Indian hedgehog gene is a target of
the Bone Morphogenetic Protein signaling pathway

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Running Title: Ihh is a target of BMP signaling pathway
Summary

Bone morphogenetic proteins (BMPs), a large subgroup of the TGF-β family of growth factors, are key mediators of many fundamental processes in embryonic development. The BMP target genes mediating these effects and the mechanism of their selective regulation are poorly characterized. In this study, we use a chromatin immunoprecipitation-based gene cloning method to identify BMP target genes in mouse embryonic carcinoma cell line. We thus identified the Indian hedgehog (Ihh) gene, a member of the mammalian hedgehog family, as a target of BMP signaling. Both reporter and RT-PCR assays revealed that Ihh is upregulated in embryonic cells upon BMP treatment. The BMP-responsive region (BRE) of the Ihh promoter contains multiple GC-rich motifs known as “Mad/Medea binding box” found in the Drosophila tinman gene and in the mouse Id1 gene. DNA binding studies revealed that Smad4 binds to these GC-rich motifs. These findings indicate that BMP stimulates Ihh expression. We also suggest expression of the Ihh gene, which contains multiple Smad-binding sites, might finely regulated by a gradient of BMP concentrations.

Introduction

The bone morphogenetic proteins (BMPs) were originally identified as peptides which induce ectopic bone and cartilage production(1). BMPs constitute the largest subfamily of the TGF-β superfamily of growth factors, and exert pleiotropic biological effects ranging from regulation of early developmental processes to organogenesis(2,3). Like other members of the TGF-β-family of growth factors, BMP signals are initiated by binding of ligand to the specific serine/threonine-kinase receptors, known as type I and type II. Upon ligand binding, type II receptor kinase phosphorylates type I receptor and
activates its kinase domain. Activated type I receptor kinase, then, phosphorylates downstream signal transducers, the Smad proteins. Upon BMP stimulation, Smad1, 5 and 8 are phosphorylated by the receptor, form a complex with Smad4, and translocate from the cytoplasm to the nucleus, where they act as transcriptional regulators (4-6). To understand the mechanism of how BMPs exert various biological effects in a cell-type specific manner, it is important to elucidate genes that are regulated by Smads and the mechanism of their regulation. The Smad complex by itself has low specificity and affinity for its DNA recognition site and it is now known to associate with various DNA-binding cofactors to achieve high-affinity and selective interactions with cognate DNAs (7). Among these cofactors, OAZ is the first identified as a DNA-binding cofactor that associates with an activated Smad1-Smad4 complex to activate the Xenopus Vent-2 gene. However, in the same tissue and at the same stage, BMP gradients can select different target genes. Therefore, the other mechanism of target gene selection dependent on the intensity of the BMP signal was investigated. In Drosophila, the homeobox protein Tinman cooperates with Mad and Medea (the orthologs of Smad1 and Smad4, respectively) in the induction of tinman itself by decapentaplegic (Dpp) (the ortholog of BMP2/4) during formation of the visceral mesoderm (8). A recent study on the promoter of the Zen gene, whose expression is regulated by Dpp during Dorso-Ventral axis formation, also suggests that multimerized Mad/Media binding sites (Mad box) sensor the intensity of the BMP signal and set a threshold for gene expression (9).

In this study, BMP responsive genomic regions were cloned by chromatin immunoprecipitation (ChIP) by targeting Smad4. Mapping of the sequence from one clone revealed that mouse Indian hedgehog (Ihh) is a target of BMP signaling, confirmed by RT-PCR in P19 cells. Ihh is a member of the hedgehog family and is one of the key regulators in endochondral bone formation. The BMP responsive region of the Ihh promoter contains 6 Mad Boxes (GCGNCGC motif) and one GCGGCC motif, which is similar to the promoter structure found in Drosophila Dpp-target genes. Our findings of
the mouse *Ihh* promoter structure suggest evolutionary conservation of BMP regulation of gene transcription and also clarify one aspect of how the two morphogens, BMP and hedgehog, interact.

**Experimental Procedures**

**Cell culture**

COS-1 and P19 cells were maintained in D-MEM media (Invitrogen) supplemented with 10% fetal bovine serum (HyClone). Recombinant BMP7 was prepared and provided by Curis Inc. Briefly, human BMP7 was overexpressed in CHO cells and purified from the culture media by column chromatography. The purity of BMP7 was monitored by SDS-PAGE.

**Chromatin immunoprecipitation cloning**

P19 cells were treated with 100 μg/ml BMP7 for 1 hr and fixed with 1% formaldehyde at room temperature for 10 min. Reaction was stopped with 0.125 M glycine and rinsed with phosphate buffered saline (PBS). After being washed with PBS, cells were resuspended in swelling buffer (10 mM HEPES-KOH [pH 7.9], 10 mM potassium chloride, 1.5 mM magnesium chloride, 0.5% NP-40, 1 mM sodium fluoride, 1 mM sodium vanadate, proteinase inhibitors), and were incubated on ice for 10 min. Cell suspension was passed through 22 μm gauze needle and the nuclei were collected by centrifugation. The nuclei were resuspended in chip buffer (20 mM Tris-HCl, pH 7.4, 1M sodium chloride, 0.5% NP-40, 1 mM EDTA, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% bovine serum albumin, proteinase inhibitors) and incubated on ice for 10 min. Genomic DNAs were sonicated on ice to an average length of 1,000 bp and then
centrifuged and the supernatant was collected. The sample was incubated at 4˚C with 5
µg of rabbit IgG for 4 hr and additionally with 20 µl of protein A agarose beads for 1 hr,
and then centrifuged. Precleared chromatin was incubated with 5 µg of anti-Smad4
rabbit polyclonal antibodies (SantaCruz, H-552), or non-specific rabbit IgG for overnight.
Immune complex was recovered using 20 µl of protein A agarose beads after washing
three times with 1 ml of chip buffer. Chromatin was eluted in 95 µl of elution buffer (0.1
M glycine, pH 1.7, 150 mM sodium chloride). The eluate was neutralized with 1 M Tris-
HCl, pH 8.0 and then diluted with chip buffer to proceed second cycle of
immunoprecipitation. The neutralized second eluate was incubated at 65˚C for over night
for reverse cross-link. RNA was removed by incubation with 10 µg RNase A at 37˚C for
30 min and protein was digested with Proteinase K treatment. DNA was extracted with
phenol-chloroform-isoamylalcohol followed by extraction with chloroform and then
ethanol-precipitated with 20 µg of glycogen. Both ends of the purified DNA were once
blunt ended with T4 DNA polymerase and then forced adenylated at 5' end for cloning
(pCR2-TOPO, Invitrogen). The sequences were mapped in the whole mouse genomic
sequence (http://genome.ucsc.edu).

Chromatin immunoprecipitation assay

The Chromatin immunoprecipitation (ChIP) assay was performed under the same conditions as
the ChIP cloning method described above except DNA fragments were sonicated to an average
length of 200-300 bp. Immunoprecipitated DNA fragments were amplified with PCR primers
specific for the promoter region of the mouse Ihh gene; Ihh(GC): 5’-
CCCCCGCGTCGCCTGGCCAAAACAAAC-3’ and 5’-

GTGCTGTCCCCCTCGCCTCGACTCTGAGC-3' and Ihh(5'): 5'-GGTAGTGGCCGGACCGCACAA-3', and 5'-TGGAAACACTGTTAAAAACAA-3', Smad6: 5'-ACCCCGCCGCGCCGCTCCAG-3' and 5'-GGGCTCTCCCGCCTGGCGTCC-3', and hypoxanthine guanine phosphoribosyl transferase (HPRT) promoter: 5'-AGCGCAAGTTGAATCTGC-3' and 5'-AGCGACAATCTACCAGAG-3'. PCR products were separated on an agarose gel and visualized by ethidium bromide staining.

**RT-PCR**

P19 cells were treated with 10 μg/ml cyclohexamide for 0.5 hr prior to BMP7 stimulation for 2hr. Total RNA was extracted by TRIzol (Invitrogen), and 5 μg of total RNA was subjected to reverse transcription using first-strand cDNA synthesis kit (Invitrogen) according to manufacturer’s manual. One tenth of the reaction mixture was used as template for each PCR reaction. The fold-change of expression level was calculated by a real-time PCR following the manufacturer’s protocol. Primers used are, 5'-CCCCAACTACAATCCCGACATC-3' and 5'-CGCCAGCAGTCCATACTTATTTCG-3' for murine Ihh. Primers for murine Smad6 and murine G3PDH were described previously(10).

**Plasmids**

To transfer genomic fragments from pCR2-TOPO to luciferase vector, the insert was digested with XhoI and HindIII and was ligated to XhoI and HindIII sites of pE1bTATA-luc vector. Deletion mutants were generated by PCR and PCR products was ligated to XhoI and HindIII sites of pE1bTATA-luc vector. The primers used were as follow.
-427/-123-Luc; 5'-CCGCTCGAGTCCATTTCCCCTCTCACTCGACCCCGGGCTG-3' and 5'-
CCCAAGCTTTGCGGGGCAGGGGGGCGGGGGGCTGGCTGGTGGCGGCGGTG-3' (IHH10), -385/-123-Luc; 5'-
CCGCTCGAGCGGCCCTCAGGCCCGCCGAGGGCGCC -3' and IHH10, -305/-
123-Luc; 5'- CCGCTCGAGGCACGAGCGCACGGCCCGCAGCGCCGCGCC -3' and
IHH10, -285/-123-Luc; 5'-
CCGCTCGAGGGGGCGGCCAGCGCGCAGCCGCGCCGCGCCGCCC -3' (IHH35) and
IHH10, -248/-123-Luc; 5'-
CCGCTCGAGGGCCAGCGCCGCGCGCCCGCCGCGCGCGCC -3' and IHH10, -285/-
146-Luc; IHH35 and 5'-
TTCCCAAGCTTTGCTGTGGCGCGCAGCGTCTGTGCTGCTGCCGCGCTC-3', -285/-
160-Luc; IHH35 and 5'-
TTCCCAAGCTTTGCTGTGGCTGCCGCTCGACTCTGAGC -3'. All the
reporters with point mutations within –285/-123 regions were generated by PCR, and
later verified by sequencing. To generate 2GC(WT)-luc which has two copies of the
–285/-123 sequence, the sequence was amplified by PCR using the primers, IHH35 and
5'-
AAACGCGTCAGCTCGCGCGCCGCGCGCGCCGCGCGCGCGCGCGCGCGCG
G-3' (IHH52) and the product was digested with XhoI and SalII, then ligated into XhoI site
in E1bTATA-luc. 2GC(MUT)-luc was generated by using the plasmid which has the
2HC8 sequence with mutation in GGCGCC sequence as a template DNA, the mutant
sequence was amplified with primers, 5'-
CCGCTCGAGCGGCCCTCAGCCCCGACGCACGACGCACCCCGACGTAGCCGCG TT-3' and IHH52 and then ligated into pE1bTATA-luc. All the constructs were verified by sequencing. Smad1, 2, 4 expression vectors and constitutive active BMP receptor type IB expression vector and constitutive active TGF-β type I receptor expression vector were described(10).

**Luciferase Assays**

P19 cells were transfected in six-well plates using Fugene6 (Roche) according to the manufacturer's instructions. Luciferase assays were carried out as described before (10). After transfection the cells were re-seeded onto 12-well plates and treated with 100 μg/ml BMP7 for 18 hr in media containing 0.2% fetal bovine serum. Luciferase assay was carried out using luciferase assay kit (Promega) and Berthold luminometer.

**Biotinylated Oligonucleotide Precipitation Assays**

48 hr after transfection with Smad1, Smad4, and constitutive active BMPRIB vectors, COS-1 cells were lysed by sonication in T-NET buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.05% of NP-40) containing protease and phosphatase inhibitors. Cell extracts were precleared with ImmunoPure streptavidin-agarose beads (Pierce) for 1 hr, and then incubated with 1 μg of biotinylated DNA probe and 5 μg of poly (dI-dC)-poly (dI-dC) for 2 hr. DNA-bound proteins were collected with streptavidin-agarose beads for 1 hr washed with T-NET buffer, separated on a SDS-PAGE gel, and identified by Western blotting. A relative intensity of bands on the Western blot was quantitated by densitometry.
Western Blotting

Smad4, Smad6, and p21/Waf1/Cip1 proteins were detected with anti-Smad4 rabbit polyclonal antibodies (H-552, SantaCruz), anti-Smad6 rabbit polyclonal antibodies (SD-6, Zymed) and rabbit anti-p21/Waf1/Cip1 polyclonal antibodies (ab7960, abCAM), respectively.

Results

Isolation of genomic DNA fragments bound to Smad4

To identify novel target genes of the BMP signaling pathway, we isolated genomic DNA fragments to which Smad4 binds using a ChIP-based cloning method(11,12). The mouse teratocarcinoma cell line P19 is well characterized as a cell line which responds to BMPs (10) and expresses some of the BMP target genes such as Smad6 (13)(Stewart, S. et al., unpublished observation). P19 cells were treated with BMP7 and were subjected to ChIP cloning using anti-Smad4 polyclonal antibodies to precipitate genomic DNA fragments bound to Smad4. Out of 60 individual clones, four clones contained putative Smad binding elements (SBE or CAGAC/G box). Out of 40 randomly selected clones which were isolated by non-specific rabbit IgG, none contained an SBE sequence suggesting that SBE containing genomic DNA fragments were selectively precipitated by anti-Smad4 antibodies.

All four fragments (2HC7, 2HC8, HE5, and H5) were cloned upstream of the luciferase gene, and transfected into P19 cells to test the BMP7 responsiveness of each DNA fragment. One construct 2HC8-luc, was activated by BMP7 about 3-fold (Fig. 1A).
Cotransfection of a BMP-specific Smad combination set, Smad1 and Smad4 augmented the 2HC8-luc reporter response in a dose dependent manner (1.5-fold induction without overexpression of Smads compared to 3-fold induction with maximal overexpression of Smads). A dose dependent expression of Smad4 protein was detected by Western blot with anti-Smad4 polyclonal antibodies (Fig. 1B). No further induction was observed upon overexpression of TGFβ-specific Smads, Smad2 and Smad4 (Fig. 1B). In addition, the 2HC8-luc reporter was not influenced by TGFβ (Fig. 1B, hatched bar), suggesting that the 2HC8 clone is a specific target of BMP signaling. By searching the mouse whole mouse genome database, the 430-bp 2HC8 sequence was mapped to the 5’ upstream region of the Indian hedgehog (Ihh) gene (Fig. 1C). This region of the Ihh gene is 91% identical between human and mouse and coincides with a CpG island, suggesting that this region has an important role in the regulation of Ihh expression.

**163-bp region of Ihh gene confers BMP responsiveness**

To define the minimum region which is required for BMP responsiveness, we constructed a series of deletion mutants of the Ihh promoter and characterized them by a luciferase assay. The minimum region that retains a BMP response equivalent to the full-length fragment (2HC8-Luc) was (-285/-123-Luc) (Fig. 2A). Within this region, we found six copies of a GCCGCNGC-like motif (GC-1-6) (Fig. 2 B and C), which is the binding consensus sequence for the Drosophila Smad1 ortholog Mad(14), and one GC-palindromic (GC-pal) sequence (GGCGGCC), which was identified in the mouse Id1 gene promoter(15,16). All the motifs were conserved between mouse and human genes (Fig. 2B). Another Smad binding element (SBE; CAGAC/G), which is bound by Smads with
relatively low specificity, was identified between the GC-5 and GC-pal motifs (Fig. 2B)(17,18).

**Multiple GC-rich motifs confer BMP responsiveness**

To determine which sequence motif is essential to respond to BMP and bind to the Smad complex, we disrupted each one of the GC-rich motifs (GC-1-6) or the GC palindromic sequence (GC-pal) and measured the response of the resulting constructs to BMP using the luciferase assay. Disruption of GC-1, GC-2, GC-3, or GC-6 showed a significant reduction of the BMP7 response, while mutation of GC-4 or GC-5 did not (Fig. 3A). This is presumably due to a GC-rich sequence remaining in the complementary strand of the GC-4 and GC-5 (See Fig. 2C and Fig. 3A). When we mutated the GC-rich sequence in both strands of GC-5 \((\text{GC-5}^{\text{dbl}})\), a marked reduction of the BMP response was observed (37% of the activity of WT). Disruption of the GC-pal motif alone reduced the BMP response to 12% of the wild type sequence. These results suggest that both the GC-rich and the GC-pal motifs are essential for BMP responsiveness. Mutations of multiple motifs such as \((\text{GC-1, GC-2, GC-5}^{\text{dbl}})\), \((\text{GC-3,GC- GC-5}^{\text{dbl}})\), and \((\text{GC-1, GC-2, GC-pal})\) showed marked reduction of the BMP7 responsiveness compared to single mutations of each motif (Fig. 3A) These results suggest that each GC-rich and GC-pal motif contributes quantitatively to BMP responsiveness and all the elements are necessary for maximum level of responsiveness. A mutation of the SBE had no effect on BMP responsiveness, suggesting the SBE is dispensable in this promoter (Fig. 3A, SBE).

**GC-rich motifs are essential for BMP responsiveness**
Two tandem repeats of a fragment spanning the six GC-rich motifs and GC-pal motif has been generated (2GC(WT)-luc). This construct showed strong activation by BMP7 (24-fold). Overexpression of Smad1 and Smad 4 further augmented the response upon BMP stimulation or cotransfection of constitutive active BMP type IB receptor (BMPR1B\textsuperscript{CA}) but not in cotransfection of the constitutive active TGF\textbeta type I receptor (T\textbeta RI\textsuperscript{CA}) (Fig. 3B). Mutation of the motifs GC-1, GC-2, GC-3 and GC-pal (2GC(MUT)-luc) completely abolished response to BMP7, suggesting that the six GC-rich motifs and the GC-pal motif are necessary and sufficient for BMP specific upregulation of the Ihh gene (Fig. 3B).

**GC-rich motifs mediate BMP-dependent Smad4 binding**

A ChIP assay was carried out to confirm that Smad4 binds to GC-rich motifs within the -285/-123 region of the Ihh gene upon BMP7 stimulation in vivo. Genomic DNA fragments from BMP7-untreated or treated P19 cells were isolated and sonicated to an average size of 200-300 bp (Fig. 3C, left panel). DNA fragments were then immunoprecipitated with anti-Smad4 antibodies. Rabbit non-specific IgG was used as a negative control for the chromatin immunoprecipitation. The presence of the GC-rich motif region of the Ihh gene promoter bound to Smad4 was analyzed by semi-quantitative PCR and real-time PCR using specific pairs of primers spanning the GC-rich motif region (-258/-160) of the Ihh promoter [Ihh(GC)] and the 5’ upstream region of the GC-rich motif region (-1030/-1230), which is not responsible for BMP-dependent regulation of \textit{Ihh} [Ihh(5’)] (Fig. 3C). Primer sets designed to amplify the BMP-response element (BRE) of the Smad6 gene promoter, which is known to bind to Smads (13) and the 5’ upstream region of the hypoxanthine guanine phosphoribosyl transferase (HPRT)
promoter were used as positive and negative control, respectively (Fig. 3C). As shown in Fig. 3C and D, stimulation with BMP7 induced an increase in the binding of Smad4 to the GC-rich motif region of the Ihh and BRE of the Smad6 gene promoters. However, no increase was observed in Smad4 association with the 5' upstream region of the Ihh [Ihh(5')] or the HPRT gene promoters upon BMP7 stimulation (Fig. 3C and D). These results confirm that BMP7 stimulation triggers endogenous Smad4 to bind specifically to a short region of the endogenous Ihh gene promoter which contains multiple GC-rich motifs.

A DNA-pull down assay was carried out to examine whether lack of activation of 2GC(MUT)-luc by BMP7 is due to lack of Smad4 binding to GC-1, GC-2, GC-3 and GC-pal motifs (Fig. 3B). The DNA fragment corresponding to -285/-123 was biotinylated, followed by incubation with Cos 1 cell extract overexpressing both Smad 1 and Smad4. Binding of Smad4 to the wild type -285/-123 fragment was observed in extracts from cells transfected with constitutively active type IB BMP receptor (BMPR1B<sup>CA</sup>) (Fig. 3E, upper panel, WT lanes). The -285/-123 fragment with mutations in GC-1, GC-2, GC-3, and GC-pal precipitated approximately 3-fold less Smad4 (Fig. 3E, upper panel, MUT lanes). This result suggests that Smad4 binds to the GC-rich motifs within the 163-bp fragment of the promoter.

**Ihh mRNA is upregulated by BMP7 stimulation of P19 cells**

To examine whether the Ihh mRNA is increased by BMP in vivo, we performed a semi-quantitative RT-PCR analysis using total RNA extracted from P19 cells treated or untreated with BMP7 for 2hr. The Ihh mRNA level was increased 3.9-fold (calculated by
real time PCR) increased by BMP7 stimulation, similar to the induction of Smad6 mRNA (Fig. 4), which was shown to be induced by BMP7 (13) (Stewart et al., unpublished observation). Blocking protein synthesis with cyclohexamide abolished both the accumulation of Smad6 protein upon BMP7 stimulation and the expression of p21\(^{\text{Waf1/Cip1}}\), which is known to have a half-life of approximately 60 min (Fig. 4, WB) (19). Cyclohexamide had no effect on the upregulation of mRNA for both \(\text{Ihh}\) and \(\text{Smad6}\) by BMP7 (Fig. 4, RT-PCR). These results confirm that \(\text{Ihh}\) is regulated by Smad4 at the transcriptional level.

**Discussion**

Using a ChIP-based cloning method, we found that the \(\text{Ihh}\) gene is a direct target of the BMP-Smad signaling pathway in the mouse embryonic carcinoma, P19 cell line. The promoter region of mouse \(\text{Ihh}\) gene contains multiple GC-rich motifs, which bind to Smad4, and are responsible for BMP-dependent transcriptional activation of the gene.

**Ihh gene as BMP target**

BMP is first identified as an activity that induces ectopic cartilage and endochondral bone when implanted in experimental animals(1). Several members of the BMP family including BMP 2, 3, 4, 5 and 7 and their receptors are expressed in developing cartilage and were predicted to serve as key regulators of endochondral bone formation. The exact role of BMPs in endochondral bone formation, however, is poorly understood. Several lines of evidence suggested that BMP signaling pathways have at least two distinct roles during endochondral bone formation; i) cell fate determination and ii) regulation of the overall speed of cartilage development. \(\text{Ihh}\), a member of the conserved
hedgehog family of signaling molecule, is known to regulate several aspects of bone development. During mouse embryonic development, Ihh expression is first detected at embryonic stage 11.5 among chondrocytes at a stage of early cartilaginous condensation. Ihh knockout mice revealed severe skeletal defects, which include short limb bones at birth(20). Further analysis revealed that this is due to a decreased rate of chondrocyte proliferation, suggesting two possible roles of Ihh in the regulation of chondrocyte differentiation; i) upregulating parathyroid hormone-related protein (PTHrP), a secreted peptide that regulates chondrocyte differentiation, and ii) control of chondrocyte proliferation. An important question in the mechanism of bone development is the relationship between BMPs and Ihh/PTHrP signaling pathways. It has been proposed that BMPs might serve as secondary signals of Ihh because the expression pattern of BMPs flanks the Ihh expression domain. Recently, Vortkamp’s group reported that BMP signaling modulates the expression level of Ihh, thereby integrating the regulation of chondrocyte proliferation and the onset of hypertrophic differentiation during bone formation(21,22). Our finding, that Ihh as a target of BMP signaling, adds a molecular explanation of how BMP modulates chondrocyte growth and differentiation through modifying Ihh expression.

**GC-rich motif as a conserved Smad-binding sequence between the Drosophila Dpp signal and mammalian BMP signal**

The observation that Smads can directly bind to DNA and modulate its transcription was first reported by studying the BMP ortholog Drosophila Dpp responsive genes, such as vestigial, labial, and ultrabithorax (14). The Drosophila Smad homologues, Mad and Medea, were shown to bind to a GC-rich consensus binding sequence, GCCGNCGC (Mad box). We identified multiple Mad box-like, GC-rich sequences in the promoter region of mlhh, which are essential and sufficient for the BMP response and Smad binding. Previously, Mad box-like sequences were found in the promoter region of
mammalian BMP target genes, *Id1* and *Smad6*(13,15,16). GGCGCC (GC-pal) is another GC-rich motif important for BMP-responsiveness first reported in the mammalian *Id1* gene promoter(15,16). The Ihh promoter region also contains a GC-pal motif, which is critical for the BMP-responsiveness of Ihh. Thus the GC-pal motif is a common motif among BMP target genes. Interestingly, the more common Smad binding element (SBE) does not play a significant role in regulation of *Ihh* by BMP because deletion of the SBE had no effect on BMP response (Fig. 3A, SBE).

**Mechanism to sensor the gradient of BMPs**

BMP signaling regulates gene expression to exert a wide range of biological activities. Two factors have been proposed to regulate the temporal and spatial-specific cell responses to BMP signaling. One is the competency for BMP signaling, conferred by cell-type specific transcriptional partners of Smad proteins(10) and the other is the intensity of the signal, that is, the gradient of BMP as a morphogen. Each cell senses gradients of morphogens and integrates the information into its responses, such as regulation of gene expression. Multiple GC-rich motifs have been reported in the promoters of the *Drosophila* *tinman* and *zen* genes and it has been proposed that the multiple Mad boxes in the promoters of target genes translate the BMP gradient into a gene expression pattern(8,9). Eight and six Mad-binding motifs are present in a 448-bp promoter region of *zen* and a 230-bp enhancer region of the *tinman* gene, respectively. All the Mad-binding motifs in the *zen* promoter are indispensable for its expression in full and the correct pattern at the mid-late cellularisation stage(9). The *Ihh* promoter contains multiple Mad box-like sequences, which has a cumulative effect on the BMP-response. The structural resemblance between the mouse BMP-target gene Ihh, and *Drosophila* Dpp-target genes, *zen* and *tinman* suggest that the promoter structure of these genes is conserved during evolution in order for genes to respond to different concentrations of BMPs in vivo.
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References


Figure Legend

Fig. 1 The genomic fragment 2HC8 is located in the promoter of the Ihh gene.

A. Genomic fragment 2HC8-luciferase construct is positively regulated by BMP7. Four independent genomic fragments, which contain putative Smad binding element(s), were inserted upstream of the luciferase gene. P19 cells transfected with these reporter constructs were treated with (black bars) or without (gray bars) 100 ng/ml BMP7 for 18 hr prior to analysis for luciferase activity. Only the 2HC8-luc reporter construct responded to BMP7 treatment. B. Induction of 2HC8 is specific for the BMP7 signaling pathway. The 430-bp genomic fragment 2HC8-luciferase construct was cotransfected with increasing amounts (500, 1000, 1500 ng/well) of expression vectors of Smad1 and Smad4, or Smad2 and Smad4. The total amount of DNA transfected into the cells was kept constant by addition of the appropriate amounts of empty vector. To examine Smad4 protein expression, part of the cell lysates used for the luciferase assay was examined by Western blot using anti-Smad4 polyclonal antibodies. C. Mapping of the genomic fragment (2HC8) and sequence conservation between mouse and human. The 2HC8 sequence was mapped to the complete sequence of the mouse genome, and was found to reside within a CpG island.

Fig. 2. A 163-bp region of 2HC8 is necessary and sufficient for BMP-dependent activation.

A. Mapping of the minimal BMP response region of 2HC8. A series of deletion mutants was transfected into P19 cells followed by luciferase assay. The result of a luciferase
assay is shown in the right panel. Fold-induction of the luciferase activity by BMP7 stimulation is indicated with +/- under “BMP response” column in the left panel. A 163-bp fragment (-285/-123-Luc) retains the full activity of 2HC8; any further deletion from either end of the fragment reduces the activity. B. Sequence comparison of the 163-bp region of the mouse (upper lanes) and human (lower lanes) Ihh genes. Nucleotides not conserved between the two species are indicated in bold. Multiple recognizable functional elements (six degenerate GCCGNCGC motifs (GC box), one GGCGCC palindromic motif (GC-pal) and one SBE box) are all conserved. C. Six GC box-like sequences are present within a 163-bp region of the Ihh promoter. GC-boxes 4 and 5 overlap on complementary DNA strands.

**Fig. 3 Multiple GC-rich motifs contribute additively to the BMP responsiveness of the Ihh promoter.**

A. Mutations were introduced into highly conserved nucleotides within each GC box or GC-pal motif, individually or in combination. BMP induction for each mutant construct is expressed as percentage of the response of the wild type (WT) 2HC8-163-luc construct. In the GC-5<sup>dbl</sup>-mut reporter, mutations were introduced into both strands of the Mad box-like sequence. B. GC-box 1-6 and GC-pal motifs are sufficient for the BMP response. A reporter construct containing two copies of the 163-bp promoter fragment, either wild type (GCx2(WT)-luc) or mutated (GCx2(MUT)-luc), was transfected into P19 cells alone or together with vectors expressing Smad1, Smad4 and constitutively active BMP receptor type IB. Lane 4, 9: control cotransfection with Smad2 and Smad4 vectors. Lane 5, 10: control cotransfection with Smad2, Smad4 and constitutively active TGF-β receptors.
receptor type I vectors. To examine Smad4 protein expression, part of the cell lysates used for the luciferase assay was examined by Western blot using anti-Smad4 polyclonal antibodies. C. Smad4 binds to the GC-rich region of the endogenous Ihh promoter upon BMP7 stimulation. Genomic DNA was isolated from P19 cells after BMP7 treatment for 2 hr. Genomic DNA samples before and after sonication were separated on an agarose gel and were visualized by ethidium bromide staining (Left panel). Sonicated genomic DNA fragments were then immunoprecipitated with anti-Smad4 polyclonal antibodies or rabbit non-specific IgG (negative control). Precipitated genomic DNA fragments were amplified using pairs of primers that cover two independent regions of the Ihh gene promoter [Ihh(GC) and Ihh(5′)]. Smad6 and HPRT gene promoters were examined as positive and negative control for binding to Smad4, respectively (Right panel). D. Quantitative real-time PCR analysis of enrichment of Smad4 to the GC-rich motifs of the Ihh promoter. The Y axis represents the relative content of the specific region of the gene promoter shown in the X axis compared with input samples. E. Smad4 binds to the GC-rich motifs of the Ihh promoter upon BMP7 stimulation. Two biotinylated 163-bp DNA oligonucleotide probes were synthesized, one corresponding to the wild type sequence, and one harboring mutations of GC-box 1, 2, 3, and GC-pal. COS-1 cells were transfected with expression vectors of Smad1 and Smad4. A vector expressing constitutively active BMP receptor type IB (BMPRIB\textsuperscript{CA}) was cotransfected as indicated. 48 hr after transfection, the cells were lysed. Cell lysates were incubated with biotinylated wild type or mutant oligos. Both total cell lysates (direct western) and the precipitated proteins (DNA pull down) were subjected to SDS-PAGE followed by immunoblot using anti-Smad4 antibodies.
Fig. 4 Induction of endogenous Ihh gene by BMP7 stimulation

P19 cells were stimulated with 100 ng/ml BMP7 for 2 hr. Control samples were treated with cyclohexamide for 30 min prior to BMP7 treatment. Total RNA was extracted and subjected to RT-PCR using primers of Ihh, Smad6, and G3PDH. Smad6 is the BMP7-induced control. G3PDH is the loading control. Smad6 and p21Waf1/Cip1 protein expression were examined by immunoblotting total cell lysates prepared from the same samples.
Seki et al. Fig. 2
Fig. 3A & B Seki et al.
Fig. 3C-E Seki et al.
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<th>Cyclohexamide</th>
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<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP7</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**RT-PCR**
- **Ihh**
- **Smad6**
- **G3PDH**

**WB**
- **Smad6**
- **p21/Waf1**

Fig. 4 Seki et al.
Indian hedgehog gene is a target of the bone morphogenetic protein signaling pathway
Kenji Seki and Akiko Hata

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