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Smad9 is a new type of transcriptional regulator in bone morphogenetic protein signaling

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Smad1, Smad5 and Smad9 (also known as Smad8) are activated by phosphorylation by bone morphogenetic protein (BMP)-bound type I receptor kinases. We examined the role of Smad1, Smad5, and Smad9 by creating constitutively active forms (Smad^{DVD}). Transcriptional activity of Smad9^{DVD} was lower than that of Smad1^{DVD} or Smad5^{DVD}, even though all three Smad^{DVD}s associated with Smad4 and bound to the target DNA. The linker region of Smad9 was sufficient to reduce transcriptional activity. *Smad9* expression was increased by the activation of BMP signaling, similar to that of inhibitory Smads (I-Smads), and Smad9 reduced BMP activity. In contrast to I-Smads, however, Smad9 did not inhibit the type I receptor kinase and suppressed the constitutively active Smad1^{DVD}. Smad9 formed complexes with Smad1 and bound to DNA but suppressed the transcription of the target gene. Taken together, our findings suggest that Smad9 is a new type of transcriptional regulator in BMP signaling.

B one morphogenetic proteins (BMPs) are the largest subfamily of the transforming growth factor-β (TGF-β) family, which also includes growth and differentiation factors (GDFs) and activin. These proteins have multiple functions that are associated with growth, differentiation and cell death during embryonic development[1,2]. The implantation of demineralized bone matrix or BMPs in skeletal muscle induces heterotopic bone formation in vertebrates[3]. This osteogenic activity is found in some BMPs and GDFs but not in TGF-βs, activin or other growth factors[4,5]. The in vitro treatment of myoblasts with osteogenic BMPs inhibits myogenic maturation and induces the differentiation of myoblasts into cells expressing typical markers of mature osteoblasts, including alkaline phosphatase (ALP), parathyroid hormone receptor and osteocalcin[6]. In contrast, non-osteogenic members of the TGF-β family do not induce these phenotypic osteoblast markers in myoblast cultures in vitro, although non-osteogenic family members inhibit myogenesis[6]. Thus, osteogenic BMPs activate specific intracellular signaling pathways in target cells.

The intracellular signaling of TGF-β family members, including BMPs, is transduced by two types of transmembrane serine/threonine receptor kinases[7,8]. Type II receptors phosphorylate the intracellular domain of type I receptors to activate receptor kinases, thereby activating downstream signaling pathways[7,8]. Substitution mutations at the phosphorylation sites of type I receptors constitutively activate receptor kinases[9]. The overexpression of a constitutively activated form of type I BMP receptors (such as ALK2, BMPR-IA and BMPR-IB) induces the osteoblastic differentiation of myoblasts in cell culture in the absence of BMPs[10,11]. Fibrodysplasia ossificans progressiva (FOP) is a rare genetic disorder that is characterized by heterotopic bone formation in skeletal muscle similar to the effects of BMP implantation[12,13]. Our group and others have demonstrated that the ALK2 mutations in patients with FOP are natural gain-of-function mutants of type I BMP receptors[14–17]. A kinase inhibitor of BMP type I receptors, LDN-193189, suppresses artificial bone formation in soft tissues in mice expressing constitutively activated ALK2[18]. These findings suggest that the substrates and downstream effectors of BMP type I receptors are critical for the bone-inducing activity of BMPs.

Smad proteins (Smads 1 through 9) are transcriptional regulators that are important for intracellular TGF-β family signaling[19–21]. Smads are classified into 3 subgroups (R-Smad, Co-Smad and I-Smad) based on their

structures and biochemical functions[20,22–24]. Smads have conserved N-terminal MH1 and C-terminal MH2 domains that are connected by a linker region[25]. Type I receptors phosphorylate R-Smads at the C-terminal serine-valine-serine (SVS) or serine-methionine-serine (SMS) motifs of the MH2 domains[26]. Osteogenic BMPs induce the phosphorylation of Smad1, Smad5, and Smad9 (also known as Smad8), whereas, non-osteogenic members of the TGF-β family induce the phosphorylation of Smad2 and Smad3[20,27]. I-Smads, Smad6 and Smad7, bind to type I receptors and inhibit receptor kinase activity[28–31]. The expression of I-Smad mRNAs is increased in response to ligand stimulation, indicating that these mRNAs form negative feedback loops in the intracellular signaling among the TGF-β family[32]. Smad6 has been shown to interact with co-repressors, such as Hoxc-8 and CtBP, to inhibit BMP induced transcription activity[33,34]. Phosphorylated R-Smads form complexes with Smad4, a Co-Smad, and bind to the responsive element to regulate the transcription of target genes[20]. The *Id1*, *Id2* and *Id3* genes are typical early-response genes in BMP signaling and have a conserved GGCGCC sequence in their 5′ upstream BMP-responsive elements (BREs) that is required for Smad binding[35–40]. We identified a novel BMP-responsive non-coding RNA called BMP-inducible transcript-1 (*Bit-1*) by utilizing the conserved BMP-responsive elements among the *Id* genes, suggesting that the GGCGCC sequence is important for the BMP-induced transcription[40].

The specific role of each R-Smad on BMP activity remains unclear because the activation of a type I receptor simultaneously activates all the Smad and/or non-Smad signaling pathways[24,41]. We reported a constitutively active mutant of Smad1, Smad1[DVD], in which the C-terminal SVS motif is mutated to an aspartic acid-valine-aspartic acid (DVD) sequence[42]. Smad1[DVD] is recognized by an antibody that is specific for phosphorylated Smad1, Smad5 and Smad9 and activates BMP-induced biological events in vivo and in vitro in the absence of BMPs[42]. This constitutively active mutation allowed us to examine the specific role of Smad1 on BMP activity in the absence of the activation of the other signaling pathways. We used this strategy to examine the influence of Smad5 and Smad9 on BMP activity. We found that Smad9 was less active than Smad1 and Smad5 and that this lower activity depended on a deletion and a unique sequence in the linker region of Smad9. Moreover, Smad9 inhibited BMP signaling in cell culture similar to I-Smads. However, in contrast to I-Smads, Smad9 did not inhibit the phosphorylation of R-Smads by type I receptors. Smad9 formed complexes with BMP-regulated R-Smads and bound to the BRE but suppressed the transcriptional activity in a dominant negative manner. Thus, the inhibition of transcription is a previously uncharacterized role for Smad9 in BMP signaling.

Results

Construction and characterization of the constitutively active forms of R-Smads. We reported that the overexpression of Smad1[DVD] under the control of the cytomegalovirus (CMV) promoter in the pFLAG-CMV2 vector induces the ligand-independent activation of BMP signaling, such as the induction of alkaline phosphatase (ALP) activity in C2C12 mouse myoblast cells[42]. The abundance of FLAG-Smad1[DVD] and ALP activity were greater in the C2C12 cells that were transfected with Smad1[DVD] in the pcDEF3 vector, which contains an elongation factor-1 promoter, compared to those transfected with pFLAG-CMV2 (Supplemental Fig. 1A and 1B). This increased signal-to-noise ratio enabled the identification of potentially weak activation. Therefore, we used the pcDEF3 vector.

We examined the effects of inhibitors on the BMP activity that is induced by a constitutively active BMPR-IA receptor or Smad1[DVD] in C2C12 cells. In contrast to the constitutively active BMPR-IA, the activity of Smad1[DVD] was almost unchanged, even in the presence of

receptor inhibitors, including LDN-193189 (Supplemental Fig. 1C) and Smad6 (Supplemental Fig. 1D). However, the overexpression of a major component of NF-κB, p65, which inhibits the DNA binding of the Smad complex to a BRE[43], suppressed the activity of the constitutively active BMPR-IA and Smad1[DVD] (Supplemental Fig. 1E). These data confirm our previous finding that Smad1[DVD] acts as a constitutively active Smad1 in BMP signaling[42].

To determine the role of each R-Smad in BMP signaling, we introduced serine-to-aspartate mutations in the residues that were homologous to the C-terminal SVS motif in Smad1 in other R-Smads, including Smad5, Smad9, Smad2 and Smad3. The overexpression of Smad1[DVD], Smad5[DVD], and Smad9[DVD], but not Smad2[DMD] or Smad3[DVD], in C2C12 cells increased the activity of two independent BMP-specific luciferase reporters, Id1WT4F-luc (Fig. 1A) and BIT1WT4F-luc (Fig. 1B), relative to that of the cells that were transfected with the empty vector or the corresponding wild-type Smad. The overexpression of the DVD forms of Smad1, Smad5, and Smad9 induced a typical marker of osteoblastic differentiation of C2C12 cells, ALP activity (Fig. 1C). These activities were lower with Smad9[DVD] than with Smad1[DVD] or Smad5[DVD], even under similar abundance conditions (Supplemental Fig. 2). As we previously reported for Smad1[DVD] [42], a specific antibody against phosphorylated Smad1, Smad5 and Smad9 recognized Smad1[DVD], Smad5[DVD] and Smad9[DVD] (Fig. 1D), suggesting that the C-terminal structures of these proteins mimic the conformational changes in the Smad proteins that were activated through phosphorylation.

Transcriptional activity of Smad9 is weaker than that of Smad1 and Smad5 despite having a greater DNA-binding activity. Because Smad9 showed lower activities than Smad1 or Smad5, we examined the biochemical characteristics of Smad9. The abundance of Smad9 was lower than that of Smad1 (Fig. 2A, bottom panel). However, more endogenous Smad4 was co-immunoprecipitated with FLAG-Smad9 than with FLAG-Smad1 from lysates of human embryonic kidney (HEK) 293 cells that were exposed to BMP-4 (Fig. 2A and 2B). FLAG-Smad9[DVD] coprecipitated more endogenous Smad4 than did FLAG-Smad1[DVD] and FLAG-Smad5[DVD] (Fig. 2C). A specific antibody against Smad4 coprecipitated more endogenous Smad9 than endogenous Smad1 and Smad5 (Fig. 2E). The co-expression of Myc-Smad4 increased the transcriptional activity of Smad1[DVD], Smad5[DVD], and Smad9[DVD], but that of Smad9[DVD] was significantly lower (Fig. 2D). We further examined the DNA-binding capacity of Smad9 in a DNA pull-down assay using a BRE in the *Id1* gene. Although the abundance of FLAG-Smad9 was less than that of FLAG-Smad1, more Smad9 than Smad1 bound to the BRE (Fig. 2F). Endogenous Smad9 also bound to the BRE in response to BMP-4 stimulation (Fig. 2G). The binding of both endogenous and FLAG-tagged Smad9 and Smad1 to the BRE was sequence-specific, as these proteins did not bind to the mutant sequence in which the Smad-binding GGCGCC core was substituted with TTTGCC (Fig. 2F and 2G).

The linker region of Smad9 suppresses the transcriptional activity of Smads. We constructed a series of chimeric Smad proteins in which the MH1 domain, linker region and MH2 domain of one Smad protein was replaced with those of another Smad protein. The overexpression of the chimeric Smad proteins in which the MH1 (Smad9-1-1[DVD]) or MH2 (Smad1-1-9[DVD]) domains in Smad1 were replaced with those from Smad9 resulted in an equivalent or increased activation of the Id1WT4F-luc reporter compared to that of Smad1[DVD] (Fig. 3A and 3B), suggesting that the MH1 domain of Smad9 has a higher DNA-binding affinity than that of Smad1. In contrast, Smad1-9-1[DVD], which had the Smad9 linker region in Smad1[DVD], exhibited a significantly reduced ability to activate the reporter, similar to Smad9[DVD] (Fig. 3A and 3B). We generated similar results with the overexpression of the chimeric Smads that were created using the domains of Smad5[DVD] and Smad9[DVD] (Fig. 3C
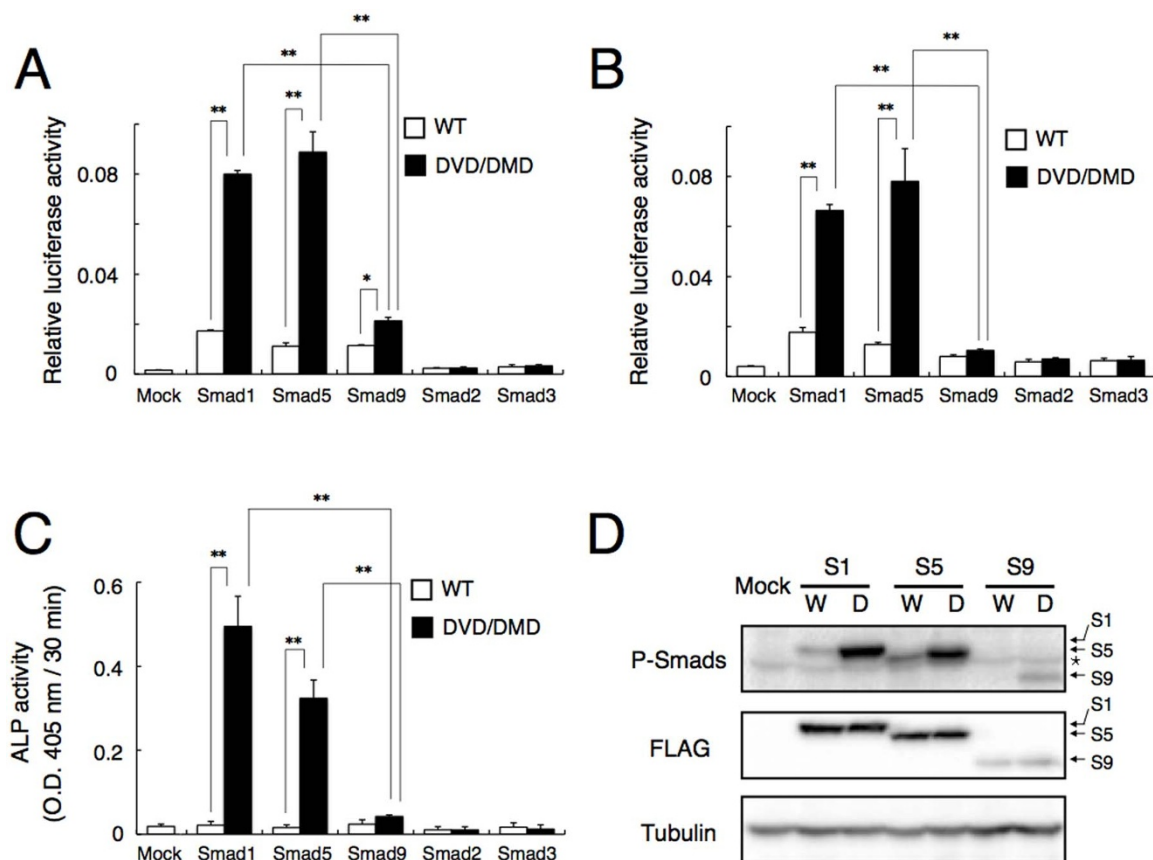
Figure 1 | Biological activities of the wild-type and DVD/DMD forms of R-Smads. (A–C) The BMP-specific luciferase reporter activity using Id1WT4F-luc (A) and BIT1-WT4F-luc (B) reporters and the ALP activity (C) were determined in C2C12 cells transiently overexpressing the wild-type (open bars) and DVD/DMD form (solid bars) of each R-Smad. The data are expressed as the mean $\pm$ SD (N = 3). *, $p < 0.05$; **, $p < 0.01$. (D) Western blot analysis of the whole-cell extracts that were prepared from the C2C12 cells that were transfected with the wild-type (W) and DVD forms (D) of Smad1 (S1), Smad5 (S5) and Smad9 (S9). The asterisks indicate non-specific bands.

and 3D), suggesting that the linker region of Smad9 suppressed its ability to activate BMP signaling.

Although Smad9, Smad1 and Smad5 share greater than 91% amino acid sequence similarity in the MH1 and MH2 domains, the linker regions share less than 40% similarity, including a deletion at the C-terminal portion of the linker in Smad9 compared to Smad1 and Smad5 (Supplemental Fig. 3). Thus, we inserted the sequence from this region of Smad1 [Smad9^{DVD} + S1L] or Smad5 [Smad9^{DVD} + S5L] into Smad9^{DVD} and found that the overexpression of either construct induced ALP activity (Fig. 3E and 3F) and activated the BMP reporter (Supplemental Fig. 4) to a similar degree as Smad1^{DVD} and Smad5^{DVD}. The PPAY motif in the linker region of Smad1 is recognized by E3 ubiquitin ligases, and this motif is not present in Smad9[44]. However, Smad9^{DVD} + S1L(AAAY), in which the PPAY motif has been substituted with the sequence AAAY, exhibited similar activity to Smad9^{DVD} + S1L (Fig. 3E and 3F), suggesting that the PPAY motif is not involved in Smad9 activity.

Smad9 mRNA is increased by BMP-4 stimulation similar to Smad6 mRNA. Transcription-dependent feedback loops are often involved in the regulation of signal transduction pathways, including BMP signaling[32]. We found that the expression of *Smad9* increased in C2C12 myoblasts, C3H10T1/2 fibroblasts, MC3T3-E1 osteoblastic cells, 3T3-L1 adipocytes, and primary calvarial osteoblasts that were exposed to BMP-4 (Fig. 4A). The expression of *Smad9* mRNA, similar to *Smad6* but not *Smad1*, *Smad5*, or *Smad4*, increased within one hour of BMP-4 stimulation in C2C12 cells (Fig. 4B). This increase was blocked by adding LDN-193189, a kinase inhibitor of BMP receptors, to the cell culture medium (Fig. 4C). In contrast, *Smad7*

but not *Smad9* increased in the cells that were exposed to TGF-β1 (Fig. 4D), suggesting that *Smad9* is induced by BMP-specific intracellular signaling. A BMP-induced increase in Smad9 protein levels was observed in C3H10T1/2 cells 24 h after stimulation (Fig. 4E). Furthermore, the increase in *Smad9* mRNA levels was not blocked by pretreatment with cycloheximide, suggesting that *Smad9* is an early response gene in the BMP-Smad axis (Supplemental Fig. 5). In addition, the abundance of *Smad9* mRNA sharply decreased between E7.0 and E11.0 of developing mouse embryos, similar to *Smad6* and *Smad7* but not *Smad1*, *Smad5* or *Smad4* (Fig. 4F).

Smad9 inhibits BMP signaling. Our data suggest that Smad9 may act as an I-Smad similar to Smad6 and Smad7. Therefore, we tested whether the overexpression of wild-type Smad9 could inhibit the upstream activation of BMP signaling. As expected, the overexpression of Smad6 or Smad7 inhibited the BMP-specific Id1WT4F-luc activity that was induced by BMP-4 stimulation (Fig. 5A) or by the overexpression of a constitutively active form of BMPR-IA (Fig. 5B). Similarly, the overexpression of Smad9 suppressed the BMP activity that was induced by BMP-4 (Fig. 5A) or the constitutively active BMPR-IA (Fig. 5B). Overexpression of Smad9 suppressed the expression of endogenous *Osterix* mRNA induced by the constitutively active BMPR-IA, and these results were similar to those observed with Smad6 (Supplemental Fig. 6).

We examined the effect of siRNA knockdown of endogenous Smad9 on BMP activity. The transfection of two independent siRNAs against Smad9 in C2C12 cells that were exposed to BMP-4 decreased *Smad9* mRNA (Fig. 5C) and increased ALP activity (Fig. 5D).
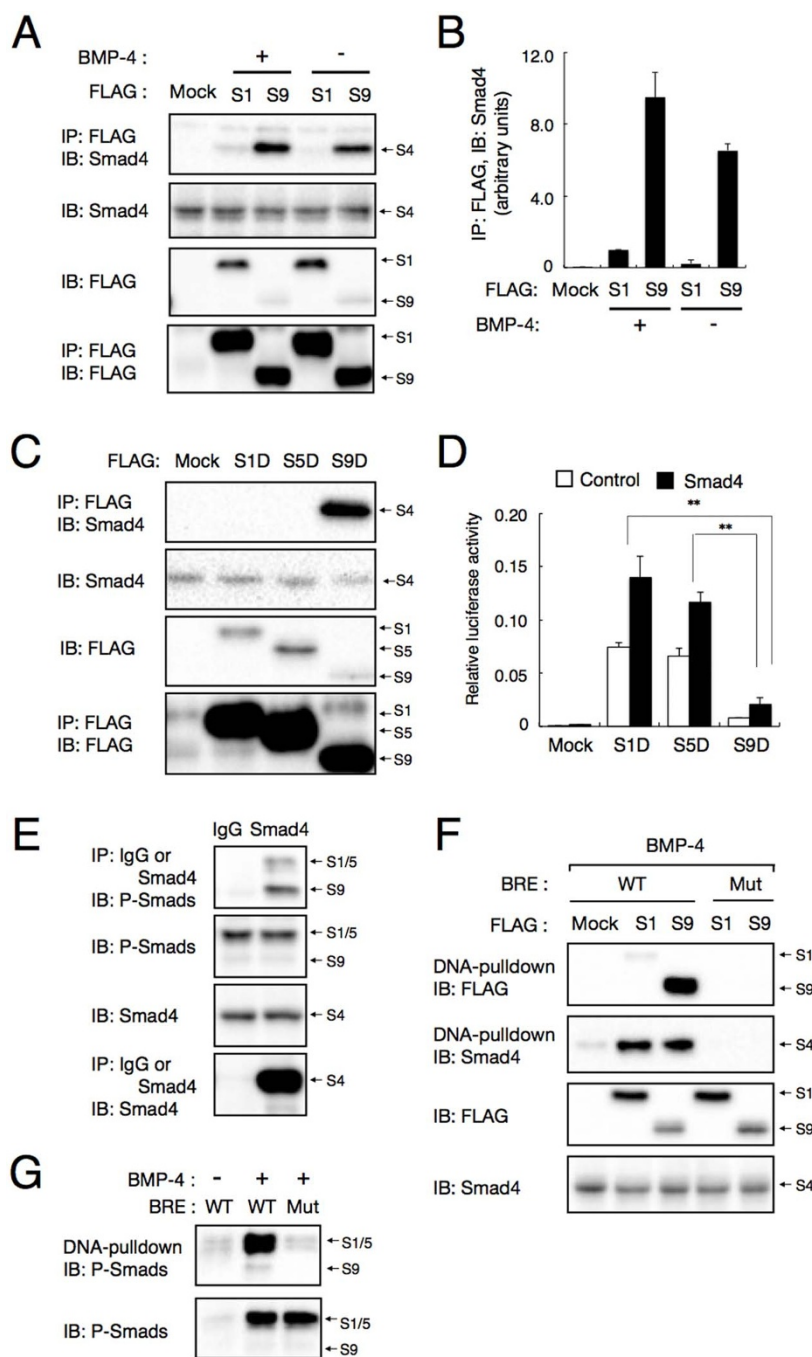
Figure 2 | Smad9 forms a complex with Smad4 and binds to the target DNA. (A and B) Co-immunoprecipitation of FLAG-Smad1 and FLAG-Smad9 with endogenous Smad4. FLAG-Smad1 and FLAG-Smad9 were transiently overexpressed in HEK293 cells, and whole-cell extracts were prepared from the cells that were stimulated with or without 50 ng/ml BMP-4 for 1 h. The precipitates obtained with an antibody against the FLAG tag were analyzed by Western blot analysis using an antibody against Smad4. The arrows indicate the positions of Smad1 (S1), Smad9 (S9) and Smad4 (S4). The data are representative of N = 4 blots. The blots in A are quantified in B. (C) Co-immunoprecipitation of endogenous Smad4 with FLAG-Smad1^{DVD}, FLAG-Smad5^{DVD} and FLAG-Smad9^{DVD}. The precipitates obtained with an antibody against the FLAG tag were analyzed by Western blot analysis using an antibody against Smad4. The arrows indicate the positions of Smad1 (S1), Smad5 (S5), Smad9 (S9) and Smad4 (S4). (D) BMP-specific luciferase assay in C2C12 cells co-expressing FLAG-tagged Smad1^{DVD}, Smad5^{DVD} and Smad9^{DVD} in the absence (open bars) or presence (solid bars) of Myc-Smad4. The data are expressed as the mean + SD (N = 3). **, $p < 0.01$. (E) Co-immunoprecipitation of endogenous Smad4 with endogenous Smad1, Smad5 and Smad9. The precipitates obtained with an antibody against Smad4 were analyzed by Western blot analysis using an antibody against phosphorylated Smad1, Smad5 and Smad9. The arrows indicate the positions of Smad1 and Smad5 (S1/5), Smad9 (S9) and Smad4 (S4). (F) DNA pull-down assay of Smad1 and Smad9. Whole-cell extracts were prepared from BMP-4-stimulated (50 ng/ml for 1 h) HEK293 cells overexpressing FLAG-Smad1 (S1) or FLAG-Smad9 (S9). The extracts that were incubated with biotinylated wild-type (WT) and mutant (Mut) BRE were analyzed by Western blot using antibodies against the FLAG tag and Smad4. (G) DNA pull-down assay of endogenous Smad1, Smad5 and Smad9. Whole-cell extracts were prepared from BMP-4-stimulated (50 ng/ml for 1 h) 10T1/2 cells. The extracts that were incubated with a biotinylated wild-type (WT) and mutant (Mut) BRE were analyzed by Western blot using an antibody against phosphorylated Smad1, Smad5 and Smad9.
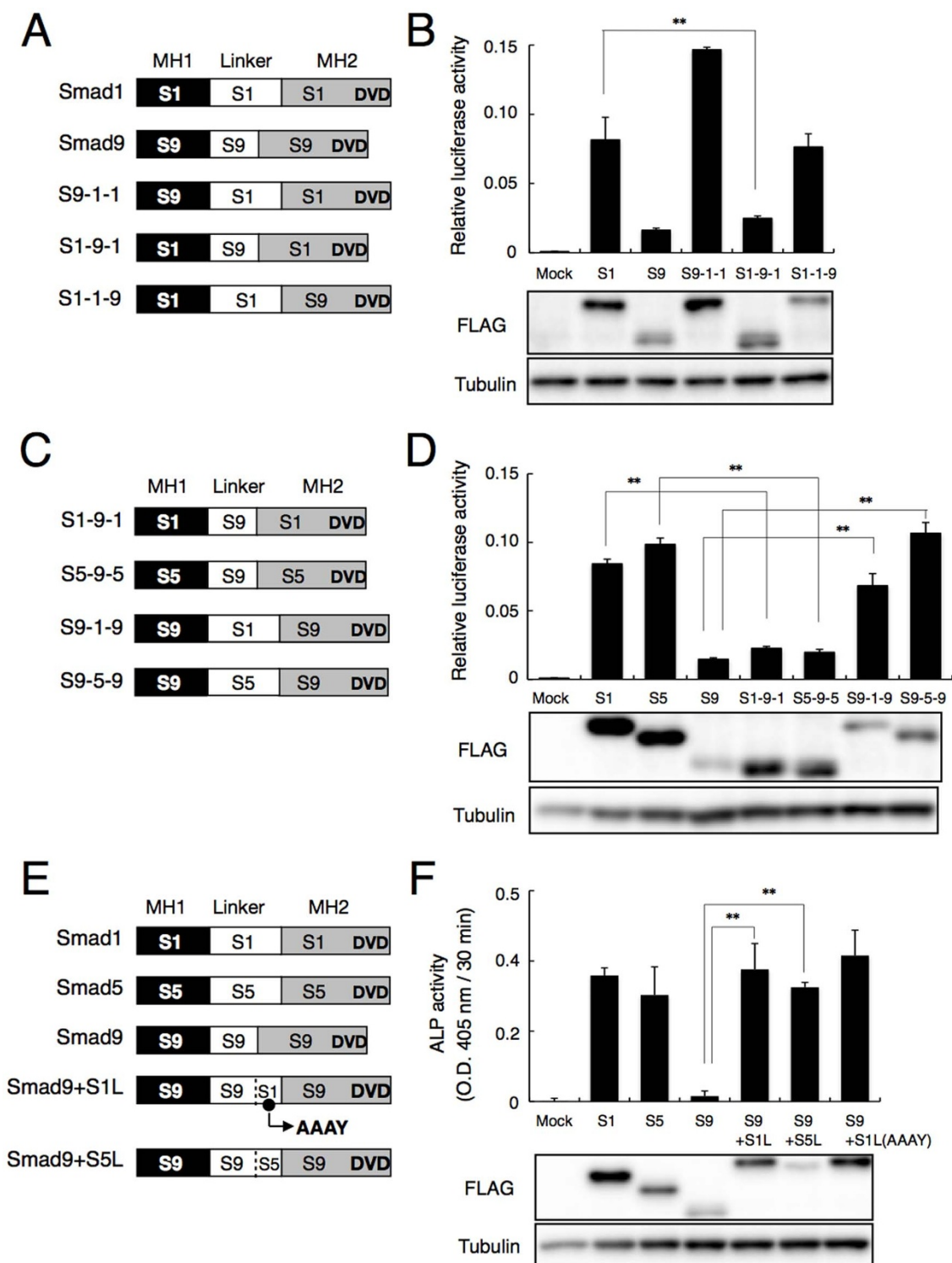
Figure 3 | The linker regions of Smad1, Smad5 and Smad9 regulate transcriptional activity. Transcriptional activity analysis of chimeric Smad^{DVD} in C2C12 cells. Chimeras were constructed by replacing the MH1, linker or MH2 domains between the DVD forms of Smad1 and Smad9 (A and B), as well as by replacing the linker regions between the DVD forms of Smad1, Smad5 and Smad9 (C and D). Id1WT4F-luc was co-transfected with each chimeric Smad in C2C12 cells. The data are expressed as the mean ± SD (N = 3). **, $p < 0.01$. The Western blots were performed using antibodies against the FLAG tag and tubulin. (E and F) The insertion of a C-terminal region of the Smad1 or Smad5 linker to Smad9^{DVD} restored the osteoblastic differentiation-inducing activity in C2C12 cells. The data are expressed as the mean ± SD (N = 3). **, $p < 0.01$. The Western blots were performed using antibodies against the FLAG tag and tubulin.

Smad9 functioned as an inhibitor of the BMP-specific Id1WT4F-luc activity similar to Smad6 and Smad7. In contrast to Smad6 and Smad7, however, Smad9 was phosphorylated and did not inhibit the phosphorylation of Smad1 that was induced by the constitutively active BMPR-IA receptor (Fig. 5E). These results suggest that Smad9 suppressed BMP activity via a novel molecular mechanism.
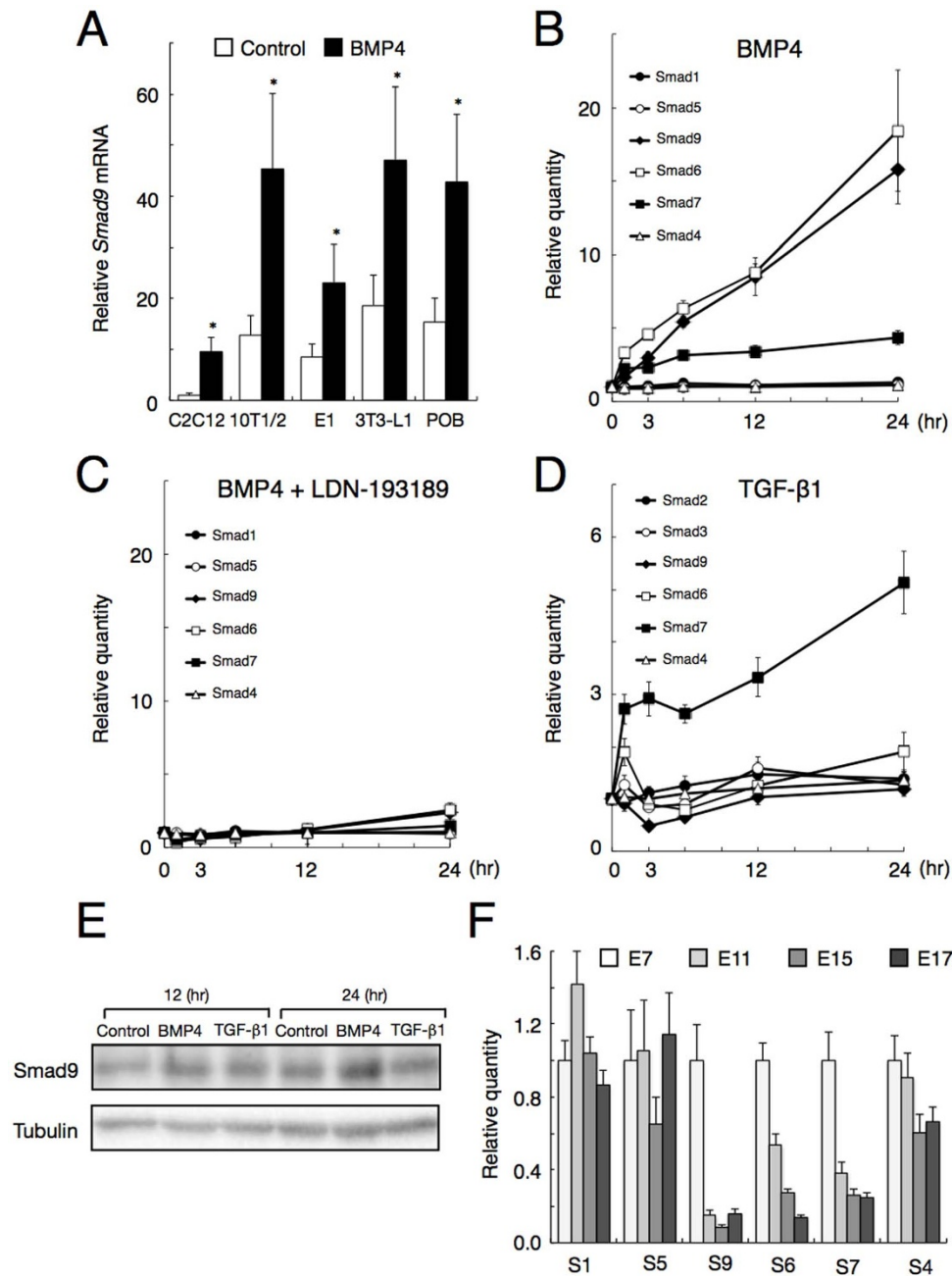
Figure 4 | The expression of *Smad9* mRNA is increased by BMP signaling. (A) *Smad9* mRNA expression was increased in various types of cells. The indicated cells were stimulated with (closed bars) or without (open bars) 50 ng/ml BMP-4 for 24 h. The data are expressed as the mean $\pm$ SD (N = 8). *, $p < 0.05$. [Wilcoxon's signed rank test] (B and C) Time course changes in the mRNA levels of *Smad1*, *Smad5*, *Smad9*, *Smad6*, *Smad7* and *Smad4* in C2C12 cells that were stimulated with 50 ng/ml BMP-4 in the absence (B) and presence (C) of 100 nM LDN-193189. The mRNA levels were determined by qRT-PCR. The data are expressed as the mean $\pm$ SD (N = 3). (D) Time course changes in the mRNA levels of *Smad2*, *Smad3*, *Smad9*, *Smad6*, *Smad7* and *Smad4* in C2C12 cells that were stimulated with 10 ng/ml TGF-β1. The data are expressed as the mean $\pm$ SD (N = 3). (E) BMP-4-induced increases in Smad9 protein. C3H10T1/2 cells were treated for 12 h and 24 h with 50 ng/ml of BMP-4 or 10 ng/ml of TGF-β1. Whole cell lysates were analyzed via Western blot using an antiserum against Smad9. (F) Changes in the mRNA levels of *Smad1*, *Smad5*, *Smad9*, *Smad6* *Smad7* and *Smad4* during mouse development. The total RNA of whole mouse embryos at E7, E11, E15 and E17 was analyzed by qRT-PCR. The data are expressed as the mean $\pm$ SD (N = 3).

Smad9 acts as a dominant negative Smad for Smad1. We examined the effects of Smad9 on the BMP activity that was induced by Smad1^{DVD}. The overexpression of either R-Smads (Smad1 and Smad5) or I-Smads (Smad6 and Smad7) inhibited the BMP-specific Id1WT4F-luc reporter activity that was induced by Smad1^{DVD} (Fig. 6A). However, Smad9 significantly suppressed the reporter activity in the presence of Smad1^{DVD} (Fig. 6A), suggesting

that Smad9 functions downstream of the phosphorylation of R-Smads to inhibit BMP signaling.

To examine the molecular mechanisms of suppression by Smad9, we investigated whether Smad9 inhibited the ability of Smad1 to bind to a BRE in a DNA pull-down assay. More Smad9 bound to the BRE, even though the amount of Smad9 protein was lower than Smad1 (Fig. 6B). The amount of bound Smad9 was not decreased in Smad4-
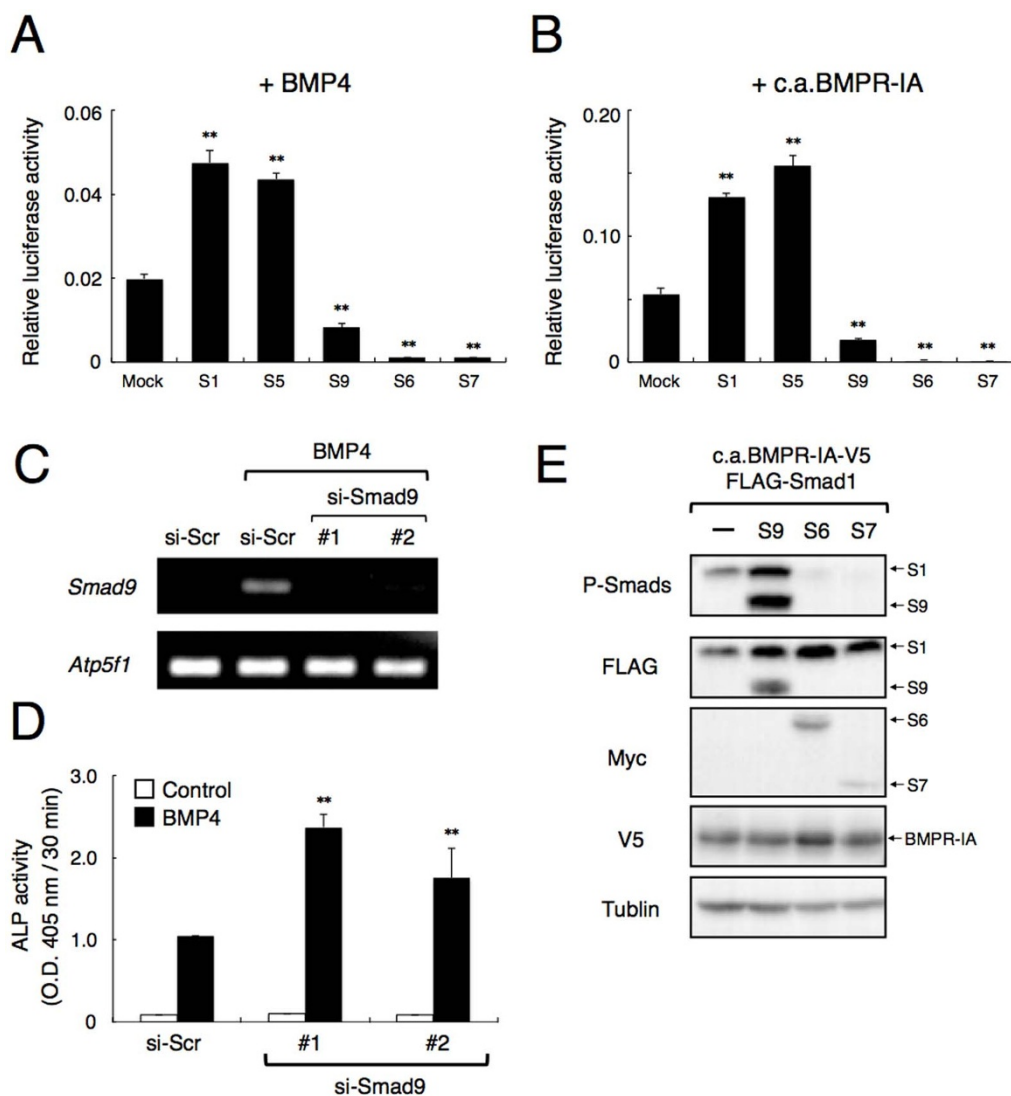
Figure 5 | Smad9 represses BMP activity without inhibiting the phosphorylation levels of Smad1. (A) The effects of Smad1 (S1), Smad5 (S5), Smad9 (S9), Smad6 (S6) and Smad7 (S7) on the BMP-specific luciferase activity that was induced by BMP-4 in C2C12 cells. The data are expressed as the mean ± SD (N = 3). **, $p < 0.01$. (B) The effects of Smad1 (S1), Smad5 (S5), Smad9 (S9), Smad6 (S6) and Smad7 (S7) on the BMP-specific luciferase activity that was induced by constitutively active BMPR-IA (c.a.BMPR-IA) in C2C12 cells. The data are expressed as the mean + SD (N = 3). **, $p < 0.01$. (C and D) Effects of scrambled (si-Scr) and Smad9 siRNAs (si-Smad9; #1 and #2) on the *Smad9* mRNA levels (C) and ALP activity (D) in the presence of 50 ng/ml BMP-4. The data are expressed as the mean ± SD (N = 3). **, $p < 0.01$. (E) Effects of Smad9 (S9), Smad6 (S6) and Smad7 (S7) on the phosphorylation levels of Smad1. The C2C12 cells were transfected with V5-tagged constitutively active BMPR-IA (c.a.BMPR-IA-V5), FLAG-tagged Smad1, FLAG-tagged Smad9, Myc-tagged Smad6 or Myc-tagged Smad7. Whole-cell lysates were analyzed by Western blot using an antibody against phosphorylated Smad1, Smad5 and Smad9 (P-Smads).

knockdown HEK293T cells, suggesting that the DNA-binding activity of Smad9 does not require Smad4 (Supplemental Fig. 7). The amounts of bound Smad9 and Smad1 were unchanged in the cells expressing both Smad9 and Smad1 simultaneously (Fig. 6B), suggesting that these proteins did not inhibit the DNA binding of each other.

We assessed whether Smad9 formed a complex with Smad1. We found that FLAG-Smad9 co-immunoprecipitated with Myc-Smad1 to a greater degree than FLAG-Smad1, even in Smad4-knockdown HEK293 cells (Fig. 6C). Endogenous Smad9 formed complexes with endogenous Smad1 or FLAG-Smad1 in BMP-4-stimulated cells (Fig. 6D). We examined the DNA-binding capacity of the Smad9-Smad1 complex. We co-immunoprecipitated FLAG-Smad9 and Myc-Smad1 from cell lysates using beads that were crosslinked with an antibody against the FLAG tag, eluted the complexes with an excess amount of FLAG peptide, and then applied the eluents to a

DNA pull-down assay using the BRE. Myc-Smad1 co-precipitated with the BRE in the presence of FLAG-Smad9 in HEK293 cells that were exposed to BMP-4 and decreased in the cells that were exposed to both BMP-4 and LDN-193189 (Fig. 6E), suggesting that the Smad9-Smad1 complex was formed and bound to the BRE in response to BMP signaling.

Discussion

In this study, we found that Smad9 is distinct from other Smads. The constitutively active form of Smad9 exhibited less activity than the BMP-regulated R-Smads, namely Smad1 and Smad5, even though more Smad9 associated with Smad4 and bound to the BRE. Moreover, we found several common characteristics among Smad9 and I-Smads, especially Smad6; 1) their transcription increased in response to BMP stimulation; 2) their mRNA levels sharply decreased during early embryonic development; 3) and they inhib-
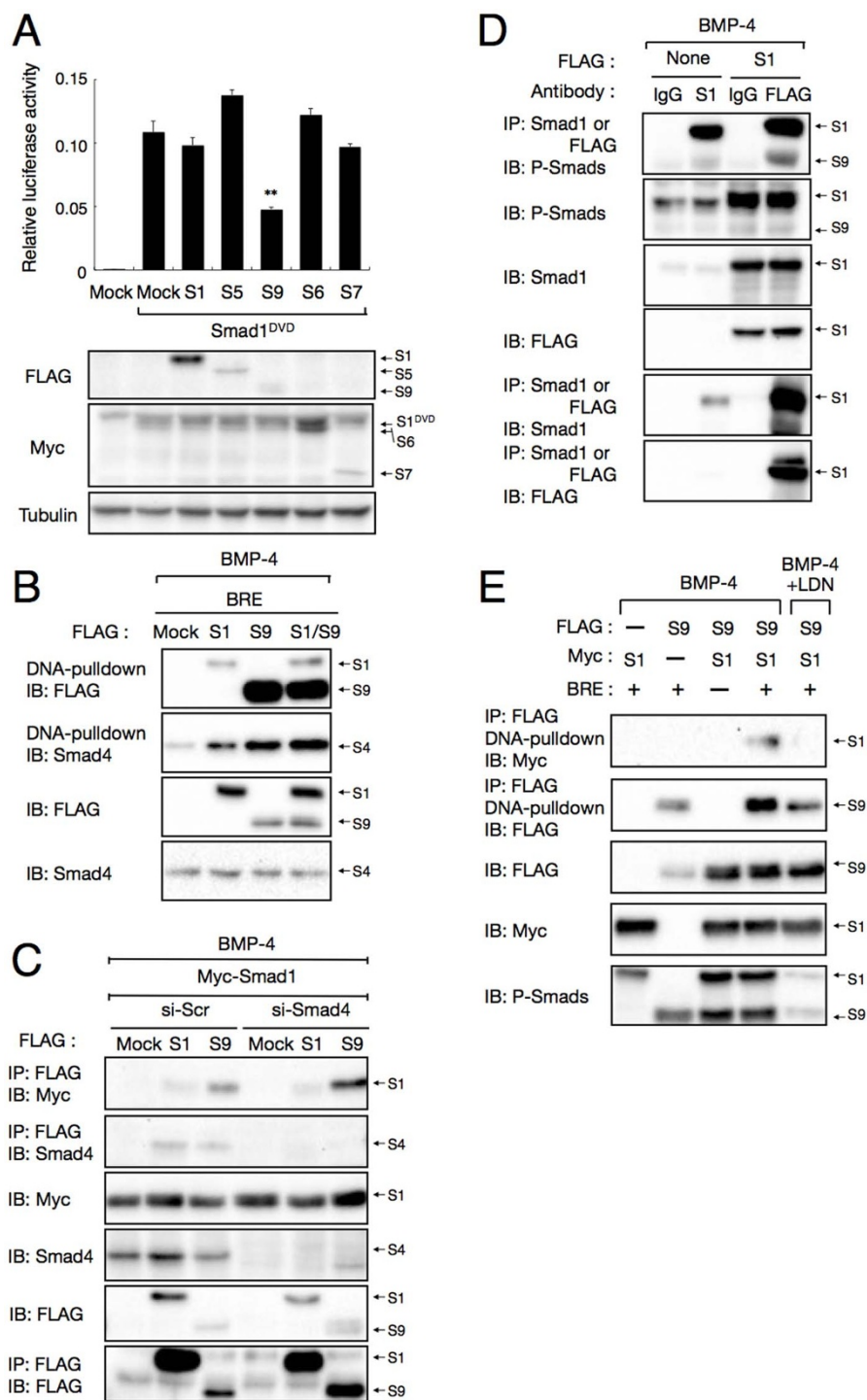
Figure 6 | Smad9 represses BMP activity by acting as a dominant negative Smad of Smad1. (A) The effects of Smad1 (S1), Smad5 (S5), Smad9 (S9), Smad6 (S6) and Smad7 (S7) on the BMP-specific Id1WT4F-luc reporter activity that was induced by Smad1^{DVD} in C2C12 cells. The data are expressed as the mean $\pm$ SD (N = 3). **, $p < 0.01$. Protein levels were analyzed via Western blots using antibodies against FLAG-tag and Myc-tag. (B) The DNA-binding activity of Smad1 and Smad9 in response to BMP-4 stimulation. HEK293 cells overexpressing FLAG-tagged Smad1 (S1) and/or Smad9 (S9) were stimulated with 50 ng/ml BMP-4 for 1 h. Whole-cell extracts were precipitated by a biotinylated BRE and analyzed by Western blot using antibodies against the FLAG tag and Smad4. (C) Interaction between Smad1 and Smad9 in HEK293 cells. Myc-tagged Smad1 was co-expressed with FLAG-tagged Smad1 (S1) or Smad9 (S9) in parental or Smad4-knockdown HEK293 cells and stimulated with BMP-4. Whole-cell lysates were analyzed via a co-immunoprecipitation assay followed by Western blot analysis. (D) Interaction between Smad1 and endogenous Smad9 in 10T1/2 cells. Whole-cell lysates that were prepared from 10T1/2 cells with or without the overexpression of FLAG-Smad1 were co-immunoprecipitated with a control IgG or a specific antibody against Smad1 or the FLAG tag, followed by Western blot analysis using an antibody against phosphorylated Smad1, Smad5 and Smad9. (E) The DNA-binding activity of the Smad1-Smad9 complex. A Smad1-Smad9 complex was prepared from HEK293 cells overexpressing Myc-tagged Smad1 and FLAG-tagged Smad9 that were stimulated with BMP-4 in the absence or presence of LDN-193189. The complex was analyzed via a DNA pull-down assay using a BRE followed by Western blot analysis using antibodies against the Myc-tag and the FLAG tag.

ited the BMP activity that was induced via type I receptors. These findings suggest that Smad9 shares biochemical characteristics with both BMP-regulated R-Smads and I-Smads. However, Smad9 and I-Smads exhibited distinct molecular mechanisms in the inhibition of BMP activity. I-Smads inhibit BMP activity by blocking the phosphorylation of R-Smads via direct interaction with type I receptors[28–31]. Smad9 inhibited BMP activity without inhibiting the phosphorylation of R-Smads by BMP type I receptors. We found that Smad9 suppressed the transcription of a target gene via a novel mechanism. Because Smad9 itself exhibited less transcriptional activity, even though it bound to the BRE, the hetero-oligomeric complexes that formed with Smad1 or Smad5 would reduce the transcriptional activity in a dominant negative manner. Because Smad9 mRNA increased within 1 h of BMP stimulation, this novel type of inhibition may act as part of the negative feedback loop to regulate the fine-tuning of BMP signaling (Fig. 7). Taken together, our findings suggest that Smad9 is a new type of transcriptional repressor of BMP signaling that shares several biochemical characteristics with both BMP-regulated R-Smads and I-Smads but still has its own unique functions (Fig. 7). Further studies are required to elucidate the physiological role of Smad9 in BMP signaling.

Establishing the constitutively activated forms of Smads allowed us to examine the molecular mechanism of the low activity of Smad9. By constructing a series of chimeric Smad proteins, we found that the lower transcriptional activity of Smad9 was due to the shorter linker region compared to the regions of Smad1 and Smad5. The linker region of Smad9 inhibited the transcriptional activity of Smad1 and Smad5. The insertion of the fragment from the C-terminal linker region of Smad1 or Smad5 restored the transcriptional activity of Smad9. To examine the function of this fragment in the linker region, we deleted the short region from the Smad1 linker and analyzed its activity. Unexpectedly, the Smad1 containing the deletion still showed transcriptional activity in the BMP-specific reporter assay using Id1WT4F-luc (Supplemental Fig. 8), suggesting that the remaining portion of the linker region of Smad9 negatively affected BMP signaling. The linker region of Smad6 interacts with CtBP, a transcriptional co-repressor[34]. Indeed, the linker region of Smad9 shares less than 40% homology with that of Smad1 and Smad5. Smad9, but not Smad1, interacts with ANA, which is a co-repressor in the TOB/BTG family that decreases the bone-inducing activity of BMP-2 in mice[45]. Because more Smad9 interacted with Smad4, Smad1 and the BRE, the short linker likely allows Smad9 to interact with other molecules, including co-activators, co-repressors, and target DNAs. The insertion of a fragment to the C-terminal region of the Smad9 linker may disrupt these interactions. Although mouse
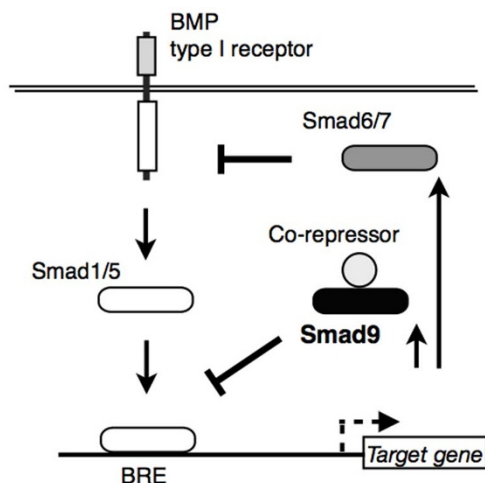
Smad9 is only a short form, the human genome has a short form and a long form of Smad9, Smad9b and Smad9a, respectively[46]. Their linker regions share less than 51% homology with that of human Smad1 (Supplemental Fig. 9). Human Smad9a^{DVD} and Smad9b^{DVD} had significantly lower transcriptional activity of Id1WT4F-luc (Supplemental Fig. 10), supporting our hypothesis that the Smad9 linker region negatively affects BMP signaling. Additional studies are needed to elucidate the molecular mechanisms of the linker-dependent regulation of the transcriptional activities of Smad1, Smad5 and Smad9.

In conclusion, we found that although Smad9 shares common structures with positive effectors of BMP signaling, namely Smad1 and Smad5, it represses intracellular BMP signaling as a dominant negative Smad. Thus, Smad9 is a new type of transcriptional regulator in BMP signaling.

Experimental Procedures

Plasmids. A constitutively active form of Smad1, Smad1^{DVD}, in pFLAG-CMV2 has been previously reported[42]. The wild-type and the DVD or DMD (only Smad2) forms of the mouse Smad1, Smad2, Smad3, Smad5, Smad9, Smad4, Smad6, Smad7 (Smad4, Smad6 and Smad7 were wild-type only), and human Smad1, Smad9a (long form), Smad9b (short form) cDNAs except for the start codon (ATG), were amplified from a cDNA pool that was prepared from C2C12 cells and qPCR Human Reference Total RNA (Clontech, Palo Alto, CA), respectively, by standard RT-PCR using reverse primers containing wild-type, DMD or the DVD-mutated sequences (Supplemental Table 1) and inserted into pcDEF3 downstream of the FLAG-tag or Myc-tag sequence[47]. Chimeric Smads with the structures that are described in the text and figures were constructed by ligating DNA fragments encoding each domain of the Smad in-frame into pcDEF3. Each Smad was divided into an MH1 domain, linker region and MH2 domain as follows (the numbers indicate the amino acid positions): Smad1, 2–132, 133–264 and 265–465; Smad5, 2–133, 134–264 and 265–465; and Smad9, 2–136, 137–227 and 228–428, respectively. The linker regions of Smad1(218–264) and Smad5(217–264) were inserted downstream of the linker of Smad9^{DVD} to obtain Smad9^{DVD} + S1L and Smad9^{DVD} + S5L, respectively. The linker region of human Smad1(218–264) was deleted in S1ΔLC^{DVD}. The final constructs were confirmed by DNA sequencing. The expression plasmids for p65 and constitutively active BMPR-IA were previously described[16,43]. For the BMP-specific luciferase assay, Id1WT4F-luc[35] or BIT1WT4F-luc[40] was co-transfected with phRL-SV40 (Promega, Madison, WI).

Cell culture, transfection, luciferase assay and ALP assay. Mouse C2C12 myoblasts were cultured as previously described[6]. The cells were inoculated at 1.5×10^4 cells/cm^2 in 96-well plates 1 day before transfection. After overnight culture, the cells were transfected with 200 ng of plasmid DNA using 0.5 μl of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, and the culture medium was changed after 2.5 h[35]. The luciferase assay was performed on day 1 using the Dual-Glo Luciferase Assay System (Promega), and the data are expressed as the luciferase activity of firefly relative to that of *Renilla*[15,35]. The ALP activity was determined on day 3 using *p*-nitrophenylphosphate as the substrate as previously described[48]. For the siRNA-mediated knockdown experiments, a scrambled control (si-Scr) (Invitrogen), two independent Smad9-specific (si-Smad9) siRNAs (#1: GGGCUUCUCUC AAAUGUAAACAGAA, and #2: GGCACAUUGGAAAGGGUG-UGGAUUU) (Invitrogen) or Smad4-specific (si-Smad4) siRNA[42] were transfected using the Lipofectamine RNAi Max reagent (Invitrogen) according to the manufacturer's instructions.

Co-immunoprecipitation, DNA pull-down and Western blot analysis. Western blot analysis was performed as previously

Figure 7 | A schema of our proposed mechanism of Smad9 action in BMP signaling.

described[16,42]. In brief, whole cell extracts were prepared using lysis buffer (10 mM Tris-HCl, pH 7.8, 1% Triton X-100 and protease inhibitor cocktail; Nacalai Tesque, Kyoto, Japan), separated using sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes.

For co-immunoprecipitation assays, the cell extracts were incubated with magnet beads that were conjugated to an antibody against the FLAG tag (Sigma-Aldrich, St. Louis, MO) for 12 h at 4°C. The FLAG-epitope-containing complex was precipitated using a magnet and eluted by adding FLAG peptide (Sigma-Aldrich).

For the DNA pull-down assays, whole cell extracts were incubated for 30 min on ice with biotinylated DNA of wild-type or mutant BMP-responsive element (BRE), which was prepared from Id1WT4F-luc or Id1MutB4F-luc[35], respectively, by standard PCR using a set of biotinylated primers[49]. The DNA-protein complex was precipitated using streptavidin-conjugated magnet beads (Dynabeads M-280 streptavidin; Invitrogen) and applied to Western blot analysis as described above.

The following antibodies were used in these experiments: mouse monoclonal antibody against the FLAG tag (clone M2, Sigma-Aldrich), mouse monoclonal antibody against Smad4 (Cell Signaling Technology, Beverly, MA), mouse monoclonal antibody against the V5-tag (clone V5005; Nacalai Tesque), rabbit polyclonal antibody against the Myc-tag (Cell Signaling Technology), rabbit polyclonal antibody against phosphorylated Smad1, Smad5 and Smad9 (Cell Signaling Technology), and rabbit polyclonal antibody against α-tubulin (Cell Signaling Technology) and rabbit normal IgG (Cell Signaling Technology). The target proteins were detected using a horseradish peroxidase-conjugated antibody against mouse or rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Chemiluminescence was detected by a ChemiDoc XRS+ System (Bio-Rad, Hercules, CA).

Rabbit antiserum against mouse Smad9 was produced by regularly injecting a rabbit with a peptide corresponding to the mouse Smad9 linker region (a.a. 153–167).

Reverse transcription-polymerase chain reaction and quantitative RT-PCR. Total RNA was prepared using a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) and reverse-transcribed with Superscript III (Invitrogen) according to the manufacturer's instructions. The PCR assays were performed with GoTaq DNA polymerase (Promega) with the listed primer sets (Supplemental Table 1). Quantitative RT-PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (TaKaRa, Shiga, Japan) with a Thermal Cycler Dice TP800 (TaKaRa). The total RNA of mouse embryos was purchased from Clontech Laboratories (Mountain View, CA). The primers that were used are listed in Supplemental Table 1. The data are expressed as the relative expression levels compared to those of Atp5f1 as a control[49].

Statistical analysis. Comparisons were made using an unpaired ANOVA with Tukey-Kramer post-hoc test and Wilcoxon's signed rank test. The results are shown as the mean ± S.D. The statistical significance is indicated as follows: *, $p < 0.05$ and **, $p < 0.01$.

1. Katagiri, T., Suda, T. & Miyazono, K. The bone morphogenetic proteins. In: TGF-β Family, Derynck, R. & Miyazono, K. eds. (Cold Spring Harbor Press, 2008), pp. 121–149.
2. Miyazono, K., Kamiya, Y. & Morikawa, M. Bone morphogenetic protein receptors and signal transduction. J. Biochem. 147, 35–51 (2010).
3. Urist, M. R. Bone: formation by autoinduction. Science 150, 893–899 (1965).
4. Sampath, T. K., Muthukumaran, N. & Reddi, A. H. Isolation of osteogenin, an extracellular matrix-associated, bone-inductive protein, by heparin affinity chromatography. Proc. Natl. Acad. Sci. U. S. A. 84, 7109–7113 (1987).
5. Wozney, J. M. et al. Novel regulators of bone formation: molecular clones and activities. Science 242, 1528–1534 (1988).
6. Katagiri, T. et al. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J. Cell. Biol. 127, 1755–1766 (1994).
7. Mueller, T. D. & Nickel, J. Promiscuity and specificity in BMP receptor activation. FEBS Lett. 586, 1846–1859 (2012).
8. Katagiri, T. & Tsukamoto, S. The unique activity of bone morphogenetic proteins in bone: a critical role of the Smad signaling pathway. Biol. Chem. 394, 703–714 (2013).
9. Wieser, R., Wrana, J. L. & Massague, J. GS domain mutations that constitutively activate TβR-I, the downstream signaling component in the TGF-β receptor complex. EMBO J. 14, 2199–2208 (1995).
10. Akiyama, S. et al. Constitutively active BMP type I receptors transduce BMP-2 signals without the ligand in C2C12 myoblasts. Exp. Cell. Res. 235, 362–369 (1997).
11. Fujii, M. et al. Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. Mol. Biol. Cell. 10, 3801–3813 (1999).
12. Glaser, D. L. et al. In vivo somatic cell gene transfer of an engineered Noggin mutein prevents BMP4-induced heterotopic ossification. J. Bone. Joint. Surg. Am. 85-A, 2332–2342 (2003).
13. Katagiri, T. Recent topics in fibrodysplasia ossificans progressiva. J. Oral. Biosci 54, 119–123 (2012).
14. Shore, E. M. et al. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat. Genet. 38, 525–527 (2006).
15. Fukuda, T. et al. A unique mutation of ALK2, G356D, found in a patient with fibrodysplasia ossificans progressiva is a moderately activated BMP type I receptor. Biochem. Biophys. Res. Commun. 377, 905–909 (2008).
16. Fukuda, T. et al. Constitutively activated ALK2 and increased SMAD1/5 cooperatively induce bone morphogenetic protein signaling in fibrodysplasia ossificans progressiva. J. Biol. Chem. 284, 7149–7156 (2009).
17. Ohte, S. et al. A novel mutation of ALK2, L196P, found in the most benign case of fibrodysplasia ossificans progressiva activates BMP-specific intracellular signaling equivalent to a typical mutation, R206H. Biochem. Biophys. Res. Commun. 407, 213–218 (2011).
18. Yu, P. B. et al. BMP type I receptor inhibition reduces heterotopic ossification. Nat. Med. 14, 1363–1369 (2008).
19. Wrighton, K. H., Lin, X. & Feng, X. H. Phospho-control of TGF-β superfamily signaling. Cell. Res. 19, 8–20 (2009).
20. Massague, J. TGFβ signalling in context. Nat. Rev. Mol. Cell. Biol. 13, 616–630 (2012).
21. Wakefield, L. M. & Hill, C. S. Beyond TGFβ: roles of other TGFβ superfamily members in cancer. Nat. Rev. Cancer. 13, 328–341 (2013).
22. Moustakas, A. & Heldin, C. H. The regulation of TGFβ signal transduction. Development 136, 3699–3714 (2009).
23. Zi, Z., Chapnick, D. A. & Liu, X. Dynamics of TGF-β/Smad signaling. FEBS Lett. 586, 1921–1928 (2012).
24. Kokabu, S., Katagiri, T., Yoda, T. & Rosen, V. Role of Smad phosphatases in BMP-Smad signaling axis-induced osteoblast differentiation. J. Oral. Biosci 54, 73–78 (2012).
25. Bruce, D. L. & Sapkota, G. P. Phosphatases in SMAD regulation. FEBS Lett. 586, 1897–1905 (2012).
26. Qin, B. Y. et al. Structural basis of Smad1 activation by receptor kinase phosphorylation. Mol. Cell. 8, 1303–1312 (2001).
27. Wu, M. Y. & Hill, C. S. TGF-β superfamily signaling in embryonic development and homeostasis. Dev. Cell. 16, 329–343 (2009).
28. Hayashi, H. et al. The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. Cell 89, 1165–1173 (1997).
29. Imamura, T. et al. Smad6 inhibits signalling by the TGF-β superfamily. Nature 389, 622–626 (1997).
30. Nakao, A. et al. Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling. Nature 389, 631–635 (1997).
31. Zhang, X. et al. Fine-tuning BMP7 signalling in adipogenesis by UBE2O/E2-230K-mediated monoubiquitination of SMAD6. EMBO J. 32, 996–1007 (2013).
32. Itoh, S. & ten Dijke, P. Negative regulation of TGF-β receptor/Smad signal transduction. Curr Opin Cell Biol. 19, 176–184 (2007).
33. Bai, S., Shi, X., Yang, X. & Cao, X. Smad6 as a transcriptional corepressor. J. Biol. Chem. 275, 8267–8270 (2000).
34. Lin, X. et al. Smad6 recruits transcription corepressor CtBP to repress bone morphogenetic protein-induced transcription. Mol Cell Biol. 23, 9081–9093 (2003).
35. Katagiri, T. et al. Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. Genes. Cells. 7, 949–960 (2002).
36. Korchynskyi, O. & ten Dijke, P. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J. Biol. Chem. 277, 4883–4891 (2002).
37. Lopez-Rovira, T., Chalaux, E., Massague, J., Rosa, J. L. & Ventura, F. Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. J. Biol. Chem. 277, 3176–3185 (2002).
38. Shepherd, T. G., Theriault, B. L. & Nachtigal, M. W. Autocrine BMP4 signalling regulates ID3 proto-oncogene expression in human ovarian cancer cells. Gene 414, 95–105 (2008).

39. Kurooka, H., Nakahiro, T., Mori, K., Sano, K. & Yokota, Y. BMP signaling is responsible for serum-induced Id2 expression. *Biochem. Biophys. Res. Commun.* **420**, 281–287 (2012).

40. Shin, M. *et al.* Identification of a novel bone morphogenetic protein (BMP)-inducible transcript, BMP-inducible transcript-1, by utilizing the conserved BMP-responsive elements in the Id genes. *J. Bone. Miner. Metab.* **31**, 34–43 (2013).

41. Nohe, A., Keating, E., Knaus, P. & Petersen, N. O. Signal transduction of bone morphogenetic protein receptors. *Cell. Signal.* **16**, 291–299 (2004).

42. Nojima, J. *et al.* Dual roles of smad proteins in the conversion from myoblasts to osteoblastic cells by bone morphogenetic proteins. *J. Biol. Chem.* **285**, 15577–15586 (2010).

43. Yamazaki, M. *et al.* Tumor necrosis factor alpha represses bone morphogenetic protein (BMP) signaling by interfering with the DNA binding of Smads through the activation of NF-kB. *J. Biol Chem* **284**, 35987–35995 (2009).

44. Sapkota, G., Alarcon, C., Spagnoli, F. M., Brivanlou, A. H. & Massague, J. Balancing BMP signaling through integrated inputs into the Smad1 linker. *Mol Cell.* **25**, 441–454 (2007).

45. Miyai, K. *et al.* ANA deficiency enhances bone morphogenetic protein-induced ectopic bone formation via transcriptional events. *J. Biol Chem* **284**, 10593–10600 (2009).

46. Arnold, S. J., Maretto, S., Islam, A., Bikoff, E. K. & Robertson, E. J. Dose-dependent Smad1, Smad5 and Smad8 signaling in the early mouse embryo. *Dev Biol* **296**, 104–118 (2006).

47. Goldman, L. A., Cutrone, E. C., Kotenko, S. V., Krause, C. D. & Langer, J. A. Modifications of vectors pEF-BOS, pcDNA1 and pcDNA3 result in improved convenience and expression. *Biotechniques* **21**, 1013–1015 (1996).

48. Kodaira, K. *et al.* Purification and identification of a BMP-like factor from bovine serum. *Biochem. Biophys. Res. Commun.* **345**, 1224–1231 (2006).

49. Ohte, S. *et al.* Identification and functional analysis of Zranb2 as a novel Smad-binding protein that suppresses BMP signaling. *J. Cell. Biochem.* **113**, 808–814 (2012).

Author contributions

S.T. and T.K. designed the experiments. S.O., K.O., E.M., E.J., S.K. and T.K. conducted the experiments. S.T., T.M., M.F., S.O., K.O., A.M., K.Y. and T.K. performed the experiments and analyzed the data. S.T. and T.K. wrote the paper.

Additional information