Function of the Two Xenopus Smad4s in Early Frog Development*

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Signals from the transforming growth factor β family members are transmitted in the specific receptor-activated Smads and a common partner Smad4. Two Smad4 genes (α and β/10, or smad4 and smad4.2) have been isolated from Xenopus, and conflicting data are reported for Smad4/β/10 actions in mesodermal and neural induction. To further understand the functions of the Smad4s in early frog development, we analyzed their activities in detail. We report that Smad10 is a mutant form of Smad4β that harbors a missense mutation of a conserved arginine to histidine in the MH1 domain. The mutation results in enhanced association of Smad10 with the nuclear transcription corepressor Ski and leads to its neural inducing activity through inhibition of bone morphogenetic protein (BMP) signaling. In contrast to Smad10, both Smad4α and Smad4β enhanced BMP signals in ectodermal explants. Using antisense morpholino oligonucleotides (MOs) to knockdown Smad4β was required for both activin- and BMP-mediated mesodermal induction in animal caps, whereas Smad4α affected only the BMP signals. Neither Smad4 was involved directly in neural induction. Expression of Smad4β-MO in early frog embryos resulted in reduction of mesodermal markers and defects in axial structures, which were rescued by either Smad4α or Smad4β. Smad4α-MO induced only minor deficiency at late stages. As Smad4β, but not Smad4α, is expressed at high levels maternally and during early gastrulation, our data suggest that although Smad4α and Smad4β may have similar activities, they are differentially utilized during frog embryogenesis, with only Smad4β being essential for mesoderm induction.

Members of the TGFβ2 superfamily of signaling molecules regulate diverse processes both during embryogenesis and in adult tissue homeostasis. In vertebrate embryos, TGFβ family ligands play crucial roles in generation and patterning of mesodermal and endodermal tissues, in gastrulation, neural induction and patterning, and organogenesis (1–5). Two branches of the growth factor family function differently in various processes. TGFβ/activin/nodal-like molecules are involved in induction and patterning of early mesendoderm and left-right axis specification, whereas bone morphogenetic proteins (BMPs) control early dorsoventral patterning in all germ layers and subsequently modulate the formation of multiple organ systems. In adults, one predominant function of the TGFβ signals is to regulate cell proliferation, and mutations in many signaling components of this pathway are associated with tumor formation (6, 7).

TGFβ signals are transmitted through two types of transmembrane serine/threonine kinase receptors. In the ligand-induced receptor complex, the type II receptor phosphorylates and activates the type I receptor, which in turn phosphorylates Smad proteins, the cytoplasmic signal transducers. Growth factors from the TGFβ/activin/nodal subfamily act through their specific type I receptors (ALK4, -5, and -7) to activate Smad2 and -3, whereas BMPs stimulate Smad1, -5, and -8/9 through ALK2, -3, and -6. These receptor-activated Smads (R-Smads) show similar organization of their sequences, which contain the conserved N-terminal MH1 and the C-terminal MH2 domains. R-Smads from both classes interact with a common partner, the co-Smad Smad4, to regulate downstream gene expression. Smad4 also has the MH1 and MH2 domains, but it lacks the C-terminal SSXS motif to be phosphorylated by the type I receptors. Smad4 responds to TGFβ signals by forming a hetero-oligomer complex with activated R-Smads, translocating into the nucleus and binding to DNA elements, frequently with other sequence-specific transcription factors and co-factors to influence expression of target genes. This canonical TGFβ signal transduction model thus places Smad4 as an important component for signals from all TGFβ family members (8).

Although studies with dominant negative Smad4 support a pivotal role of Smad4 in both TGFβ/activin/nodal- and BMP-mediated processes (9), recent evidence from Smad4-deficient mouse embryos and cells indicate that certain responses to TGFβ signals may occur in the absence of Smad4. Thus chimeric mouse embryos, containing Smad4-null epiblast cells, form patterned mesoderm that gives rise to the heart, trunk somites, and lateral plate mesoderm, a phenotype different from that expected if both nodal and BMP signals are impaired (10). Murine fibroblast cells deficient in Smad4 still respond to TGFβ/activin-mediated growth inhibition and induction of extracellular matrix genes (11). Human cell lines with depleted Smad4 only lose a subset of TGFβ responsive gene expression and can undergo TGFβ-induced epithelial to mesenchymal

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2 The abbreviations used are: TGF, transforming growth factor; BMP, bone morphogenetic protein; R-Smad, receptor-activated Smad; MO, morpholino oligonucleotides; UTR, untranslated region.
Smad4 homolog, lead to defective regulation of a subset of the processes affected by mutations in Mad (13). These results imply that Smad4 may not be an obligatory component of all TGFβ signals and may be dispensable in particular TGFβ-responsive processes. Currently, it is unclear what determines a process or a target gene to be Smad4-dependent or Smad4-independent.

In *Xenopus*, two Smad4 genes have been identified (14–16). They show 92 and 90% identity in the MH1 and MH2 domains, respectively. However, the linker region between the two MH domains is divergent in the two genes. Smad4α is closely related to the mammalian Smad4, whereas Smad4β/10 has a linker sequence that is only 34% identical to that of the human Smad4 (15, 16). Characterization of the activities of the two Smad4 proteins by different groups has led to conflicting conclusions. On one hand, both Smad4s have been shown to form hetero-oligomer complex with Smad1 and Smad2 in response to BMP and activin signals, respectively, and overexpression of either gene enhances both Smad1- and Smad2-dependent mesoderm induction in ectodermal explants. Both Smad4s therefore act similarly to the conventional mammalian Smad4 as co-Smads (15, 16). On the other hand, the divergent Smad4, Smad4β/10, is found to induce neural markers directly without the presence of mesoderm in animal cap explants (14). Unlike other direct neural inducers that induce neural tissues via inhibition of ectodermal BMP signals in *Xenopus* (17, 18), Smad4β/10 has been claimed to act independently of BMP signals. Furthermore, using antisense morpholino oligonucleotides (MOs) to knockdown endogenous expression, Smad4β/10 was shown to be required for neural but not mesodermal tissue formation in early *Xenopus* embryos and had no effect on Smad1- and Smad2-mediated mesoderm induction in animal caps (19). The sequence responsible for the neural inducing activity of Smad4β/10 was further mapped not to the divergent linker region but to the conserved MH1 and MH2 domains. Based on these results, it was claimed that Smad4β/10 does not act in the TGFβ pathway, but has a novel neuralizing function in the Spemann organizer, separate from the dorsalizing and neuralizing effect of the BMP antagonists (14, 19).

Whereas the studies on *Xenopus* Smad4 genes are intriguing, the questions of how Smad4β/10 might transduce signals and what pathway(s) it may be involved in are open. It is also unknown whether Smad4α has a distinct function from Smad4β/10 in terms of mesodermal and neural induction, and what are the *in vivo* activities of the two Smad4s. To resolve the outstanding questions on the function of the two Smad4 proteins in early frog development, we undertook biological and biochemical analyses of their roles in TGFβ-responsive processes. We have addressed: 1) whether Smad4β/10 induces neural tissues without affecting TGFβ signaling; 2) what may be the mechanism underlying the activities of Smad4β/10; and 3) what *in vivo* roles the two Smad4 proteins play during frog embryogenesis. Our data reveal that Smad10 is a mutant Smad4β that induces neural markers through interaction with Ski and inhibition of BMPs; and that although Smad4α and -4β may have analogous activities, they play different roles in mesodermal formation due to their differential expression during early developmental stages.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction, RNA Synthesis, and Antisense Morpholino Oligonucleotides**—The Smad10 construct was obtained from Dr. Graff (University of Texas, Southwestern, GenBank accession number AF104232), and Smad4α and Smad4β plasmids were provided by Dr. Nishida (Kyoto University, GenBank accession numbers AB022721 and AB022722, respectively). The mutant Smad4β(R132H), the hemagglutinin-tagged Smad4s, and the FLAG-tagged Ski were all constructed by a PCR-based cloning strategy and inserted into the vector pCS105. The plasmids were linearized with AscI, and RNA was synthesized using the Message Machine kit from Ambion. The Smad4 antisense MO was designed (19), and Smad4α-MO is as the following: 5′-TGT-TTGTGATGGACATATTGTCGGT-3′. A standard control MO from Gene Tools was also used in this study.

**Protein Co-immunoprecipitation Assay**—RNAs encoding hemagglutinin-tagged Smad4s and FLAG-tagged Ski were injected into the animal poles of two-cell stage embryos. Protein extract was made from gastrula embryos and co-immuno-
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A

B

C

FIGURE 2. Smad4α and Smad4β differ from Smad10 in regulation of the TGFβ signals, and a point mutation in the MH1 domain of Smad4β converts it to a Smad10-like molecule. A, sequence difference between Smad4β and Smad10 in the MH1 domain of the molecules. Mutation of the conserved arginine has been identified in human cancer patients. B, Smad4α and Smad4β induce the blood marker globin, whereas Smad10 and Smad4β/R132H induce the neural marker NRP-1 and the cement gland marker XAG-1. 4 ng of RNAs were used. C, at high doses, Smad4α and Smad4β ventralize mesodermal induction by activin, whereas Smad10 and Smad4β/R132H inhibit activin and BMP. 2 ng of Smad4/10, 1 pg of activin, and 10 pg of BMP4 RNAs were used. The animal caps were harvested at stages 11 or 32.

precipitation assays were performed as previously described (20, 21).

In Situ Hybridization and Whole Mount Immunohistochemistry—Embryos were stained by in situ hybridization and immunohistochemistry as described (22, 23). 12/101 antibody was obtained from Developmental Studies Hybridoma Bank (University of Iowa).

Reverse Transcription-PCR—RNAs were injected into animal poles of two-cell stage embryos. Animal caps were dissected at blastula stage 9 and harvested at gastrula or tadpole stages. Reverse transcription-PCR was performed as described (23), with 25 cycles of PCR at 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s.

RESULTS

Smad10 Induces Neural Markers via Inhibition of BMP Signals—Smad4β and Smad10 cDNAs have been isolated by different groups, and the injected mRNA was reported either to enhance Smad1-dependent mesoderm induction or to promote neural tissue formation independent of BMP/Smad1 inhibition (14–16). To examine whether Smad4β and Smad10 indeed have different activities, we analyzed the activity of the original Smad4β and Smad10 cDNA clones. Overexpression of Smad10 mRNA induced neural markers in the absence of mesoderm in a dose-dependent manner (Fig. 1A). To address whether Smad10 neuralizes by BMP inhibition, we first tested whether Smad10 blocked BMP activity in an independent assay. Indeed, Smad10 inhibited induction of globin expression by BMP2 at similar doses to those required for neural induction (Fig. 1B). Furthermore, neural markers were suppressed when Smad10 was coexpressed with BMP2 (Fig. 1B), supporting the notion that Smad10 inhibits BMP signals.

A Smad4β isoform lacking the N-terminal 32-amino acid extension was previously used to demonstrate that Smad4β enhanced Smad1 activities (15), so we sought to examine whether the discrepancy on Smad4β/10 function might be due to the presence or absence of this N-terminal region. A short form of Smad10 (Smad10ΔN) with its N-terminal 32 amino acids removed had the same activities as the full-length Smad10 in animal caps, and could also induce neural markers through inhibition of BMPs (Fig. 1). Our results confirm that overexpression of Smad10 at high levels can indeed induce neural markers; but contrary to the previous report, we find that induction of neural tissues is associated with inhibition of BMP signaling.

Smad4α and Smad4β Have Different Activities from Smad10, and a Point Mutation in Smad4β Converts It to Smad10—Smad10 is nearly identical to Smad4β (Fig. 2A), but Smad4β has been reported to have a different activity (15, 16). To understand the potential differences between the two molecules, we overexpressed Smad4β and Smad10 in animal caps and compared their function side by side. Unlike Smad10, Smad4β did not induce neural markers, but instead stimulated the expression of globin (Fig. 2B). When coexpressed with activin, Smad4β ventralized the mesodermal induction at high doses, although at low doses it enhanced the marker induction by activin (Fig. 2C and data not shown). The behavior of Smad4β is similar to that of Smad4α in regulation of TGFβ signals (Fig. 2).
This agrees with previous reports (15, 16) and suggests that although these two Smad4s have divergent linker regions, they have comparable functions. In contrast to Smad4/H9251 and -4/H9252, Smad10 blocked both activin- and BMP-dependent mesodermal induction when expressed at high levels (Fig. 2C). Our data demonstrate that despite the nearly identical sequences of Smad10 and Smad4/H9252, they have different activities in modulation of TGFβ signaling.

Sequence comparison between Smad10 and Smad4/H9252 reveals that they differ at amino acid 132 in the MH1 domain, with Smad10 encoding histidine and Smad4/H9252 encoding arginine at this position (Fig. 2A). All mammalian Smad4s as well as Xenopus Smad4α encode arginine at the corresponding position, and mutation of this conserved arginine is found in human cancer patients (24–27). We therefore examined the possibility that the change of this residue in Smad10 was responsible for its distinct activity. We introduced the same point mutation into Smad4/H9252 and analyzed the function of the resulting mutant Smad4/H9252(R132H). As shown in Fig. 2, alteration of the single amino acid reversed the action of Smad4/H9252(R132H), so that it inhibited BMP signaling and induced neural markers. The behavior of Smad4β(R132H) mimicked that of Smad10, suggesting that the missense mutation in Smad10 changed it from a cytoplasmic transducer to an inhibitor of TGFβ signals.

To further investigate whether Smad10 might be a natural allele of Smad4/H9252 that is expressed during early frog development, we PCR cloned the corresponding fragment of Smad4/H9252 from both maternal (stage 2) and gastrula (stage 10) stage embryos. Sequence analyses of randomly picked clones revealed that all eight maternal and 12 out of 13 gastrula Smad4/H9252 clones encoded arginine at the relevant position, with only one sequence from gastrula Smad4/H9252 clones encoded histidine. We also examined both Xenopus laevis and Xenopus tropicalis EST databases and found that all 33 overlapping Smad4/H9252 EST sequences contained the codon for arginine at the pertinent position. We thus conclude that Smad10 may not be present abundantly to influence mesoderm induction and patterning during early frog embryogenesis.

Enhanced Interaction of Smad10 with the Ski Transcription Corepressor—To understand how Smad10 may differ from Smad4α and -4β in regulation of TGFβ signals, we performed
biochemical studies. As Smad10 blocked both activin and BMPs at high doses, we reasoned that it might interact with transcriptional repressors to suppress TGFβ target genes. A possible candidate is the nuclear onc protein Ski, which has previously been demonstrated to bind to the mammalian Smad4 and act as a corepressor to inhibit both TGFβ and BMP signals (28–32). We thus constructed epitope-tagged Smad4, Smad10, and Ski and examined whether these Smads had differential abilities to bind to Ski in a co-immunoprecipitation assay. As shown in Fig. 3, neither Smad4α nor Smad4β associated with Ski in the absence of the TGFβ signals, whereas Smad10 interacted with Ski strongly (Fig. 3, compare lanes 6 and 7 with lane 8). Activation of the TGFβ pathway by activin or BMP4 did not significantly alter the interaction of Smad10 with Ski (not shown). Our results indicate that the point mutation in Smad10 leads to enhanced binding of Smad10 to the transcriptional corepressor Ski, and that this interaction may contribute to its ability to repress activin and BMP signals when expressed at high levels in early frog embryos.

Differential Requirement for Smad4α and Smad4β in Activin- and BMP-stimulated Mesodermal Induction—Overexpression experiments show that the two Xenopus Smad4s may have analogous activities in mediating signals from TGFβ ligands. The requirement for endogenous Smad4s in TGFβ-regulated processes, however, is unknown. To address this issue, we performed loss-of-function studies, using antisense MOs to knockdown the endogenous Smad4 protein levels. In vitro translation assays showed that both Smad4α-MO and Smad4β-MO specifically reduced protein synthesis from their respective RNA templates that contained the corresponding 5′-untranslated region (UTR), but they did not block protein synthesis from the templates that contained modified upstream sequences, nor did they inhibit protein translation from the other Smad4 gene (Fig. 4A). When coexpressed with activin, Smad4α-MO did not block mesodermal induction in animal caps (Fig. 4, B and C, lane 4), but Smad4β-MO dramatically inhibited activin-induced marker expression (Fig. 4, B and C, compare lanes 2 and 3). The result demonstrates that Smad4β is required for activin-mediated mesoderm induction in animal caps. We next coexpressed the MOs with BMP4 and discovered that both Smad4α-MO and Smad4β-MO blocked the induction of globin by BMP4 (Fig. 4C, lanes 5–7). The data implies that Smad4α and Smad4β are differentially utilized in activin- and BMP-dependent mesodermal induction.

The Smad4β-MO, which is the same as the Smad10-MO, has previously been used to demonstrate that Smad10 is essential for neural induction in both animal caps and early frog embryos (19). To test whether this is the case and whether Smad4α is also critical for neural induction, we coexpressed the Smad4 MOs with the neural inducer noggin and assayed for neural markers in animal caps by reverse transcription-PCR. As shown in Fig. 4, B and C, noggin induced the anterior neural marker Otx2 and the pan-neural marker NRP-1; coexpression with Smad4 MOs did not significantly alter the induction of these genes. Our results thus indicate that neither Smad4 protein is involved directly in the neural induction process.

To examine whether MO-mediated inhibition of mesodermal induction by activin and BMPs can be rescued with Smad4s, we coexpressed Smad4 MOs with the 5′-UTR-modified Smad4α or Smad4β RNAs. At high doses (above 0.5 ng), both Smad4α and -4β rescued ventral markers without restoration of dorsal transcripts in activin-induced mesodermal formation (not shown); this result is consistent with our finding that overexpression of Smad4α and -4β ventralizes mesoderm induction by activin (Fig. 2). At low doses (50–100 pg) these Smad4s rescued expression of both dorsal (chordin at gastrula stages, type II collagen and muscle actin at tadpole stages) and ventral (XWnt8 and globin at gastrula and tadpole stages, respectively) markers, although the efficiency of rescue of these regional markers by the two Smad4s varied slightly in different experiments (Fig. 4D, left panel, and data not shown). The result indicates that although depletion of Smad4α does not
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Smad4 is required for mesoderm formation in early frog embryos. To address the in vivo functions of these Smad4s, we next injected the MOs into early frog embryos and observed the morphology and marker gene expression in the resulting embryos. When injected into the animal poles of two-cell stage embryos, Smad4β-MO induced head defects with missing eyes in most embryos, and the tails of the injected embryos were often short and bent. In contrast, embryos injected with Smad4α-MO showed relatively normal axis formation, although the dorsal fin was expanded. A control MO injected in parallel did not cause any obvious defects in the embryos (Fig. 5A). To further assess the activities of endogenous Smad4s, we also injected the MOs into the marginal zone regions of two-cell stage embryos at 20–40-ng doses. As shown in Fig. 5B, whereas neither the control MO nor the Smad4α-MO induced dramatic defects, Smad4β-MO caused severe malformation of the body axis. Gastrulation was delayed, and open blastopore was observed in late stage embryos. The head and tail of the morphant embryos were reduced or missing, and the body axis was shortened (Fig. 5B). When the two Smad4 MOs were co-injected into early embryos, the resulting morphants showed the phenotypes similar to that when Smad4β-MO was injected alone, although more embryos displayed gastrulation defects and shortened body axis (not shown). The phenotype of the MO-injected embryos indicates that Smad4β plays an important role in body axis formation during early frog development.

To examine how the mesodermal and neural transcripts are affected when the embryos are depleted of endogenous Smad4s, we assayed for expression of marker genes by in situ hybridization. Injection of Smad4β-MO into two-cell stage embryos led to reduction of multiple mesodermal genes at gastrula stages (Fig. 6 and 7). The general dorsal-ventral patterning of the mesoderm was not disrupted, but the expression of the pan-mesodermal gene Brachury (Xbra) as well as the dorsal and ventral mesodermal markers, such as Chordin, MyoD, and Wnt8, were reduced in Smad4β morphant embryos. Neither the control MO nor Smad4α-MO affected the expression of these genes (Figs. 6A and 7). When both MOs were injected into early embryos, the expression of the marker genes was only

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| A | Control-MO | Smad4β-MO | Smad4β-MO + Smad4α(UTR*) | Smad4β-MO + Smad4β(UTR*) |
|---|------------|------------|---------------------------|---------------------------|
| XBra | ![Image](image1.jpg) | ![Image](image2.jpg) | ![Image](image3.jpg) | ![Image](image4.jpg) |
| Chordin | ![Image](image5.jpg) | ![Image](image6.jpg) | ![Image](image7.jpg) | ![Image](image8.jpg) |
| MyoD | ![Image](image9.jpg) | ![Image](image10.jpg) | ![Image](image11.jpg) | ![Image](image12.jpg) |
| Wnt8 | ![Image](image13.jpg) | ![Image](image14.jpg) | ![Image](image15.jpg) | ![Image](image16.jpg) |
| B | MyoD | ![Image](image17.jpg) | ![Image](image18.jpg) | ![Image](image19.jpg) |
| Sox2 | ![Image](image20.jpg) | ![Image](image21.jpg) | ![Image](image22.jpg) | ![Image](image23.jpg) |

**FIGURE 7. Rescue of Smad4β morphant embryos with Smad4α or Smad4β.** Both Smad4α and Smad4β (with modified UTR sequences) rescued the marker expression in Smad4β morphant embryos at gastrula (A) and neurula (B) stages. Embryos in A were viewed from the vegetal side with the dorsal quadrant to the right, whereas embryos in B were viewed from the dorsal side with the anterior to the left. 20 – 40 ng of Smad4β-MO were injected, and 100 pg of Smad4α or Smad4β RNAs were used in the rescue experiments.

Slightly reduced when compared with the embryos received only Smad4β-MO (Fig. 6). This implies that Smad4β, but not Smad4α, is the major co-Smad used during mesodermal induction. At neurula stages, Xbra transcripts were localized around the blastopore and in the notochord. Similar patterns were observed in embryos injected with the control MO or Smad4α-MO. However, in embryos injected with Smad4β-MO, the notochordal expression of Xbra was reduced, although its expression around the blastopore remained high (Fig. 6B). In addition, the paraxial mesodermal marker MyoD and the pan-neural marker Sox2 were greatly down-regulated in Smad4β morphant embryos, although they were relatively normal in Smad4α morphants (Fig. 6B). When the two Smad4 MOs were both present, there was further reduction of notochordal Xbra and paraxial mesodermal MyoD expression, and the Sox2 level was also reduced (Fig. 6B). Our data reveal that Smad4β plays a primary role during early mesodermal induction, whereas Smad4α may be involved in maintenance of axial and paraxial mesoderm at later stages. In addition, although Smad4β affects neural induction, it most likely does so indirectly through its regulation of dorsal mesoderm formation.

In explant assays, Smad4α is shown to be important for induction of the blood marker by BMPs. To see whether Smad4α also plays a role in blood development in vivo, we analyzed the expression of globin in embryos injected with Smad4 MOs. As shown in Fig. 6C, both Smad4α-MO and Smad4β-MO greatly reduced the level of globin transcripts, suggesting that blood formation is sensitive to the levels of Smad4s.

To see whether the defects induced by Smad4β-MO can be rescued by Smad4α, we coexpressed the MO with the 5’-UTR-modified Smad4α or Smad4β RNAs. Consistent with our in vitro results, both Smad4α and Smad4β rescued marker expression in Smad4β morphant embryos (Fig. 7). At gastrula stages, the level of Xbra and Chordin expression was restored, whereas the expression of MyoD and Wnt8 was expanded toward the animal pole by Smad4α and -4β (Fig. 7A). Similarly, at neurula stages both the mesodermal marker MyoD and the neural marker Sox2 were rescued by either Smad4s (Fig. 7B). Our data indicate that the defects induced by Smad4β-MO are specific, and that Smad4α and -4β may have equivalent activities when overexpressed.

**Reduction of Neural Markers in Smad4β/10 Morphant Embryos Is Associated with the Reduction of Mesodermal Transcripts**—It has previously been reported that depletion of Smad10, the mutant form of Smad4β, leads to reduction of neural markers in the absence of defects in the axial/paraxial mesoderm (19). However, our results above suggest that knockdown of Smad4β/10 results in mesodermal defects, and the reduction of the neural genes may be a secondary effect. To examine further the requirement for Smad4β/10 in mesodermal and neural development, we injected the Smad4β-MO (i.e. Smad10-MO) into one blastomere of two-cell stage embryos, together with the lineage tracer the nuclear β-galactosidase mRNA. The injected side was then identified by staining with a red substrate (Red-Gal) at tailbud stages. In these embryos, we assayed for the presence of the paraxial mesoderm by whole mount immunohistochemistry study with an antibody (12/101) against the muscle epitope and the presence of the neural tissue by in situ hybridization with the Sox2 probe. As shown in Fig. 8, 12/101 and Sox2 staining are both present in the uninjected and
injected sides in embryos expressing the control MO or Smad4α-MO (Fig. 8, a–f). In embryos injected with Smad4β-MO, the expression of the 12/101 epitope and Sox2 was normal on the control side, but both markers were absent on the injected side (Fig. 8, g–i). We did not observe the loss of Sox2 without losing the 12/101 epitope also. Our result thus supports the notion that the absence of the neural tissues in the morphant embryos correlates with the absence of the dorsal mesoderm, and that Smad4β is primarily involved in formation of the mesoderm rather than neural structures.

DISCUSSION

In TGFβ signal transduction, Smad4 has been proposed to be the common Smad that associates with activated R-Smads in response to growth factor stimulation. The subsequent nuclear translocation of Smad4/R-Smad complexes allows interaction of these Smads with DNA and other transcription factors to regulate expression of TGFβ downstream genes (8). Two Xenopus Smad4 genes have recently been isolated. Whereas some studies showed that both Smad4s behave like their mammalian counterpart to cooperate with R-Smads (15, 16), one group

argued that the Smad4β/10 protein may act independently of the activin/BMP pathways to regulate neural induction (14, 19). The reason underlying the discrepancy on Smad4β/10 function has not previously been understood, nor is it known whether Smad4α and Smad4β/10 regulate similar or different processes during early frog development.

In our present study, we undertook detailed analyses of the functions of the Xenopus Smad4s in early frog embryogenesis, using both explant and whole embryo assays. We demonstrate that although Smad4β and Smad10 represent the same gene isolated by different groups, they do not have identical activities. Smad10 induced neural markers directly in the absence of mesoderm when expressed at high levels; whereas Smad4β induced globin expression and ventralized mesodermal induction. We attribute the functional differences to a single point mutation that changes a conserved arginine to a histidine in the MH1 domain of Smad10. Alteration of this arginine to histidine in Smad4β converts it to a Smad10-like molecule with neural inducing ability. Mutations of this conserved arginine in both Smad2 and Smad4 have been identified in human cancer patients (24, 33). In the case of Smad4, the tumorigenic missense mutation of arginine to threonine leads to increased autoinhibitory interaction of MH1 with MH2 domains (25), impaired ability for Smad4 nuclear translocation and DNA binding (26), and decreased protein stability through degradation by the ubiquitin-proteosome pathway (27). The RT mutation in Smad4 thus leads to an inactive molecule that cannot mediate TGFβ signaling. In comparison, the RH mutation in Smad10 is a dominant negative mutation that inhibits both activin and BMP signals when overexpressed. Our mechanistic studies reveal that the mutation leads to enhanced association of Smad10 with the nuclear oncoprotein Ski. Ski has been shown to be a transcription corepressor in the TGFβ pathway. It binds to Smad2, -3, and -4 and helps to recruit histone deacetylase to Smad-responsive promoters to block both activin and BMP downstream target genes (28–32, 34–37). Because Ski has low affinity to Smad1, binding of Ski to Smad4 is indispensable for suppression of BMP signals by Ski in mammalian cells (38). In Xenopus, Smad4β resides constitutively in the nucleus regardless of the status of TGFβ activation (15, 39). The enhanced association of Smad10 with Ski can thus serve to
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inhibit BMP activities constitutively, blocking both basal and activated BMP signaling. Potentially if Smad10 is a natural allele of Smad4β and is present during early development, it may act to restrict BMP signaling levels in frog embryos. However, analyses of randomly picked Smad4β fragments amplified from maternal and gastrula stage cDNA show that most (20/21) of Smad4β sequences present during the time of mesodermal formation encode an arginine at the corresponding position. EST data base searches also revealed that all 33 Smad4β EST clones have the conserved arginine. The data suggests that Smad10 is likely a rare allele of Smad4β.

In previous reports (14, 19), Smad10 is shown to induce neural markers without blocking BMP signaling and to be required for endogenous neural tissue formation in Xenopus. Our current finding indicates that Smad10 inhibits marker induction by BMPs in a dose-dependent manner, and the doses required of Smad10 for neural induction are the same as those when it inhibits BMPs. The result implies that Smad10 induces neural tissue not by a novel mechanism as proposed, but via inhibition of BMP signaling. Depletion of endogenous Smad10 (i.e. Smad4β) indeed decreases neural marker expression; however, the effect seems to be secondary, as dorsal mesodermal markers are also reduced. The involvement of Smad10 in neural induction in vivo is thus indirect.

What is the endogenous function of Smad4β/10, and how does it compare with that of Smad4α? Using the MO-mediated knockdown approach, we discover that Smad4β plays an essential role in mesodermal induction and axis formation, and this may rely on the ability of Smad4β to mediate endogenous nodal and BMP signals. Depletion of Smad4β does not alter the dorsal-ventral patterning of the mesoderm, but causes a general reduction of all mesodermal markers assayed. In contrast, Smad4α is dispensable for mesodermal induction at gastrula stages, although at neurula stages it may participate in maintenance of axial and paraxial mesodermal structures. The differences in requirement of the two Smad4s in early frog embryogenesis seem to reflect their different expression patterns rather than their different activities. Overexpression experiments in animal caps as well as rescue assays in explants and whole embryos demonstrate that the two Smad4s can functionally replace each other. However, Smad4β, but not Smad4α, is expressed at high levels maternally and during early gastrulation; whereas Smad4α transcripts are low initially and increased only at mid- to late gastrula stages (15, 16). The expression profile is consistent with our conclusion that Smad4β plays a more vital role than Smad4α in mesodermal induction. Currently, it is unclear whether all aspects of early TGFβ signals are affected in Smad4 knockdown frog embryos. In mouse, specific elimination of Smad4 in epiblast cells leads to defects in only a subset of TGFβ- and BMP-regulated processes (10, 11). In Drosophila, the Smad4 homolog Medea also potentiates most but not all Dpp-dependent responses (13). In mammalian cells, a nuclear protein TIF1γ interacts with the activated Smad2/3 and mediates different processes from the Smad4-Smad2/3 complex in response to TGFβ signals (40). Future research is required to determine whether one or both Smad4s are dispensable in certain TGFβ-/BMP-modulated developmental events in frog.

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