Smad1 Interacts with Homeobox DNA-binding Proteins in Bone Morphogenetic Protein Signaling*

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Bone morphogenetic proteins (BMP) transduce their signals into the cell through a family of mediator proteins known as Smads. Upon phosphorylation by the BMP receptors, Smad1 interacts with Smad4 and translocates into the nucleus where the complex recruits DNA-binding protein(s) to activate specific gene transcription. However, the DNA-binding protein(s) involved in BMP signaling has not been identified. Using a yeast two-hybrid approach, we found that Smad1 interacts with Hoxc-8, a homeodomain transcription factor. The interaction between Smad1 and Hoxc-8 was confirmed by a “pull-down” assay and a co-immunoprecipitation experiment in COS-1 cells. Interestingly, purified Smad1 inhibited Hoxc-8 binding to the osteopontin Hoxc-8 site in a concentration-dependent manner. Transient transfection studies showed that native osteopontin promoter activity was elevated upon BMP stimulation. Consistent with the gel shift assay, overexpression of Hoxc-8 abolished the BMP stimulation. When a wild type or mutant Hoxc-8 binding element was linked to an SV40 promoter-driven reporter gene, the wild type but not the mutant Hoxc-8 binding site responded to BMP stimulation. Again, overexpression of Hoxc-8 suppressed the BMP-induced activity of the wild type reporter construct. Our findings suggest that Smad1 interaction with Hoxc-8 dislocates Hoxc-8 from its DNA binding element, resulting in the induction of gene expression.

Transforming growth factor-β (TGF-β)1-related molecules, or BMPs, regulate embryonic development, vertebral patterning, and mesenchymal cell differentiation (1, 2). BMP-2 and -4 have been identified as bone inductive growth factors and are important signaling molecules during the development of the skeleton in vertebrates (1, 3, 4). Signal transduction in the TGF-β superfamily requires the interaction of two types of serine/threonine transmembrane receptor kinases (5). The signaling is mediated by direct phosphorylation of Smad proteins. Specifically, Smad2 and Smad3 are phosphorylated by TGF-β and activin receptors (5, 6), whereas phosphorylation of Smad1 is induced by BMPs (7, 8). Upon phosphorylation, these Smads interact with a common partner, Smad4, which then translocates to the nucleus where the complexes recruit DNA-binding protein(s) to activate specific gene transcription (5, 7, 9). The downstream DNA-binding proteins in the TGF-β signaling pathway, such as Fast-1, Fast-2, and TFE3, have been reported (10–12). However, little is known about the downstream DNA-binding protein(s) beyond Smad1 in the BMP signal transduction machinery.

It has been suggested that homeobox genes play a role in downstream events in BMP signaling (13, 14). In vertebrates, there are 39 Hox transcription factor genes organized into four separated chromosome clusters, which play critical roles in the patterning of vertebrate embryonic development (15). These 39 genes are subdivided into 13 paralogous groups on the basis of duplication of an ancestral homeobox cluster during evolution, sequence similarity, and position within the cluster (16). Each paralog group has been demonstrated to be responsible for the morphogenesis of a particular embryonic domain or structure (15).

Hoxc-8, as one of the three members of paralog VIII, is predominantly expressed at a high level in the limbs, backbone, and spinal cord in early mouse embryos (17, 18). Null mutant mice showed that Hoxc-8 is expressed in the neuron, chondrocyte, fetal liver, and adult bone marrow (19, 20). Bending and fusion of the ribs, anterior transformation of the vertebrae, and abnormal patterns of ossification in the sternum were observed in adult Hoxc-8 null mice (20). Studies published recently demonstrated that tissue-specific overexpression of a Hoxc-8 transgene inhibits chondrocyte maturation and stimulates chondrocyte proliferation (21). The other two members in the Hox VIII group are Hoxb-8 and Hoxd-8. Hoxb-8 has been shown to activate the Sonic hedgehog gene, an essential mediator in forelimb development (22, 23), whereas generalized expression of Hoxc-8 modifies Drosophila anterior head segments (24).

Despite the fact that homeobox genes are DNA-binding proteins, little has been learned about their natural DNA response elements and role in transcription (25). In the current study, we report that Smad1 interacts with Hoxc-8, and this interaction specifically activates the osteopontin gene transcription in response to BMP stimulation. Our data suggest that Hoxc-8 functions as a transcription repressor and that the interaction of Smad1 with Hoxc-8 dislocates Hoxc-8 binding from its element resulting in initiation of gene transcription.

EXPERIMENTAL PROCEDURES

Two-hybrid Library Screening—A full-length Smad1 coding sequence from pBluescript-Smad1(9) was cloned into SacII/PstI sites of pGBT9 (CLONTECH) to generate the pGBT9/Smad1 bait plasmid. The human U2 OS osteoblast-like pACT2 cDNA library was screened according to the manufacturer’s instruction (CLONTECH). To confirm the interaction between Hoxc-8 and Smad1, a full-length mouse Hoxc-8 cDNA (18) was subcloned into pACT2 vector (CLONTECH) between the
XhoI and EcoRI sites. The pACT/Hox-8 was co-transformed with pBGT9/Smad1 into Y190, and the colonies were assayed for the production of β-galactosidase using both filter lift and liquid assays. Expression and Purification of Glutathione S-transferase (GST) Fusion Proteins—GST fusion constructs of GST-Smad1 and -Smad3 were generated by restriction digest of pBGT-Smad1 (SalI/HindIII) and pCMV5-Smad3 (SalI/SalI) and subsequently inserted into the pGEX-KG vector, respectively. GST-Smad2 and -Smad4 were digested with EcoRI/SalI from pCMV5-Smad2 and pCMV5-Smad4 (9) and inserted into the EcoRI/SalI sites of the pGEX-5X-2 and pGEX-5X-1 vector (Amerham Pharmacia Biotech), respectively. The GST-Hox-8 and GST-Hoxa-9 (26) were amplified by polymerase chain reaction using high fidelity Fnu-Turbo DNA polymerase (Stratagene) and cloned in the BamHI/EcoRI and BamHI/XhoI sites of the pGEX-KG vector, respectively. The GST-Max-1 and -Max-2 expression plasmids (27) were provided by Dr. C. Abate-Shen (Center for Advanced Biotechnology and Medicine, Piscataway, NJ). The GST constructs described above were transformed into BL21. The expression and purification of the fusion proteins were performed as described (28).

**GST Pull-down Assay**—[35S]Methionine-labeled Hox-8 protein was synthesized using the TNT-coupled transcription and translation system (Promega) with linearized pBluescript-Hox-8 plasmid according to the manufacturer's instruction. The production of labeled protein was verified by SDS-polyacrylamide gel electrophoresis. An equivalent amount (1 μg) of purified GST or GST-Smad1 fusion protein was pre-incubated with 35S-labeled Hox-8 protein (5 μl) for 30 min on ice. Following the addition of GST-agarose, the samples were incubated for another 30 min at 4 °C. The agarose beads were washed four times in a phosphate-buffered saline, 0.1% Triton X-100 solution, and bound proteins were eluted by boiling in 2× SDS buffer for 5 min before loading onto 10% SDS-polyacrylamide gel electrophoresis.

**Immuno precipitation and Western Blot**—HA-tagged Hox-8 was subcloned from pACT2/Hox-8 into a mammalian expression vector pcDNA3 (Invitrogen) at the EcoRI and XhoI sites. Expression vectors for FLAG-tagged Smad1 and Smad4 were provided by Dr. Rik Derynck (University of California, San Francisco, CA). Expression plasmids for FLAG-Smad2 and -Smad4 were digested with EcoRI/HindIII and subsequently cloned into the pGEX-5X-2 and pGEX-5X-1 vector (Amersham Pharmacia Biotech), respectively. The GST-Hox-8 and GST-Hoxa-9 (26) were amplified by polymerase chain reaction using high fidelity Fnu-Turbo DNA polymerase (Stratagene) and cloned in the BamHI/EcoRI and BamHI/XhoI sites of the pGEX-KG vector, respectively. The GST-Max-1 and -Max-2 expression plasmids (27) were provided by Dr. C. Abate-Shen (Center for Advanced Biotechnology and Medicine, Piscataway, NJ). The GST constructs described above were transformed into BL21. The expression and purification of the fusion proteins were performed as described (28).

**Gel Shift Assay**—Gel shift assays were performed as described previously (29). In brief, DNA fragments OPN-1, OPN-2, and OPN-3 were generated by polymerase chain reaction using primers specific for the osteonectin promoter. The double-stranded oligomers were created by annealing the pairs of synthetic oligonucleotides (only top strands are shown) as follows: 5'-CATGACCCCAATTAGCTGGCAGCA-3' (probe-Mi); 5'-CCCTTCTTATGGATCCCTG-3' (OPN-4); 5'-GGTAGT- AAAGATCTTGCTTATGAC-3' (OPN-5); 5'-GCTACGGCGACATGT- CTTAG-3' (OPN-5); and 5'-GACATGTTCATCGAATCTGGT- G-3' (OPN-6). Mutated nucleotides in OPN-5 are bolded. These DNA fragments were radiolabeled with T4 polynucleotide kinase and [γ-32P]ATP.

**Transfection**—The osteonectin promoter from region −266 to −1, relative to the transcription start site, was amplified by polymerase chain reaction using primers localized to pGL3-control vector (Promega). The Hox recognition core TAAT was replaced with GCCG in Hox-pGL3 by polymerase chain reaction to create mutant Hox-pG3L (mHox-pGL3). CH10T1/2 cells (2.5 × 105 cells/60-mm dish) were transfected using Tfx-50 with 0.5 μg of luciferase reporter plasmid (OPN-266, Hox-pGL3, or mHox-pG3L) and different expression plasmids as indicated. Total DNA was kept constant by the addition of pSV-β-galactosidase plasmid. Luciferase activities were assayed 48 h post-transfection using the dual luciferase assay kit (Promega) according to the manufacturer's direction. Values were normalized with the Renilla luciferase activity expressed from pRL-SV40 reporter plasmid. Luciferase values shown in the figures are representative of transfection experiments performed in triplicate at least three independent experiments.

**RESULTS AND DISCUSSION**

**Yeast Two-hybrid Library Screening**—To investigate the transcription mechanism in BMP-induced gene activation, we used a yeast two-hybrid system to identify transcription factors that interact with Smad1. An intact Smad1 cDNA fused with the Gal4 DNA-binding domain was used as a bait plasmid to screen a human U-2 OS osteoblast-like cell cDNA library constructed in the pACT2 plasmid. After two rounds of screening, we obtained 25 positive clones. DNA sequence analysis identified one clone as Hox-8 and two clones as Smad4. Because our objective is to identify downstream transcription factors in the BMP signaling pathway and Hox-8 is a homeodomain DNA-binding protein, we chose the Hox-8 cDNA clone for further study. Cloning of Smad4 provided a positive control for the two-hybrid library screening because the interaction between Smad1 and Smad4 is known. The other 22 clones were not characterized.

The initial Hox-8 cDNA clone (Fig. 1B, clone 19) encodes amino acids 68−237 of a 242-amino acid Hox-8 protein. Fig. 1A shows the growth properties of the two-hybrid clones, suggesting that there is an interaction between Smad1 and Hox-8 in vitro. The yeast bearing both Smad1 and Hox-8 plasmids grew on medium deficient in Trp, Leu, and His. The interaction between Hox-8 and Smad1 was further confirmed with a β-galactosidase filter lift assay (data not shown) and quantified by a liquid β-galactosidase assay (Fig. 1B). When the full length of Hox-8 fused with the Gal4 transcriptional activation domain was tested in the two-hybrid system, it showed an interaction similar to clone 19. The assays of both the empty prey vector (pACT2) with Smad1 in the bait plasmid and the empty bait vector (pBGT9) with full-length Hox-8 in the prey vector showed very little activity. Compared with the interaction between Smad1 and Smad4, the interaction of Smad1 with Hox-8 is weaker in the yeast two-hybrid β-galactosidase assay (Fig. 1B).

**Smad1 Interacts with Hox-8 in Vitro and in COS-1 Cells**—The interaction between Smad1 and Hox-8 was examined in an in vitro pull-down experiment using [35S]methionine-labeled Hox-8 and purified GST-Smad1 or GST alone. As shown in Fig. 2A, Hox-8 was precipitated with the purified GST-Smad1 fusion protein (lane 3) but not with GST alone (lane 2), demonstrating a direct interaction between the two proteins in vitro.

BMP-2 stimulates phosphorylation of Smad1, and phosphorylated Smad1 in turn binds to Smad4 and takes the complex into the nucleus. It is of interest whether Smad1, Smad4, or the complex of Smad1 and Smad4 also interacts with Hox-8 in cells. COS-1 cells were transiently co-transfected with expression plasmids for FLAG-Smad1, FLAG-Smad4, HA-Hox-8, and/or constitutively active BMP type 1A receptor ALK3 (Q233D). The cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-FLAG antibody to look for the interaction of Smad1 and Hox-8 in cells. Co-transfection of ALK3 (Q233D) enhanced the interaction of Smad1 (lane 4) or Smad4 (lane 6) with Hox-8. However, ALK3 (Q233D) did not significantly enhance the interaction of Smad1 and Smad4 complex with Hox-8 (lane 8). These results show both Smad1 and Smad4 interact with Hox-8 in COS-1 cells with or without BMP stimulation, indicating that the phosphorylation of Smad1 is not required for its interaction with Hox-8. If this is the case, the BMP-dependent regulation of the interaction is inherent in the intracellular localization of the proteins. Hox proteins are homeodomain transcription factors localized in the nucleus (30), whereas both Smad1 and Smad4 are cytoplasmic (5). It is likely that the interaction
occurs only when Smad1 or the complex translocates to nucleus upon its phosphorylation induced by BMP receptors.

**Osteopontin Promoter Contains a Hoxc-8 Binding Element**—To examine the effect of the interaction between Smad1 and Hox-8 on Hox-8 DNA binding activity, we turned our attention to BMP-2 inducible genes. Putative Hox binding sites that have served as markers for osteogenic differentiation were found in four BMP-2 inducible genes, including bone sialoprotein, osteopontin, osteonectin, and osteocalcin (3, 31). These genes have served as markers for osteoblast differentiation. The osteopontin promoter was examined for this purpose because its mRNA expression is rapidly induced in response to BMP-2 treatment in C3H10T1/2 mesenchymal cell (3). Five putative Hox binding sites with a core sequence of Tt/aAT (32) were identified within the first 382 base pairs of the 5' flanking region in the osteopontin gene (Fig. 3A). When a 212-base pair DNA fragment from -382 to -170 (OP-1 in Fig. 3A) containing all five putative Hox sites was used for a gel shift assay with purified GST-Hoxc-8 protein, one shifted band (Fig. 3B, lane 3) was observed. This band was not present in lane 1, containing probe only, or in lane 2, containing probe with GST (Fig. 3B). This result indicates that there is only one Hoxc-8
binding site in this osteopontin promoter fragment. Further gel shift assays with shorter probes (OPN-2 and OPN-3 in Fig. 3A) indicated that OPN-2 contains this Hoxc-8 binding element (Fig. 3B, lane 6). When three single putative Hox binding probes (OPN-4, -5, and -6, Fig. 3A) were used, Hoxc-8 only bound to OPN-5, located at -206 to -180 (Fig. 3C, lane 8). Neither GST alone nor GST-Smad1 fusion protein could bind to any of the probes used in this series of gel shift assays (Fig. 3B, lanes 2, 5, and 8, and C, lanes 2, 3, 6, 7, 10, and 11). When the TAAT core sequence of Hoxc-8 binding site in OPN-5 was mutated to GCCG (mOPN-5), Hoxc-8 binding was abolished (Fig. 3C, lane 15).

The specificity of the Hoxc-8 binding to the DNA was determined by a gel shift competition assay. Unlabeled Hoxc-8 DNA binding element inhibited the shifted band in a concentration dependent manner (Fig. 3D, lanes 3–5) in which a 100-fold excess of the specific cold probe eliminated the Hoxc-8 binding, whereas a 100-fold excess of the Msx-2 DNA binding element (33) did not (Fig. 3D). Msx-2 is a homeodomain-containing protein, but it does not belong to the HOX family. The Msx-2 DNA binding element was identified from the osteocalcin promoter, and its flanking regions of the core sequence is different from Hoxc-8 binding site.

There are three TAAT and two TTAT putative Hox sites identified from the osteopontin promoter. Hoxc-8 binds to only one of the TAAT core sequences (−206 to −180), suggesting that the flanking regions are also important for Hoxc-8 binding. The Hoxc-8 binding site, including its flanking regions, is highly conserved in chicken, mouse, pig, and human. The other four putative Hox sites may be involved in other homeodomain protein binding or may not be authentic Hox binding sites.

Smad1 Inhibits Binding of Hox Proteins to DNA—Purified GST-Smad1 was examined for the effect of its interaction with Hoxc-8 on Hoxc-8 DNA binding activity. When GST-Hoxc-8 protein and its DNA binding element (OPN-5) were incubated with increasing amounts of GST-Smad1 protein, the binding of Hoxc-8 to the DNA probe was inhibited in a concentration-dependent manner (Fig. 4A, lanes 5–7). The same amount of

**Fig. 3.** Characterization of a Hoxc-8 DNA-binding site from the osteopontin promoter. A, DNA fragments of osteopontin promoter used for gel shift assays in panels B, C, and D. Nucleotides are numbered relative to the transcription start site.Filled boxes indicate putative Hox binding sites. The striped box represents a putative Hox binding site containing the mutated core sequence. B, OPN-2 DNA fragment contains a Hoxc-8 binding site. A gel shift assay was performed using different 32P-labeled DNA fragments as indicated. C, OPN-5 is the Hoxc-8 binding element. A gel shift assay was performed using shorter DNA fragments as marked. The Hoxc-8 binding element is located from −206 to −180. D, Hoxc-8 specifically binds to OPN-5. A gel shift assay was performed using OPN-5 alone (lane 1) or with GST-Hoxc-8 (lanes 2–8). Lanes 3–5 and 6–8 contained 5-, 25-, and 100-fold molar excess of unlabeled OPN-5 and MSX-2 DNA binding element (Probe-M), respectively.
GST protein did not have an effect on Hoxc-8 binding activity (Fig. 4, lane 4). These results suggest that the interaction of Smad1 with Hoxc-8 dislodges Hoxc-8 from its response element.

Because the signaling networks of the TGF-β superfamily are very complex, it is important to understand the specificity of the interaction between Hox and Smad proteins. Hoxa-9 was chosen as a well characterized homeobox DNA-binding protein (34, 35) to examine its interaction with different Smad proteins. Two other homeodomain proteins, Msx-1 and Msx-2, were also used for gel shift assays for the same purpose. Msx-1 and Msx-2, found at different loci than the Hox gene clusters, are involved in development of teeth. The expression of both genes is coordinately regulated by BMP-2 and BMP-4 (36–38).

To estimate the relative strength of the interactions between the Smads and homeodomain proteins, the same amounts of Hoxc-8 and Hoxa-9 or Msx-1 and Msx-2 proteins were used in each of the gel shift assays with a fixed amount of different Smad proteins (Fig. 4, B and C). Smad1 and Smad4 inhibited both Hoxc-8 and Hoxa-9 binding, and the inhibition was enhanced when both Smad proteins were added together (Fig. 4, lanes 7 and 14). In contrast, neither Smad2 nor Smad3 interacted with these two Hox proteins. Fig. 4C showed that neither of the Msx proteins interacted with any of the four Smad proteins. GST did not affect Hox or Msx protein binding (Fig. 4C, lanes 4 and 10). The homeodomain, a well conserved DNA binding motif, is the region highly conserved between Hoxc-8 and Hoxa-9, suggesting that Smad1 interacts with other Hox proteins involved in BMP signaling.

**Fig. 4. Smad1 inhibits binding of Hox proteins to DNA.** A, Smad1 inhibits the binding of Hoxc-8 to OPN-5 in a concentration-dependent manner. Gel shift assays were performed using OPN-5 alone (lane 1) or with 1.5 μg of GST (lane 2), 1.5 μg of GST-Smad1 (lane 3), or 0.2 μg of GST-Hoxc-8 protein (lanes 4–7) and different amounts of GST-Smad1 (1.5, 3, and 4.5 μg for lanes 5–7, respectively). B, Hox proteins interact with Smad1 and -4 but not Smad2 and -3. Hoxa-9 and Hoxc-8 GST fusion proteins (0.2 μg) were tested for their ability to interact with Smad1, -2, -3, and -4 or GST (3 μg) in a gel shift assay. C, Smads do not inhibit binding of Msx-1 and Msx-2 homeodomains containing proteins to their cognate DNA element. Purified GST-Msx-1 or -Msx-2 (0.5 μg) was incubated together with probe-M and different Smads (3 μg).
moter fragment containing the Hoxc-8 binding site into the pGL3-basic luciferase reporter vector to generate an OPN-266 reporter plasmid (Fig. 5A). Transfection of the OPN-266 construct in C3H10T1/2 mesenchymal cells with Hoxc-8, Smad1, or Smad4 plasmids alone or in a combination of all three in the presence or absence of ALK3 plasmid. C, the osteopontin Hox binding site mediates BMP-induced transcription. Hox-pGL3 construct was co-transfected with ALK6 or ALK3 in C3H10T1/2 mesenchymal cells. D, mutation of Hox binding site abolishes BMP stimulation. Hox-pGL3 construct or mHox-pGL3 pGL3 control plasmid was co-transfected with ALK6, ALK3, or Hoxc-8 plasmids in C3H10T1/2 mesenchymal cells. Cell lysates in B, C, and D were assayed for luciferase activity normalized to Renilla luciferase levels 48 h after transfection. Experiments were repeated twice in triplicates.

**FIG. 5.** BMP-induced osteopontin gene transcription is mediated by Hoxc-8 binding site. A, schematic description of the constructs used in the transfection assays: OPN-266 is the native osteopontin construct; Hox-pGL3 contains the osteopontin Hox binding site linked to SV40 promoter; mHox-pGL3 contains the mutated osteopontin Hox binding site. B, BMP activates the osteopontin promoter. The OPN-266 plasmid was co-transfected in C3H10T1/2 mesenchymal cells with Hoxc-8, Smad1, or Smad4 plasmids alone or in a combination of all three in the presence or absence of ALK3 plasmid. C, the osteopontin Hox binding site mediates BMP-induced transcription. Hox-pGL3 construct was co-transfected with ALK6 or ALK3 in C3H10T1/2 mesenchymal cells. D, mutation of Hox binding site abolishes BMP stimulation. Hox-pGL3 construct or mHox-pGL3 pGL3 control plasmid was co-transfected with ALK6, ALK3, or Hoxc-8 plasmids in C3H10T1/2 mesenchymal cells. Cell lysates in B, C, and D were assayed for luciferase activity normalized to Renilla luciferase levels 48 h after transfection. Experiments were repeated twice in triplicates.

To further define the transcription activity of the Hoxc-8 binding site, we linked a shorter osteopontin promoter fragment containing the Hoxc-8 binding site to a luciferase reporter vector under the control of the SV40 promoter (Fig. 5A). Transfection of the OPN-266 construct in C3H10T1/2 mesenchymal cells showed that the reporter activity was stimulated moderately when Smad1 or Smad4 expression plasmids were co-transfected. The luciferase activity was significantly enhanced when the OPN-266 reporter construct was co-transfected with ALK3 (Q233D), Smad1, and Smad4 expression plasmids. Furthermore, the ALK3 (Q233D)-induced transcriptional activity was completely abolished when Hoxc-8 was overexpressed (Fig. 5B).

To further define the transcription activity of the Hoxc-8 binding site, we linked a shorter osteopontin promoter fragment containing the Hoxc-8 binding site to a luciferase reporter vector under the control of the SV40 promoter (Fig. 5A). When the Hox-pGL3 construct was co-transfected in C3H10T1/2 cells with ALK3 (Q233D) or ALK6 (Q203D), luciferase reporter activity was induced more than 13- and 11-fold, respectively. Most importantly, overexpression of Hoxc-8 suppressed the ALK3 (Q233D)-induced or ALK6 (Q203D)-induced reporter activity (Fig. 5C). These results suggest that the Hox binding site mediates BMP signaling and that Hoxc-8 functions as a transcription repressor. In comparison with the osteopontin native promoter, the Hox-pGL3 construct does not require overexpression of Smad1 and -4 in responding to BMP stimulation. This is an SV40 promoter-driven construct with a much shorter osteopontin promoter fragment, which does not contain many other transcription elements like the native osteopontin promoter construct.

To validate whether the Hoxc-8 site mediates BMP signaling, we mutated the core nucleotides of the Hoxc-8 binding site from TAAT to GCCG to create mHox-pGL3 (Fig. 5A). Transfection of the mutant construct, mHox-pGL3, completely abolished the ALK3 (Q233D)-induced or ALK6 (Q203D)-induced reporter activity and eliminated Hoxc-8 inhibition in C3H10T1/2 cells (Fig. 5D). These results confirm that the osteopontin Hox binding site is a BMP response element.

Several Smad downstream transcription factors have been characterized in the TGF-β pathway. Here, we first show that Hoxc-8 interacts with Smad1 as a downstream DNA-binding protein in the BMP pathway. Our data demonstrate that Hoxc-8 binds to the osteopontin promoter and represses the gene transcription. BMP stimulation activates gene transcription by derepressing the Hoxc-8 protein through the interaction...
of Smad1 with the Hoxc-8 protein. The direct interaction between Smad1 and Hox protein(s) suggests their functional relationship and the mechanisms in BMP-induced skeleton development.

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