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Spemann organizer activity of Smad10

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SUMMARY

The Spemann organizer induces neural tissue, dorsalizes mesoderm and generates a second dorsal axis. We report the isolation and characterization of Smad10, which has all three of these Spemann activities. Smad10 is expressed at the appropriate time to transduce Spemann signals endogenously. Like the organizer, Smad10 generates anterior and posterior neural tissues. Smad10 appears to function downstream of the Spemann organizer, consistent with a role in mediating organizer-derived signals. Interestingly, Smad10, unlike previously characterized mediators of Spemann activity, does not appear to block BMP signals. This finding, coupled with the functional activity and expression profile, suggests that Smad10 mediates Spemann action in a novel manner.

Key words: Transforming Growth Factor-β superfamily, *Xenopus laevis*, Neural Induction, Signal Transduction, Smads, Spemann organizer

INTRODUCTION

The vertebrate nervous system contains a vast number of cell types and connections. Before the staggering complexity of the nervous system is generated, cells are first instructed to become neural tissue (reviewed in Sasai and De Robertis, 1997). This neural induction occurs during gastrulation when dorsal ectodermal cells are instructed by underlying mesoderm to change from an epidermal to a neural fate. Neural induction was discovered by Spemann and Mangold in the 1920s with amphibian embryos (Hamburger, 1988; Spemann, 1938). Transplantation of a small piece of dorsal tissue, containing dorsal mesoderm, to the ventral side of a host embryo induces a second dorsal axis composed largely of host tissue (reviewed in Smith, 1989). Thus, the donor graft instructs host ventral ectoderm, normally fated to become skin, to form a second organized nervous system with anterior and posterior pattern. Similarly, the graft redirects host ventral and lateral mesoderm to form dorsal mesoderm in a process known as dorsalization. Because of these striking activities, Spemann named this dorsal region of the amphibian embryo the ‘organizer’ (Spemann organizer); the homologous region in the chick and the mouse is called the node (reviewed in Graff, 1997).

Recently, a number of unrelated secreted factors – noggin, chordin, follistatin, Xnr3, Cerberus, and Gremlin – have been demonstrated to have direct neural inducing and dorsalizing activity (Bouwmeester et al., 1996; Hansen et al., 1997; Hemmati-Brivanlou et al., 1994; Hsu et al., 1998; Lamb et al., 1993; Sasai et al., 1995). All of these factors function antagonistically by blocking active, ventral-inducing bone morphogenetic protein (BMP) signals rather than by actively promoting neural fates (Hansen et al., 1997; Hemmati-Brivanlou and Melton, 1997; Hsu et al., 1998; Piccolo et al., 1996; Yamashita et al., 1995; Zimmerman et al., 1996). The BMP inhibitors induce only anterior fates, whereas the organizer induces both anterior and posterior neural tissue (Hansen et al., 1997; Hemmati-Brivanlou et al., 1994; Hsu et al., 1998; Lamb and Harland, 1995; Lamb et al., 1993; Sasai et al., 1995). Thus, if the BMP inhibitors function endogenously, they do so in concert with other signals (Lamb and Harland, 1995).

The same molecules, BMP4 and the BMP inhibitors, are thought to be the endogenous arbiters of the dorsal-ventral cell fate decision in both mesoderm and ectoderm (Graff, 1997; Hsu et al., 1998). However, at least for the mesoderm, a separate inducing signal is required in addition to blockade of BMP signals. This additional signal almost certainly involves a transforming growth factor β (TGFβ) signaling cascade (Hemmati-Brivanlou and Melton, 1992; Lagna et al., 1996). As the same BMP signals and inhibitors pattern mesoderm and ectoderm and as a TGFβ signal appears necessary to induce dorsal mesoderm, it is plausible that a dorsal ectodermal (neural) inducer will function via TGFβ signaling.

TGFβ signals are transduced from serine kinase receptors to the nucleus via the *Smad* gene family (reviewed in Derynck and Zhang, 1996; Massague, 1996; Wrana and Attisano, 1996; Heldin et al., 1997; Zhang et al., 1996). To date, nine vertebrate Smads (Smad1-Smad9) have been described and can be placed into three general classes (reviewed in Heldin et al., 1997). The first class of Smads (Smad1, Smad2, Smad3, Smad5 and Smad9) contains carboxy-terminal serines (SSXS) which are phosphorylated upon ligand-stimulation (Kretzschmar et al., 1997; Macias-Silva et al., 1996). Smad4, the only known member of the second class, is a common partner for the

pathway-restricted (SSXS) Smads and associates with the phosphorylated SSXS Smads. Then, the complex translocates to the nucleus and activates gene transcription (Kretzschmar et al., 1997; Macias-Silva et al., 1996). Smad6, Smad7 and Smad8 constitute the structurally and functionally distinct third class, which inhibit, rather than activate, TGFβ signaling (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997).

Of central importance, Smads function in distinct and specific signaling pathways (Graff et al., 1996; Heldin et al., 1997). Smad1 and the highly related Smad5 transduce the BMP signaling pathway while Smad2 and its close homolog, Smad3, transduce activin and TGFβ signals (reviewed in Heldin et al., 1997). Of note, all functional results obtained with Smads in *Xenopus* embryos are confirmed by other functional and biochemical studies (Eppert et al., 1996; Hoodless et al., 1996).

Here, we describe a novel Smad, Smad10, that directly induces neural tissue, dorsalizes mesoderm and generates second dorsal axes. These results imply that Smad10 might mediate organizer actions. The structure of Smad10 and its functional attributes suggests that Smad10 may lie downstream of an organizer signal. Previously characterized molecules that mimic Spemann function do so by blocking BMP signaling and induce only anterior neural tissue (Lamb and Harland, 1995). In contrast, Smad10 does not block BMP signals and, like the Spemann organizer, induces both anterior and posterior neural fates. Therefore, our studies suggest that the Spemann organizer may function, via Smad10, by an additional mechanism.

MATERIALS AND METHODS

Cloning Smad10 cDNA

Smad10 was cloned from a *Xenopus* oocyte cDNA library (Rebagliati et al., 1985) as described (Graff et al., 1996). Smad10 was sequenced on both strands.

Formation of synthetic mRNA for microinjection

The open reading frame of Smad10 was subcloned into the plasmid pCS2 (a gift of Richard Harland). pCS2-Smad10 was linearized with *Not*I and capped mRNA was transcribed in vitro as described (Krieg and Melton, 1987).

To generate synthetic mRNA encoding Smad10Δ, amino acids 1-555 were amplified with Vent DNA polymerase, cloned into p64TNE and sequenced. The sequence of the primers, 5′ to 3′, are:

Upstream: CGGGATTCATGGCGTTTGCCAGCCTAG

Downstream: CGGAATTCTTAAGGGCCCCAGCCCTTCAC

The plasmid, p64TNE-Smad10Δ, was linearized with *Xba*I and transcribed.

Generation of synthetic mRNA encoding Smad1, Smad2, Smad4Δ, noggin, BMP4 and β-gal are described elsewhere (Graff et al., 1994, 1996; Candia et al., 1997; Smith and Harland, 1991, 1992).

Embryological methods

Embryos were obtained, microinjected and cultured, and animal caps or marginal zones dissected as described (Graff et al., 1994, 1996). Embryos were either uninjected (control) or injected with mRNA as described in the figure legends. Histological sections were processed and stained with hematoxylin and eosin as described (Graff et al., 1994, 1996; Allen, 1992). All embryos were staged according to Nieuwkoop and Faber (1967). For second axes, 8-cell embryos were injected into one ventral vegetal blastomere which was identified by pigmentation differences. β-galactosidase was assayed with X-gal.

Analysis of RNA by RT-PCR

RNA extraction and RT-PCR analyses have been described previously (Graff et al., 1994; Wilson and Melton, 1994). The conditions for the PCR detection of RNA transcripts and the primer sequences for specific markers have been described previously:

Marker	Reference
brachyury	Smith et al., 1991
chordin	Sasai et al., 1994
EF-1α	Krieg et al., 1989
endodermin	Sasai et al., 1996
engrailed, HoxB9, Krox20, NCAM	Hemmati-Brivanlou and Melton, 1994
follistatin	Hemmati-Brivanlou et al., 1994
globin	Graff et al., 1994
goosecoid	GenBank/EMBL M63782
muscle actin	Wilson and Melton 1994
noggin	Smith and Harland, 1992
otx2	Lamb and Harland, 1995
siamois, Xnr3	Darras et al., 1997
Xvent1	Lagna et al., 1996
Xwnt-8	Smith and Harland, 1991

The Smad10 primers were used for 25 cycles.

Smad10 Upstream: GCCCCTCTCTCCCTCTGT

Downstream: CCCCAGCCCTTCACAAAAC

Immunostaining and in situ hybridization

Immunostaining was used to detect neural tissue with the antibody 6F11 (Lamb et al., 1993) or muscle with the antibody 12/101 (Ryan et al., 1996). In whole-mount mRNA in situ hybridizations, NRP-1 marks neural tissue (Knecht et al., 1995) and muscle actin detected dorsal mesoderm (Mohun et al., 1984). For histological sections, NCAM labels neural tissue (Kintner and Melton, 1987; Lemaire and Gurdon, 1994).

RESULTS

Cloning of Smad10

Degenerate oligonucleotides were used in a PCR-based approach to clone novel vertebrate Smads and a cDNA clone of Smad10 was obtained from a *Xenopus* oocyte library. Data base analysis revealed that Smad10 was unique, had low identity to the antagonistic Smads (Smad6, Smad7, Smad8) and was only 63% identical to Smad4 (Fig. 1A,B). Unlike Smad4, Smad10 contains carboxy-terminal serines in the sequence SSVN (Fig. 1A, bold). This sequence is similar to the carboxy-terminal SSVS phosphorylation site motif of Smad1 and Smad5 or SSMS in Smad2 or Smad3 (Heldin et al., 1997). The sequence similarity to Smad4 and the presence of carboxyl-terminal serines, coupled with the very low similarity to the inhibitory Smads, are consistent with Smad10 functioning positively to activate transcription rather than working as an antagonist (Liu et al., 1997).

As Smad10 is a hybrid of the SSXS Smads and Smad4, we sought to determine whether Smad10 had similar activity. As an initial attempt, we utilized the observations of Candia et al. (1997) that carboxy-terminal truncated forms of SSXS Smads or Smad4 all block BMP4 activity in the animal cap. So, we constructed a form of Smad10, Smad10Δ, that was truncated at the analogous position. We synthesized mRNA encoding Smad10Δ and injected it into the animal pole of *Xenopus* embryos alone or with mRNA encoding BMP4. As a positive

control for blockade, we also injected the truncated form of Smad4 (Smad4Δ), with and without BMP4. After injection, animal caps were explanted and cultured. In this assay, BMP4 induced expression of globin, a ventral mesoderm marker (Fig. 1C) (Graff et al, 1994). Notably, while Smad4Δ blocked BMP4-dependent expression of globin, Smad10Δ had no effect. This suggests that Smad10 functions via a novel signaling pathway that does not involve interaction with Smad1, Smad2 or Smad4.

Smad10 directly forms neural tissue

To assay Smad10 function, we synthesized mRNA encoding full-length Smad10, injected the mRNA into the animal pole, explanted animal caps and analyzed them as described (Graff et al., 1994, 1996). When Smad10 was expressed in the animal cap, the explants underwent a morphological change (Fig. 2A). Some Smad10-injected caps developed cement glands, anterior ectodermal derivatives often induced in parallel with neural tissue, suggesting that Smad10 might itself be able to generate neural tissue.

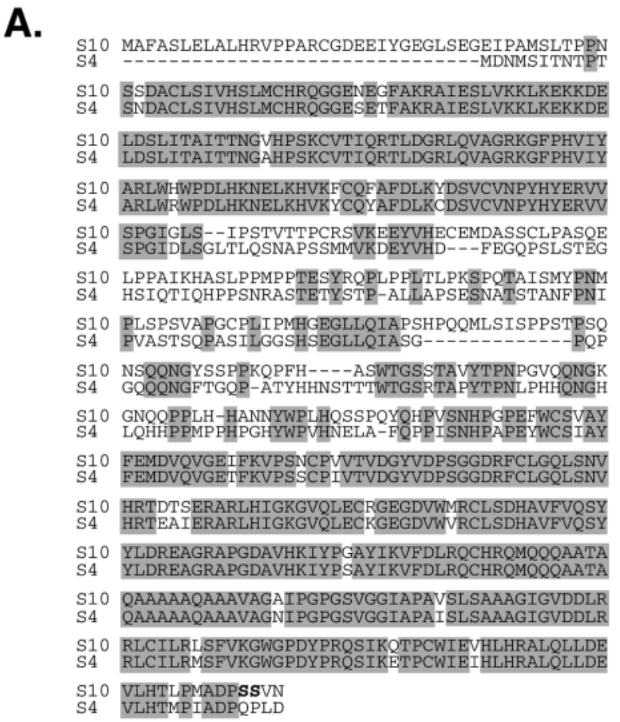
Neural tissue can be induced either directly or indirectly. In indirect, or secondary, neural induction, dorsal mesoderm is formed first and then mimics the Spemann organizer, sending a signal that induces neural tissue. In contrast, the bona fide neural inducer forms neural tissue directly. Direct is defined as neural induction in the absence of dorsal mesoderm formation.

To determine if Smad10 induced neural tissue, we analyzed animal caps for expression of the neural marker NCAM (Kintner and Melton, 1987). To determine if any such neural induction was direct, we also assayed for the expression of the dorsal mesodermal marker, muscle actin (Mohun et al., 1984), and the ventral mesodermal marker, globin (Hemmati-Brivanlou et al., 1990). Smad10 induced expression of NCAM but neither mesodermal marker (Fig. 2B). This has been confirmed in 22 independent experiments. In a small minority of experiments, we have observed a low level of expression of muscle actin by RT-PCR. This has also been reported for the direct neural inducer noggin (Lamb et al., 1993). However, this effect is uncommon and not reproducible, and we have not detected mesodermal derivatives by whole-mount RNA or antibody staining (see below). Therefore, Smad10 appears to directly generate neural tissue.

To confirm that Smad10-mediated formation of neural tissue was direct, we evaluated neural and mesodermal markers by in situ hybridizations and immunohistochemistry. As a specificity control, we expressed the dorsal mesodermal inducer, Smad2, a secondary (indirect) neural inducer (Graff et al., 1996). All Smad10-injected animal caps expressed the neural marker, NRP-1, but not the dorsal mesodermal marker, muscle actin (Fig. 2C). Smad2-injected caps expressed high levels of actin and, through secondary neural induction, some NRP-1 (Fig. 2C).

The conclusion that Smad10 directly forms neural tissue is strengthened by antibody staining. All the Smad10-injected animal caps stained with the neural-specific antibody, 6F11, but not with the muscle-specific antibody, 12/101 (Fig. 2C). In contrast, all Smad2-injected caps reacted with both the neural- and the muscle-specific antibodies (Fig. 2C).

To determine whether Smad10 led to the formation of neural tubes, we examined Smad10-injected animal caps histologically. Hematoxylin- and eosin-stained sections



B.

Smad	% Identity to Smad10
1	32
2	33
3	30
4	63
5	31
6	13
7	18
8	16

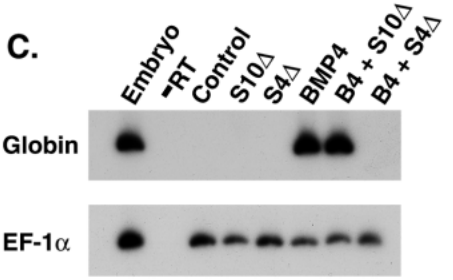


Fig. 1. Amino acid sequence of Smad10 and relationship to other Smads. (A) Alignment of the predicted protein sequence of *Xenopus* Smad10 with human Smad4. Identical residues are indicated by the shaded background. The accession number for Smad10 is AF104232. (B) Percent identity between Smad10 and other Smads. (C) Animal poles of 1-cell embryos were injected with synthetic mRNA encoding Smad10Δ (S10Δ, 4 ng), dominant negative Smad4 (S4Δ, 3 ng), BMP4 (2 ng), or a mixture of BMP4 (B4) with either of the truncated Smads. Animal caps were dissected and cultured. At stage 27, total RNA was harvested and analyzed by RT-PCR. EF-1α is ubiquitously expressed and serves as a loading control (Krieg et al., 1989). RNA from whole embryos (Embryo) is a positive control; the negative control (–RT) is identical to the embryo lane except reverse transcriptase is omitted.

revealed the presence of organized tubes within Smad10-injected animal caps, but none in control caps (Fig. 2D). In situ hybridzations with NCAM revealed vigorous staining within these tubular structures (Fig. 2D). Some of these tubes appear strikingly similar to an endogenous neural tube (Fig. 2D, lower right panel).

Dose response

The results presented in Fig. 2 established that expression of Smad10 forms neural tissue, but not muscle, in animal caps. However, it remained possible that Smad10 induced other mesodermal derivatives, which might then initiate secondary neural induction. To evaluate this possibility, we determined whether any dose of Smad10 could induce expression of the early mesodermal markers brachyury (marker of general mesoderm and the dorsal derivative, notochord, Smith et al., 1991), goosecoid (marker of dorsal mesoderm, Cho et al., 1991) and Xwnt8 (marker of ventral and lateral mesoderm, Christian et al., 1991; Smith and Harland, 1991) or the late markers muscle actin, globin and NCAM (Graff et al., 1994). Smad10-injected animal caps began to express the neural marker NCAM at approximately 500 pg (Fig. 3A). No concentration of Smad10 induced expression of any mesodermal marker (Fig. 3A). In contrast, 1 ng of Smad2 induced all the dorsal mesodermal markers and, through secondary induction, a low level of NCAM (Fig. 3A). Therefore, expression of Smad10 forms neural tissue in a direct, dose-dependent manner.

Smad10 dorsalizes ventral mesoderm

The Spemann organizer directly induces neural tissue and also alters ventral mesoderm to more dorsal fates in a process known as dorsalization (Slack, 1994). To determine if Smad10 had dorsalizing activity, we assessed Smad10 action in the marginal zone, the endogenous site of mesoderm induction and patterning. Control VMZs expressed the ventral mesodermal marker, globin, but not the dorsal mesodermal marker, muscle actin (Fig. 3B). In VMZ explants, Smad10 induced the ectopic expression of muscle actin and eliminated the expression of globin (Fig. 3B). Thus, like the organizer, Smad10 dorsalizes ventral mesoderm.

Smad10 produces secondary axes

The Spemann organizer was defined for its ability to induce a second dorsal axis (Hamburger, 1988; Spemann, 1938). Fig. 4A,B shows that virtually all embryos injected with Smad10 formed secondary axes which as demonstrated by in situ hybridization, contained muscle and neural tissue (data not shown). Histological analysis revealed dorsal axis structures such as muscle, a notochord, and a neural tube within the secondary axes (Fig. 4C).

Smad10 appears to function downstream of the organizer

In principle, Smad10 could carry out Spemann activity in one of two ways. First, it could act within future organizer cells to

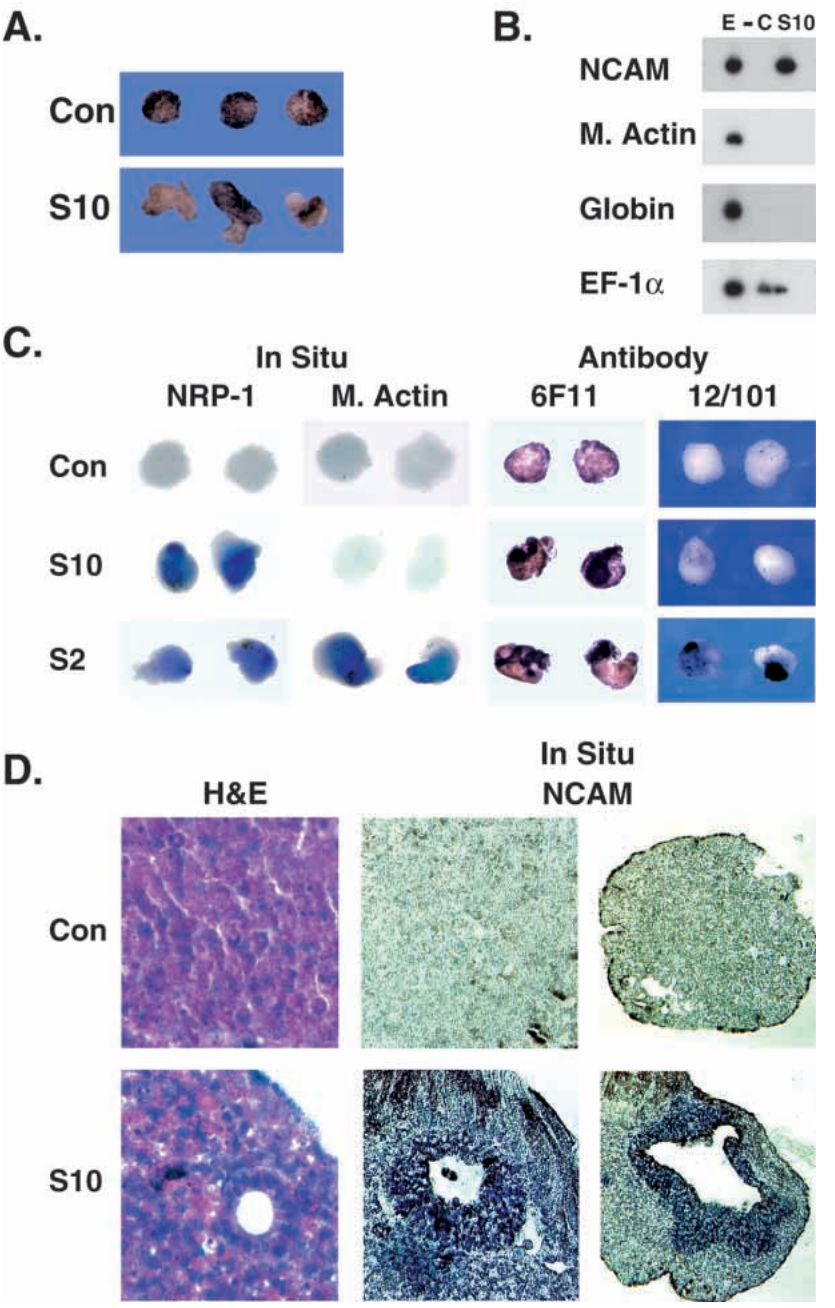


Fig. 2. Smad10 forms neural tissue directly. (A) Synthetic mRNA encoding Smad10 (4 ng) was injected into animal poles of fertilized eggs, and blastula stage animal caps were dissected and cultured until stage 19. Control (Con) animal caps were round while Smad10 (S10)-injected animal caps had an altered shape. (B) Smad10-injected (4 ng) animal caps were cultured until tadpole stage 38. Total RNA was harvested and analyzed by RT-PCR for the presence of the indicated transcripts. C, Control; NCAM, general neural marker; M. Actin, marker of dorsal mesoderm; globin, marker of ventral mesoderm. (C) Animal caps expressing either Smad10 (S10, 4 ng) or Smad2 (S2, 1 ng) were cultured until stage 38 and in situ hybridization and immunohistochemistry performed. Con, control. (D) Control (Con) or Smad10-injected (S10, 4 ng) caps were cultured until stage 34 and then fixed and sectioned for histological analysis (hematoxylin and eosin) and in situ hybridizations.

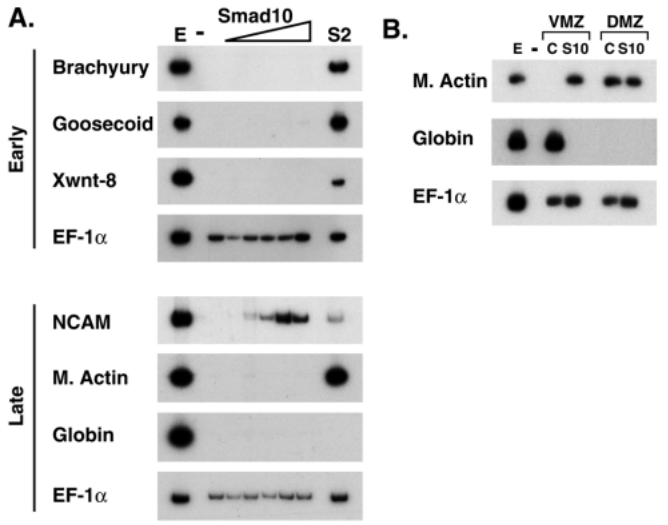


Fig. 3. Smad10 mimics Spemann organizer function. (A) Animal poles expressing Smad10 in a 2-fold dilution series from 250 pg to 4 ng or Smad2 (1 ng) were cultured until either gastrula stage 11 (Early) or tadpole stage 38 (Late) and analyzed by RT-PCR. Markers and lanes are as described in the Fig. 2 legend and the results. (B) 2-cell embryos were injected with Smad10 (S10, 2 ng) into the equatorial region of both blastomeres and, upon formation of the dorsal blastopore lip, ventral marginal zones (VMZ) and dorsal marginal zones (DMZ) were dissected and cultured. At stage 38, RNA was extracted and analyzed by RT-PCR for the presence of muscle actin, globin, and EF-1α. The lanes are as described in Fig. 2.

mediate organizer inducing Nieuwkoop signals or, second, it could act within prospective neural and dorsal mesodermal cells to transduce organizer-generated signals. To distinguish these two possibilities, we determined whether Smad10 localizes to and is active in the cells of the second axis; if so, then it may function downstream of organizer signals. To determine the fate of the cells in which Smad10 is active, Smad10 mRNA was coinjected with the lineage tracer *lacZ*. We found that staining localized to the second axis (Fig. 4D). Histological sections revealed β-galactosidase staining in all cell types of the second axis (not shown). Thus, Smad10 is present in the cells that form the second axis, suggesting that Smad10 is downstream of an organizer signal.

To further test the position of Smad10 in organizer activity, we evaluated whether Smad10 could induce the formation of the organizer. Presumably, if Smad10 mediates Spemann signals, organizer-specific genes will not be expressed. Smad10-injected animal caps were analyzed by RT-PCR for expression of the organizer genes: chordin, noggin, siamois, Xnr3, follistatin and goosecoid (Lamb et al., 1993; Smith et al., 1993; Sasai et al., 1994, 1995, 1996; Hemmati-Brivanlou et al., 1994; Smith et al., 1995; Carnac et al., 1996; Cho et al., 1991). As a postive control for organizer formation, we also injected mRNA encoding Xwnt8 (Smith and Harland, 1991). Xwnt8 induced expression of the organizer-specific genes: chordin, siamois, Xnr3 and noggin (Fig. 4E). In contrast, Smad10 did not induce the expression of any of the organizer genes (Fig. 4E), suggesting that Smad10 acts downstream of the organizer.

We also examined the ability of Smad10 to induce the expression of organizer genes in the marginal zone, the endogenous site of organizer formation. As described above, Smad10 converts VMZs to terminally differentiated dorsal fates (Fig. 3B). However, those experiments did not address whether the Smad10 dorsalization was due to ectopic organizer formation or whether the dorsalization was conferred downstream of the organizer. To discriminate between these two possibilities, Smad10 or Xwnt8 mRNA was injected into the prospective marginal zones of two-cell embryos and ventral marginal zones were explanted and analyzed. In VMZs, Xwnt8 induced the expression of all the organizer genes examined (Fig. 4F). In contrast, Smad10 did not promote expression of any of the organizer genes in the VMZ (Fig. 4F). Notably, in the same assay, blockade of BMP signaling does induce expression of organizer-specific genes in the VMZ (Graff et al., 1994, data not shown). These VMZ studies parallel the animal cap data and, coupled with the lineage tracing experiments, suggest that Smad10 neither induces formation of an organizer nor functions within the organizer. Rather, it appears to exert its effects downstream of the organizer.

Smad10 is expressed at the appropriate time and place to convey organizer signals

For Smad10 to play an endogenous role in mediating organizer signals, it must be expressed in the cells that receive signals from the organizer during normal development. These are the cells of the dorsal side of the embryo adjacent to the organizer. However, if Smad10 transduces Spemann signals, it must also be present on the ventral side of the embryo, as these tissues respond to the organizer when it is grafted to ectopic locations. By semi-quantitative RT-PCR (Fig. 5A), Smad10 is expressed through gastrulation, when Spemann signaling is active, and then decreases (Fig. 5B). In contrast, EF-1α expression increased during development. To localize Smad10 transcripts, we dissected specific regions of embryos. During gastrulation, Smad10 is expressed at approximately equal levels in all regions of the embryo (Fig. 5C). Whole-mount and section in situ hybridization studies confirmed this ubiquitous expression of Smad10 (not shown). The peak of expression through gastrulation and the equality of expression of Smad10 throughout the embryo are consistent with the ability of both ventral and dorsal tissues to respond to the organizer signal.

Smad10 induces both anterior and posterior neural fates

Smad10 mimics the organizer's ability to directly induce neural tissue and to dorsalize ventral mesoderm. The molecules previously described to exhibit these two activities, the BMP inhibitors, induce neural tissue that is only anterior in character (Hansen et al., 1997; Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1997; Lamb et al., 1993). In contrast, the Spemann organizer induces the entire spectrum of anterior and posterior neural tissue (Lamb and Harland, 1995). To examine the character of the neural tissue induced by Smad10, we analyzed Smad10-injected animal caps for induction of the anterior neural marker otx2 (Lamb et al., 1993) and the posterior spinal cord marker HoxB9 (Wright et al., 1990), as well as two markers of intermediate neural fates; engrailed, which marks the midbrain/hindbrain junction (Hemmati-Brivanlou et al., 1991), and Krox20, which marks rhombomeres 3 and 5 of the hindbrain (Bradley et al., 1993). As a control, we also expressed noggin, a prototypic BMP inhibitor, at a dose that induced NCAM expression roughly

equal to that induced by Smad10 (Fig. 6). The noggin-injected caps expressed the anterior neural marker otx2 but not the more posterior markers, as reported (Fig. 6) (Lamb et al., 1993). Strikingly, Smad10 induced expression of all the anterior-posterior neural markers (Fig. 6). Thus, Smad10, like the organizer, generates both anterior and posterior neural tissues.

Smad10 does not block BMP signals

The ability of Smad10 to induce the expression of anterior and posterior neural markers suggested that it might not be a BMP inhibitor. BMP4 induces formation of ventral mesoderm (Graff et al., 1994). To test whether Smad10 blocks BMP4 signaling, we examined the expression of mesodermal markers in animal caps injected with BMP4 with or without Smad10 (Fig. 7A). As a positive control for BMP inhibition, noggin was expressed at a dose that leads to roughly the same level of NCAM expression as does Smad10. BMP4 induced the mesodermal markers brachyury and Xwnt-8. Noggin completely eliminated all mesoderm induced by BMP4. In contrast, Smad10 did not block BMP4-dependent expression of brachyury or Xwnt-8.

Smad10 functions intracellularly whereas BMP4 and noggin are secreted. It is possible that Smad10 overcomes BMP signals in cells that express a high amount of Smad10 mRNA. In contrast, cells that received little or no Smad10 mRNA may still be responsive to BMP signaling and express brachyury and Xwnt-8. Thus, the RT-PCR assay would indicate that BMP-signaling was not inhibited in the animal cap as a whole while Smad10 may actually be inhibiting BMP function in individual cells. To address this possibility, we examined the effects of coinjecting Smad10 mRNA with Smad1 mRNA into the animal cap (Fig. 7B). Smad1 transduces BMP signals and, like Smad10, is an intracellular molecule (Graff et al., 1996). Therefore, in coinjection experiments, Smad1 and Smad10 should be present and function in the same cells. Smad1 induced expression of globin, a ventral mesodermal marker. Smad10 coinjection did not reduce the level of Smad1-dependent globin expression, again suggesting that Smad10 does not block BMP signaling.

To further evaluate whether Smad10 and BMP signaling function independently, we used the marginal zone assay. Both Smad10 and BMP inhibitors dorsalize ventral mesoderm. The dorsalization induced by BMP inhibitors is reversed by addition of BMP4 (Piccolo et al., 1996; Re'em-Kalma et al., 1995; Sasai et al., 1995). As our data imply that Smad10 functions in a distinct manner, we determined whether BMP4 could also reverse Smad10 dorsalization of the marginal zone. Control VMZs expressed globin and not muscle actin. Smad10 dorsalized VMZs as demonstrated by the elimination of globin expression and the ectopic induction of muscle actin. Notably, BMP4 did not alter the dorsalization induced by Smad10, providing further evidence that Smad10 and BMP signaling function independently.

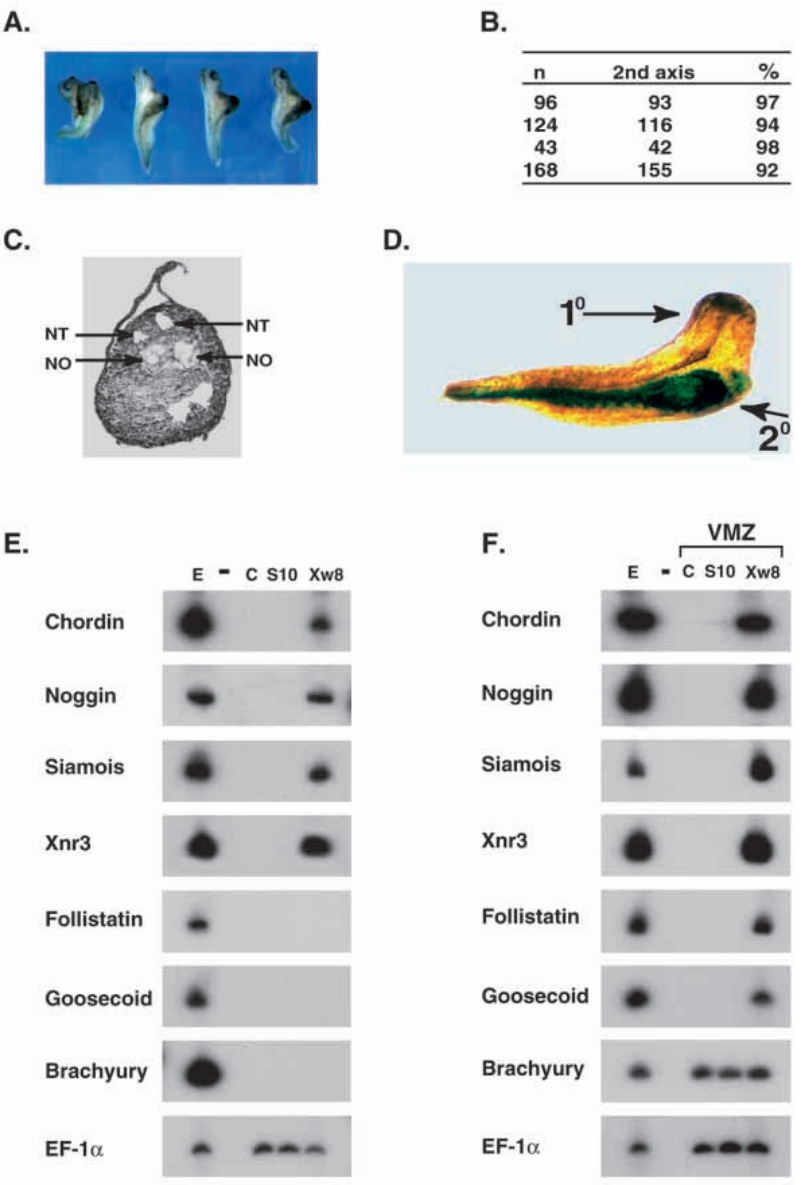


Fig. 4. Smad10 produces secondary dorsal axes. (A) Synthetic mRNA encoding Smad10 (4 ng) was injected at the 8-cell stage into one ventral vegetal blastomere. The injected embryos developed secondary dorsal axes, a few of which were complete (embryo to left) and a majority of which were partial (three embryos to right). (B) Greater than 90% of Smad10 injected embryos develop secondary axes. Embryos were scored for secondary axis formation at stage 16 from 4 independent and representative experiments. (C) Histological analysis of embryos with Smad10-dependent secondary axes revealed the presence of two patterned neural tubes (NT) and two notochords (NO). (D) Smad10 is localized to the secondary axes. Smad10 (4 ng) was co-injected with mRNA encoding β-gal (0.2 ng) into a single ventral vegetal blastomere at the 8-cell stage and the embryos were stained for β-galactosidase activity. The β-galactosidase staining localized to the secondary (2°) axis. (E) Smad10- (S10, 4 ng) or Xwnt8- (80 pg) injected animal caps were analyzed at stage 10.5 by RT-PCR, for expression of organizer markers. (F) 2-cell embryos were injected with Smad10 (S10, 4 ng) or Xwnt8 (80 pg) into the prospective marginal zone of both blastomeres and VMZs were isolated and analyzed at stage 10.5.

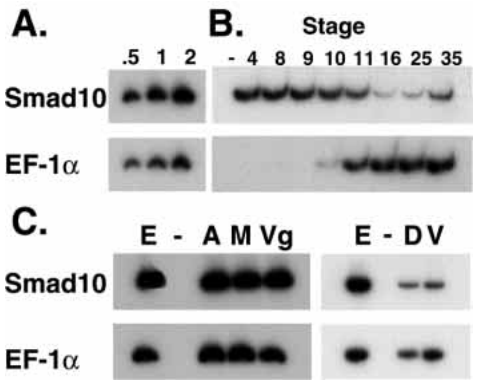


Fig. 5. Smad10 expression during *Xenopus* development. (A) Semiquantitative assay for Smad10 levels. In the RT-PCR based assay, the amount of Smad10 or EF-1α amplified reflected the amount of cDNA (0.5 ul, 1 ul, or 2 ul of the Stage 4 cDNA of Fig 5B) added to the reaction. (B) Developmental time course of Smad10 expression. Embryos were analyzed by RT-PCR for the presence of either Smad10 or EF-1α at each stage. (C) Early gastrula embryos were dissected into roughly equal thirds (animal (A), marginal (M), or vegetal (Vg)) or into dorsal (D) and ventral (V) marginal zones and total RNA was harvested. The RNA was analyzed by RT-PCR for the presence of Smad10 or EF-1α, a loading control.

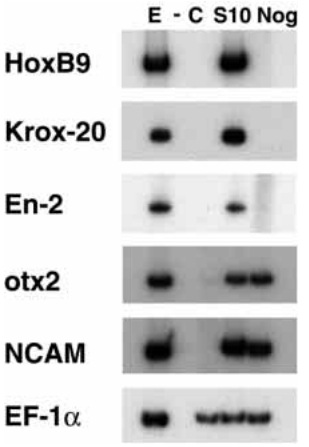


Fig. 6. Smad10 generates anterior and posterior neural fates. Smad10 (S10, 2 ng) or noggin (Nog, 1 ng) mRNA was injected into animal poles and animal caps were dissected, cultured and analyzed by RT-PCR. Expression of Smad10 and noggin generated approximately equal levels of NCAM. The BMP inhibitor, noggin, only induced the anterior neural marker otx2. In contrast, Smad10-injected animal caps expressed all the anterior and posterior neural markers.

As discussed previously, BMP inhibitors are able to induce the formation of neural tissue when expressed in the animal cap (Bouwmeester et al., 1996; Hansen et al., 1997; Hemmati-Brivanlou et al., 1994; Hsu et al., 1998; Lamb et al., 1993; Sasai et al., 1995). Coinjection of BMP4 with the BMP inhibitors reverses this neural induction (Hsu et al., 1998; Piccolo et al., 1996; Sasai et al., 1995). To determine whether BMP4 counteracts Smad10-mediated neural induction, we coinjected Smad10 and BMP4. We found that BMP4 does not block neural induction by Smad10 in the animal cap (Fig. 7D).

The coinjection studies indicated that Smad10 did not block BMP signaling. To further address BMP-pathway/Smad10 interactions, we evaluated the expression of Xvent (Gawantka et al., 1995; Onichtouk et al., 1996). The Xvent promoter is thought to be a direct target of BMP signaling via Smad1 (Candia et al., 1997; Gawantka et al., 1995; Lagna et al., 1996). To examine any potential Smad10 effects on BMP regulation of Xvent, we injected BMP4 into animal caps with and without Smad10 and evaluated the level of Xvent expression (Fig. 7E). We also co-injected BMP4 with noggin as a control for BMP inhibition. A low level of Xvent is present in animal caps and is thought to mark ventral ectodermal fates (Fig. 7E,F) (Gawantka et al., 1995; Onichtouk et al., 1996; Lagna et al., 1996). Both Smad10 and noggin reduced the level of Xvent expression; presumably, because both convert the fate of the cap from ventral ectoderm to dorsal ectoderm (neural tissue) in which Xvent is not expressed (Gawantka et al., 1995; Onichtouk et al., 1996; Candia et al., 1997). BMP4 induced the expression of Xvent (Fig. 7E). Noggin, the BMP inhibitor, completely eliminated BMP4-dependent expression of Xvent (Fig. 7E). In contrast, Smad10 did not block Xvent expression in BMP4-injected animal caps (Fig. 7E). As a control for effects due to the intracellular location of Smad10 (see above), we evaluated whether Smad10 would block Smad1-dependent induction of Xvent. In the animal cap, Smad10 did not interfere with Xvent expression mediated by the intracellular BMP signal transducer, Smad1 (Fig. 7F). These results again demonstrate that Smad10 does not function like a BMP inhibitor.

The BMP inhibitors induce expression of endodermal markers in the animal cap assay (Sasai et al., 1996; Hsu et al., 1998). As a final step to discriminate between Smad10 function and BMP blockade, we determined whether Smad10 could induce endoderm. As reported, microinjection of noggin mRNA led to expression of the endodermal marker endodermin (Fig. 7G) (Sasai et al., 1996; Hsu et al., 1998). In contrast, Smad10 did not induce endoderm.

DISCUSSION

In this study, we describe Smad10, a new member of the Smad family. The Smad family of molecules are intracellular transducers of TGFβ superfamily signals and function downstream of the ligand-receptor complex (reviewed in Derynck and Zhang, 1996; Massague, 1996; Wrana and Attisano, 1996; Heldin et al., 1997). The primary structure of Smad10 is most closely related to the common-partner Smad, Smad4. Smad10 also contains carboxyl-terminal serines which are the sites of phosphorylation in the ligand-activated Smads. Therefore, Smad10 may be a hybrid of these two classes of Smads and function in a new manner. In support of this, Smad10Δ, a form of Smad10 that was truncated in the same position as dominant negative forms of Smad1, Smad2 and Smad4 does not block BMP4 activity as the other truncated Smads do. This suggests that Smad10 functions via a different signaling pathway.

To determine the function of Smad10, we used *Xenopus* embryos. Expression of Smad10 in *Xenopus* embryos directly forms both anterior and posterior neural fates, dorsalizes mesoderm and generates secondary dorsal axes in greater than 90% of experimental embryos-mimicking the Spemann

organizer. In addition, Smad10 functions in the cells of the second axes and does not promote the formation of an organizer, implying that it acts in the cells that receive the Spemann organizer signal. Taken together, these data are consistent with the idea that Smad10 mediates Spemann signaling.

In the current model, Spemann signaling results from inhibiting BMP signals (reviewed in Graff, 1997; Hemmati-Brivanlou and Melton, 1997; Sasai and De Robertis, 1997). A number of experimental findings support this view. For example, previously described molecules that dorsalize and induce neural tissue – follistatin, noggin, chordin, cerberus, gremlin and XNR3-block BMP signals (Hemmati-Brivanlou and Melton, 1997; Hsu et al., 1998). Additional evidence for such a model is provided by studies with dissociated animal cap explants. Intact animal caps form epidermis; however, when dissociated, the cells express neural markers. When BMP protein is added to the dissociated animal caps, they return to an epidermal fate (reviewed in Hemmati-Brivanlou and Melton, 1997; Sasai and DeRo bertis, 1997).

Smad10 functions in a manner consistent with playing a role in Spemann action. However, six lines of evidence indicate that Smad10 induces neural tissue in a manner distinct from the BMP inhibitors. First, the primary sequence of Smad10 is most similar to positively acting Smads (Smad4 and the SSXS Smads). Second, in contrast to the BMP inhibitors, Smad10 does not block gene expression induced by BMP signaling in the animal cap assay. Third, unlike the BMP inhibitors, BMP4 and Smad10 function independently in the marginal zone. Fourth, although the BMP blockers induce endodermal markers, Smad10 does not. Fifth, although BMP4 signaling counteracts neural induction by BMP inhibitors, it does not reverse Smad10-mediated neural induction. Finally, in contradistinction to the molecules that block BMP signals and like the organizer, Smad10 induces both anterior and posterior neural markers. Taken together, these data suggest that Smad10 does not block BMP signals. Therefore, our results suggest that neural induction and dorsalization may occur, not just in the absence of BMP signals, but also as the result of a signal transduced by Smad10.

Although, we favor the view that Smad10 does not block BMP signals, to date, we have no direct biochemical evidence for how Smad10 acts. In principle, this means that Smad10 might block BMP signals in a manner that we cannot detect. Alternatively, Smad10 could function downstream of a BMP inhibitor in an active fashion. In such a case, Smad10 could play a role in BMP inhibitor-mediated neural induction without interfering with BMP activity. This seems unlikely to explain all of the actions of Smad10, as Smad10, unlike the BMP inhibitors, induces both anterior and posterior neural fates.

As discussed in the Introduction, absence of BMP signaling

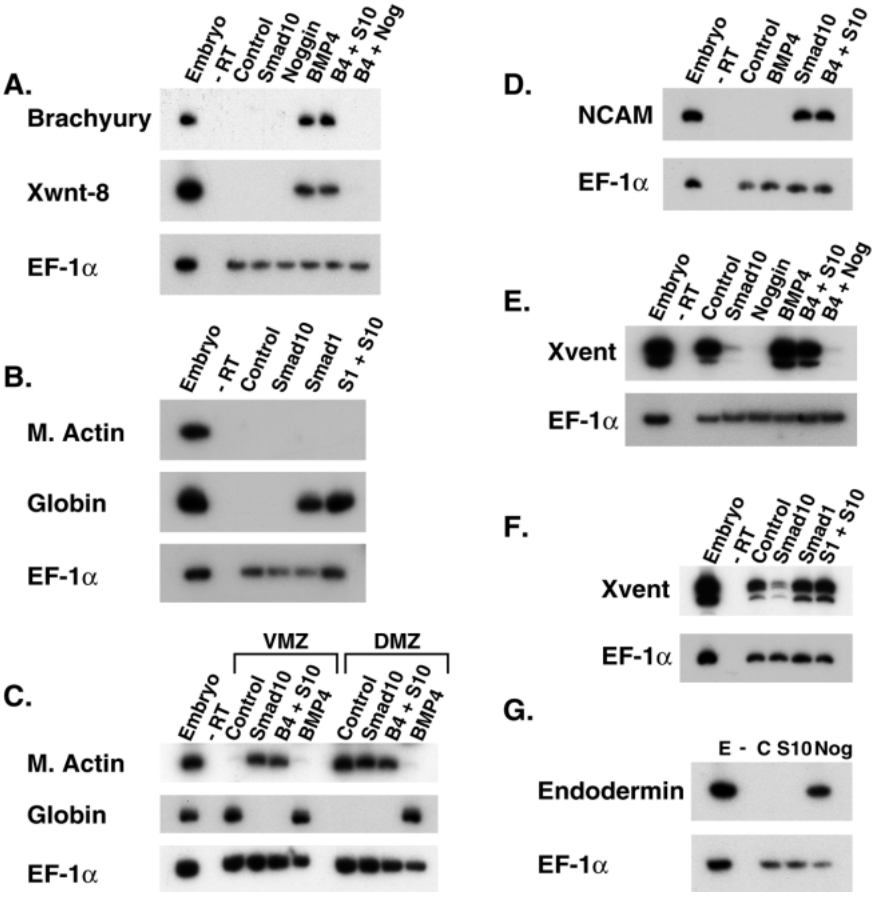


Fig. 7. Smad10 does not block BMP-signaling. (A) Embryos were injected with mRNA encoding Smad10 (2 ng), noggin (1 ng), BMP4 (2 ng), BMP4 (B4) mixed with Smad10 (S10, 2 ng of each), or BMP4 (2 ng) mixed with noggin (Nog, 1 ng). Animal caps were dissected and cultured until stage 10.5 and RNA was analyzed by RT-PCR. The assay, transcripts and lanes are as described previously. (B) Embryos were injected with mRNA encoding Smad10 (2 ng), Smad1 (2 ng), or Smad1 mixed with Smad10 (S1, S10, 2 ng of each), animal caps were cultured until stage 38, and RNA was extracted and then analyzed by RT-PCR. (C) Smad10 (2 ng), Smad10 (S10) mixed with BMP4 (B4, 2 ng of each) or BMP4 (2 ng) were injected and marginal zones explanted and analyzed by RT-PCR as described in the Fig. 3B legend. (D) Smad10 mRNA (4 ng), BMP4 mRNA (2 ng), or a mixture of both (S10, 4 ng; B4, 2 ng) were injected into animal caps and caps were cultured until stage 27. Then, RNA was analyzed by RT-PCR. (E) Embryos were injected with mRNA encoding Smad10 (2 ng), noggin (1 ng), BMP4 (2 ng), BMP4 (B4) mixed with Smad10 (S10, 2 ng of each), or BMP4 (2 ng) mixed with noggin (Nog, 1 ng), and animal caps were dissected, cultured until stage 11.5 and analyzed by RT-PCR. (F) Embryos were injected with mRNA encoding Smad10 (2 ng), Smad1 (2 ng), Smad1 and Smad10 (S1, S10, 2 ng of each), and animal caps cultured until stage 11.5. Then, RNA was extracted and analyzed by RT-PCR. Smad1 induced expression of Xvent. Smad10 did not block Smad1-dependent expression of Xvent. (G) Embryos were injected with mRNA encoding Smad10 (2 ng) or noggin (1 ng) and animal caps cultured until tadpole stage 38 when RNA was extracted and analyzed by RT-PCR for expression of the general endodermal marker endodermin (Sasai et al., 1996).

is not the only condition necessary for dorsal mesodermal induction. A TGFβ signal transduced via a Smad is thought essential for induction of dorsal mesoderm (Hemmati-Brivanlou and Melton, 1992; Lagna et al., 1996). Our data suggest that the parallel may extend to complementary actions of BMP and other active TGFβ signals in neural induction and patterning. It is plausible that Smad10 is the transducer of this signal and that the pattern of the induced neural tissue is then modified by factors such as the BMP inhibitors, FGF signals, or Wnt signals (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; McGrew et al., 1995). The data presented here are also consistent with Smad10 functioning in other roles in neural induction or anterior-posterior patterning of the neuraxis. For example, Smad10 may modify the neural tissue induced by other factors such as the BMP inhibitors. Alternatively, it is possible that Smad10 mediates a signal generated by one or a combination of the above-mentioned factors. That is, Smad10 activity or expression may be induced by one or several of these factors. One drawback with this notion is that Smad10 uniquely induces anterior and posterior neural fates.

Taken together, our results suggest that Smad10 plays a role in mediating the action of the Spemann organizer. Presumably, Smad10 functions downstream of a signaling cascade. Discovery of a ligand that activates Smad10 will provide insights into this novel signaling cascade. Detailed structural analysis coupled with loss-of-function or genetic studies will be required to understand the mechanism of Smad10 action. As Smad10 probably induces neural tissue by activating a cohort of genes, elucidation of its downstream targets may allow one to connect the early inductive events with the later neurogenic program that specifies neuronal fates.

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