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Xenopus Smad8 acts downstream of BMP-4 to modulate its activity during vertebrate embryonic patterning

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SUMMARY

Bone morphogenetic proteins (BMPs) participate in the development of nearly all organs and tissues. BMP signaling is mediated by specific Smad proteins, Smad1 and/or Smad5, which undergo serine phosphorylation in response to BMP-receptor activation and are then translocated to the nucleus where they modulate transcription of target genes. We have identified a distantly related member of the *Xenopus* Smad family, Smad8, which lacks the C-terminal SSXS phosphorylation motif present in other Smads, and which appears to function in the BMP signaling pathway. During embryonic development, the spatial pattern of expression of *Smad8* mirrors that of *BMP-4*. We show that an intact BMP signaling pathway is required for its expression. Overexpression of Smad8 in *Xenopus* embryos phenocopies the effect of blocking BMP-4 signaling, leading to induction of a secondary axis on the ventral side of intact embryos and to direct neural induction in ectodermal explants. Furthermore, Smad8 can block BMP-4-mediated induction of ventral mesoderm-specific gene expression in ectodermal explants. Overexpression of Smad8 within dorsal cells, however, causes patterning defects that are distinct from those reported in BMP-4-deficient embryos, suggesting that Smad8 may interact with additional signaling pathways. Indeed, overexpression of Smad8 blocks expression of *Xbra* in whole animals, and partially blocks activin signaling in animal caps. In addition, Smad8 inhibits involution of mesodermal cells during gastrulation, a phenotype that is not observed following blockade of activin or BMPs in *Xenopus*. Together, these results are consistent with the hypothesis that Smad8 participates in a negative feedback loop in which BMP signaling induces the expression of *Smad8*, which then functions to negatively modulate the amplitude or duration of signaling downstream of BMPs and, possibly, downstream of other transforming growth factor-β (TGF-β) family ligands.

Key words: Smad8, BMP, Neuralization, TGF-β, Spina bifida

INTRODUCTION

Members of the transforming growth factor-β (TGF-β) family of cell-cell signaling molecules play critical roles in specifying cell fate during embryogenesis. For example, BMP-4 and its *Drosophila* homolog, decapentaplegic (Dpp), function in a conserved pathway that establishes the dorsoventral axis of both vertebrate and invertebrate species (reviewed by Ferguson, 1996). In *Xenopus*, expression of *BMP-4* is restricted to cells on the ventral side of gastrula stage embryos. Blocking BMP-4 activity in the ventral part of the *Xenopus* embryo eliminates blood formation and induces formation of a secondary dorsal axis. In addition, when BMP-4 signaling is blocked in prospective epidermal cells, these cells instead differentiate into neural tissue. Thus, BMP-4 is required for ventral mesoderm formation and for repression of neural fate (reviewed by Graff, 1997).

The receptors for BMPs, as for all TGF-β ligands, consist of complexes of type I and type II transmembrane serine-threonine kinases to which BMP ligands bind cooperatively (reviewed by Yamashita et al., 1996). Following ligand binding, type II receptors transphosphorylate the type I receptors, which then propagate the signal. Multiple members of each class of receptor have been identified.

A novel group of structurally related proteins, referred to as Smads, has been shown to transduce signals downstream of BMP and other TGF-β family receptors (reviewed by Massagué et al., 1997). The first member of the Smad family was identified in a genetic screen in *Drosophila* and encodes a protein that transduces signals downstream of Dpp. A *Xenopus* homolog, Smad1, which mediates BMP-4 signaling in frogs, was subsequently isolated. Overexpression of Smad1 (Graff et al., 1996; Thomsen, 1996), or of the closely related protein, Smad5 (Suzuki et al., 1997), produces patterning defects that are identical to those observed following misexpression of BMP-4, i.e. ventralization and repression of neural fate. Two

other vertebrate Smads, Smad2 and Smad3, have been shown to mediate signaling by activin and TGF-β. In addition, Smad4 has been identified as a protein that heteroligomerizes with all of the above pathway-restricted Smads (reviewed by Massagué et al., 1997).

Most Smad proteins are direct substrates for activated TGF-β family receptors. Phosphorylation of pathway-restricted Smads, which occurs on carboxy-terminal serine residues in the sequence SSXS (Macías-Silva et al., 1996; Kretzschmar et al., 1997), leads to association with Smad4 followed by translocation of the complex from the cytoplasm to the nucleus. Within the nucleus, Smad hetero-oligomers associate with unrelated DNA binding proteins and function to modulate transcription of TGF-β family target genes.

We have identified a novel, distantly related member of the *Xenopus* Smad family, Smad8, which lacks the carboxy-terminal SSXS phosphorylation motif found in other Smads. Expression of *Smad8* is positively regulated by BMP signaling but, surprisingly, Smad8 appears to function as an intracellular antagonist of the BMP pathway. We hypothesize that at least one function of Smad8 is to participate in a negative feedback loop that is designed to modulate the amplitude or duration of BMP signaling during embryonic development.

MATERIALS AND METHODS

Molecular cloning of the *Xenopus* Smad8 cDNA

Degenerate PCR primers were designed based on sequence motifs that are conserved between a Smad-related *Drosophila* protein (Dad; Tsuneizumi et al., 1997) and other Smad family members. Total oocyte RNA was reverse transcribed as described (Cui et al., 1996). cDNAs were amplified using the oligonucleotide pair, downward: 5′-G(G/A/T/C) GT(G/T) GG(A/T/C) GA(G/A) TT(T/C) TT(TC) CA-3′ (coding for (R)VGEFF(H), amino acids 411-417 of Dad), and upward: 5′-(G/A/T)CC CCA (G/A/T)CC (T/C)TT (G/A/T)CC (G/A)AA-3′ (coding for FGKGWG, amino acids 540-545 of Dad) to prime the PCR (95°C for 5 minutes, followed by 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute and 72°C for 10 minutes). PCR products were subcloned into pGEM-T (Promega) and sequenced. A cDNA encoding a partial length Smad-related protein (Smad8) was identified and used to screen a *Xenopus* oocyte cDNA library (kindly provided by D. Melton). 10^6 independent phage were screened and ten clones rescreened positive through three rounds of purification. One of these was fully sequenced on both strands and was found to contain a single open reading frame that initiated with an ATG and terminated with a stop codon. The 5′ region of an overlapping clone, which includes sequence 5′ of the putative ATG start codon, revealed the presence of stop codons in all three frames upstream of this ATG, thereby confirming that our original cDNA contains the full coding region of Smad8. The accession number for Smad8 sequence is AF026125. This cDNA was recloned into pCS2+ (Rupp et al., 1994) in both directions to yield pCS2+Smad8-sense and pCS2+Smad8-antisense.

Embryo culture and manipulation

Xenopus eggs were obtained, embryos injected with synthetic mRNAs and cultured as described (Moon and Christian, 1989). Embryonic stages are according to Nieuwkoop and Faber (1967). Capped synthetic RNA was generated by in vitro transcription of pCS2+Smad8-sense and -antisense, pSP64T-XBMP-4, pCS2+nβgal, pCS2+MT, pCS2+Flag-Smad7 and pSP64T-tBR. The tBR cDNA was amplified from embryonic cDNA using the PCR and published primers (Graff et al., 1994), and was sequenced to verify that errors had not been introduced into the coding region. Animal caps were isolated and cultured as described (Cui et al., 1996). Activin A was obtained from the National Hormone and Pituitary Program (Rockville, MD).

Immunostaining, in situ hybridization and histological analysis

Whole-mount immunocytochemical analysis using the muscle-specific monoclonal antibody 12/101 or the notochord-specific monoclonal antibody Tor70 (Bolce et al., 1992; a gift of R. Harland) was performed as described (Moon and Christian, 1989). Whole-mount in situ hybridization was performed as described (Harland, 1991) using BM Purple AP Substrate (Boehringer Mannheim). Following in situ hybridization, some embryos were dehydrated in methanol, embedded in paraffin and sections 14-20 μm thick were cut and counterstained with eosin (Christian and Moon, 1993).

Analysis of molecular markers by RT-PCR

RNA was isolated and reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed as described (Cui et al., 1996) using the following PCR conditions: 95°C for 5 minutes, followed by a variable number of cycles (determined empirically to be in the linear range for each primer pair using RT product of control embryo RNA) at 94°C for 30 seconds; 55°C for 30 seconds and 72°C for 30 seconds. Each RNA sample was isolated from 10 pooled caps or from 5-15 pooled VMZ or DMZ for each experiment. The sequences of *Xwnt-8* (Cui et al., 1996), *EF1-α*, *N-CAM*, *α-actin*, *OtxA*, *XAG1* and *Xbra* (Tsuneizumi et al., 1997) primers have been reported. Smad8 primers were as follows: downward, 5′-ATGTTCAGGACCAAACGATC-3′; upward, 5′-CTCCTTCAGCTTCTTCAGGAC-3′. The PCR products were visualized with a Molecular Dynamics phosphorimager and quantified using the MacIntosh IP lab gel program. Each experiment was repeated at least once with similar results.

RNase protection assay

RNase protection analysis of RNA isolated from whole embryos (20 μg), from dissected regions of cleaving embryos (10 μg), or from five animal caps was subjected to RNase protection analysis as described (Cui et al., 1995). Protected bands were visualized with a Molecular Dynamics phosphorimager and were quantified using the MacIntosh IP lab gel program. Each experiment was repeated at least once with similar results.

RESULTS

Molecular cloning and structural characterization of Smad8

A partial length cDNA encoding *Xenopus* Smad8 was obtained using a PCR-based strategy (see Materials and methods) and was subsequently used to isolate cDNAs containing the full coding region of Smad8 from a *Xenopus* cDNA library. The predicted sequence of Smad8 protein is shown in Fig. 1. Smad8 is most closely related to the recently reported vertebrate Smad7 (74% amino acid identity) (Hayashi et al., 1997; Nakao et al., 1997), but shares less overall identity (26%-28%) with other *Xenopus* Smads reported to date (Graff et al., 1996; Thomsen, 1996) (Fig. 1). Smad proteins typically consist of three modules: highly conserved amino- (termed MH1) and carboxy-terminal (MH2) domains, and a poorly conserved linker region (reviewed by Massagué et al., 1997). *Xenopus* Smad1 and 2 share 55% and 72% identity within MH1 and MH2 domains respectively, whereas Smad8 shares 20-25% and 28-30% identity with Smad1 and 2 within these same domains. Smad8, like Smad6 (Imamura et al. 1997), Smad7 and *Drosophila* Dad (Tsuneizumi et al., 1997), is truncated at the

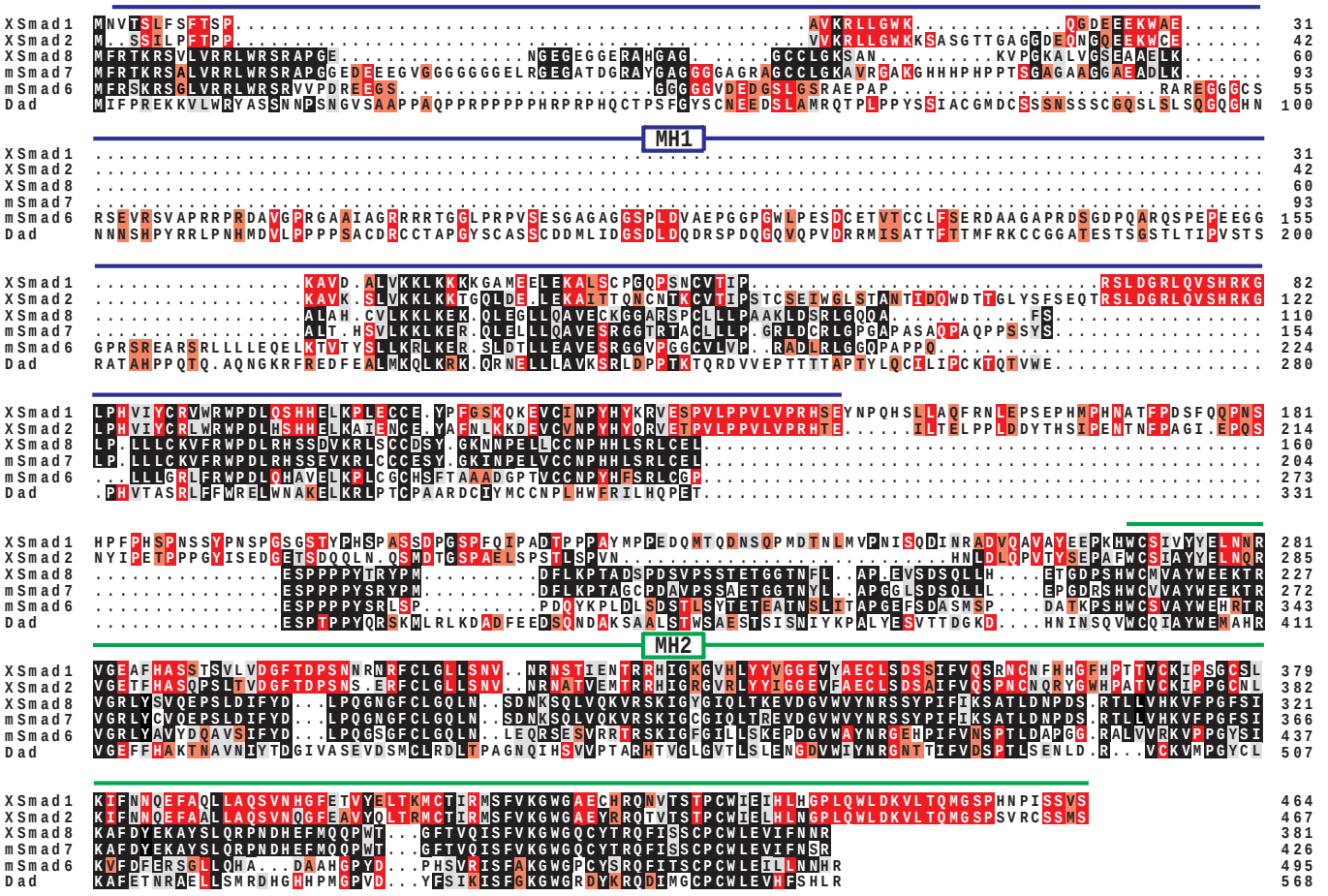

Fig. 1. Comparison of deduced amino acid sequences of selected Smads. The complete amino acid sequences of *Xenopus* Smad (XSmad) 1, 2 and 8, mouse Smad (mSmad) 6 and 7, and *Drosophila* Dad are shown aligned with each other. Amino acid identity between Smad8 and other genes is indicated by black boxes while conservative substitutions are represented by gray boxes. Identical residues that are conserved among Smads other than Smad8 are shown in red and conservative substitutions are indicated by light red shading. MH1 and MH2 domains are shown by purple and green lines, respectively.

C terminus relative to other Smad proteins and therefore lacks the C-terminal SSXS phosphorylation motif that is present in Smad1, 2, 3 and 5. These C-terminally truncated Smads also contain a conserved stretch of amino acids within the linker region (ESP(P/T)PPYXR; amino acids 161-169 of Smad8), which is not shared with other Smads (Fig. 1). Thus, *Smad8* encodes a novel protein that is most closely related to mammalian Smad7.

The embryonic spatio-temporal patterns of expression of Smad8 are similar to those of BMPs

Ribonuclease (RNase) protection analysis of RNA isolated from whole embryos, or from dissected embryonic fragments, revealed the presence of maternally derived *Smad8* transcripts as well as fairly steady levels of transcripts throughout embryonic development. *Smad8* RNAs are uniformly distributed across the dorsal-ventral and animal-vegetal axes of cleavage stage embryos (Fig. 2).

The spatial pattern of expression of *Smad8* was examined by whole-mount in situ hybridization of digoxygenin-labeled *Smad8* riboprobes to developmentally staged albino *Xenopus* embryos (Fig. 3). *Smad8* transcripts are uniformly distributed across the dorsoventral axis of early gastrulae (Fig. 3A), but become progressively restricted to cells on the ventral side during gastrulation (Fig. 3B). The ventral enrichment of *Smad8* transcripts was confirmed by RT-PCR analysis of *Smad8* expression in tissues explanted from the dorsal or ventral marginal zone of gastrula stage embryos (Fig. 4C). In early neurulae, *Smad8* transcripts remain ventrally restricted and are especially concentrated in cells near the anterior end (Fig. 3C). In tailbud through tadpole stage embryos, expression of *Smad8* becomes increasingly restricted to the developing heart (Fig. 3D-F,I, closed arrowheads), eye (Fig. 3D-F,I, black arrows), olfactory placode (Fig. 3G, asterisk), otic vesicle (Fig. 3F,G, white arrows) and dorsal-most parts of selected regions of the brain and neural tube (Fig. 3D-F,H,I, open arrowheads).

The spatio-temporal patterns of expression of *Smad8* resemble those reported for *BMP-4*. *BMP-4* is expressed maternally, although at very low levels (Köster et al., 1991; Dale et al., 1992) and, like Smad8 (Fig. 3B), zygotic expression becomes progressively restricted to ventral regions during gastrulation (Fainsod et al., 1994; Schmidt et al., 1995). In tadpoles, BMP-4 is expressed in the eye, otic vesicle, heart (Fig. 3G; Fainsod et al., 1994; Schmidt et al., 1995; Hemmati-

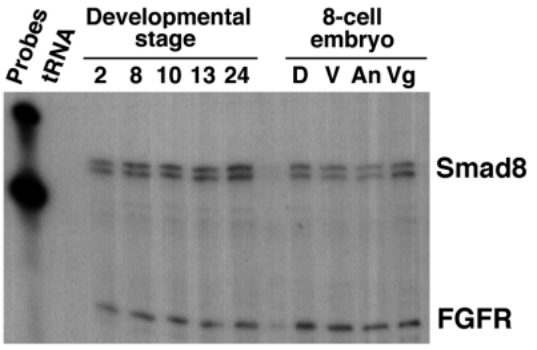

Fig. 2. *Xenopus* Smad8 transcripts are present throughout development. Equivalent amounts of RNA isolated from developmentally staged embryos, or from dissected animal (An) and vegetal (Vg), or dorsal (D) and ventral (V) halves of 8-cell embryos were analyzed for expression of *Smad8* and *fibroblast growth factor receptor* (*FGFR*, as a control for equal loading) in an RNase protection assay.

Brivanlou and Thomsen, 1995) and in dorsal regions of the brain (S. S. G., T. N. and J. L. C., unpublished data). The domains of expression of *Smad8* appear to be somewhat broader than those of *BMP-4* (Fig. 3G).

An intact BMP-4 signaling pathway is required for expression of Smad8 in vivo

The overlap in the dynamic expression patterns of *BMP-4* and *Smad8* led us to examine the possibility that BMP signaling is necessary for expression of *Smad8*. Initially we blocked or upregulated BMP function in animal pole cells by microinjection of 1 ng of RNA encoding a dominant negative form of the BMP-2/4 receptor (tBR) or 1 ng of RNA encoding BMP-4, respectively, into 2-cell embryos. Animal caps were isolated at the late blastula stage and expression of *Smad8* was analyzed at stage 12 by RNase protection (Fig. 4A). In several independent experiments, blockade of BMP signaling led to at least a threefold decrease, whereas upregulation of BMP signaling led to at least a 1.9-fold increase, in levels of *Smad8* transcripts when normalized to levels of *FGFR* transcripts (Fig. 4A).

We further analyzed the ability of overexpressed BMP-4 to upregulate expression of *Smad8* in whole embryos (Fig. 4B) and in explanted tissues (Fig. 4C). As shown in Fig. 4B, when 500 pg of BMP-4 mRNA was injected near the animal pole of 2-cell embryos, localized regions of intense staining were observed following in situ hybridization of Smad8 riboprobes to whole gastrulae. Furthermore, microinjection of 500 pg of BMP-4 mRNA near the dorsal midline of 4-cell embryos led to a 2.7- to 4.0-fold increase in levels of Smad8 transcripts in dorsal marginal zone explants relative to explants isolated from uninjected embryos (values from two independent experiments; normalized to levels of *EF-1α*) (Fig. 4C). In contrast, blockade of BMP function in ventral cells, by injection of 300 pg of tBR RNA, led to a 2.7- to 8.5-fold reduction in Smad8 transcripts in gastrula stage ventral marginal zone explants (values from two independent experiments) (Fig. 4C). These results demonstrate that BMP signaling is required for expression of *Smad8*, and that *Smad8* is a target gene, either directly or indirectly, downstream of BMP signaling.

Overexpression of Smad8 in isolated animal caps results in direct neural induction

The observation that expression of *Smad8* is regulated by BMP-4 raises the possibility that Smad8 might act downstream of BMP-4 to mediate its effects. If Smad8 is sufficient to transduce a BMP-4-like signal, then overexpression of Smad8

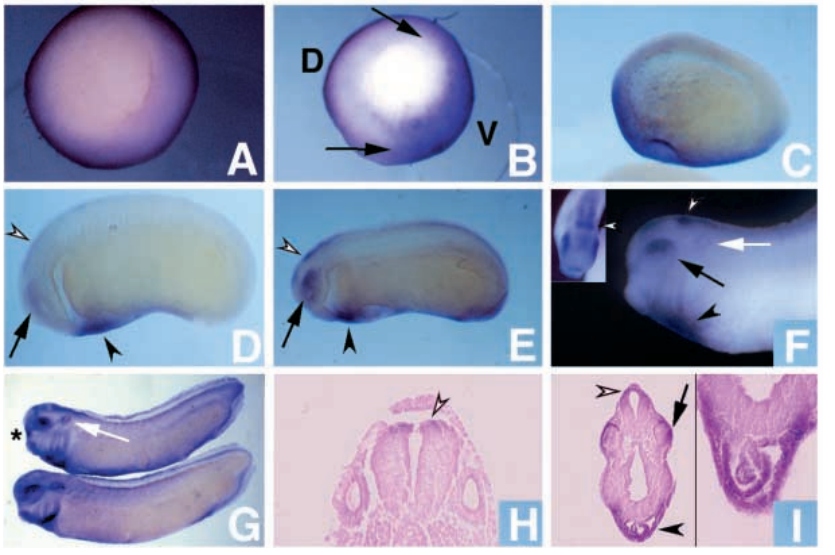

Fig. 3. The spatial pattern of expression of *Smad8* in developing *Xenopus* embryos is similar to that of *BMP-4*, as analyzed by whole-mount in situ hybridization. (A,B) Vegetal views of gastrulae hybridized with a Smad8 antisense riboprobe. *Smad8* transcripts are uniformly distributed at the onset of gastrulation (stage 10) (A) but become restricted to cells on the ventral (V) side by the stage 11 (B). Arrows indicate the border of expression between ventral and dorsal (D) cells. (C) Lateral view of a neurula (stage 15) stage embryo (anterior to the left). *Smad8* transcripts are ventrally restricted and concentrated in cells near the anterior end. (D-F) Lateral views of stage 22 (D), stage 25 (E) and stage 27 (F) embryos (anterior to the left, ventral down). During these stages, expression of *Smad8* becomes increasingly restricted to the heart anlage (closed arrowhead), to dorsal regions of the eye (arrow), and to the brain (open arrowhead). (F) In tailbud stage embryos, weak expression of *Smad8* is observed in the otic vesicle (white arrow), and discrete bands of *Smad8*-expressing cells are observed in the anterior central nervous system (inset). (G) Lateral views of stage 30/31 embryos hybridized with Smad8 (top) and BMP-4 (bottom) probes. Both genes are expressed in the eye, the otic vesicle (white arrow), the brain and the heart anlage, while *Smad8* (asterisk), but not *BMP-4*, is expressed in the olfactory placode. (H) Transverse section of a stage 27 embryo at the level of the hindbrain. Expression of *Smad8* is restricted to the dorsal part of the brain (open arrowhead). (I) Transverse sections of stage 34/35 embryos at the level of the forebrain. (Left) Expression of *Smad8* in the dorsal forebrain (open arrowhead), eye (arrow) and heart (closed arrowhead) is observed. (Right) Higher magnification view demonstrates expression throughout the heart. Embryos shown in (C-E,G) were cleared in benzyl benzoate:benzyl alcohol (2:1).

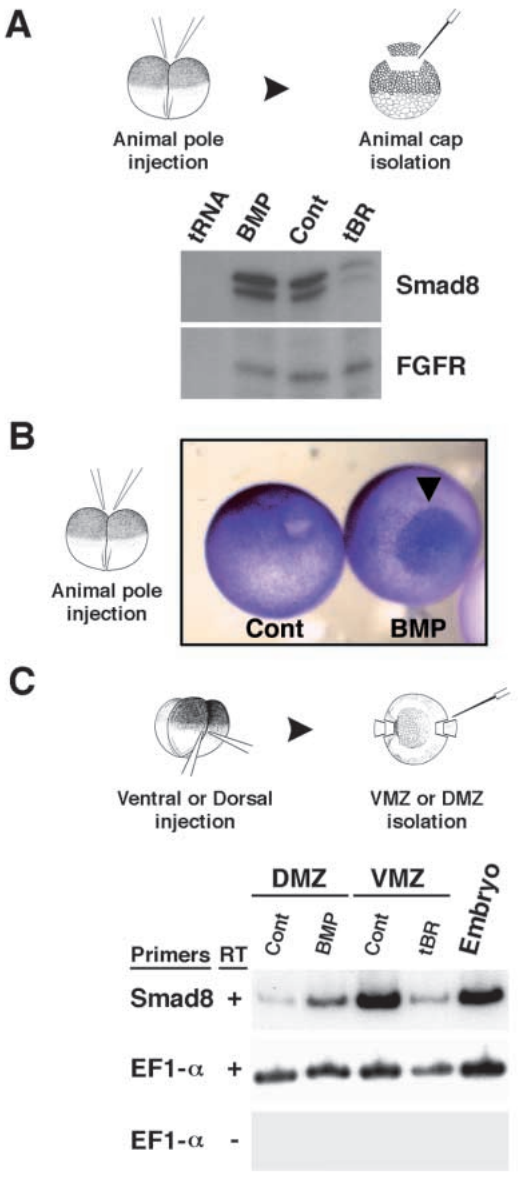

Fig. 4. BMP signaling is required for *Smad8* expression. (A) Animal caps made to overexpress either BMP-4 or a truncated BMP receptor (tBR) were isolated at the blastula stage and cultured until stage 12, at which time expression of *Smad8* and *FGFR* was analyzed by RNase protection. Overexpression of BMP-4 or blockade of BMP-4 signaling led to upregulation or downregulation, respectively, of expression of *Smad8* relative to *FGFR*. (B) BMP-4 RNA was injected into the animal pole of 2-cell embryos and expression of endogenous *Smad8* was analyzed by whole-mount in situ hybridization at stage 12. BMP-4 RNA-injected embryos showed localized upregulation of *Smad8* expression (arrowhead) relative to uninjected (Cont) embryos. (C) Dorsal or ventral marginal zone cells (DMZ or VMZ) made to overexpress BMP-4 or tBR, respectively, were isolated at stage 10 and cultured until stage 12, at which time expression of *Smad8* and *EF-1α* was analyzed by RT-PCR. Signal was not observed in the absence of reverse transcriptase (RT–). *Smad8* transcripts are much more abundant in VMZ than in DMZ explants isolated from control (Cont) embryos. Injection of BMP-4 (BMP) RNA upregulated *Smad8* expression in DMZ explants, while blockade of BMP activity (tBR) downregulated *Smad8* expression in VMZ explants.

in isolated animal caps should phenocopy the effects of overexpressing BMP-4, i.e. should lead to ventral mesoderm formation (reviewed by Graff, 1997). To test this possibility, we upregulated expression of Smad8 in isolated animal caps by injection of synthetic RNA (200 pg). Ectodermal explants (animal caps) made to overexpress Smad8 formed well-developed cement glands (Fig. 5B, arrowheads), while explants from control embryos retained a rounded epidermal appearance (Fig. 5A). Examination of molecular markers showed that Smad8-injected explants expressed cement gland-specific (*XAG*) and neural-specific (*N-CAM*, *OtxA*) genes but not a dorsal mesodermal gene, *α-actin* (Fig. 5C), nor a pan-mesodermal gene, *Xbra* (Fig. 5D, see below), indicating that Smad8 can directly mediate neural induction in the absence of mesoderm. This phenotype is identical to that produced by blockade, rather than activation, of the BMP signaling pathway (reviewed by Graff, 1997).

To further test the possibility that Smad8 can antagonize BMP function, we injected RNA encoding either BMP-4 (3 ng) or Smad8 (200 pg), or co-injected both RNAs, into the animal pole of 2-cell embryos. Blastula stage ectodermal explants were isolated and analyzed at the late gastrula stage for expression of two BMP-inducible mesodermal genes: *Xwnt-8* (Hemmati-Brivanlou and Thomsen, 1995) and *Xbra* (Schmidt et al., 1995). Expression of *Xwnt-8* and *Xbra* is not observed in control or Smad8-injected caps (Fig. 5D). BMP-4 induced expression of both *Xwnt-8* and *Xbra* (Fig. 5D), but induction of expression of both genes was almost completely repressed by co-injection of Smad8 RNA (Fig. 5D). This suggests that Smad8 can inhibit BMP-4-mediated mesoderm induction.

Overexpression of Smad8 in ventral cells induces formation of a partial secondary axis

In addition to directly inducing ectodermal cells to form neural tissue, BMP antagonists can induce ventral mesodermal cells to adopt a dorsal fate (reviewed by Graff, 1997). To further test the function of Smad8 as a BMP-antagonist, we overexpressed Smad8 on the ventral side of cleaving embryos. When Smad8 RNA (100 pg) was injected near the marginal zone of two ventral blastomeres of 4-cell embryos, 65% of injected embryos (n=121) formed a partial secondary dorsal axis and 6% of the induced axes included a fused or cyclopic eye. Injection of 200 pg of Smad8 RNA led to secondary axis formation in 88% of embryos (n=86), 59% of which contained a fused or cyclopic eye (Fig. 6B, arrow). Immunstaining with notochord- or muscle-specific antibodies revealed that the secondary axes induced by Smad8 lacked a notochord (Fig. 6D) but most contained some muscle (Fig. 6F), which was often disorganized. Embryos injected with antisense Smad8 RNA (100-200 pg) appeared morphologically normal (data not shown).

Ectopic expession of Smad8 in dorsal cells causes spina bifida and eye defects

We also examined embryos that had been made to overexpress Smad8 in dorsal cells for phenotypic defects. As shown in Fig. 7, over 90% of embryos injected dorsally with Smad8 RNA (100-200 pg) developed spina bifida, a condition in which the neural folds fail to fuse. Overexpression of Smad8 in dorsal cells also caused variable eye defects (Fig. 7A, arrows), ranging from complete loss of eyes to formation of a single fused or cyclopic eye. Axial structures including notochord

(Fig. 7B), muscle (Fig. 7C) and neural tissue (data not shown) were present in Smad8-injected embryos but were split along either side of the cleft caused by the defect in neural tube closure. Spina bifida and eye defects have not been reported in BMP-deficient *Xenopus* embryos.

Overexpression of Smad8 blocks *Xbra* expression in embryos and partially blocks activin signaling in animal caps

The observation that overexpression of Smad8 causes defects that are not observed following overexpression of BMP-specific antagonists raises the possibility that Smad8 might also modulate signaling downstream of other members of TGF-β family. The recent finding that Smad7, a structurally related Smad protein, can inhibit transduction of TGF-β/activin-like signals (Hayashi et al., 1997; Nakao et al., 1997) led us to examine whether Smad8 can do the same. Blockade of endogenous activin/Vg1 signaling in early *Xenopus* embryos leads to a loss of mesoderm formation, which is reflected in the loss of expression of the mesoderm-specific gene, *Xbra* (Hemmati-Brivanlou and Melton, 1992). When 200 pg of Smad8 mRNA was injected near the equator of one blastomere of 2-cell embryos, *Xbra* expression was abolished on one side of the embryo, consistent with the possibility that Smad8 can inhibit endogenous activin/Vg1-like signals. A complete ring of *Xbra*-expressing cells was observed in embryos injected with 200 pg of a control mRNA encoding six copies of a Myc-epitope tag (Fig. 8A).

In order to directly test the ability of Smad8 to block signaling downstream of activin, animal caps were isolated from noninjected, Smad7 RNA-injected, or Smad8 RNA-injected embryos at the blastula stage and were cultured in the presence or absence of activin (20 ng/ml) until stage 18. Noninjected control caps elongated (data not shown) and expressed the mesodermal markers *Xbra* and *α-actin* in the presence, but not in the absence, of activin (Fig. 8B). In contrast, caps injected with either Smad7 or Smad8 mRNA (400 pg) showed less elongation in the presence of activin (data not shown). Smad7-injected caps expressed 85±6% (mean ± s.e.m. from three independent experiments) and 82±4% (n=3) fewer *α-actin* and *Xbra* transcripts, respectively, than did noninjected caps when normalized to levels of *EF1-α*. Smad8-injected caps expressed 21±11% (n=3) and 69±3% (n=3) fewer *α-actin* and *Xbra* transcripts, respectively, than did controls. These results are consistent with the published function of Smad7 (Hayashi et al., 1997; Nakao et al., 1997), and suggest that Smad8 can also antagonize activin-like signaling. It is not clear whether the apparently weaker blockade of activin signaling by Smad8 relative to Smad7 is due to intrinsic properties of the two proteins, or whether this reflects lower levels of expression of Smad8 protein. Notably, Smad8 does produce a more complete blockade of BMP signaling (Fig. 5D) than it does of activin signaling (Fig. 8B), despite injecting twice as much RNA in the activin blockade experiment.

Overexpression of Smad8 inhibits dorsal mesodermal involution during gastrulation

To better understand the phenotypes caused by overexpression of Smad8, we traced the lineage of Smad8-injected cells by co-injection of RNA encoding a nuclearly localized form of β-

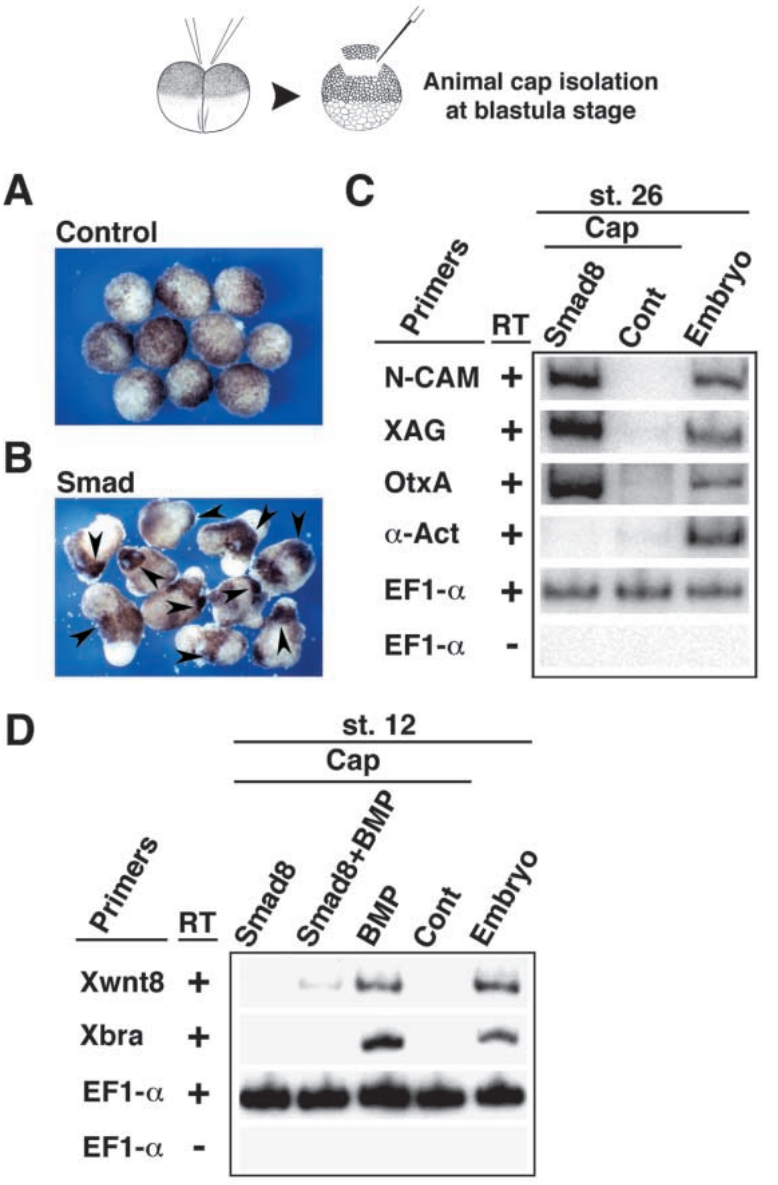

Fig. 5. Smad8 antagonizes BMP signaling in isolated animal caps. (A-C) Animal caps were isolated at the blastula stage from control embryos, or from embryos that had been injected with Smad8 RNA at the 2-cell stage, and were cultured until sibling embryos reached stage 26. Control caps (A) differentiated into epidermis, while Smad8-injected caps (B) formed cement glands (arrowheads). (C) Smad8-injected, but not control explants expressed cement gland-specific (*XAG*) and neural-specific (*N-CAM*, *OtxA*) genes but failed to express the dorsal mesodermal marker *α-actin* (α-Act) as analyzed by RT-PCR. Signal was not observed in the absence of reverse transcriptase (RT–). (D) Smad8 and BMP-4 RNAs were injected alone, or together, near the animal pole of 2-cell embryos and animal caps were explanted at the blastula stage. Expression of *Xwnt-8*, *Xbra* and *EF-1α* was analyzed by RT-PCR in the presence (+) and absence (–) of reverse transcriptase (RT) when explants reached control stage 12. Co-expression of Smad8 blocked BMP-4-mediated induction of the ventral mesodermal marker (*Xwnt-8*) and pan-mesodermal marker (*Xbra*) in isolated animal caps.

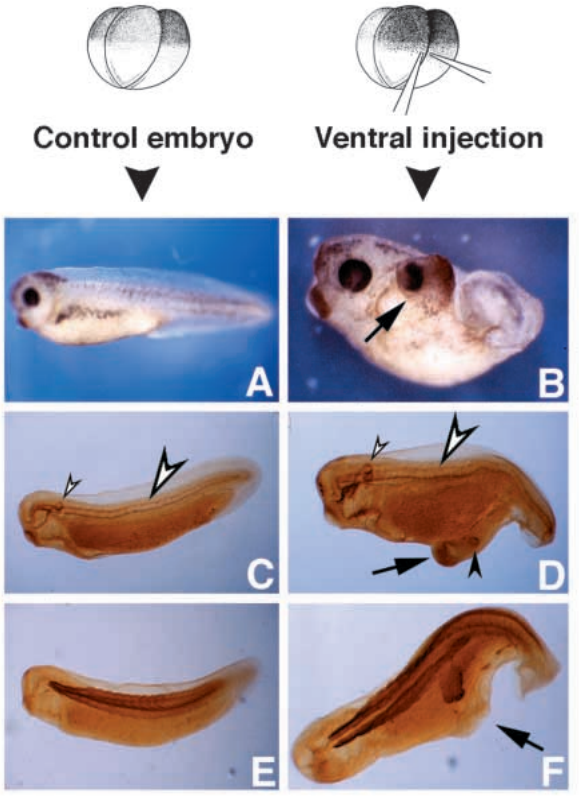

Fig. 6. Smad8 induces a partial secondary dorsal axis when overexpressed on the ventral side of the embryo. Tadpole stage control embryos (A,C,E) and sibling embryos that had been injected ventrally with Smad8 RNA at the 4-cell stage (B,D,F) are shown. Smad8 induces the formation of a partial secondary axis (arrows, B,D,F) that often contains a cyclopic eye (B). Immunostaining with Tor70 antibodies reveals the presence of notochord in control embryos (large arrowhead, C) and in the primary axis (large arrowhead, D) but not in the secondary axis (closed arrowhead, D) of Smad-injected embryos. In contrast, an otic vesicle is detected in both primary (small open arrowheads, C,D) and in secondary (closed arrowhead, D) axes. Immunostaining with a muscle-specific antibody (E,F) reveals the presence of disorganized muscle in the secondary axis of some Smad8-injected embryos.

galactosidase (nβ-gal) (Fig. 9). When nβ-gal mRNA was injected near the dorsal midline of 4-cell embryos, nβ-gal-expressing cells converged toward the midline during gastrulation, forming a thin stripe of β-galactosidase positive cells (Fig. 9E), and involuted toward the future anterior end (black arrowhead, Fig. 9I). When Smad8 and nβ-gal RNAs were co-injected into dorsal cells, these cells failed to converge toward the midline during gastrulation, yielding a much broader stripe of X-gal staining cells (Fig. 9F, compare to 9E). In addition, involution of the dorsal mesoderm was severely retarded (Fig. 9J, black arrowhead, compare to Fig. 9I). The observation that Smad8 inhibits convergence and involution of dorsal cells during gastrulation raises the possibility that a mechanical defect underlies the later failure of the neural folds to close. At the tadpole stage, cells injected with nβ-gal mRNA alone were distributed along the entire length of the dorsal axis (Fig. 9A) while cells made to co-express Smad8 were concentrated near the anterior end, but were also present in dorsal structures on either side of the split neural tube (Fig. 9B).

When nβ-gal mRNA alone was injected into ventral cells, these cells did not converge toward the midline during gastrulation (Fig. 9G), and they involuted much less extensively (Fig. 9K, white arrowhead) than did their dorsal counterparts (Fig. 9K, black arrowhead). Ventral cells that were co-injected with Smad8 and nβ-gal also failed to converge (Fig. 9H) and, in many cases, ventral lip formation and involution was completely absent (Fig. 9L, white arrowhead). Thus, Smad8 can inhibit the morphogenetic movements that drive gastrulation on both dorsal and ventral sides of the embryo. At the tailbud stage, β-galactosidase-positive cells were restricted to posterior and ventral tissues in control embryos (Fig. 9C), while cells derived from Smad8-injected ventral blastomeres were concentrated near the anterior end of the induced secondary dorsal axis (Fig. 9D). Blockade of BMP-and activin signaling, achieved by overexpression of a dominant negative receptor that is shared by both ligands, causes a similar dorsoanterior relocation of ventral cells (Hemmati-Brivanlou and Melton, 1994).

DISCUSSION

The recent identification and characterization of Smad proteins has greatly enhanced our understanding of the pathway leading from activation of cell surface TGF-β family receptors to control of target gene expression in the nucleus. In the present studies, we describe a novel role for Smad8 as an antagonist, rather than a transducer, of BMP and activin signaling.

BMP signaling is regulated at multiple levels during embryonic development

BMPs exemplify a recurrent theme in developmental biology: diverse cell fates are often specified by the temporally and spatially regulated activation of a single signaling pathway. For example, BMPs act early in embryogenesis to specify ventral fate within the mesoderm and ectoderm, but they are also required at later stages for specification and/or patterning of the skin, limbs, lungs, teeth, gut and nearly all other organs and tissues (reviewed by Hogan, 1996).

Consistent with their multifunctional nature, the expression and biological activity of BMPs is highly regulated (reviewed by Hogan, 1996). Recent studies have revealed the existence of two secreted proteins (noggin and chordin) that bind extracellular BMP-2 and -4 with high affinity and thereby block BMP-mediated activation of cognate cell-surface receptors (reviewed by Graff, 1997). A third potential BMP antagonist, Xnr3, has been proposed to operate intracellularly to block synthesis and/or secretion of an active BMP ligand (Hansen et al., 1997).

In *Xenopus*, noggin, chordin and Xnr3 are expressed only in dorsal cells. Presumably, they promote dorsal fates by neutralizing BMP-4 that is secreted from ventral cells, and by directly inhibiting the function of BMP-4-related proteins that are synthesized in dorsal cells (reviewed by Thomsen, 1997). In contrast, the spatial pattern of expression of *Smad8* is similar to that of *BMP-4* and, in fact, we have shown that an intact BMP signaling pathway is required for its expression. These results suggest that Smad8 functions directly within BMP-responsive cells to modulate the amplitude or duration of BMP signaling. This may be an important mechanism for fine-tuning

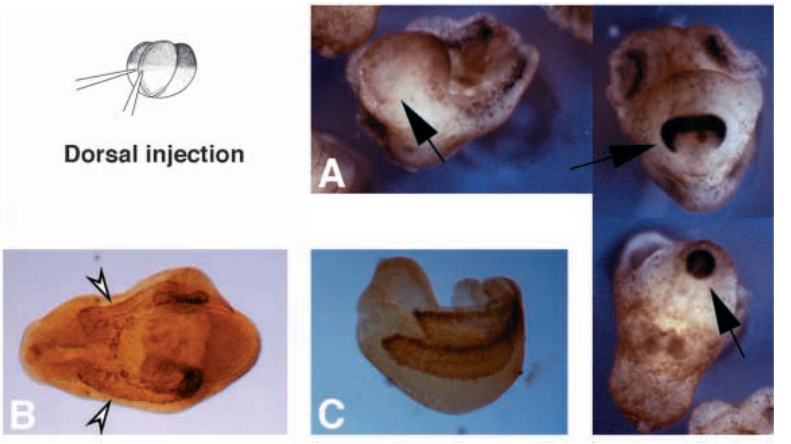

Fig. 7. Ectopic expression of Smad8 in dorsal cells of *Xenopus* embryos causes spina bifida and eye defects. Smad8 RNA was injected into the dorsal marginal zone of 4-cell embryos, which were then cultured to the tadpole stage. Embryos developed with spina bifida along with variable eye defects (A, arrows) ranging from complete loss of eyes (top left, dorsoanterior view, anterior to the left) to formation of a single fused (top right, dorsoanterior view, anterior down) or cyclopic eye (bottom right, head-on view). (B) Immunostaining with notochord-specific (B, dorsal view) or muscle-specific (C, dorsolateral view) antibodies revealed that notochord and muscle were intact in Smad8-injected embryos, but were split along either side of the cleft caused by the defect in neural tube closure.

the activity of a signaling pathway that performs multiple and varied functions throughout embryogenesis.

A number of analogous cases, in which activation of a signaling cascade induces the expression of a negative modulator of the same pathway, have been described. For example, activation of the *Drosophila* EGF receptor (DER) within the ectoderm specifies ventral fate and induces the expression of ARGOS, a secreted inhibitor of ligand binding to the DER. ARGOS is hypothesized to antagonize the EGF pathway in lateral cells, thereby maintaining the distinction between ventral and ventrolateral fate (reviewed by Schweitzer and Shilo, 1997). Similarly, activation of the Hedgehog (HH) pathway, in both vertebrates and invertebrates, induces expression of the transmembrane protein Patched, which binds HH and thereby restricts its range of action (reviewed by Hammerschmidt et al., 1997). Finally, recent studies have identified proteins whose expression is induced by cytokine-mediated stimulation of the JAK/STAT signaling pathway, but which then function as negative regulators of this pathway (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). Similar negative feedback circuits may play important roles in fine-tuning the activity of many signaling molecules which, like BMPs, are used over and over again during embryogenesis to specify very different cells fates. In fact, a novel *Drosophila* gene, *Dad*, was recently identified whose expression is induced by Dpp signaling, but which functions as an antagonist of Dpp activity (Tsuneizumi et al., 1997). Although *Dad* encodes a member of the Smad family, it shares only limited sequence homology with Smad8.

Smad8 may interact with signaling pathways distinct from those initiated by BMPs

When dominant negative BMP receptors or ligands are used to inactivate the BMP pathway within dorsal cells of *Xenopus* embryos, embryos are reported to develop normally (Ishikawa et al., 1995) or with enlarged heads (Hawley et al., 1995). In contrast, overexpression of the putative BMP antagonist Smad8 leads to eye defects, raising the possibility that Smad8 interacts with signaling pathways other than those

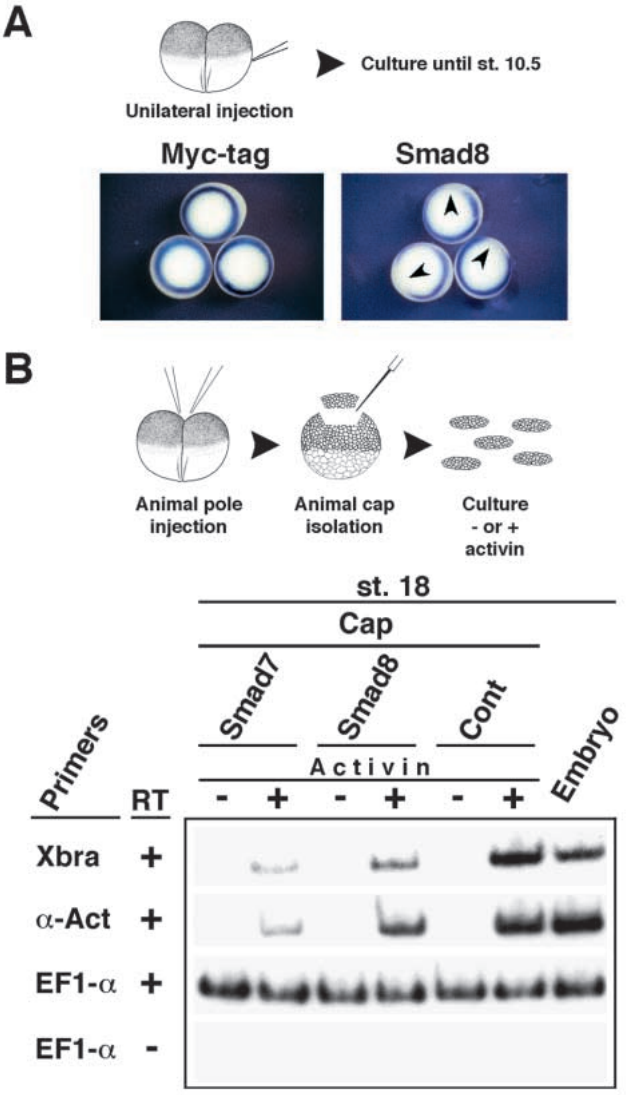

Fig. 8. Smad8 blocks *Xbra* expression in embryos and partially antagonizes activin signaling in animal caps. (A) Smad8 or Myc-epitope tag mRNA (200 pg) was injected into one blastomere of 2-cell embryos near the equator and the embryos were cultured until stage 10.5. *Xbra* expression was analyzed by whole-mount in situ hybridization. Arrowheads indicate loss of *Xbra* expression in Smad8-injected embryos. (B) Smad7 or Smad8 mRNA (400 pg) was injected into the animal pole of 2-cell embryos. Animal caps were isolated at the blastula stage and cultured in the presence or absence of human activin A (20 ng/ml) until sibling embryos reached stage 18, at which time expression of *Xbra, α-actin and EF1-α* was analyzed by RT-PCR.

initiated by BMPs. This possibility is supported by our demonstration that, at higher doses, Smad8 can partially antagonize activin-like signaling. Inhibition of activin signaling has been reported to cause deficiencies in anterior differentiation (Dyson and Gurdon, 1997), and thus, this might explain the loss of eyes following overexpression of Smad8. Alternatively, Smad8 might provide a more potent or persistent blockade of BMP signaling than can be achieved by overexpression of other BMP-specific antagonists. Consistent with this possibility, eye development initiates normally in mice carrying a targeted deletion of the BMP-7 gene, but terminal differentiation is blocked (Dudley et al., 1995; Luo et al., 1995). Thus, the loss of eyes that is observed in Smad8-overexpressing embryos might be explained by a Smad8-mediated blockade of BMP-7 signaling that persists throughout the terminal differentiation phase of eye development.

The spina bifida that is observed following dorsal overexpression of Smad8 is preceded by abnormalities in the morphogenetic movements of gastrulation. Specifically, dorsal cells fail to converge toward the midline during gastrulation, formation of the dorsal lip of the blastopore is delayed or absent, and involution of the dorsal mesoderm is inhibited, resulting in asymmetrical formation and closure of the blastopore ring. Gastrulation defects have been observed to correlate with, and may play a causal role in, the later failure of the neural folds to fuse (Keller, 1984; Lee and Gumbiner, 1995).

Interestingly, a distantly related member of the TGF-β family, *Xnr3*, is expressed on the dorsal side of *Xenopus* embryos and has been proposed to be involved in promoting the morphogenetic cell movements that drive gastrulation (Smith et al., 1995). Recently, a number of T-box genes, whose expression can be induced by members of TGF-β family, have also been implicated in driving gastrulation (Lustig et al., 1996; Zhang and King, 1996; Stennard et al., 1996; Horb and Thomsen, 1997). Thus, it is possible that the Smad8-induced gastrulation defects, and subsequent spina bifida, are due to antagonism of Xnr3, or of other TGF-β family members that can induce expression of these T-box genes, although this remains to be tested. If so, it is possible that one of the physiological roles of Smad8 is to antagonize the activity of Xnr3 or other TGF-β family members within ventral cells, thereby limiting the involution of ventral, relative to dorsal, mesodermal cells during gastrulation.

Although overexpressed Smad8 is capable of partially blocking activin signaling, the physiological relevance of this inhibition is not clear. One attractive possibility is that ventrally localized Smad8 inhibits dorsalizing signals provided by activin (or a related molecule), thereby promoting ventral development. Several observations argue against this hypothesis. First, the observation that activin-like activity is uniformly distributed across the dorsoventral axis (Watabe et al., 1995) does not support the existence of a ventral-specific inhibitor. Second, Smad8 does not become ventrally restricted until mid- to late-gastrula stages, at which time activin cannot function as a dorsalizing agent (Lettice and Slack, 1993). Third, doses of Smad8 that inhibit endogenous BMP signaling and partially block induction of *Xbra*, do not cause a loss of dorsal derivatives such as notochord and muscle (Fig. 7B,C). While it is possible that Smad8 functions as a general inhibitor of TGF-β signaling throughout development, our observations that Smad8 more potently inhibits BMP signaling than it does activin signaling, that BMP signaling is necessary and sufficient for expression of endogenous *Smad8*, and that the expression patterns of *Smad8* closely resemble those of *BMP-4* lead us to favor the hypothesis that Smad8 functions primarily in a negative feedback loop to modulate BMP activity. Loss of function studies will be required to fully test this hypothesis.

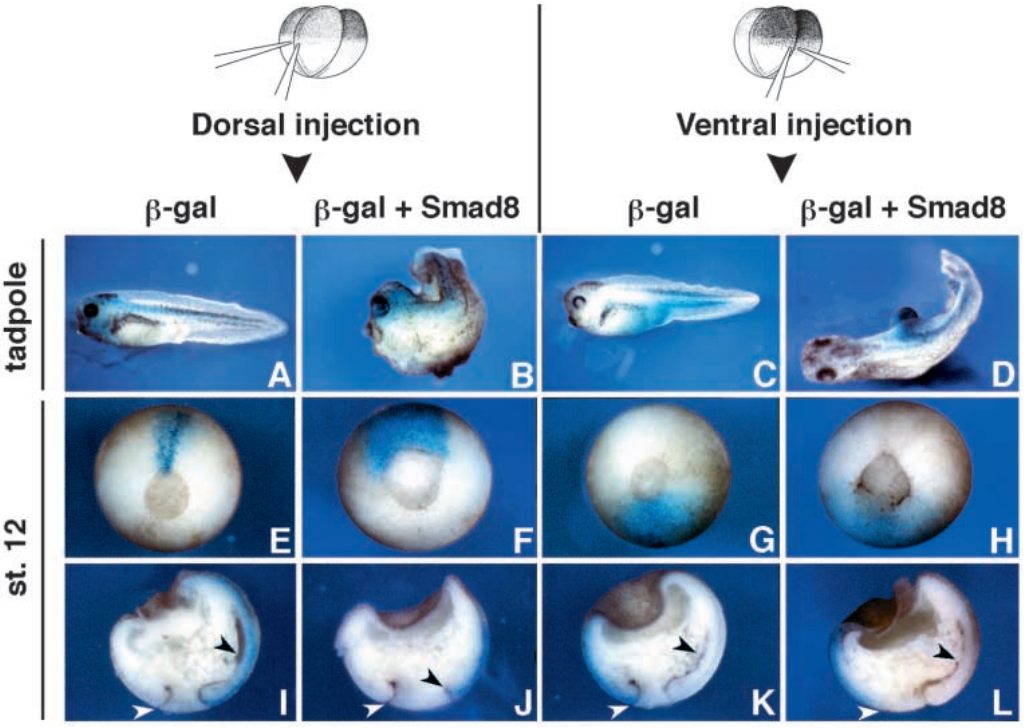

Fig. 9. Overexpression of Smad8 inhibits morphogenetic movements during gastrulation and causes a dorsoanterior relocation of ventral cells. nβ-gal mRNA (100 pg) was injected with (B,D,F,H,J,L) or without (A,C,E,G,I,K) Smad8 mRNA (200 pg) into the dorsal (A,B,E,F,I,J) or ventral (C,D,G,H,K,L) marginal zone of 4-cell embryos. (A-D) Localization of nβ-gal-expressing cells (stained in light blue) in tadpole-stage embryos. (E-H) Vegetal views of localization of nβ-gal-expressing cells during gastrulation (stage 12). Dorsal side is up. (I-L) Sagittal sections of embryos shown in E-H. Black arrowheads indicate involution of dorsal cells, white arrowheads denote the position of the ventral lip of the blastopore. Ventral is to the left and the animal pole is at the top in each panel.

Molecular mechanism of action of Smad8

Smad8 could potentially act at any step within the BMP signal transduction cascade to block signal output. Vertebrate genes that, like Smad8, encode C-terminally truncated Smad proteins (Smad6 and 7) and which function as antagonist of TGF-β signaling have recently been reported (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997). Smad6 and 7 can form a stable complex with the activated TGF-β type I receptor, thereby blocking the association, phosphorylation, and activation of Smad2 and/or 3. Smad6 can also associate with, and block signal transduction downstream of some type I BMP receptors (Imamura et al., 1997). These findings support the possibility that Smad8 acts in an analogous fashion, i.e. that it stably associates with the BMP type I receptor to block Smad1- and/or 5-mediated transduction of BMP signals. It is likely that a novel class of Smad proteins exist which antagonize, rather than transduce, signals downstream of TGF-β family ligands.

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Note added in proof

While this manuscript was in press, Chen et al (*Proc. Natl. Acad. Sci. USA* **94**, 12938-12943), published an article describing the cloning and characterization of a cDNA encoding a Smad-related protein, designated Smad8, which mediates signaling downstream of ALK2. This protein is distinct from the *Xenopus* Smad8 described here.