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Molecules and Cells

Hoxc8 Represses BMP-Induced Expression of *Smad6*

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Proper regulation of bone morphogenetic protein (BMP) signaling is critical for correct patterning and morphogenesis of various tissues and organs. A well known feedback mechanism is a BMP-mediated induction of Smad6, an inhibitor of BMP signaling. Hoxc8, one of the *Hox* family transcription factors, has also been shown to negatively regulate BMP-mediated gene expression. Here we add another level of Hoxc8 regulation on BMP signaling. Our results show that Hoxc8, when over-expressed in C3H10T1/2 or C2C12 cells, suppressed basal *Smad6* promoter activity and its mRNA expression. Activation of *Smad6* transcription either by BMP2 treatment or Smad1 over-expression was also abolished by Hoxc8. When chromatin was precipitated from mouse embryos with anti-Smad1 or anti-Hoxc8 antibody, *Smad6* promoter sequence was enriched, suggesting that Hoxc8 proteins make complexes with Smad1 in the *Smad6* promoter region. Yet, a lack of Hox binding motifs in the *Smad6* promoter sequence suggests that instead of directly binding to the DNA, Hoxc8 may regulate *Smad6* expression via an indirect mechanism. Our results suggest that the Smad6-mediated negative feedback mechanism on BMP signaling may be balanced by the repression of *Smad6* expression by Hoxc8.

INTRODUCTION

The Hox family of transcription factors modulates various aspects of patterning and morphogenesis during embryonic development (Krumlauf, 1994; Pearson et al., 2005). *Hoxc8* is one of the three members of paralogue VIII within the *Hox* gene cluster and is well known to be important for correct patterning of the axial skeleton along the anteroposterior axis during early embryogenesis (Juan et al., 2006; van den Akker et al., 2001). Hoxc8 is also involved in both cartilage and bone differentiation in late embryonic and postnatal stages (Juan et al., 2006). In particular, Hoxc8 is shown to contribute to skeletal development by acting as a transcriptional repressor of genes that mediate bone morphogenetic protein (BMP) signaling (Shi et al., 1999; Yang et al., 2000; Yueh et al., 1998).

BMPs, the largest group in the transforming growth factor-β superfamily, mediate their signals via transmembrane serine/threonine receptor kinases, which phosphorylate and activate downstream effectors, Smad transcription factors (Li and Cao, 2006). When a subgroup of receptor-regulated Smads including Smad1 becomes phosphorylated, they form complexes with the common partner, Smad4. The complexes then translocate into the nucleus where they regulate gene expression either by directly binding to defined DNA elements or by associating with other transcription factors. Among the genes induced by BMP-Smad pathway is *Smad6*, which in turn acts as a negative regulator of BMP signaling (Ishida et al., 2000; Takase et al., 1998).

Thus, both Hoxc8 and Smad6 act as negative regulators of BMP signaling, yet inhibitory mechanisms of the two factors are different. Hoxc8, when BMP signaling is low, directly binds to the promoter sequence of BMP target genes such as *osteoprotegerin* and *osteopontin* and inhibits their transcription (Shi et al., 1999; Yang et al., 2000; Yueh et al., 1998). This repressive action of Hoxc8 is relieved when BMP activates Smad1 which interacts with and dislodges Hoxc8 from the promoter region. Meanwhile, Smad6 suppresses BMP signaling primarily by preventing Smad1 phosphorylation and activation. In addition, Smad6 is also shown to reinforce the repressive action of Hoxc8 on BMP target genes by directly interacting with Hoxc8, which prevents Smad1 from interacting with and dislodging Hoxc8 from DNA. Thus, Hoxc8 and Smad6 cooperate to negatively regulate BMP signaling (Bai et al., 2000).

Here, we show that the cooperative negative action by Hoxc8 and Smad6 proteins may be balanced by repression of *Smad6* transcription by Hoxc8. We observed that BMP-induced *Smad6* expression was repressed by Hoxc8 in murine mesenchymal cell lines. The repressive action of Hoxc8 on *Smad6* transcription is mediated through interacting with Smad1 and inhibiting its transcriptional activity.

MATERIALS AND METHODS

Plasmid construction

To construct a luciferase reporter regulated by Smad6 promoter, we isolated 5′ upstream promoter sequence of Smad6 by PCR using the following primers, 5′-AGCAG TCCAG AGCAC AGGTA TC-3′ (forward) and 5′-GGCTA CATGG ATCAC GATGG-3′ (reverse), from genomic DNA of C3H10T1/2 cells. The ampli-

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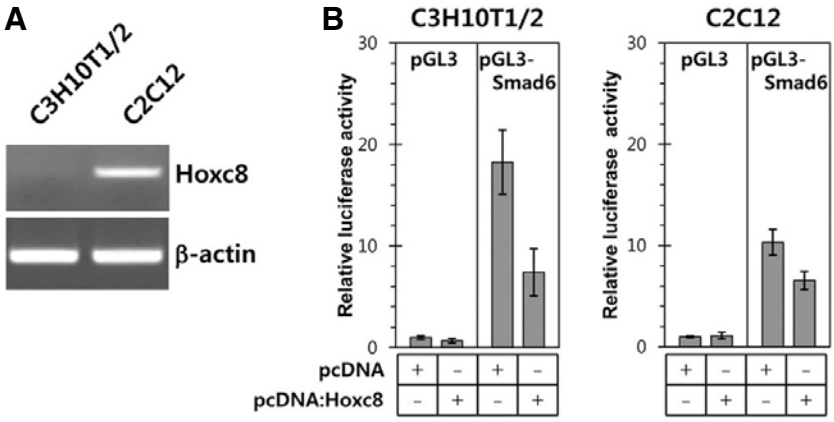

Fig. 1. Hoxc8 represses the *Smad6* promoter activity in mesenchyme-derived cell lines. (A) Semi-quantitative RT-PCR shows that C2C12 cell line has higher endogenous levels of *Hoxc8* transcripts compared to C3H10T1/2 cells. β-actin was used as an internal control. (B) Luciferase activities measured from the lysates of the two cell lines that were transfected with either pGL3-Smad6 reporter plasmid or pGL3 control plasmid together with expression plasmids for Smad1 and/or Hoxc8. Total amount of DNA transfected was kept constant by adding pcDNA3 empty vector. Basal *Smad6* promoter activities in C3H10T1/2 cells were higher than those in C2C12 cells, and both were repressed by co-transfection of Hoxc8. Values are the means ± S.D. taken from at least three independent experiments.

fied 2-kb DNA fragment was first cloned into the pGEM-T easy vector (Promega), and subsequently into the pGL3 luciferase reporter plasmid by PCR amplifying the fragment using a set of primers containing *Nhe*I and *Xho*I sites at the ends: 5′-GGCTA GCAGC AGTCC AGAGC ACAGG TATC-3′ (forward) and 5′-CCTCG AGGGC TACAT GGATC ACGAT GG-3′ (reverse). To construct Smad1 expression vector, Smad1 cDNA was isolated by RT-PCR using primers 5′-GGGAT CCGCT ATGAA TGTGA CC-3′ (forward) and 5′-GCTCG AGACG GAAGC CACAG GT-3′ (reverse), and cloned into a mammalian expression vector, pcDNA3 (Invitrogen). Construction of mouse Hoxc8 expression plasmid was previously described (Kwon et al., 2003).

Cell cultures, transfection and reporter assays

C2C12 myoblastic cells and C3H10T1/2 murine mesenchymal progenitor cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin (Welgene) in a 5% CO2 humidified atmosphere. One day after plating C2C12 cells (2×10^4) and C3H10T1/2 cells (1×10^4) onto 24-well plates, cells were transiently transfected with luciferase reporter plasmid (100 ng/well) and expression vectors (400 ng/well) using FuGENE HD (Roche Applied Science) according to the manufacturer's instructions. For all transfections, 50 ng of the TK-*Renilla* luciferase vector (TK-*Renilla*) (Promega) was co-transfected as an internal control for transfection efficiency. Six hours after transfection, 100 ng/ml of BMP2 (R&D Systems) was added to the cells when needed. Cells were harvested 48 h post-transfection, and luciferase activity was measured using the dual luciferase assay kit (Promega) and with a Glomax 20/20 luminometer (Promega). Luciferase activity was normalized by the activity of TK-*Renilla* luciferase to correct for the differences in transfection efficiencies between experiments. All luciferase assays were performed in triplicates and in three independent experiments.

RNA isolation and RT-PCR

Total cellular RNA was extracted using the RNA Bee reagent (AMS Biotechnology) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA using cDNA synthesis kit iScript (Bio-Rad). After a reverse transcription reaction, PCR was carried-out with the following primer sets: Hoxc8: 5′-CCTAT TACGA CTGCC GGTTC-3′ (forward) and 5′-TTGGC GGAGG ATTTA CAGTC-3′ (reverse); β-actin: 5′-CATGT TTGAG ACCTT CAACA CCCC-3′ (forward) and 5′-GCCAT CTCCT GCTCG AAGTC TAG-3′ (reverse); Smad6: 5′-CCACT TGGAG ACTTC TTCTT CTTCG-3′ and 5′-ATGTA GGGCA GCATC TGGTG CG-3′; and Smad1: 5′-ATGAA TGTGA CCAGC TTGTT T-3′ (forward) and 5′-TGCT TGGAA CCAAA TGGGA A-3′ (reverse). PCR conditions were as follows: Smad6 (59°C, 29 or 21 cycles), Smad1 (56°C, 29 cycles), β-actin (58°C, 29 cycles) and Hoxc8 (58°C, 26 cycles). Quantitative real time PCR was performed using an Applied Biosystems Sequence Detection system with the same sets of primers

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) assay was performed using anti-Hoxc8 monoclonal antibody (Covance MMS-266R) and anti-Smad1 polyclonal antibody (Santa Cruz). Cells prepared from E11.5 mouse embryos were fixed with 1% formaldehyde for 5 min at room temperature, and treated with 125 mM of glycine for 5 min. After washing with ice-cold PBS, cells were lysed in SDS buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.0) containing protease inhibitors (Sigma). The cell lysates were then sonicated to an average fragment size of 0.7 kb. After centrifuging for 10 min at 4°C, the supernatant was diluted with 0.9 ml of ChIP dilution buffer (0.01% SDS, 1.1% TritonX-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0, 167 mM NaCl) and pre-cleared with Protein G-agarose beads (Upstate Biotechnology). Immunoprecipitation was performed with either anti-Hoxc8 or anti-Smad1 antibody at 4°C for 12 h. The immuno-complexes were collected with protein G-agarose beads, and then cross-linking was reversed by incubating the eluate for at least 6 h at 65°C. DNA from immuno-complexes was purified by phenol chloroform extraction. Enrichment of the Smad6 promoter sequence was examined by PCR using the following primers 5′-GGGCC AATCC TGACT TTACA-3′ (forward) and 5′-ATCGA GGGAG CTCCA GGATA G-3′ (reverse).

RESULTS AND DISCUSSION

Repression of basal *Smad6* expression by Hoxc8

To examine whether Hoxc8 proteins regulate *Smad6* transcription, we chose two different cell lines, C3H10T1/2 embryonic mesenchymal cells and C2C12 myoblastic cells, both of which are known to respond to BMP signaling and differentiate into osteoblasts. We first checked the endogenous expression levels of *Hoxc8* in these cell lines. C2C12 cells displayed a high level of endogenous *Hoxc8* expression, while C3H10T1/2 cell showed no or little *Hoxc8* expression (Fig. 1A). Embryonic fibroblasts isolated from 12.5 dpc mouse embryos and F9 mouse teratocarcinoma cells also showed high levels of *Hoxc8* transcripts comparable to that of C2C12 cells (data not shown).

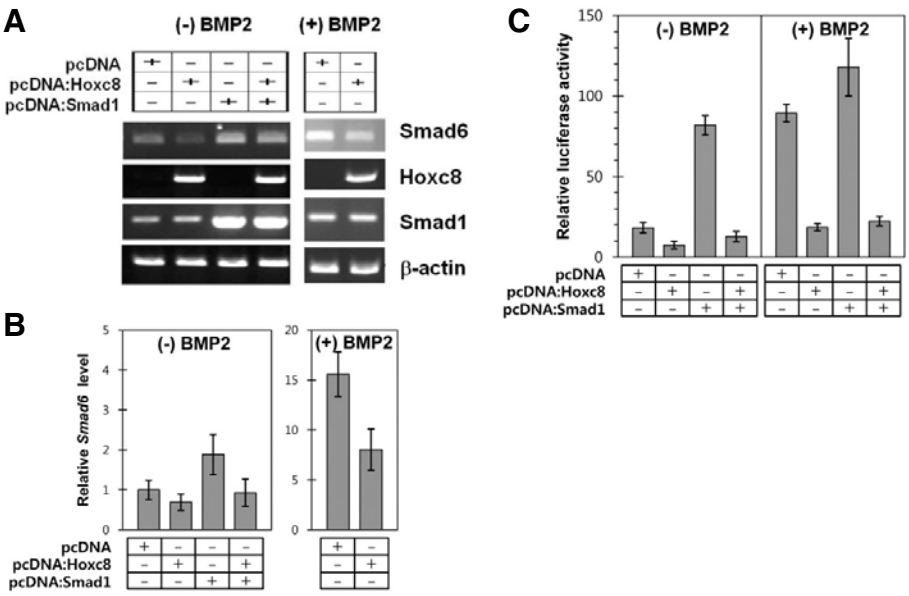

Fig. 2. Hoxc8 represses BMP2- or Smad1-induced *Smad6* transcription. (A) Semi-quantitative RT-PCR analysis in C3H10T1/2 cells transfected with Hoxc8- and/or Smad1-expression vectors in the presence or absence of BMP-2 (200 ng/ml). Smad1- or BMP2-induced *Smad6* expression was repressed by Hoxc8. Of note, since BMP2 dramatically increased *Smad6* transcript levels, *Smad6* mRNAs from BMP2-treated cells were amplified at 21 cycles, whereas 29 cycles were used for non-BMP2-treated cells. (B) Quantitative real time PCR analysis in the same set of experimental conditions used for semi-quantitative RT-PCR in (A). (C) Hoxc8 down-regulates Smad1- or BMP2-induced *Smad6* promoter activities, measured by luciferase assays.

To access the promoter activity of *Smad6*, 2 kb genomic DNA fragment containing 5′ upstream promoter region of *Smad6* was cloned into a luciferase reporter plasmid (pGL3-Smad6). When transfected into C3H10T1/2 or C2C12 cells, the luciferase activity was elevated 10 to 20 fold compared to that of the empty pGL3 reporter plasmid (Fig. 1B), indicating a basal constitutive activity of the *Smad6* promoter. When *Hoxc8* expression plasmid (pcDNA:Hoxc8) was co-transfected with the reporter, the luciferase activity was reduced both in C3H10T1/2 and C2C12 cells (Fig. 1B), indicating that Hoxc8 negatively regulates the *Smad6* promoter activity. Interestingly, the reduction by exogenous Hoxc8 was less effective in C2C12 cells. This may be because endogenous Hoxc8 proteins are already present and exert their repressive action on the *Smad6* introducing moter in C2C12 cells. Thus, the effect of exogenous Hoxc8 proteins could be less obvious than that observed in C3H10T1/2 cells, in which no endogenous Hoxc8 is expressed. Thus, C3H10T1/2 cells were used for all the following experiments.

Next we examined whether expression of endogenous *Smad6* mRNAs was repressed by Hoxc8. Consistent with the basal activity of the *Smad6* promoter (Fig. 1B), *Smad6* transcripts were detected by RT-PCR in C3H10T1/2 cells (Fig. 2A). This mRNA level was decreased when pcDNA:Hoxc8 vector was co-transfected, indicating that Hoxc8 negatively regulates endogenous *Smad6* expression.

Hoxc8 represses Smad1- or BMP2-induced *Smad6* expression

The decrease in the reporter activity and endogenous *Smad6* expression by Hoxc8 prompted us to search for canonical Hoxc8-binding element(s) in the *Smad6* promoter sequence. However, we failed to recognize any of such Hoxc8-binding motifs, suggesting that Hoxc8 may elicit its repressive effect indirectly by interacting with other proteins rather than directly binding to the *Smad6* promoter. It has long been known that Hox proteins have a propensity of interacting with other protein partners, mostly transcription factors, such as Pbx, Extradenticle, or Engrailed, to improve both DNA-binding and functional specificity of Hox (Levine and Hoey, 1988). Since Smad1 has been shown to interact with Hoxc8 and relieve the repressive action of Hoxc8 on expression of *osteopontin* (Shi et al., 1999; Yang et al., 2000), it is possible that Hoxc8 suppresses *Smad6* expression by inhibiting the transcriptional activity of Smad1.

To test this possibility, we first examined whether Smad1 could up-regulate endogenous *Smad6* expression. When C3H10T1/2 cells were transfected with Smad1 expressing vector (pcDNA:Smad1), endogenous *Smad6* expression was increased. This increase was suppressed when pcDNA:Hoxc8 was co-transfected, suggesting that Hoxc8 inhibits Smad1-activated *Smad6* transcription. Since Smad1 is a mediator of BMP signaling, we next asked whether Hoxc8 could repress BMP-activated *Smad6* expression. *Smad6* transcript level was dramatically increased in cells treated with BMP2 compared to cells that were not exposed this ligand (Fig. 2A). It should be noted that Smad6 RT-PCR results obtained from BMP2-treated cells were products of 21 cycles since Smad6 signals have already reached plateau after 21 cycles in this condition (the number of PCR cycles for non-treated cells was 29 cycles). This increase of *Smad6* transcript level by BMP2 was reduced when Hoxc8 was over-expressed (Fig. 2A). Similar patterns of *Smad6* downregulation by Hoxc8 overexpression was observed by quantitative real-time PCR (Fig. 2B). Together, these results suggest that Hoxc8 represses endogenous *Smad6* expression induced by either BMP2 or Smad1, a mediator of BMP signaling.

Next, we examined whether the repressive effect of Hoxc8 on BMP2- or Smad1-induced *Smad6* expression is mediated by the action on the *Smad6* promoter. C3H10T1/2 cells were transfected with pGL3-Smad6 reporter plasmids together with pcDNA:Smad1 and/or pcDNA:Hoxc8. Consistent with the endogenous *Smad6* regulation, over-expression of Smad1 increased the *Smad6* promoter activity (Fig. 2C), and this increase was abolished by co-transfection of pcDNA:Hoxc8 (Fig. 2C). We further asked whether Hoxc8 could repress the *Smad6* promoter activity induced by BMP2. Upon BMP2 treatment, the *Smad6* promoter activity was increased to a comparable level induced by Smad1 over-expression (Fig. 2C), suggesting that Smad1 is a major mediator of BMP signaling on *Smad6* induction. The *Smad6* promoter activity was further increased by a combination of BMP2 treatment and *Smad1* over-expression (Fig. 2C). When Hoxc8 was co-transfected in these conditions, the Smad6 promoter activity was decreased

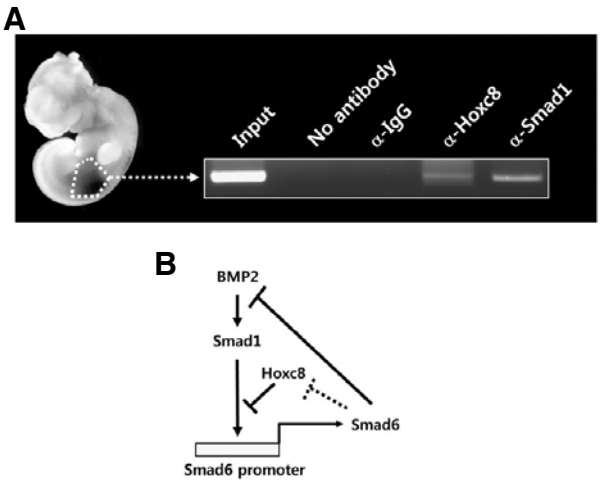

Fig. 3. Hoxc8 and Smad1 bind to the promoter region of *Smad6*. (A) Whole-mount *in situ* hybridization for *Hoxc8* in 11.5 dpc mouse embryo (Kwon et al., 2005) (left panel). Tissue lysates from the regions expressing *Hoxc8* were used for chromatin immunoprecipitation (ChIP)-PCR analysis. The *Smad6* promoter sequence was enriched from the chromatins precipitated with either anti-Hoxc8 or anti-Smad1 antibody, the intensity being stronger with anti-Smad1 antibody. Immunoprecipitants with no or anti-IgG antibody and input chromatin were included as negative and positive controls, respectively. (B) A schematic view of a canonical BMP-Smad signaling pathway and a possible regulation by Hoxc8. Upon BMP binding to its receptors, receptor-regulated Smads such as Smad1 are activated and regulate transcription of target genes, including *Smad6*. Smad6 is an inhibitory Smad that can negatively regulate BMP-signaling pathway through blockage of recruitment and activation of receptor-regulated Smads. The proposed role of Hoxc8 is the repression of *Smad6* transcription, albeit indirectly and possibly by binding to Smad1.

almost to a basal level, suggesting that Hoxc8 can repress the Smad6 promoter activity induced by BMP2 as well as its mediator, Smad1.

Hoxc8 and Smad1 bind to a region in the Smad6 promoter in the mouse embryo

Even though it is clear that the repressive action of Hoxc8 on *Smad6* expression is mediated on the *Smad6* promoter, we could not recognize Hoxc8-binding element(s) in the *Smad6* promoter sequence, suggesting that Hoxc8 may not directly bind to the *Smad6* promoter to exert its inhibitory effect. Since it has been shown that Hoxc8 proteins can make complexes with Smad1 proteins, it is possible that Hoxc8 indirectly suppresses *Smad6* expression by interacting with Smad1 and inhibiting its transcriptional activity. To test whether Hoxc8 interacts with Smad1 proteins bound on the *Smad6* promoter region, we performed chromatic immunoprecipitation (ChIP) assay. We reasoned that if Hoxc8 interacts with Smad1 on the *Smad6* promoter region, the *Smad6* promoter sequence would be enriched when precipitated either with anti-Smad1 or anti-Hoxc8 antibodies. In embryonic day 11 mouse embryos, Hoxc8 is strongly expressed in the mesoderm of trunk area (Kwon et al., 2005). As shown in Fig. 3A, the Smad6 promoter sequence was enriched from the chromatin precipitated with anti-Smad1 antibody, indicating that Smad1 proteins bind to the Smad6 promoter region *in vivo*. Interestingly, we also observed that the Smad6 promoter sequence was amplified from the chromatin precipitated with anti-Hoxc8 antibody, although the intensity was weaker than that by anti-Smad1 antibody. Since there is no Hox binding motif in the *Smad6* promoter sequence, it is most likely that Hoxc8 binds to the promoter region indirectly by interacting with Smad1. Nevertheless, we could not exclude the possibility that Hoxc8 may directly bind to the *Smad6* promoter sequence to repress Smad1-mediated transcription.

BMP signaling is regulated by multiple steps either positively or negatively to mediate correct patterning and morphogenesis of various tissues and organs including skeletal development. Hoxc8 has been shown to act as a negative regulator of BMP signaling by repressing genes that mediate BMP-induced bone differentiation such as *osteopontin* and *osteoprotegerin* (Shi et al., 1999; Yang et al., 2000). Our results show that Hoxc8 represses expression of *Smad6*, another negative regulator of BMP signaling. However, the lack of Hox binding motifs in the Smad6 promoter region suggests that the repressive action of Hoxc8 on *Smad6* expression may be different from those on *osteopontin* or *osteoprotegerin*, in which Hoxc8 directly binds to the Hox binding motifs located in their promoter region (Shi et al., 1999; Yang et al., 2000). Instead of directly binding to the promoter region, Hoxc8 appears to repress *Smad6* expression by inhibiting transcriptional activity of Smad1 bound to the *Smad6* promoter region.

Thus far, it is not clear how the negative regulation of *Smad6* by Hoxc8 contributes to skeletal differentiation mediated by BMP signaling pathway. Nevertheless, our observations demonstrate that BMP signaling is tightly regulated at multiple levels. When induced by BMP-Smad1 signaling, Smad6 and Hoxc8 negatively regulate BMP signaling at different levels. These repressive actions by Hoxc8 and Smad6 may be balanced by the negative regulation of *Smad6* expression by Hoxc8. Thus, expression of *Smad6*, a negative regulator of BMP signaling, can be regulated positively by BMP-Smad1pathway and negatively by Hoxc8.

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