Development 134, 3861-3872 (2007) doi:10.1242/dev.007179

# Neural induction requires continued suppression of both Smad1 and Smad2 signals during gastrulation

**Chenbei Chang[1] and Richard M. Harland[2]**

Vertebrate neural induction requires inhibition of bone morphogenetic protein (BMP) signaling in the ectoderm. However, whether inhibition of BMP signaling is sufficient to induce neural tissues in vivo remains controversial. Here we have addressed why inhibition of BMP/Smad1 signaling does not induce neural markers efficiently in *Xenopus* ventral ectoderm, and show that suppression of both Smad1 and Smad2 signals is sufficient to induce neural markers. Manipulations that inhibit both Smad1 and Smad2 pathways, including a truncated type IIB activin receptor, Smad7 and Ski, induce early neural markers and inhibit epidermal genes in ventral ectoderm; and co-expression of BMP inhibitors with a truncated activin/nodal-specific type IB activin receptor leads to efficient neural induction. Conversely, stimulation of Smad2 signaling in the neural plate at gastrula stages results in inhibition of neural markers, disruption of the neural tube and reduction of head structures, with conversion of neural to neural crest and mesodermal fates. The ability of activated Smad2 to block neural induction declines by the end of gastrulation. Our results indicate that prospective neural cells are poised to respond to Smad2 and Smad1 signals to adopt mesodermal and non-neural ectodermal fates even at gastrula stages, after the conventionally assigned end of mesodermal competence, so that continued suppression of both mesoderm- and epidermis-inducing Smad signals leads to efficient neural induction.

**KEY WORDS: Neural induction, BMP, Smad1, Nodal, Smad2, *Xenopus***

## INTRODUCTION

The concept that vertebrate neural tissue is induced in the ectoderm by signals from dorsal mesoderm was derived from the organizer grafting experiments reported in 1924 (Spemann and Mangold, 1924) (reviewed by Harland, 2000). Since then, equivalent organizing centers have been identified in other vertebrates, including rabbit, mouse, chick and fish (Waddington, 1932; Oppenheimer, 1936; Beddington, 1994; Shih and Fraser, 1996; Knoetgen et al., 2000; Saude et al., 2000); all of these organizers induce ectopic neural tissues when transplanted into more ventral regions. The hunt for substances that confer neural identity to the ectoderm began soon after Spemann and Mangold's discovery (Witkowski, 1985), but progress in identifying endogenous neural inducers came only about 70 years later with the application of molecular biological tools.

The first direct neural inducer cloned from a vertebrate was *noggin*, which was subsequently shown to be an organizer-specific soluble bone morphogenetic protein (BMP) inhibitor (Smith and Harland, 1992; Lamb et al., 1993; Zimmerman et al., 1996). Other organizer-secreted BMP antagonists, including chordin, follistatin, Xnr3 and Cerberus, were then identified. Manipulations involving overexpression, dominant-negative mutants and antisense depletion of these molecules as well as BMP signaling components indicate that the BMP pathway plays an essential role in neural induction (reviewed by Harland and Gerhart, 1997; Weinstein and Hemmati-Brivanlou, 1999; Harland, 2000; De Robertis and Kuroda, 2004; Vonica and Brivanlou, 2006). Taken together, the evidence suggests that ectodermal cells have an inherent tendency toward the neural identity, but constitutive BMP signaling in the ectoderm prevents realization of the neural fate. Signals emanating from the organizer disrupt the BMP pathway, so that ectodermal cells can follow their intrinsic program to adopt the neural lineage. This view is known as the default model of neural induction (Hemmati-Brivanlou and Melton, 1997).

Challenges to the default model emerged recently from studies both in *Xenopus* and in other vertebrate species, particularly in chick; and fibroblast growth factor (FGF) and canonical Wnt signals have both been implicated in the neural induction process. In *Xenopus*, blocking FGF signals by different means has been shown by some groups to prevent neural induction in explants and in embryos, although others find anterior neural induction to prevail when FGF/Ras signaling is moderately inhibited (Ribisi et al., 2000). Activation of FGF signaling was first shown to cause neural differentiation of isolated cells by Kengaku and Okamoto (Kengaku and Okamoto, 1993), and subsequently the FGF-stimulated mitogen-activated protein kinase (MAPK) pathway has been shown to cooperate with BMP inhibition to promote neural induction (Lamb and Harland, 1995; Launay et al., 1996; Hongo et al., 1999; Linker and Stern, 2004; Delaune et al., 2005; Wawersik et al., 2005). Unlike organizer-expressed BMP antagonists, no FGF ligand has been shown to be specifically localized to the region of neural induction in *Xenopus*, so it appears that low-level FGF signaling may at most be a permissive signal for general neural induction, whereas localized FGF signaling is important for posterior patterning. This contrasts with the probable localized role of FGF8 and FGF3 in the chick (Streit et al., 2000; Wilson et al., 2000). Canonical Wnt signaling, mediated by β-catenin and the TCF/Lef family of transcription factors, also participates in neural induction, although it may have opposite activities in promoting and inhibiting neural tissues at different developmental stages (Baker et al., 1999; Wessely et al., 2001; Heeg-Truesdell and LaBonne, 2006). Both FGF and Wnt pathways are reported to crosstalk with and inhibit BMP signaling. The Erk members of the MAPK family function downstream of FGF and insulin-like growth factor receptors to phosphorylate the linker

[1]Department of Cell Biology, MCLM 360, University of Alabama at Birmingham, Birmingham, AL 35294-0005, USA. [2]Department of Molecular and Cell Biology, and Center for Integrative Genomics, University of California, Berkeley, Life Sciences Addition, #3200, Berkeley, CA 94720-3200, USA.

e-mail: cchang@uab.edu; harland@berkeley.edu

**Accepted 14 August 2007**

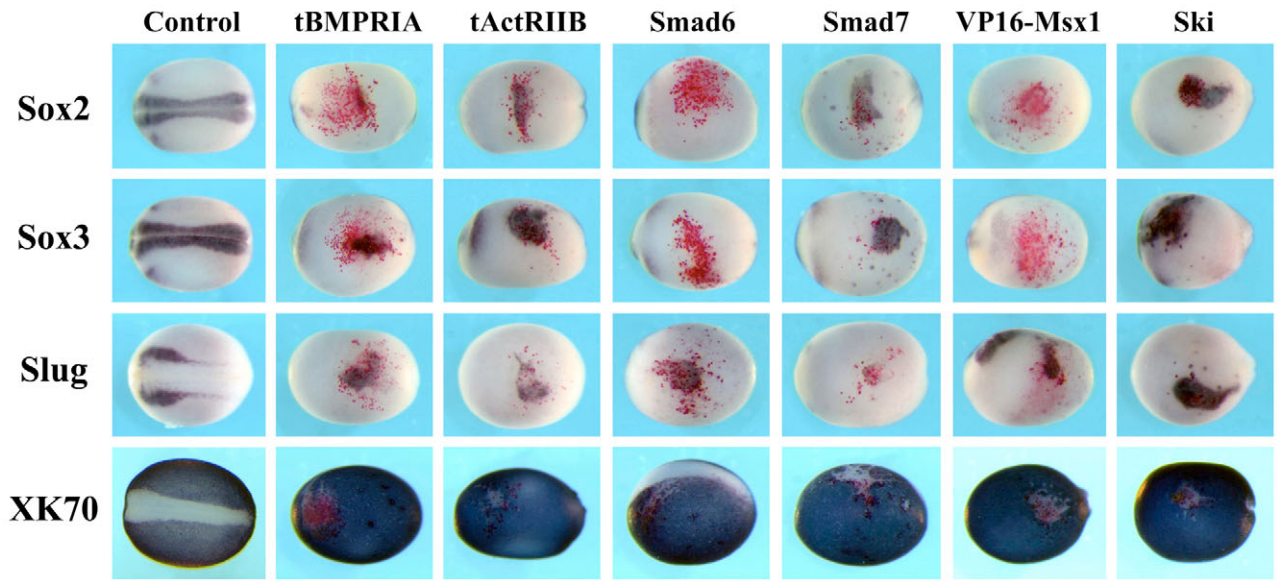


**Fig. 1. Neural induction in *Xenopus* ventral ectoderm by ectopic expression of inhibitors of TGFβ signals.** RNAs encoding transmembrane (1 ng tBMPRIA and tActRIIB), cytoplasmic (0.1 ng Smad6 and Smad7) or nuclear (0.1 ng VP16-Msx1 and Ski) inhibitors of TGFβ signaling were co-injected with nβGal (0.1 ng RNA) into one ventral animal blastomere of 32- to 64-cell stage embryos. The embryos were analyzed by Red-Gal staining (red speckled stain) and in situ hybridization of the neural (Sox2 and Sox3), neural crest (Slug) and epidermal (XK70) markers at neurula stages. Inhibitors of both Smad1 and Smad2 signaling (tActRIIB, Smad7 and Ski) induced neural markers efficiently. Among the specific inhibitors of Smad1 signaling, tBMPRIA induced Sox2 and Sox3 weakly, whereas Smad6 and VP16-Msx1 were ineffective in inducing neural markers.

region of BMP-specific Smad1, resulting in cytoplasmic retention of Smad1 and suppression of BMP signaling (Pera et al., 2003). By contrast, early Wnt signals are active over the entire dorsal domain of the embryo as a result of cortical rotation, and act both to repress transcription of BMP4 in the dorsal ectoderm and to stimulate expression of the BMP antagonists noggin and chordin, thus ensuring the clearance of BMP ligands and inhibition of BMP signals in the neural field (Baker et al., 1999; Wessely et al., 2001). Although FGF and Wnt may have BMP inhibition-independent functions, the nature of such actions is unknown.

One central piece of data arguing against the default model is that unlike in animal caps or explanted ventral ectoderm (Lamb et al., 1993), inhibition of BMP signaling by secreted antagonists, a truncated type I receptor or the inhibitory Smad6 is not sufficient to induce neural marker expression in prospective ventral epidermis of frog embryos or in the chick extra-embryonic epiblast (Linker and Stern, 2004; Delaune et al., 2005). Clearly other conditions need to be met in order for neural specification to occur in competent non-neural ectoderm in vivo. One such condition may be a low level of FGF/ras signaling, as it induces neural markers in combination with BMP inhibitors in *Xenopus* (Linker and Stern, 2004; Delaune et al., 2005; Wawersik et al., 2005). Indeed, even in the absence of localized BMP inhibitors, FGF8a expression alone can induce ectopic differentiating neurons in the ectoderm (Hardcastle et al., 2000; Fletcher et al., 2006).

Here we have addressed other potential mechanisms that may cooperate with BMP inhibition in neural induction. Proceeding from observations on the neural-inducing activities of reagents that block both Smad1 and Smad2, we have tested whether Smad2 inhibition may cooperate with Smad1 inhibition in neural induction. Our data suggest that neural induction in early *Xenopus* embryos exploits simultaneous suppression of BMP and nodal-like signals, and that combined inhibitory rather than instructive signals may be crucial for neural fate determination in vivo.

## MATERIALS AND METHODS

The pCS105-GR-Smad2 construct was made by ligation of *Bam*HI/*Eco*RI fragment of pCS2-hGRN (kindly provided by Dr Paul Wilson) with *Eco*RI/*Asc*I fragment of Smad2 PCR product (using pCS105-Smad2 as the template), and the ligation product was inserted into the *Bam*HI/*Asc*I sites of pCS105 vector. The plasmid was linearized with *Asc*I and transcribed with SP6 polymerase, using the mMessage mMachine RNA Transcription Kit (Ambion).

The embryos were injected with RNAs at 16- to 64-cell stages into one of the dorsal or ventral animal blastomeres, as indicated in the Results section. At gastrula stages, some of the embryos were treated with 2 μM dexamethasone. The injected embryos were incubated to neurula and tailbud stages and in situ hybridization was performed as described previously (Harland, 1991). For histological analyses, embryos were embedded in paraffin and sectioned at 10 μm.

## RESULTS

### Neural specification by inhibitors of Smad1 and Smad2 signals

It has previously been shown that inhibition of BMP signaling by the secreted antagonist noggin, the truncated type IA BMP receptor (tBR/tALK3) or the cytoplasmic inhibitory Smad6 did not result in neural induction in ventral ectoderm of early *Xenopus* embryos, although all these reagents could induce neural marker expression in animal caps (Linker and Stern, 2004; Delaune et al., 2005). To see whether this also holds true for nuclear proteins, we examined the ability of two nuclear BMP inhibitors to induce neural markers in the ventral ectoderm. A dominant-negative mutant of the transcription factor Msx1, VP16-Msx1, was reported to interfere with the function of wild-type Msx1, a direct target of BMP signaling, and block BMP-mediated ventralization of early frog embryos (Suzuki et al., 1997; Yamamoto et al., 2000). Ski, a nuclear oncoprotein, was also shown to inhibit BMP signal transduction. It binds to the BMP-specific Smad1 as well as the common Smad for transforming growth factor β (TGFβ) signaling, Smad4, and helps

to recruit the transcription co-repressors N-CoR and histone deacetylase (HDAC) to suppress BMP-dependent gene expression (Wang et al., 2000; Luo, 2003; Takeda et al., 2004). Both inhibitors can induce neural markers in animal caps (Amaravadi et al., 1997; Wang et al., 2000; Ishimura et al., 2000). We therefore co-injected RNAs encoding these nuclear factors with the nuclear β-galactosidase tracer (nβGal) into one ventral animal blastomere of 32- to 64-cell stage embryos. The embryos were allowed to develop to neurula stages before they were collected, stained with a βGal substrate (Red Gal, Research Organics) and assayed by in situ hybridization for marker gene expression. Interestingly, we observed that although both inhibitors blocked the expression of the type I epidermal keratin XK70 (Winkles et al., 1985) and induced the neural crest marker Slug (Mayor et al., 1995), only Ski was able to induce the neural markers Sox2 and Sox3 efficiently in the ventral epidermal region (Fig. 1; 51/51 Sox2 and 45/45 Sox3 positive for Ski, and 6/77 Sox2 and 8/69 Sox3 positive for VP16-Msx1). The induction of the neural genes was direct, as no mesodermal markers, including chordin (notochord) and MyoD (paraxial mesoderm), were expressed (see Fig. S1 in the supplementary material); although at higher doses, we obtained induction of paraxial mesoderm underlying the neural tissue (data not shown) (Mariani et al., 2001). This result was intriguing, as it suggested that inhibition of BMP signaling by Ski, but not by VP16-Msx1, was sufficient to convert prospective epidermis into neural tissue in vivo.

One explanation for our observation is that Ski may inhibit BMP signaling more effectively than VP16-Msx1. However, in the above experiments, we detected similar or more efficient inhibition of epidermal keratin by VP16-Msx1 (21/21 and 19/20 XK70 negative in VP16-Msx1- and Ski-expressing embryos, respectively), suggesting that both molecules function equally well to block BMP-mediated epidermal formation. Another explanation is that Ski may have activities other than BMP inhibition, and these activities may contribute to its neural-inducing ability. Consistent with the latter hypothesis, Ski has also been shown to block TGFβ/nodal signaling through direct binding to the downstream signal transducers Smad2/3 and recruitment of transcriptional co-repressors to Smad2/3-regulated genes (Liu et al., 2001; Luo, 2004). It is therefore possible that neural induction occurs in Ski-expressing embryos via simultaneous inhibition of both BMP and TGFβ/nodal-like signals in the ectoderm. To test this hypothesis, we assayed for neural-inducing activities of various other inhibitors of TGFβ signaling. We used two mutant membrane receptors, a truncated type IA BMP receptor tBMPRIA (also known as tBR or tALK3), which preferentially blocks the BMP pathway, and a truncated type IIB activin receptor tActRIIB, which inhibits both BMP and activin/nodal-like signals. We found that tBMPRIA induced Sox2 and Sox3 weakly in most embryos (40/65 and 53/67 embryos positive for Sox2 and Sox3, respectively), but tActRIIB induced Sox2 and Sox3 more strongly, so that darker staining was observed in the epidermal region (76/83 and 70/72 embryos positive for Sox2 and Sox3, respectively; Fig. 1). We also analyzed two cytoplasmic inhibitors of TGFβ signaling. Smad6 preferentially interferes with BMP/Smad1 signal through direct binding to Smad1 and preventing it from association with Smad4 (Hata et al., 1998); whereas Smad7 inhibits both Smad1 and Smad2 signaling through interaction with different type I receptors and the Smurf E3-ubiquitin ligases to direct the receptors to the ubiquitin-mediated degradation pathway (Hayashi et al., 1997; Kavsak et al., 2000; Ebisawa et al., 2001; Murakami et al., 2003). When expressed in ventral ectodermal cells of early frog embryos, Smad6 was unable to induce neural markers efficiently (4/69 Sox2-positive), although it induced Slug expression (39/50 Slug-positive) and could induce secondary axes effectively when expressed in the ventral marginal region (Fig. 1 and data not shown). In comparison, Smad7 was a robust neural inducer in vivo (43/50 and 42/46 positive for Sox2 and Sox3, respectively; Fig. 1). None of the TGFβ inhibitors induced mesodermal markers at the doses we used, indicating that the observed neural induction was direct (see Fig. S1 in the supplementary material).

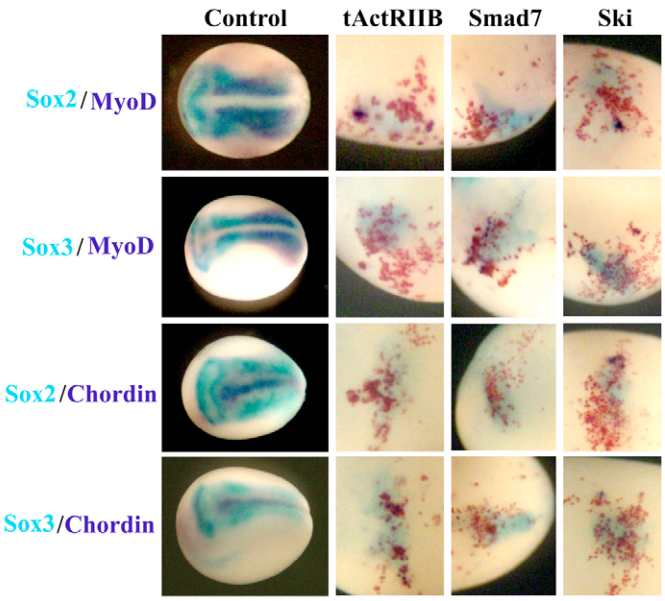


**Fig. 2. Neural induction by inhibitors of Smad1 and Smad2 signaling occurs in the absence of the mesoderm in *Xenopus*.** Double in situ hybridizations showed that neural marker induction in embryos injected with tActRIIB, Smad7 or Ski occurred in the absence of the mesodermal markers Chordin and MyoD. The red speckled staining is from the injected lineage tracer.

To further confirm that the neural markers were induced in the absence of the mesoderm, we performed double in situ hybridizations. As shown in Fig. 2, tActRIIB, Smad7 and Ski all induced Sox2 and Sox3 in the absence of chordin and MyoD, verifying that neural induction occurred without prior induction of dorsal mesoderm. Taken together, our data demonstrate that inhibitors of both BMP/Smad1 and nodal/Smad2 signals induce neural markers directly in the ventral ectoderm of early frog embryos, whereas specific inhibitors of BMP/Smad1 signals are not efficient neural inducers. This suggests that neural induction in this context may require co-inhibition of both branches of TGFβ signals.

## Simultaneous inhibition of Smad1 and Smad2 signals leads to efficient neural induction

While the experiments above are consistent with the idea that simultaneous inhibition of Smad1 and Smad2 results in neuralization, we wished to test whether the simultaneous application of reagents that are known to be Smad1- or Smad2-specific would have the same effect. To test this idea at the receptor level, we co-expressed the truncated BMP receptor with a truncated type IB activin receptor (tActRIB, or tALK4), which has been shown to be incapable of blocking BMP-mediated epidermal induction in dissociated animal cells, but was efficient in blocking *Xenopus* nodal-related ligands (Xnrs) and activin (Chang et al., 1997; Reissmann et al., 2001). When expressed alone, tActRIB did not change epidermal cell fate, whereas tBMPRIA induced neural

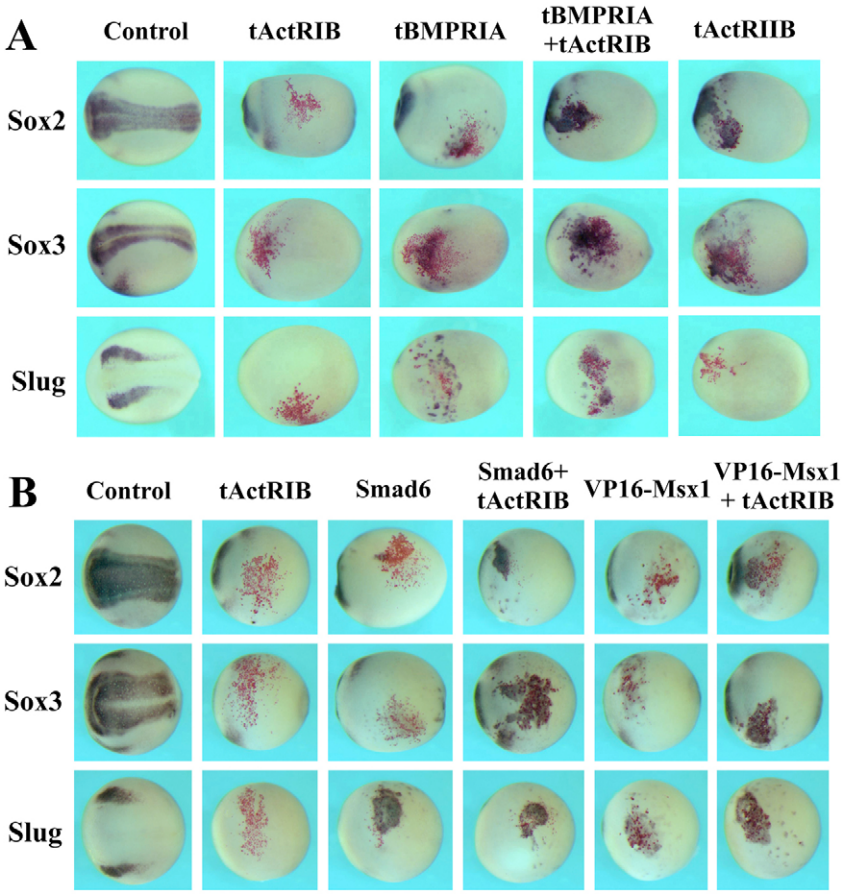


**Fig. 3. Blocking both Smad1 and Smad2 signals leads to efficient neural induction in *Xenopus* ventral ectoderm.** (**A**) At neurula stages, the truncated activin receptor tActRIB did not induce and tBMPRIA only weakly induced the neural markers Sox2 and Sox3. When the two truncated receptors were co-expressed, the neural markers were induced strongly to a level similar to that induced by the truncated type II receptor tActRIIB. One nanogram of each RNA was used. (**B**) Co-expression of tActRIB (1 ng) with Smad6 (0.1 ng) or VP16-Msx1 (0.1 ng) in ventral animal cells led to induction of the neural markers Sox2 and Sox3 in ventral ectoderm of frog neurulae.

markers weakly. Co-expression of tActRIB and tBMPRIA, however, led to strong neural induction. The neural markers Sox2 and Sox3 were detected at high levels, mimicking the situation in which the truncated type II receptor tActRIIB was ectopically expressed (48/60 and 52/63 were positive for Sox2 and Sox3, respectively; Fig. 3A). In addition, co-expression of tActRIB with either Smad6 or VP16-Msx1 led to induction of Sox2 and Sox3 in the ventral ectoderm (19/30 Sox2- and 32/40 Sox3-positive for Smad6, 15/37 Sox2- and 30/33 Sox3-positive for VP16-Msx1; Fig. 3B). The enhanced neural induction could not be attributed simply to the increased total amount of RNAs injected, as the same dose of Smad6 RNA alone was not efficient in inducing neural markers (1/11 Sox2-positive for 2 ng Smad6 injection). Our results thus support the idea that blocking both Smad1 and Smad2 signals is sufficient for neural induction in vivo.

Neural tissues can be induced in animal caps by BMP inhibitors alone, but it is possible that the effectiveness would be enhanced by simultaneous blocking of Smad2 signaling. To test this, we co-injected RNAs encoding BMP inhibitors and tActRIB into the animal region of two-cell stage embryos and analyzed gene expression in animal caps derived from the injected embryos. tActRIB enhanced neural marker induction by low doses of BMP inhibitors (Fig. 4), although it did not influence neural induction when high doses of BMP inhibitors were used (not shown). This may reflect observations that animal caps have only low levels of activin/nodal signaling and thus strong inhibition of BMP signals alone can induce neural markers. Our data are consistent with the in vivo results and suggest that inhibition of Smad2 signaling can boost the neural-inducing ability of threshold BMP inhibitors in ectodermal explants.

## Anterior, but not posterior, neural tissues are induced in the ventral ectoderm

To examine whether the neural tissues induced by the inhibitors of Smad signals exhibited anteroposterior patterning characteristics, we assayed for the expression of Otx2 (fore- and midbrain), Engrailed 2 (En2, midbrain), Krox20 (hindbrain) and HoxB9 (spinal cord) in the ectopic neural tissues. As shown in Fig. 5, tActRIIB, Smad7 and Ski all induced Otx2 (13/22, 14/30 and 23/30 positive) and En2 (3/20, 17/29 and 18/30 positive) at different efficiencies, but they failed to stimulate the expression of Krox20 (0/22, 1/30 and 1/29 positive) and HoxB9 (0/22, 0/30 and 0/32 positive). The results indicate that inhibition of both Smad1 and Smad2 signals leads to induction of anterior, but not posterior, neural tissues.

## Activation of Smad2 signaling at gastrula stages inhibits neural induction

If neural induction requires simultaneous inhibition of Smad1 and Smad2 signals, we would expect that activation of either signal in the presumptive neural tissue can interfere with neural development. Consistent with this, it has been reported that stimulation of BMP signaling via a constitutively active type I receptor leads to inhibition of neural markers with concurrent expression of epidermal genes in the neural plate (Mariani et al., 2001; Delaune et al., 2005). The consequence of activation of Smad2 signaling in the neural tissue, however, has not been examined; and this was the issue we addressed next.

To avoid the early mesoderm-inducing effects of Smad2 activation, we constructed a chimeric protein containing the hormone-binding domain of glucocorticoid receptor linked to the

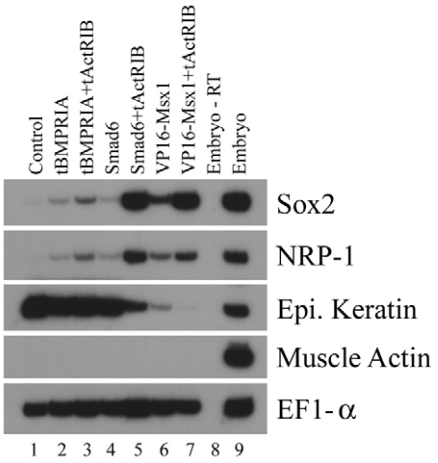


**Fig. 4. Inhibition of Smad2 signaling enhances neural induction by low doses of BMP inhibitors in *Xenopus* animal caps.** Blocking Smad2 signaling with tActRIB (1 ng) led to more efficient neural induction by low doses of BMP inhibitors tBMPRIA (0.1 ng), Smad6 (10 pg) or VP16-Msx1 (25 pg) in animal caps.

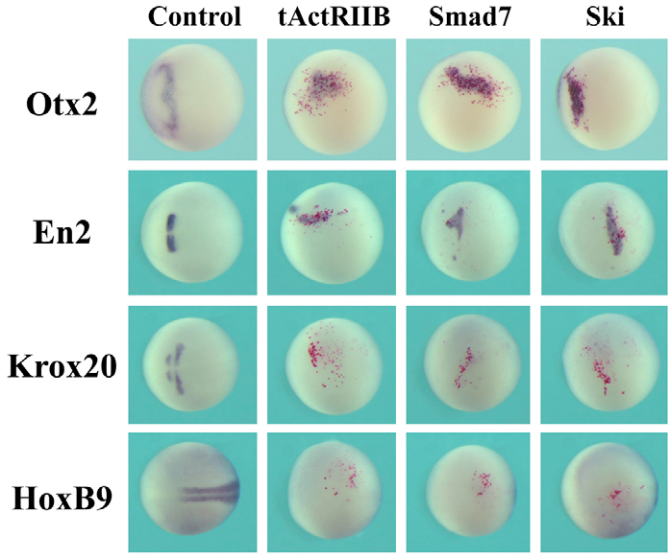


**Fig. 5. Induction of anterior, but not posterior, neural tissues in *Xenopus*.** tActIIB, Smad7 and Ski induced the fore- and mid-brain markers Otx2 and En2, but not the hindbrain and spinal cord markers Krox20 and HoxB9.

full-length Smad2. The resulting GR-Smad2 exhibited dexamethasone (DEX)-dependent activity in inducing mesodermal markers in animal caps. Consistent with the previous studies on temporal responses to activin (Green et al., 1990), the ability of GR-Smad2 to induce mesoderm declined during gastrulation, so that if activated by mid-gastrula stages, it no longer acted as a mesodermal inducer (Fig. 6A). To see whether activation of Smad2 signaling had any effect on formation of neural tissues, we injected the RNA encoding GR-Smad2 with the nβGal tracer into one of the dorsal animal cells of 16- to 32-cell stage embryos and examined the expression of Sox2 at neurula stages. In the absence of DEX, GR-Smad2 did not disturb the pattern of Sox2 transcription, so that the red-Gal labeled cells were found to express Sox2 in the neural plate. Treatment of the injected embryos with DEX at mid-gastrula stages, however, severely interfered with the expression of Sox2. The nβGal-labeled cells turned off Sox2 cell-autonomously at all positions along the anteroposterior axis, so that Sox2-negative, nβGal-positive regions were observed near the head, the trunk or the caudal end (Fig. 6B). The data indicate that activation of Smad2 signaling in neural tissues at gastrula stages impairs neural development.

To assess the fate of the cells expressing activated GR-Smad2, we next assayed for expression of neural, neural crest, epidermal and mesodermal markers in these embryos. Interestingly, although no neural inhibition or mesoderm induction was observed in the absence of DEX, we did observe ectopic expression of the neural crest marker Slug (66/106 positive) and at lower incidence the epidermal marker XK70 (11/92 positive) in the neural plate (Fig. 7A). This suggested that the construct we used was somewhat leaky, and that a low level of Smad2 activity in the neural plate was sufficient to stimulate neural crest markers without significant inhibition of neural markers. Treatment of the embryos with DEX at mid-gastrula stages resulted in inhibition of both Sox2 (68/94 negative) and Sox3 (45/72 negative) in the neural plate, but with concurrent induction of the mesodermal markers MyoD (27/62 positive) and chordin (52/68 positive; Fig. 7A; see also below). The induction of the mesodermal markers occurred in prospective neural tissue, as seen in bisected as well as sectioned embryos (Fig. 7B). The conversion of the neural to the mesodermal fate was a little surprising, as animal cap cells lost their competence to respond to the activated Smad2 by mid-gastrulation (Fig. 6). We therefore asked whether the competence to form mesoderm persisted longer in vivo, and whether GR-Smad2 always converted neural tissues to mesodermal derivatives. We thus treated the injected embryos with DEX at different stages and analyzed the expression of neural and mesodermal markers at neurula stages. As shown in Fig. 8, treatment with DEX from gastrula stages 10.5 to 11.5 onward was sufficient for GR-Smad2 to inhibit neural markers; however, if DEX was added at late gastrula to early neurula stages (stages 12-13), GR-Smad2 became ineffective in inhibition of neural markers. Indeed, chordin induction in the neural plate followed a similar temporal profile. By stage 12, activation of GR-Smad2 could neither block Sox2 and Sox3 nor induce chordin (Fig. 8). Our data imply that activation of Smad2 signaling during gastrulation can convert neural tissues to mesoderm.

In animal caps, neural markers can be induced by inhibition of BMP signaling. Activation of Smad2 signaling in naive caps, by contrast, stimulates mesodermal development, and the induced mesoderm can induce neural markers secondarily. To test whether activation of Smad2 signaling in neuralized animal caps may also boost both mesodermal and neural gene expression, we co-injected the RNAs encoding the soluble BMP antagonist noggin and GR-Smad2 into the animal poles of two-cell stage embryos. Assays for gene expression in animal caps by RT-PCR showed that GR-Smad2 did not prevent neural marker induction by noggin in the absence of DEX. However, treating the caps with DEX from blastula to early gastrula stages onward led to a strong inhibition of the neural markers NCAM and NRP-1 with concurrent induction of the mesodermal markers muscle actin and type II collagen. In addition, the neural crest markers Slug and Twist were also induced in these caps (Fig. 9A and data not shown). The ability of GR-Smad2 to inhibit neural markers and induce mesoderm declined progressively when it was activated at mid- to late gastrula stages (Fig. 9A). Our in vitro explant assays were thus

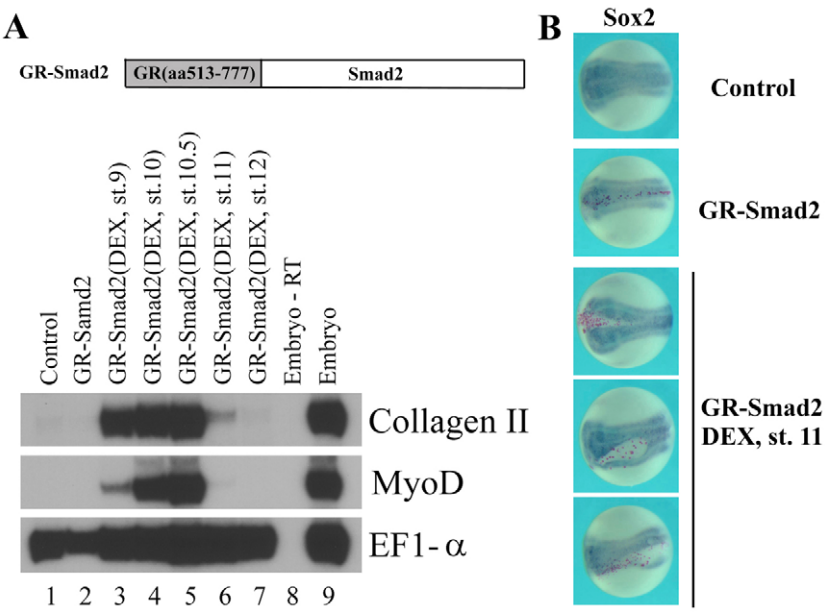


**Fig. 6. Activation of Smad2 signaling in *Xenopus* neural tissues at gastrula stages results in inhibition of neural markers.**
(**A**) Schematic representation of the chimeric protein GR-Smad2 and analyses of its activity in animal caps. GR-Smad2 RNA (0.2 ng) was used. In the absence of dexamethasone (DEX), GR-Smad2 did not induce mesodermal markers (lane 2). Activation of GR-Smad2 from blastula to early gastrula stages by DEX (2 μM) led to mesoderm induction (lanes 3 to 5). However, if activated at mid- to late gastrula stages (stages 11 to 12), GR-Smad2 no longer induced mesoderm in animal caps (lanes 6 and 7).
(**B**) Activation of GR-Smad2 in neural tissues at mid-gastrula stages led to inhibition of Sox2 at different axial levels along the anteroposterior axis.

consistent with our in vivo results to suggest that activation of Smad2 signaling can inhibit neural development and promote mesoderm formation during gastrulation.

As GR-Smad2 showed leaky activation in the absence of DEX in vivo, it was possible that this low Smad2 activity helped to condition the neural cells to respond to subsequent activation of Smad2 at mid-gastrula. To further examine whether endogenous neural tissues without any prior treatment could respond to the activation of Smad2 at gastrula stages, we dissected prospective neural explants from the dorsal ectodermal region during gastrulation and treated them with exogenously added activin protein. As shown in Fig. 9B, activin induced dorsal mesoderm and reduced neural markers in explants obtained from stage 11 and 11.5 embryos, but did not affect marker expression in explants acquired from stage 12 embryos. As reported previously, the ventral ectoderm differed in its ability to respond to exogenous activin, and at the stages showed less, or no, mesoderm induction in response (Fig. 9C and data not shown). Thus, our results support the argument that neural cells still retain their responsiveness to Smad2 activation at gastrula stages, and continued suppression of Smad2 signaling is required for neural development.

### Activation of Smad2 signaling during gastrulation leads to defective neural development

If aberrant activation of Smad2 signaling interferes with neural development, we would expect that resulting embryos would display defects in the nervous system. We therefore examined the embryos injected with GR-Smad2 and treated with DEX at gastrula stages. Tadpoles expressing GR-Smad2 without exposure to DEX developed normally; but if DEX was added at mid-gastrula stages, the resulting tadpoles showed severe head defects with malformed or missing eyes (Fig. 10A). It has been suggested that Smad2 signaling is important in anteroposterior patterning of vertebrate embryos (Piccolo et al., 1999; Sun et al., 1999; Feldman et al., 2000; Sirotkin et al., 2000; Thisse et al., 2000; Lu et al., 2001; Andersson et al., 2006). However, it has been difficult to document any direct effect of Smad2 signaling on neural patterning against a background of Smad2 induction of mesoderm, which would also secrete caudalizing signals (Eimon and Harland, 2002). We therefore addressed whether the head defects might reflect a direct effect on anteroposterior patterning by Smad2 signaling. We thus examined these embryos both histologically and by in situ hybridization. Transverse sections of the embryos revealed that the neural tube was disrupted at both the head and the trunk levels (Fig. 10B and data not shown). Ectopic notochord and mesenchyme were often observed to split the neural tube or compress the size and change the position of the neural tube (Fig. 10B). The formation of endodermal and mesodermal derivatives, including the gut, the notochord and the somites, seemed to be normal. Gene expression studies indicated that Sox2 was greatly reduced along the entire anteroposterior axis, and separate stripes of weak Sox2 could be seen in some embryos (Fig. 10C and data not shown), consistent with the neural tube defects. Otx2 was still expressed, although at lower levels. In contrast to the neural markers, the neural crest marker Twist was expressed normally, and the muscle marker MyoD was equally unaffected (Fig. 10C). The data indicate that activation of Smad2 signaling during gastrulation in the neural plate interferes with neural development, resulting in neural tube defects. However, these results are consistent with the diversion of neural tissues to a non-neural mesodermal fate by Smad2 signaling, and there remains no evidence for a direct posteriorizing role of Smad2 signals.

## DISCUSSION

With the application of molecular biological techniques, considerable advances have been made in understanding the source and the nature of signals instructing the ectoderm to become properly patterned neural tissue. One prominent view deriving from such research, especially in *Xenopus*, is the default model of neural induction. Although a large body of work supports this model, some observations are in conflict. Among outstanding questions remaining unanswered are whether BMP inhibition is sufficient for neural specification in vivo, whether other signaling pathways are also involved and provide instructive information independently of BMP inhibition, and whether the mechanisms deployed by diverse chordate species for neural induction are conserved or different. In this study, we show that in *Xenopus* neural ectoderm retains its competence to respond to nodal/Smad2 signaling to form mesoderm

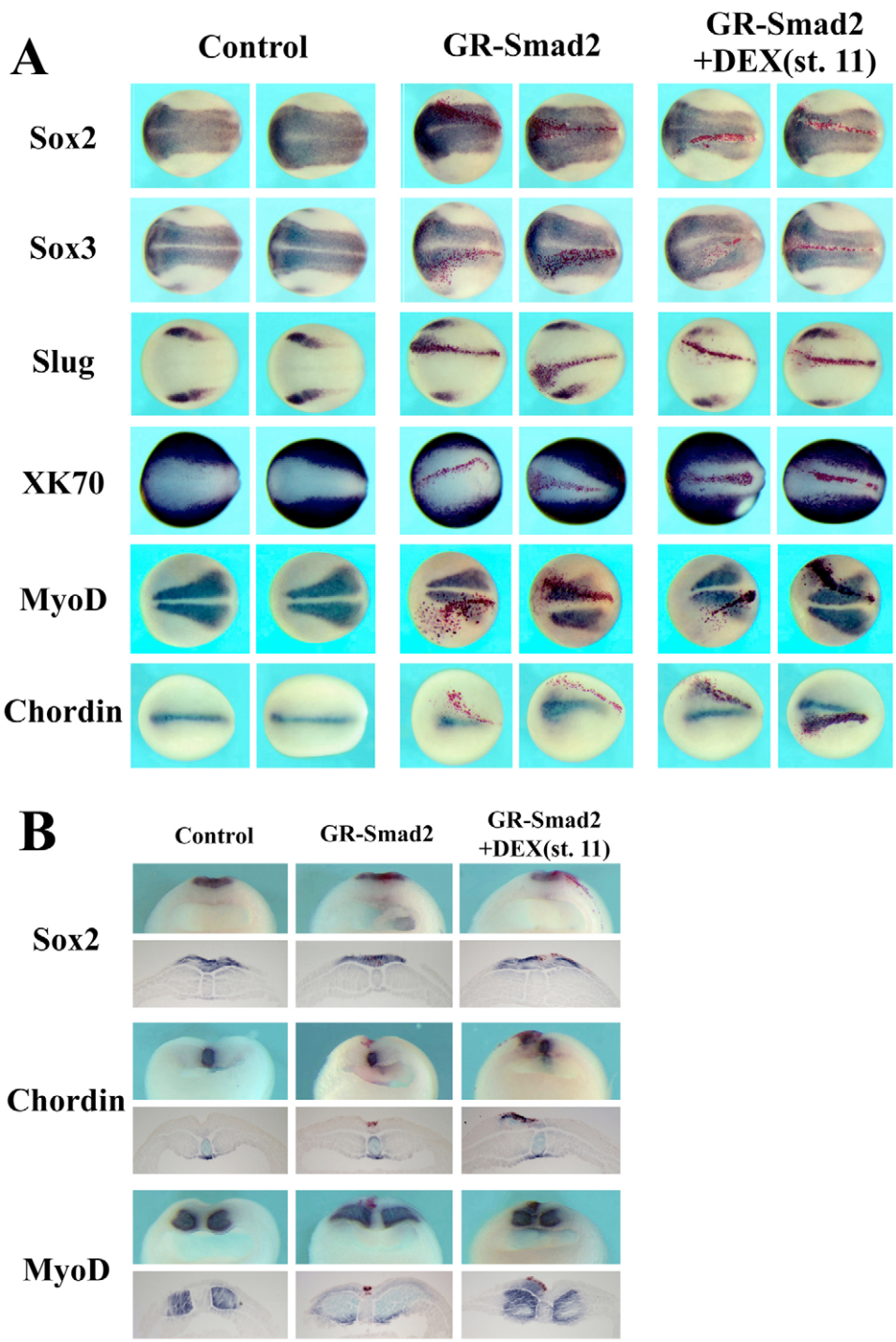


**Fig. 7. Activation of Smad2 signaling converts neural tissue to neural crest and mesodermal tissues in *Xenopus*.** (**A**) In the absence of DEX, leaky GR-Smad2 activity was sufficient for neural crest induction, but not sufficient for inhibition of neural markers or induction of mesodermal genes. Activation of GR-Smad2 by DEX (2 μM) at mid-gastrula stages led to inhibition of Sox2 and Sox3 and simultaneous induction of the mesodermal markers MyoD and Chordin in the neural plate (seen more clearly in Fig. 7B and Fig. 8). GR-Smad2 RNA (0.1-0.2 ng) was used. The embryos were orientated with the head toward the left and viewed from the dorsal side. (**B**) Induction of mesodermal markers by activated GR-Smad2 occurred in the neural plate, as shown in transversely bisected (top) or sectioned (bottom) embryos.

even at late gastrula stages (stage 11.5), and that co-suppression of epidermis- and mesoderm-inducing Smad1 and Smad2 signals is required for neural development.

### Neural induction requires simultaneous inhibition of nodal/Smad2 and BMP/Smad1 by organizer- and ectodermal-derived molecules

While the use of animal cap explants has led to the simple idea that BMP signal inhibition is sufficient for neural induction, another experimental paradigm, namely neuralization of the ventral ectoderm of the intact embryo, has led to the conclusion that BMP inhibition is insufficient for efficient neural induction (Linker and Stern, 2004; Delaune et al., 2005; Wawersik et al., 2005). To date, FGF signaling has been emphasized as the 'missing ingredient' in this. However, in our current study, we find that inhibition of both BMP/Smad1 and nodal-like/Smad2 signaling is sufficient for efficient neural induction in ventral ectoderm of early frog embryos. Single reagents, such as Ski or Smad7, which inhibit both signals, are efficient neuralizing agents (Figs 1 and 2); and separate reagents, such as the truncated nodal receptor and Smad6, which are well-characterized inhibitors of individual pathways, work together to neuralize ectoderm (Figs 3 and 4). To some extent, there is an

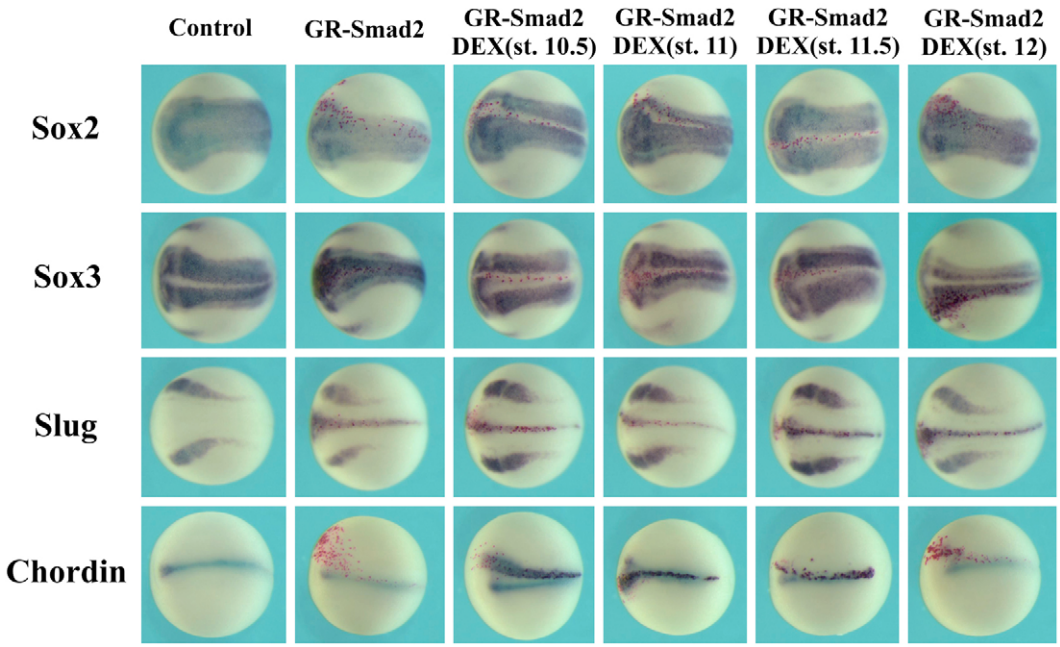


**Fig. 8. The ability to inhibit neural markers by activated Smad2 attenuates during gastrulation.** Treatment of *Xenopus* embryos expressing GR-Smad2 with DEX at different stages during gastrulation showed that activated GR-Smad2 lost its neural inhibitory activity by the end of gastrulation, at which stages it also failed to induce mesoderm in the neural plate. All the embryos were viewed from the dorsal side with the anterior to the left.

expectation that both pathways would need to be inhibited, as each will induce a non-neural tissue, epidermis or mesoderm, on its own. It has always been assumed that the epidermis has not experienced significant Smad2 signaling, and thus it was unexpected that inhibition of Smad2 signaling potentiates the neural-inducing activity of Smad1 inhibitors so strongly in the context of ventral epidermis. However, evidence that the ectoderm does experience some Smad2 activation comes from experiments in which animal caps have been cut large or late, where mesoderm is induced in the presence of the competence modifying signals from Wnts or noggin (Christian et al., 1992; Lamb et al., 1993; Sokol, 1993). In any case, our results emphasize the requirement in neural induction that all TGFβ signals need to be inhibited for neural specification to occur.

Consistent with this view, we show that neural markers are inhibited when Smad2 signaling is activated in the neural plate or in neuralized ectodermal explants during gastrula stages (Figs 6-9). Even quite late Smad2 signals cause presumptive neural cells to take on mesodermal or neural crest cell lineages. In these experiments, we observed a difference in temporal response to activated Smad2 signaling in vivo and in vitro. Whereas animal caps lost their competence to form mesoderm and failed to react to activated Smad2 to inhibit neural induction by noggin at mid-gastrula stages (stage 11), neural plate cells responded to Smad2 signaling till later gastrula stages (stage 11.5). The differences may reflect the activity of other signals in vivo that maintain the competence of neural plate cells to respond to mesoderm-inducing signals. This prolonged competence to generate mesoderm poses a stringent condition in the dorsal ectoderm for nodal-like mesoderm-inducing signals to be countered in order for neural tissue to form. In early *Xenopus* embryos, different factors may be responsible for this nodal inhibitory action. The Spemann's organizer secrets soluble nodal inhibitors, including Cerberus and Lefty/Antivin, in addition to BMP antagonists (Cheng et al., 2000; Branford and Yost, 2002; Tanegashima et al., 2004; Cha et al., 2006). In ectoderm, a Dan/Cerberus family member, Coco, moderates both BMP and nodal/activin signaling to regulate cell fate specification and competence (Bell et al., 2003). Both BMP and nodal-like signals are also inhibited by an ectodermally expressed Smad4 ubiquitin ligase, Ectodermin (Dupont et al., 2005). Furthermore, a transcription factor belonging to the foxi-class of winged-helix proteins, Xema/FoxI1e, is localized exclusively in the ectoderm and represses mesoderm induction by activin/nodal-like growth factors (Suri et al., 2005; Mir et al., 2007). Depletion of Lefty/Antivin, Ectodermin and Xema all leads to expansion of mesodermal genes toward the animal region, and in the case of Ectodermin and FoxI1e, the neural tissue is reduced (neural development in Lefty morphant embryos has not been studied in detail). Thus the available evidence is consistent with the view that ectodermal cells adopt a neural fate when both nodal-like and BMP signals are inhibited simultaneously to prevent mesodermal and epidermal development, respectively. In line with this idea, it has been reported that both phosphorylated Smad2 and Smad1 are completely cleared from the neural plate by early neurula stages in frog embryos (Schohl and Fagotto, 2002).

Despite the presence of multiple stage- and tissue-specific endogenous modifiers of Smad2 activity, the ventral ectoderm must perceive significant Smad2 signaling, as its response to BMP inhibitors is radically altered by the simultaneous blocking of Smad2 signaling. Activation of Smad2 has been studied in the relevant stages from blastula to gastrula (Faure et al., 2000; Lee et al., 2001; Schohl and Fagotto, 2002). Numerous ligands, such as Xnr1 and Derriere (Sun et al., 1999; Lee et al., 2001; Eimon and Harland, 2002), are deployed close to the ventral ectoderm, so it is not surprising that the ventral epidermis would have experienced some Smad2 signaling at the relevant stages. Consistent with this,

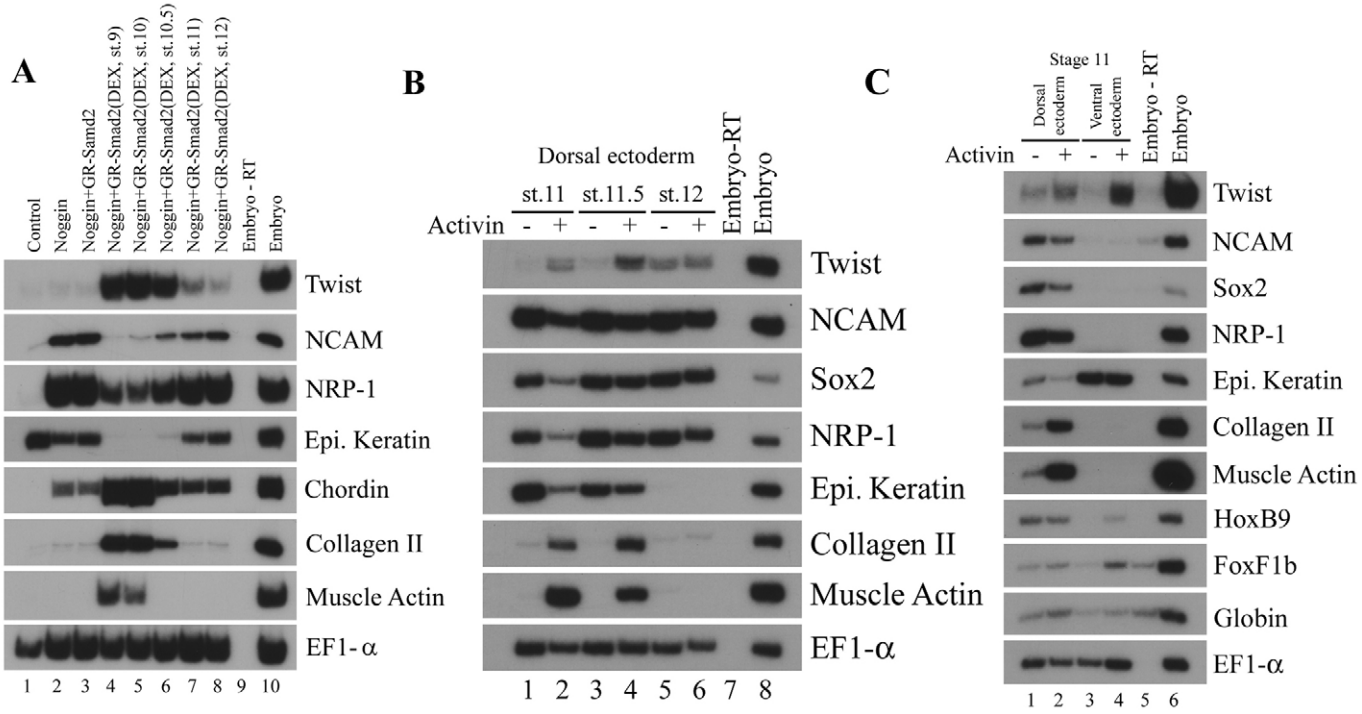


**Fig. 9. Activation of Smad2 inhibits neural induction in *Xenopus* explants.** (**A**) Activation of Smad2 by DEX at blastula to early gastrula stages suppressed neural induction by noggin and induced mesodermal markers in animal caps; but it could not do so efficiently if activated at mid- to late gastrula stages. noggin (10 pg) and GR-Smad2 (0.2 ng) RNAs were used. (**B**) Activin protein (1:50 dilution of oocyte conditioned medium) reduced neural and induced mesodermal markers in dorsal ectoderm explanted from stages 11-11.5 embryos, but not from stage 12 embryos. (**C**) Unlike dorsal ectoderm, ventral ectoderm did not respond to activin efficiently at mid-gastrula stages (stage 11 onward).

phosphorylated Smad2 has been detected in the ventral ectoderm of frog gastrulae and in the epidermis of neurula and tailbud embryos (Schohl and Fagotto, 2002). It is not clear whether this low level of signaling has an effect on epidermis; and perhaps a more detailed examination of different epidermal markers might reveal an instructive role for Smad2 in the diversion of the atypical epidermis that is induced in animal caps compared to the epidermis that develops in whole embryos.

An unexpected observation from these experiments is that a low level of Smad2 signaling, caused by the leaky activity of GR-Smad2, is capable of inducing neural crest markers in the neural plate in the absence of any mesodermal gene expression. While BMP, FGF and Wnt signaling have been implicated in neural crest induction (Barembaum and Bronner-Fraser, 2005; Basch and Bronner-Fraser, 2006), this raises the possibility that Smad2 activity may also contribute to neural crest specification.

## FGF/MAPK signaling in neural induction: inhibition of Smad1 and Smad2?

In *Xenopus* neural induction, several models have been proposed that emphasize the involvement of different signals. The default model states that BMP-free ectoderm assumes neural fate autonomously, whereas other views stress the importance of additional signaling, such as the IGF/FGF pathways. Our experiments offer an alternative explanation for several unresolved issues. For example, inhibition of BMP signaling in animal caps is sufficient for neural induction, but is not efficient in neural specification in ventral ectoderm. We now recognize the involvement of Smad2 signals in the suppression of neural induction in the whole embryo context. Another puzzle is the role of the Ras/MAPK pathway in neural induction. IGF and FGF have been shown to activate Ras/MAPK to inhibit Smad1 through phosphorylation of the linker region of Smad1, and FGF may also regulate BMP expression (Pera et al., 2003; Delaune et al., 2005; Kuroda et al., 2005). The Ras/MAPK signaling may thus converge with the BMP pathway during neural induction. However, it is also reported that FGF may have BMP-independent effects in neural specification, although the mechanism is unknown (Delaune et al., 2005). In light of our current finding and previous studies in mammalian cell culture (Kretzschmar et al., 1999), we propose that in addition to blocking Smad1, FGF/MAPK signaling may also inhibit Smad2 through linker phosphorylation, and this may contribute to the synergistic effect on neural induction by BMP inhibitors and low FGF/ras/MAPK signaling (Linker and Stern, 2004; Delaune et al., 2005; Wawersik et al., 2005). Indeed, inactivation of Smad2 by linker phosphorylation has been correlated with loss of competence of gastrula ectoderm to respond to activin-mediated mesodermal induction (Grimm and Gurdon, 2002). In this case, the relative levels of Smad2 and FGF signals may be important, as high levels of both signals are required for mesoderm induction, while low levels of both signals may lead to Smad2 inhibition and neural development.

## Conserved and divergent mechanisms of neural induction during animal evolution

When compared with other animals, we find that both similar and divergent mechanisms may be utilized during neural induction. One common, although under-emphasized, theme for cells to adopt a neural fate in all species is that cells choose not just between epidermal and neural fates, but also neural and mesendodermal fates (Harland, 2000). This is demonstrated, for example, by a

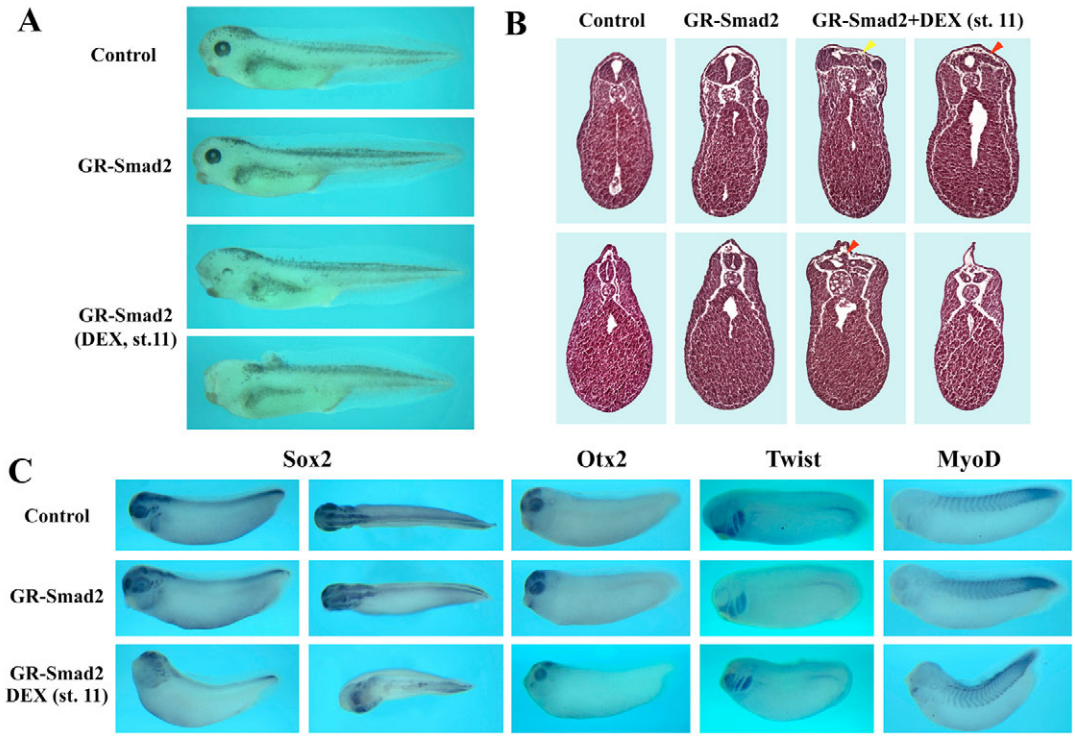


**Fig. 10. Activation of Smad2 at gastrula stages in the neural plate leads to defective neural development in *Xenopus*.** (**A**) Activation of GR-Smad2 (0.2-0.5 ng) at mid-gastrula stages (stage 11) in the neural tissue induced neural defects in frog tadpoles. Embryos showed reduced head structures and malformed or missing eyes. Embryos without DEX treatment developed normally. (**B**) Histological analyses indicated that neural development at both anterior (upper panels) and posterior (lower panels) trunk levels was defective when Smad2 signaling was activated. The neural tube was disrupted and ectopic notochord (yellow arrowhead) and mesenchyme (red arrowhead) were observed in the neural derivatives. (**C**) In situ hybridization demonstrated that Sox2 was reduced and split from the midline and Otx2 was reduced, but the neural crest marker Twist and the muscle marker MyoD were unaffected. All embryos were viewed from the lateral side with the anterior to the left, except the second column of Sox2 in panel C, which was viewed from the dorsal direction.

common precursor for spinal cord and notochord in the non-vertebrate chordate ascidian (Lemaire et al., 2002) and by conversion of dorsal mesoderm to neural ectoderm in nodal-signaling-deficient zebrafish embryos (Feldman et al., 2000; Schier and Talbot, 2001). In the chick, the transcription factor Churchill acts through upregulation of Smad-interacting-protein-1 (Sip1) to block mesoderm induction and permit neural development in competent epiblast (Sheng et al., 2003). In the mouse, the lack of the mesendodermal specification signaling factor nodal leads to precocious neural differentiation (Camus et al., 2006). All these results indicate that obstruction or loss of response to mesoderm-inducing factors may be an essential first step for cells to adopt a neural fate. A less conserved mechanism among different chordates concerns which signaling pathways are involved in neural specification. In ascidians, the Ras/MEK/Erk pathway downstream of FGFs has been shown to regulate neural development directly by modulating the promoter activities and therefore the expression of neural-specific genes (Hudson and Lemaire, 2001; Bertrand et al., 2003; Hudson et al., 2003). BMP inhibition does not appear to be important for early neural induction in ascidian (Lemaire et al., 2002), and may not be an ancestral mechanism in the chordates, as the hemichordate outgroup also shows no correlation of BMP signaling in the neural versus epidermal choice (Lowe et al., 2006). FGF signaling has been strongly implicated in neural induction in the chick (Alvarez et al., 1998; Streit et al., 2000; Wilson et al., 2000), and inhibition of Wnt signaling is also required for early neural induction in the chick (Wilson et al., 2001). In these cases, FGF and Wnt signals may crosstalk with the BMP pathway to affect neural induction. Indeed, it has been shown that in both chick and zebrafish, FGF signaling regulates expression of BMP ligands and/or BMP antagonists (Wilson et al., 2000; Furthauer et al., 2004; Londin et al., 2005). Nodal signaling may also have a direct effect on neural induction in mammals, as in human embryonic stem cells nodal inhibits neural differentiation while promoting cell maintenance in a pluripotent state (Vallier et al., 2004); and in mice deficient in nodal, anterior neural tissues form precociously (Camus et al., 2006). Although it may be surprising that different chordates have exploited different pathways as precursors to neural induction, the ultimate loss of BMP signaling and an absence of mesoderm-inducing signals in the neural precursors remain a common theme in the vertebrates.

We thank Dr Paul Wilson for providing the plasmid used in construction of GR-Smad2, Dr Francois Fagotto for sending us the supplementary data on p-Smad2 staining of early frog embryos, Drs Claudia Linker and Claudio Stern for communicating data prior to publication. The project was conceived during discussions at the embryology course at the Marine Biological Laboratory at Woods Hole, MA. We are grateful for the open, friendly and stimulating atmosphere in the course that facilitates our collaboration. This work is supported by a grant from the NIH.

**Supplementary material**

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/21/3861/DC1

**References**

**Alvarez, I. S., Araujo, M. and Nieto, M. A.** (1998). Neural induction in whole chick embryo cultures by FGF. *Dev. Biol.* **199**, 42-54.

**Amaravadi, L. S., Neff, A. W., Sleeman, J. P. and Smith, R. C.** (1997). Autonomous neural axis formation by ectopic expression of the protooncogene c-ski. *Dev. Biol.* **192**, 392-404.

**Andersson, O., Reissmann, E., Jornvall, H. and Ibanez, C. F.** (2006). Synergistic interaction between Gdf1 and Nodal during anterior axis development. *Dev. Biol.* **293**, 370-381.

**Baker, J. C., Beddington, R. S. P. and Harland, R. M.** (1999). Wnt signaling in Xenopus embryos inhibits Bmp4 expression and activates neural development. *Genes Dev.* **13**, 3149-3159.

**Barembaum, M. and Bronner-Fraser, M.** (2005). Early steps in neural crest specification. *Semin. Cell Dev. Biol.* **16**, 642-646.

**Basch, M. L. and Bronner-Fraser, M.** (2006). Neural crest inducing signals. *Adv. Exp. Med. Biol.* **589**, 24-31.

**Beddington, R. S. P.** (1994). Induction of a second neural axis by the mouse node. *Development* **120**, 613-620.

**Bell, E., Munoz-Sanjuan, I., Altmann, C. R., Vonica, A. and Brivanlou, A. H.** (2003). Cell fate specification and competence by Coco, a maternal BMP, TGFβ and Wnt inhibitor. *Development* **130**, 1381-1389.

**Bertrand, V., Hudson, C., Caillol, D., Popovici, C. and Lemaire, P.** (2003). Neural tissue in ascidian embryos is induced by FGF9/16/20, acting via a combination of maternal GATA and Ets transcription factors. *Cell* **115**, 615-627.

**Branford, W. W. and Yost, H. J.** (2002). Lefty-dependent inhibition of Nodal- and Wnt-responsive organizer gene expression is essential for normal gastrulation. *Curr. Biol.* **12**, 2136-2141.

**Camus, A., Perea-Gomez, A., Moreau, A. and Collignon, J.** (2006). Absence of nodal signaling promotes precocious neural differentiation in the mouse embryo. *Dev. Biol.* **295**, 743-755.

**Cha, Y. R., Takahashi, S. and Wright, C. V. E.** (2006). Cooperative non-cell and cell autonomous regulation of Nodal gene expression and signaling by Lefty/Antivin and Brachyury in Xenopus. *Dev. Biol.* **290**, 246-264.

**Chang, C., Wilson, P. W., Mathews, L. S. and Hemmati-Brivanlou, A.** (1997). A Xenopus type I activin receptor mediates mesodermal but not neural specification during embryogenesis. *Development* **124**, 827-837.

**Cheng, A. M., Thisse, B., Thisse, C. and Wright, C. V. E.** (2000). The lefty-related factor Xatv acts as a feedback inhibitor of nodal signaling in mesoderm induction and L-R axis development in Xenopus. *Development* **127**, 1049-1061.

**Christian, J. L., Olson, D. J. and Moon, R. T.** (1992). Xwnt-8 modifies the character of mesoderm induced by bFGF in isolated Xenopus ectoderm. *EMBO J.* **11**, 33-41.

**De Robertis, E. M. and Kuroda, H.** (2004). Dorsal-ventral patterning and neural induction in Xenopus embryos. *Annu. Rev. Cell Dev. Biol.* **20**, 285-308.

**Delaune, E., Lemaire, P. and Kodjabachian, L.** (2005). Neural induction in Xenopus requires early FGF signalling in addition to BMP inhibition. *Development* **132**, 299-310.

**Dupont, S., Zacchigna, L., Cordenonsi, M., Soligo, S., Adorno, M., Rugge, M. and Piccolo, S.** (2005). Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. *Cell* **121**, 87-99.

**Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T. and Miyazono, K.** (2001). Smurf1 interacts with transforming growth factor-β type I receptor through Smad7 and induces receptor degradation. *J. Biol. Chem.* **276**, 12477-12480.

**Eimon, P. M. and Harland, R. M.** (2002). Effects of heterodimerization and proteolytic processing on Derriere and Nodal activity: implications for mesoderm induction in Xenopus. *Development* **129**, 3089-3103.

**Faure, S., Lee, M. A., Keller, T., ten Dijke, P. and Whitman, M.** (2000). Endogenous patterns of TGFβ superfamily signaling during early Xenopus development. *Development* **127**, 2917-2931.

**Feldman, B., Dougan, S. T., Schier, A. F. and Talbot, W. S.** (2000). Nodal-related signals establish mesendodermal fate and trunk neural identity in zebrafish. *Curr. Biol.* **10**, 531-534.

**Fletcher, R. B., Baker, J. C. and Harland, R. M.** (2006). FGF8 spliceforms mediate early mesoderm and posterior neural tissue formation in Xenopus. *Development* **133**, 1703-1714.

**Furthauer, M., Van Celst, J., Thisse, C. and Thisse, B.** (2004). Fgf signalling controls the dorsoventral patterning of the zebrafish embryo. *Development* **131**, 2853-2864.

**Green, J. B., Howes, G., Symes, K., Cooke, J. and Smith, J. C.** (1990). The biological effects of XTC-MIF: quantitative comparison with Xenopus bFGF. *Development* **108**, 173-183.

**Grimm, O. H. and Gurdon, J. B.** (2002). Nuclear exclusion of Smad2 is a mechanism leading to loss of competence. *Nat. Cell Biol.* **4**, 519-522.

**Hardcastle, Z., Chalmers, A. D. and Papalopulu, N.** (2000). FGF-8 stimulates neuronal differentiation through FGFR-4a and interferes with mesoderm induction in Xenopus embryos. *Curr. Biol.* **10**, 1511-1514.

**Harland, R. M.** (1991). In situ hybridization: an improved whole-mount method for Xenopus embryos. *Methods Cell Biol.* **36**, 685-695.

**Harland, R. M.** (2000). Neural induction. *Curr. Opin. Genet. Dev.* **10**, 357-362.

**Harland, R. M. and Gerhart, J.** (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell Dev. Biol.* **13**, 611-667.

**Hata, A., Lagna, G., Massague, J. and Hemmati-Brivanlou, A.** (1998). Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev.* **12**, 186-197.

**Hayashi, H., Abdollah, S., Qiu, Y., Cai, J., Xu, Y. Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone, M. A., Jr, Wrana, J. L. et al.** (1997). The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. *Cell* **89**, 1165-1173.

**Heeg-Truesdell, E. and LaBonne, C.** (2006). Neural induction in Xenopus requires inhibition of Wnt-β-catenin signaling. *Dev. Biol.* **298**, 71-86.

**Hemmati-Brivanlou, A. and Melton, D. A.** (1997). Vertebrate neural induction. *Annu. Rev. Neurosci.* **20**, 43-60.

**Hongo, I., Kengaku, M. and Okamoto, H.** (1999). FGF signaling and the anterior neural induction in Xenopus. *Dev. Biol.* **216**, 561-581.

**Hudson, C. and Lemaire, P.** (2001). Induction of anterior neural fates in the ascidian Ciona intestinalis. *Mech. Dev.* **100**, 189-203.

**Hudson, C., Darras, S., Caillol, D., Yasuo, H. and Lemaire, P.** (2003). A conserved role for the MEK signalling pathway in neural tissue specification and posteriorisation in the invertebrate chordate, the ascidian Ciona intestinalis. *Development* **130**, 147-159.

**Ishimura, A., Maeda, R., Takeda, M., Kikkawa, M., Darr, I. O. and Maeno, M.** (2000). Involvement of BMP-4/Msx-1 and FGF pathways in neural induction in the Xenopus embryo. *Dev. Growth Differ.* **42**, 307-316.

**Kavsak, P., Rasmussen, R. K., Causing, C. G., Bonni, S., Zhu, H., Thomsen, G. H. and Wrana, J. L.** (2000). Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGFβ receptor for degradation. *Mol. Cell* **6**, 1365-1375.

**Kengaku, M. and Okamoto, H.** (1993). Basic fibroblast growth factor induces differentiation of neural tube and neural crest lineages of cultured ectoderm cells from Xenopus gastrula. *Development* **119**, 1067-1078.

**Knoetgen, H., Teichmann, U., Wittler, L., Viebahn, C. and Kessel, M.** (2000). Anterior neural induction by nodes from rabbits and mice. *Dev. Biol.* **225**, 370-380.

**Kretzschmar, M., Doody, J., Timokhina, I. and Massague, J.** (1999). A mechanism of repression of TGFβ/Smad signaling by oncogenic Ras. *Genes Dev.* **13**, 804-816.

**Kuroda, H., Fuentealba, L., Ikeda, A., Reversade, B. and De Robertis, E. M.** (2005). Default neural induction: neutralization of dissociated Xenopus cells is mediated by Ras/MAPK activation. *Genes Dev.* **19**, 1022-1027.

**Lamb, T. M. and Harland, R. M.** (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern. *Development* **121**, 3627-3636.

**Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D. and Harland, R. M.** (1993). Neural induction by the secreted polypeptide noggin. *Science* **262**, 713-718.

**Launay, C., Fromentoux, V., Shi, D. L. and Boucaut, J. C.** (1996). A truncated FGF receptor blocks neural induction by endogenous Xenopus inducers. *Development* **122**, 869-880.

**Lee, M. A., Heasman, J. and Whitman, M.** (2001). Timing of endogenous activin-like signals and regional specification of the Xenopus embryo. *Development* **128**, 2939-2952.

**Lemaire, P., Bertrand, V. and Hudson, C.** (2002). Early steps in the formation of neural tissue in ascidian embryos. *Dev. Biol.* **252**, 151-169.

**Linker, C. and Stern, C. D.** (2004). Neural induction requires BMP inhibition only as a late step, and involved signals other than FGF and Wnt antagonists. *Development* **131**, 5671-5681.

**Liu, X., Sun, Y., Weinberg, R. A. and Lodish, H. F.** (2001). Ski/Sno and TGF-β signaling. *Cytokine Growth Factor Rev.* **12**, 1-8.

**Londin, E. R., Niemiec, J. and Sirotkin, H. I.** (2005). Chordin, FGF signaling, and mesodermal factors cooperate in zebrafish neural induction. *Dev. Biol.* **279**, 1-19.

**Lowe, C. J., Terasaki, M., Wu, M., Freeman, R. M., Jr, Runft, L., Kwan, K., Haigo, S., Aronowicz, J., Lander, E., Gruber, C. et al.** (2006). Dorsoventral patterning in hemichordates: insights into early chordate evolution. *PLoS Biol.* **4**, 1603-1619.

**Lu, C. C., Brennan, J. and Robertson, E. J.** (2001). From fertilization to gastrulation: axis formation in the mouse embryo. *Curr. Opin. Genet. Dev.* **11**, 384-392.

**Luo, K.** (2003). Negative regulation of BMP signaling by the Ski oncoprotein. *J. Bone Joint Surg. Am.* **85**, 39-43.

**Luo, K.** (2004). Ski and SnoN: negative regulators of TGF-β signaling. *Curr. Opin. Genet. Dev.* **14**, 65-70.

**Mariani, F. V., Choi, G. B. and Harland, R. M.** (2001). The neural plate specifies somite size in the Xenopus laevis gastrula. *Dev. Cell* **1**, 115-126.

**Mayor, R., Morgan, R. and Sargent, M. G.** (1995). Induction of the prospective neural crest of Xenopus. *Development* **121**, 767-777.

**Mir, A., Kofron, M., Zorn, A. M., Bajzer, M., Haque, M., Heasman, J. and Wylie, C. C.** (2007). Foxl1e activates ectoderm formation and controls cell position in the Xenopus blastula. *Development* **134**, 779-788.

**Murakami, G., Watabe, T., Takaoka, K., Miyazono, K. and Imamura, T.** (2003). Cooperative inhibition of bone morphogenetic protein signaling by Smurf1 and inhibitory Smads. *Mol. Biol. Cell* **14**, 2809-2817.
**Oppenheimer, J.** (1936). Transplantation experiments on developing teleosts (*Fundulus* and *Perca*). *J. Exp. Zool.* **72**, 409-437.
**Pera, E. M., Ikeda, A., Eivers, E. and De Robertis, E. M.** (2003). Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. *Genes Dev.* **17**, 3023-3028.
**Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T. and de Robertis, E. M.** (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397**, 707-710.
**Reissmann, E., Jornvall, H., Blokzijl, A., Andersson, O., Chang, C., Minchiotti, G., Persico, M. G., Ibanez, C. F. and Brivanlou, A. H.** (2001). The orphan receptor ALK7 and the activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. *Genes Dev.* **15**, 2010-2022.
**Ribisi, S., Jr, Mariani, F. V., Aamar, E., Lamb, E. M., Frank, D. and Harland, R. M.** (2000). Ras-mediated FGF signaling is required for the formation of posterior but not anterior neural tissue in Xenopus laevis. *Dev. Biol.* **227**, 183-196.
**Saude, L., Woolley, K., Martin, P., Driever, W. and Stemple, D. L.** (2000). Axis-inducing activities and cell fates of the zebrafish organizer. *Development* **127**, 3407-3417.
**Schier, A. F. and Talbot, W. S.** (2001). Nodal signaling and the zebrafish organizer. *Int. J. Dev. Biol.* **45**, 289-297.
**Schohl, A. and Fagotto, F.** (2002). β-catenin, MAPK and Smad signaling during early Xenopus development. *Development* **129**, 37-52.
**Sheng, G., dos Reis, M. and Stern, C. D.** (2003). Churchill, a zinc finger transcriptional activator, regulates the transition between gastrulation and neurulation. *Cell* **115**, 603-613.
**Shih, J. and Fraser, S. E.** (1996). Characterizing the zebrafish organizer-microsurgical analysis at the early-shield stage. *Development* **122**, 1313-1322.
**Sirotkin, H. I., Dougan, S. T., Schier, A. F. and Talbot, W. S.** (2000). bozozok and squint act in parallel to specify dorsal mesoderm and anterior neuroectoderm in zebrafish. *Development* **127**, 2583-2592.
**Smith, W. C. and Harland, R. M.** (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* **70**, 829-840.
**Sokol, S. Y.** (1993). Mesoderm formation in Xenopus ectodermal explants overexpressing Xwnt8: evidence for a cooperating signal reaching the animal pole by gastrulation. *Development* **118**, 1335-1342.
**Spemann, H. and Mangold, H.** (1924). Uber die Induktion von Embryoanlagen durch Implantation artremder Organisatoren. *Willhelm Roux Arch. Entwickl. Mech. Org.* **100**, 599-638.
**Streit, A., Berliner, A. J., Papanayotou, C., Sirulnik, A. and Stern, C. D.** (2000). Initiation of neural induction by FGF signalling before gastrulation. *Nature* **406**, 74-78.
**Sun, B. I., Bush, S. M., Collins-Racie, L. A., LaVallie, E. R., DiBlasio-Smith, E. A., Wolfman, N. M., McCoy, J. M. and Sive, H. L.** (1999). derriere: a TGF-β family member required for posterior development in Xenopus. *Development* **126**, 1467-1482.
**Suri, C., Haremaki, T. and Weinstein, D. C.** (2005). Xema, a foxi-class gene expressed in the gastrula stage Xenopus ectoderm, is required for the suppression of mesendoderm. *Development* **132**, 2733-2742.
**Suzuki, A., Ueno, N. and Hemmati-Brivanlou, A.** (1997). Xenopus Msx1 mediates epidermal induction and neural inhibition by BMP4. *Development* **124**, 3037-3044.
**Takeda, M., Mizuide, M., Oka, M., Watabe, T., Inoue, H., Suzuki, H., Fujita, T., Imamura, T., Miyazono, K. and Miyazawa, K.** (2004). Interaction with Smad4 is indispensable for suppression of BMP signaling by c-Ski. *Mol. Biol. Cell* **15**, 963-972.
**Tanegashima, K., Haramoto, Y., Yokota, C., Takahashi, S. and Asashima, M.** (2004). Xantivin suppresses the activity of EGF-CFC genes to regulate nodal signaling. *Int. J. Dev. Biol.* **48**, 275-283.
**Thisse, B., Wright, C. V. E. and Thisse, C.** (2000). Activin- and Nodal-related factors control antero-posterior patterning of the zebrafish embryo. *Nature* **403**, 425-428.
**Vallier, L., Reynolds, D. and Pedersen, R. A.** (2004). Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. *Dev. Biol.* **275**, 403-421.
**Vonica, A. and Brivanlou, A. H.** (2006). An obligatory caravanserai stop on the silk road to neural induction: inhibition of BMP/GDF signaling. *Semin. Cell Dev. Biol.* **17**, 117-132.
**Waddinton, C. H.** (1932). Experiments on the development of chick and duck embryos, cultivated in vitro. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **221**, 179-230.
**Wang, W., Mariani, F. V., Harland, R. M. and Luo, K.** (2000). Ski represses bone morphogenic protein signaling in Xenopus and mammalian cells. *Proc. Natl. Acad. Sci. USA* **97**, 14394-14399.
**Wawersik, S., Evola, C. and Whitman, M.** (2005). Conditional BMP inhibition in Xenopus reveals stage-specific roles for BMPs in neural and neural crest induction. *Dev. Biol.* **277**, 425-442.
**Weinstein, D. C. and Hemmati-Brivanlou, A.** (1999). Neural induction. *Annu. Rev. Cell Dev. Biol.* **15**, 411-433.
**Wessely, O., Agius, E., Oelgeschlager, M., Pera, E. M. and De Robertis, E. M.** (2001). Neural induction in the absence of mesoderm: β-catenin-dependent expression of secreted BMP antagonists at the blastula stage in Xenopus. *Dev. Biol.* **234**, 161-173.
**Wilson, S. I., Graziano, E., Harland, R., Jessell, T. M. and Edlund, T.** (2000). An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. *Curr. Biol.* **10**, 421-429.
**Wilson, S. I., Rydstrom, A., Trimborn, T., Willert, K., Nusse, R., Jessell, T. M. and Edlund, T.** (2001). The status of Wnt signalling regulates neural and epidermal fates in the chick embryo. *Nature* **411**, 325-330.
**Winkles, J. A., Sargent, T. D., Parry, D. A., Jonas, E. and Dawid, I.** (1985). Developmentally regulated cytokeratin gene in Xenopus laevis. *Mol. Cell. Biol.* **5**, 2575-2581.
**Witkowski, J. A.** (1985). The hunting of the Organizer: an episode in biochemical embryology. *Trends Biochem. Sci.* **10**, 379-381.
**Yamamoto, T., Takagi, C. and Ueno, N.** (2000). Requirement of Xmsx-1 in the BMP-triggered ventralization of Xenopus embryos. *Mech. Dev.* **91**, 131-141.
**Zimmerman, L. B., de Jesus-Escobar, J. M. and Harland, R. M.** (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86**, 599-606.