous to human AML-1 gene found in acute myeloid leukemia (27, 28). They specifically recognize a consensus DNA binding sequence, PuAC-CPuCA. The similar response elements exist in the promoter regions of osteoblastic phenotype-related genes including the one encoding osteocalcin (29–32). Furthermore, null mutation of CBFA1 gene in mice resulted in complete lack of ossification of their bones, indicating that CBFA1 plays a critical role in the regulation of osteoblastic differentiation (33, 34).

In the present work, the expression and functions of Smad2 were examined in osteoblast-like ROS17/2.8 cells and primary rat calvaria (PRC) cells. Our data indicate that Smad2 regulates expression of Smad4 and that Smad2 also controls the expression of CBFA1 and osteocalcin genes in these osteoblast-like cells and PRC cells.

**MATERIALS AND METHODS**

**Cell Culture**—Rat osteoblastic osteosarcoma ROS17/2.8 cells were kindly provided by Dr. G. Rodan (Merck Research Laboratories, West Point, PA) and were maintained in modified F-12 medium supplemented with 5% FBS. Transient DNA transfections using Smad2-MH2 (lane 1) or pBS (lane 2, control) were performed as described under “Materials and Methods.” Total cellular RNA was extracted after 72 h of transfection. Northern blot analysis was conducted as described under “Materials and Methods.” The data represent one of three independent experiments with similar results. a, overexpressed Smad2 mRNA levels; b–d, Smad4 (b), Smad3 (c), and Smad1 (d) mRNA levels in Smad2-MH2-overexpressed ROS17/2.8 cells (lane 1) and control cells (lane 2); e, overexpressed Smad2 mRNA levels; f, Smad4 mRNA levels in Smad2-MH2-overexpressed primary rat calvaria cells (lane 1) and control cells (lane 2). Smad2, Smad2-MH2-overexpressed cells (lane 1); pBS, pBluescript SK+-transfected cells (lane 2). The positions of Smad2*, Smad4, Smad3, Smad1, GAPDH, and 28 S and 18 S ribosomal RNA are indicated. Asterisk (*) indicates the level of exogenous Smad2.

**FIG. 1.** Effects of Smad2 overexpression on Smad genes expression in ROS17/2.8 cells and primary rat calvaria cells. ROS17/2.8 cells (a, b, c, and d) or primary rat calvaria cells (e and f) were maintained in modified F-12 medium supplemented with 5% FBS. Transient DNA transfections using Smad2-MH2 (lane 1) or pBS (lane 2, control) were performed as described under “Materials and Methods.” Total cellular RNA was extracted after 72 h of transfection. Northern blot analysis was conducted as described under “Materials and Methods.” The data represent one of three independent experiments with similar results. a, overexpressed Smad2 mRNA levels; b–d, Smad4, Smad3, and Smad1 mRNA levels in Smad2-MH2-overexpressed ROS17/2.8 cells (lane 1) and control cells (lane 2); e, overexpressed Smad2 mRNA levels; f, Smad4 mRNA levels in Smad2-MH2-overexpressed primary rat calvaria cells (lane 1) and control cells (lane 2). Smad2, Smad2-MH2-overexpressed cells (lane 1); pBS, pBluescript SK+-transfected cells (lane 2). The positions of Smad2*, Smad4, Smad3, Smad1, GAPDH, and 28 S and 18 S ribosomal RNA are indicated. Asterisk (*) indicates the level of exogenous Smad2.
tute (Cambridge, MA).

**Transient DNA Transfection**—Cells were plated at 6 x 10^4 cells/cm² 24 h before transfection. Transfection of plasmid DNA into ROS17/2.8 cells was performed by using DNA-lipid complexes (LipofectAMINE, Life Technologies, Inc.). pBluescript SK⁺ (pBS) plasmid was used as a negative control, Smad2-MH2 expression vector or pBluescript SK⁺ (pBS) as a negative control. The cells were exposed to a complex of DNA (0.2 µg/cm²) and LipofectAMINE for 8 h in serum-free medium. The cells were then cultured in fresh medium supplemented with 5% FBS and were harvested after 72 h of transfection.

cDNAs—The cDNAs encoding human Smad2 and Smad1 linker regions, Smad2SA and Smad1SA, were described elsewhere (4, 10). A 264-base pair fragment of the Smad2 linker region and a 376-base pair fragment of the Smad1 linker region were excised with SacII/AccI and were used as specific probes for Smad2 and Smad1, respectively. Smad4 plasmid was provided by Dr. Kern (11), and BanHI/EcoRI fragment was used as a probe. A fragment of about 1 kb excised by EcoRI/SalI from Smad3 plasmid was used (35). Osteocalcin (OC) and alkaline phosphatase (AP) cDNAs were a gift from Dr. G. Rodan, and EcoRI fragments were used as probes. CBFA1(PEBP2αa) and CBFB-(PEBP2β) cDNAs were described elsewhere (24, 36), and fragments were used as probes.

**RNA Isolation and Northern Blot**—Total cellular RNA was prepared according to the acid guanidium thiocyanate-phenol-chloroform method (37). Aliquots of 10–15 µg of the total RNA per lane were electrophoresed on 1.0% agarose gels containing 0.66 M formaldehyde, then transferred to nylon filters (Hybond-N, Amersham Pharmacia Biotech) by electroblotting. Filters were prehybridized overnight at room temperature. Each cDNA was labeled by random primer method using Klenow fragment (Amersham Pharmacia Biotech) and [α-32p]dCTP (NEN Life Science Products). Hybridization was performed at 42 °C for 18 h. Filters were washed in 1x SSC, 0.1% SDS for 15 min at room temperature, and 0.2x SSC, 0.1% SDS for 20 min at 65 °C. Washed filters were exposed to x-ray film using intensifying screens at −80 °C for several days.

**CAT Assay**—The cells were cotransfected with a CAT reporter plasmid (38) containing a fragment of rat osteocalcin promoter (−1094 to +147) and Smad2-MH2 expression vector or pBS as a negative control. pSV2CAT plasmid was used as a positive control. Cell extracts were prepared and used for the analysis of CAT activity. Protein concentrations in the lysates were determined by the Coomassie Brilliant Blue G method (39). Equivalent amounts of the cellular proteins were incubated in a reaction buffer (0.25 M Tris-HCl, 40 mM acetyl CoA, 10 mM DTT, 264-base pair fragment of the Smad2 linker region and a 375-base pair EcoRI fragment of the linker region were excised with EcoRI/I and SalI, respectively. Smad4 plasmid was provided by Dr. Kern (11), and BanHI/EcoRI fragment was used as a probe. A fragment of about 1 kb excised by EcoRI/SalI from Smad3 plasmid was used (35). Osteocalcin (OC) and alkaline phosphatase (AP) cDNAs were a gift from Dr. G. Rodan, and EcoRI fragments were used as probes. CBFA1(PEBP2αa) and CBFB-(PEBP2β) cDNAs were described elsewhere (24, 36), and fragments were used as probes.

**RESULTS**

**Overexpression of Smad2 Enhances Expression of Smad4 mRNA Levels in ROS17/2.8 Cells and Primary Rat Calvaria Cells**—We previously showed that TGF-β regulates expression of osteoblast-related genes in ROS17/2.8 cells (17). To elucidate whether Smad2 is expressed in these cells, we first examined its mRNA levels. Smad2 mRNA expression was hardly detectable in ROS17/2.8 cells by Northern blot analysis (Fig. 1a, lane 2). Even at a low level of Smad2 may be still functional, we further examined Smad2 function by overexpressing an active domain of Smad2 (Smad2-MH2) in ROS17/2.8 cells. ROS17/2.8 cells where Smad2 was overexpressed showed exogenous Smad4 mRNA expression (Fig. 1a, lane 1; exogenous Smad2 is indicated by an asterisk (*)). Overexpression resulted in one major 3.6-kb and one minor 1.1-kb transcript in Smad2-overexpressed ROS17/2.8 cells (Fig. 1a, lane 1). Smad2 untransfected ROS17/2.8 cells express two Smad4 mRNA (a major 3.6 kb and a minor 7.8-kb) species at moderate levels (Fig. 1b, lane 2). Smad2 overexpression enhanced the levels of the major 3.6-kb Smad4 mRNA expression; the abundance of the minor 7.8-kb band was slightly reduced (Fig. 1b, lane 1). This effect was specific to Smad4, as Smad3 and Smad1 mRNA levels were not altered (Fig. 1, c and d).

**Smads, CBF, and Osteoblastic Differentiation**
Overexpression of Smad2 Suppresses Expression of CBFA1 mRNA but Enhances CBFB mRNA Levels in ROS17/2.8 Cells and Primary Rat Calvaria Cells—In order to explore whether expression of CBFs is regulated by molecules involved in TGF-β signaling pathway, we examined CBFA1 and CBFB expression levels in Smad2-overexpressing cells. We found that CBFA1 mRNA was expressed as a 5.5-kb band in ROS17/2.8 cells (Fig. 4a, lane 2) and overexpression of Smad2 suppressed its expression by about 50% (Fig. 4a, lane 1, and b). CBFB was also expressed as a 3.5-kb band in these ROS17/2.8 cells, although at a low level (Fig. 5a, lane 2). In contrast to the suppression on CBFA1 mRNA level, Smad2 overexpression enhanced CBFB expression over 20-fold for the 3.5-kb mRNA species (Fig. 5, a, lane 1, and b). In the Smad2-overexpressed cells, a faint high molecular weight mRNA species (8.5 kb) was also observed (Fig. 5a, lane 1).

In PRC cells, CBFA1 mRNA was expressed as a major 5.5-kb band and a minor 9.5-kb band (Fig. 4c, lane 2). Overexpression of Smad2 in PRC cells suppressed 5.5-kb mRNA level, but enhanced 9.5-kb mRNA level (Fig. 4c, lane 1). Comparison of the sum of quantified values of these two bands in lane 1 with the band in the control (lane 2) indicated suppression by Smad2 overexpression in PRC cells (Fig. 4d). CBFB mRNA was barely expressed in PRC cells (Fig. 5c, lane 2), while Smad2 overexpression enhanced 3.5-kb mRNA expression (Fig. 5, c, lane 1, and d). In addition, expression of two higher molecular weight species, 8.5- and 12.0-kb mRNA bands, were induced by Smad2 overexpression (Fig. 5c, lane 1).

TGF-β Enhances Smad4 Expression while Co-treatment with BMP Results in Loss of the TGF-β Effect—To examine whether Smad4 expression is also regulated by ligand-dependent signals, effects of TGF-β was examined. As shown in Fig. 6, TGF-β treatment enhanced Smad4 expression in PRC cells (Fig. 6, lane 1 versus lane 4). Interestingly, BMP treatment also enhanced Smad4 expression in these cells (Fig. 6, lane 2 versus lane 4). Correspondingly, Smad1 overexpression also enhanced Smad4 expression in PRC cells (data not shown) as well as Smad2 overexpression (Fig. 1f, lane 1). In order to examine possible interplay between TGF-β signals and BMP signals, cotreatment with TGF-β and BMP was conducted. Enhancement of Smad4 expression by either of the two cytokines alone was blocked by the cotreatment with TGF-β and BMP (Fig. 6, lane 3). On the other hand, neither TGF-β nor BMP affected Smad4 mRNA levels in ROS17/2.8 cells (data not shown).

Data are presented as mean ± S.D. Statistically significant difference existed between Smad2/OC-CAT cotransfection group and pBS/OC-CAT control (p < 0.01; indicated by an asterisk). There was no difference between the two positive control groups. c, CAT assay were carried out by using the lysates prepared from PRC cells prepared as described under “Materials and Methods.” d, quantification of the data presented in c. The results were obtained in triplicate from three independent cell extract preparations. Since Smad2-MH2 cotransfection did not affect transcriptional activity of pSV0,CAT (Fig. 3, a, lanes 7–12, and b). Although osteocalcin mRNA level was very low in the PRC cells, activity of the transfected 1-kb osteocalcin promoter was detectable. The basal level was relatively low; however, overexpression of Smad2 still suppressed the osteocalcin promoter activity in this in vitro system by about 50% (Fig. 3c, lanes 4–6 compared with lanes 1–3; Fig. 3d), similarly to the observation in ROS17/2.8 cells.

![Image](55x139 to 291x729)

**FIG. 3. Effects of Smad2 overexpression on the transcriptional activity of osteocalcin promoter.** ROS17/2.8 cells (a and b) and PRC cells (c and d) were maintained in modified F-12 medium supplemented with 5% FBS and were cotransfected with Smad2-MH2 expression vector and 1-kb OC-CAT reporter gene plasmid (lanes 4–6). pBS plasmid was used as a negative control (lanes 1–3). pSV0,CAT plasmid was used as a positive control (lanes 7–12 in a and lanes 7–10 in c). Transient DNA transfections were performed as described under “Materials and Methods.” a, CAT assays were carried out by using the lysates prepared from ROS17/2.8 cells as described under “Materials and Methods.” b, quantification of the data presented in a. The results were obtained in triplicate from three independent cell extract preparations.
Fig. 4. Effects of Smad2 overexpression on CBFA1 mRNA expression. ROS17/2.8 cells (a and b) or primary rat calvaria cells (c and d) were maintained in modified F-12 medium supplemented with 5% FBS. Transient DNA transfections using Smad2-MH2 (lane 1) or pBS (lane 2, control) were performed as described under “Materials and Methods.” Total cellular RNA was extracted after 72 h of transfection. Northern blot analysis was conducted as described under “Materials and Methods.” a, CBFA1 mRNA expression in Smad2-MH2-overexpressed ROS17/2.8 cells (lane 1) and pBS-transfected control ROS17/2.8 cells (lane 2). b, quantification of CBFA1 mRNA levels normalized against the GAPDH mRNA levels. Data were obtained from three independent experiments and are presented as mean ± S.D. Asterisk indicates the statistically significant difference (p < 0.01). c, CBFA1 mRNA expression in Smad2-MH2-overexpressed primary rat calvaria cells (lane 1) and pBS-transfected control ROS17/2.8 cells (lane 2). d, quantification of CBFA1 mRNA levels normalized against the GAPDH mRNA levels shown in c (the densities of the bands in lane 1 were combined). Data were obtained from three independent experiments and are presented as mean ± S.D. Asterisk indicates the statistically significant difference (p < 0.01). Smad2, Smad2-MH2-overexpressed cells (lane 1); pBS, pBluescript SK+-transfected cells (lane 2). The positions of CBFA1, GAPDH, and 28S and 18S ribosomal RNA are indicated.

DISCUSSION

In the present report, we showed that Smad2 overexpression enhanced Smad4 mRNA expression and suppressed CBFA1 expression in ROS17/2.8 cells as well as PRC cells. These observations suggest that overexpressed Smads could be triggering a positive feedback system since Smad4 is their partner to form heteromers to be fully active. Although Smad molecules mediate signals for diverse members of TGF-β superfamily, Smad4 is the only common partner for the other Smads (Smad1, 2, 3, and 5). It appears that Smad4 may play an important role in adjusting different Smad pathways. A recent report in which Smad4 acts as TGF-β-inducible DNA-binding protein further indicates its key role in signal transduction (41).

Smad3 is a close homologue of Smad2 (42–44), which has been reported to play a role similar to Smad2 in mediating TGF-β signal transduction. Another Smad family member, Smad1, is considered to mediate BMP signals. We observed that Smad3 and Smad1 were expressed constitutively in ROS17/2.8 cells; however, the levels of Smad3 and Smad1 were not affected by the overexpression of Smad2-MH2, indicating the specificity of the effects of Smad2 overexpression on Smad4 levels. These observations also indicate that Smad2 and Smad3 might have independent signaling pathways, which could mediate different aspects of TGF-β actions.

Our results indicate not only that cross-talks among the different Smads families members are present but also that CBFA1 gene is the downstream target of these Smads, TGF-β suppresses osteocalcin production (17), and osteocalcin promoter activity is under the control of CBFA1 as reported previously (33, 34, 45). Whether Smad2 suppression of CBFA1 could be involved at least in part in osteocalcin promoter suppression by TGF-β is being investigated by using cells derived from CBFA1 knock-out mice. We also observed that TGF-β inhibited CBFA1 mRNA expression in the presence of BMP, which enhanced CBFA1 expression in ROS17/2.8 cells and PRC cells (data not shown). Although treatment with TGF-β alone did not suppress CBFA1 mRNA levels, it appears that under physiological condition where both BMP and TGF-β are likely to be present at the same time, the role of TGF-β would be to inhibit BMP-induced enhancement of CBFA1 expression as a part of the cytokine network.

With regard to the CAT assay, ROS17/2.8 cells showed relatively high osteocalcin promoter activity, and it was suppressed by Smad2 overexpression. On the other hand, although PRC cells revealed low activity of osteocalcin promoter, Smad2 overexpression still suppressed the activity. Thus, Smad2 suppression was observed in relatively immature PRC cells as well as in relatively mature ROS17/2.8 cells.

The role of CBFB has been described as a binding partner of CBFA to enhance its binding affinity to DNA. CBFB knockout mice show similar phenotype to CBFA2 mutant mice, which are embryonic lethal due to the failure in fetal hematopoiesis and to the hemorrhage in central nervous system (46, 47). The enhancement of CBFB mRNA level by Smad2 overexpression suggest that CBFB may also be a downstream target of Smad2 and it may contribute to the modulation of the transcriptional mechanism, such as facilitating the association of CBFA1 to other transcription factors. It has been reported that CBFB is mainly located in cytoplasm and its level is increased by the differentiation of skeletal myogenic cells (48). CBFB has also been reported to interact at a high affinity with cytoskeleton (49), suggesting that CBFB could likely function in as yet unidentified aspects besides the function as a subunit of CBF transcription factors. It is also possible that Smad2 activates other CBFA isoform actions via the increase in CBFB; however, this is still a speculation and needs to be elucidated.

Similar to the Smad2 enhancement of Smad4 expression,
were cultured to confluence and were treated with TGF-
expression in primary rat calvaria cells. Primary rat calvaria cells
(ROS17/2.8 cells) were maintained in modified F-12 medium supplemented
with 5% FBS. Transient DNA transfections using Smad2-MH2 (lane 1) or pBS
(lane 2, control) were performed as described under "Materials and Methods." Total cellular RNA was extracted after
72 h of transfection. Northern blot analysis was conducted as described under
"Materials and Methods." a, CBFB mRNA expression in Smad2-MH2-overexpressed
ROS17/2.8 cells (lane 1) and pBS-transfected control ROS17/2.8 cells (lane 2). b, quantification of CBFB mRNA levels
(quantitated densities of the 3.5- and 8.5-kb bands were combined) normalized
against the GAPDH mRNA levels. Data were obtained from three independent
experiments and are presented as mean ± S.D. Asterisk indicates the statistically
significant difference (p < 0.05). c, CBFB mRNA expression in Smad2-MH2-overexpressed
primary rat calvaria cells (lane 1) and pBS-transfected control cells (lane 2).
Intriguingly, BMP alone enhanced Smad4 expression. At
this point, the mechanism of the observed inhibitory actions
of the BMP against TGF-β is not known. Either alterations in phosphorylation status of the
Smad members or the interaction among the pathway-re-
stricted Smads and inhibitory Smads (such as Smad6 or
Smad7) should be examined to elucidate the interactive phe-
nomenon between TGF-β and BMP in regulation of Smad4
expression. On the other hand, treatment with TGF-β and/or
BMP did not show regulation of Smad4 mRNA levels in ROS17/2.8 cells, suggesting the presence of different signaling pathways at the level of ligand/receptor in ROS17/2.8 cells compared
with PRC cells (data not shown).

It has been well described that Smad1, 2, 3, and 5 have the
carboxyl-terminal phosphorylation sites which are directly phosphorylated by type I receptor. Smad4 does not have this site and cannot be phosphorylated by type I receptor (9). It has also been reported that the mRNA levels of these Smads are
not regulated by the treatment with their ligands (40, 50). It seems that alteration in phosphorylation status may be more efficient and quicker in response to ligands binding. We also observed that Smad1 and Smad2 mRNA levels were not regulated
by TGF-β or BMP treatment (data not shown). Interest-
ingly, Smad4 mRNA levels were regulated by TGF-β and/or
BMP treatment in PRC cells. Combining with the data that
Smad4 was specifically enhanced in Smad2-overexpressed
cells, Smad4 plays a key role in regulating signal transduction. Recent studies showed that the inhibitory Smads, such as
Smad6 and Smad7, are involved in negative feedback of TGF-
β-related signals and their mRNA levels are up-regulated by
different ligands (40, 51, 52).

We also observed that Smad6 expression was dramatically
enhanced by BMP treatment in PRC cells (data not shown).
Although TGF-β treatment did not regulate Smad6 expression
in these cells, it abolished the enhancement of Smad6 expres-
sion by BMP (data not shown). This feature is similar to that of
Smad4, indicating both Smad4 and Smad6 would be involved
in the regulation of different Smad pathways. We assume that
Smad-dependent TGF-β signals might be regulated at least at three different levels: 1) the phosphorylation status of pathway-restricted Smads by transient binding; 2) the up-regulation of the transcription of inhibitory Smads, which can form stable association with type I receptor and block the phosphorylation of pathway-restricted Smads as a negative feedback mechanism; 3) regulation of the levels of the common-mediator Smad4, as our data showed, to be a positive feedback. The enhancement of Smad4 mRNA probably contributes to TGF-β signal transduction in a ligand-dependent manner. It remains to be elucidated how Smad4 exerts its balancing function between TGF-β and BMP signals.

TGF-β is a suppressor of growth in many types of cancer. Disruption of the TGF-β pathway in cancer has been demonstrated in several types of cancers. Inactivation mutations in Smad2 and Smad4/DPC4 were reported in colon cancers as well as other cancers (10–13). In ROS17/2.8 cells, the level of Smad2 expression was not detected by Northern blot analysis, and this may be related to tumorigenic phenotype of these cells. However, Smad2 mRNA level was also undetectable by Northern blot in another type of osteoblast-like cell line MC3T3E1 (data not shown) as well as in PRC cells. Although Smad2 mRNA level was also undetectable by Northern blot analysis in several types of cancers. Inactivation mutations in Smad2 expression was not detected by Northern blot analysis, as well as other cancers (10–13). In ROS17/2.8 cells, the level of Smad2 expression was not detected by Northern blot analysis, and this may be related to tumorigenic phenotype of these cells. However, Smad2 mRNA level was also undetectable by Northern blot in another type of osteoblast-like cell line MC3T3E1 (data not shown) as well as in PRC cells. Although Smad2 mRNA expression is difficult to detect by Northern blot, this result does not exclude the possibility that only a small number of such molecules may be enough to mediate TGF-β actions, such as those that our data on the Smad2 overexpression suggested in this paper.

In summary, we showed that Smad2 regulates the expression of Smad4 as well as CBFA1 in the osteoblastic osteosarcoma ROS17/2.8 and primary rat calvaria cells.

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