Conditional BMP inhibition in *Xenopus* reveals stage-specific roles for BMPs in neural and neural crest induction

Stefan Wawersik, Christina Evola, Malcolm Whitman*

Department of Cell Biology, Harvard Medical School, Boston, MA 02215, United States

Received for publication 5 April 2004, revised 3 October 2004, accepted 4 October 2004

Available online 27 October 2004

**Abstract**

Bone morphogenetic protein (BMP) inhibition has been proposed as the primary determinant of neural cell fate in the developing *Xenopus* ectoderm. The evidence supporting this hypothesis comes from experiments in explanted “animal cap” ectoderm and in intact embryos using BMP antagonists that are unregulated and active well before gastrulation. While informative, these experiments cannot answer questions regarding the timing of signals and the behavior of cells in the more complex environment of the embryo. To examine the effects of BMP antagonism at defined times in intact embryos, we have generated a novel, two-component system for conditional BMP inhibition. We find that while blocking BMP signals induces ectopic neural tissue both in animal caps and in vivo, in intact embryos, it can only do so prior to late blastula stage (stage 9), well before the onset of gastrulation. Later inhibition does not induce neural identity, but does induce ectopic neural crest, suggesting that BMP antagonists play temporally distinct roles in establishing neural and neural crest identity. By combining BMP inhibition with fibroblast growth factor (FGF) activation, the neural inductive response in whole embryos is greatly enhanced and is no longer limited to pre-gastrula ectoderm. Thus, BMP inhibition during gastrulation is insufficient for neural induction in intact embryos, arguing against a BMP gradient as the sole determinant of ectodermal cell fate in the frog.

© 2004 Elsevier Inc. All rights reserved.

**Keywords:** Gastrulation; Inhibition; Embryo

**Introduction**

Signaling by the bone morphogenetic proteins (BMPs), a subset of the TGF-β superfamily of cytokines, has been implicated in numerous developmental processes, including patterning of the limb, tooth, kidney, and somites (Zhao, 2003). BMPs bind as dimers to two classes of serine-threonine kinase receptors, designated type I and type II (Massague et al., 2000; Shi and Massague, 2003; Whitman, 1998). Upon ligand binding, activated type II receptors transphosphorylate type I receptors, which propagate the signal by phosphorylating receptor regulated Smad (R-Smad) proteins. Within the TGF-β family, BMP receptors phosphorylate the R-Smads Smad1, Smad5, or Smad8, while the receptors for nodal-like ligands phosphorylate Smad2 or Smad3.

BMPs are critical in the differentiation of pluripotent ectodermal cells, and have been proposed as the primary determinant of neural identity (Hawley et al., 1995; Liem et al., 1995; Wilson and Hemmati-Brivanlou, 1995). In frogs, the organizer expresses secreted BMP antagonists, and blocking BMP signaling in vivo or in ectodermal explants induces neural differentiation (Lamb et al., 1993; Piccolo et al., 1996; Sasai et al., 1995; Zimmerman et al., 1996). In situ observations reveal high BMP activity in the ventral ectoderm and low activity in dorsal ectoderm (Faure et al., 2000; Kurata et al., 2001; Schohl and Fagotto, 2002), and studies in animal caps indicate that discrete BMP signaling levels induce three cell identities within the early ectoderm: epidermis, neural crest, and neural (Marchant et al., 1998; Wilson et al., 1997). Collectively, these observations...
suggest a model in which diffusible, organizer-derived BMP antagonists establish an activity gradient in the ectoderm. In this model, low BMP signaling near the organizer specifies neural cell fate in the dorsal ectoderm, intermediate BMP levels induce neural crest in the marginal ectoderm, and high BMP activity results in epidermal fate in the ventral ectoderm.

Despite the evidence for a functional BMP gradient, several observations suggest a more complicated mechanism for determining neural and neural crest fate. Some neural-inducing genes are expressed prior to organizer formation, and neural tissue can be induced in the absence of a definitive organizer (Kroll et al., 1998; Kuroda et al., 2004; Mizuseki et al., 1998; Penzel et al., 1997; Wessely et al., 2001). In avian embryos, BMP inhibitors fail to induce neural identity in blastula or gastrula stage non-neural ectoderm, though BMP activity is excluded from the presumptive neural plate during gastrulation and activation of BMP signaling represses neural formation (Faure et al., 2002; Levin, 1998; Storey et al., 1992; Streit and Stern, 1999; Streit et al., 1998, 2000; Wilson et al., 2000, 2001). Together, these data suggest that neural induction may be governed by the integration of multiple signaling mechanisms.

Members of the fibroblast growth factor (FGF) family of growth factors have emerged as important candidates for regulators of additional signaling important for neural induction. FGFs have been implicated in neural induction and patterning in a variety of organisms (Domingos et al., 2001; Holowacz and Sokol, 1999; Hongo et al., 1999; Ishimura et al., 2000; Koshida et al., 2002; Ribisi et al., 2000; Streit and Stern, 1999; Wilson et al., 2000), and FGFs and BMPs act antagonistically in the development of a variety of embryonic structures (Bei and Maas, 1998; Chen et al., 2000; Zuniga et al., 1999). Furthermore, MAP Kinase (MAPK), a transducer of FGF signaling, phosphorylates several serine residues in the linker region of Smad1, and Smad1 mutations that block MAPK-dependent phosphorylation act as dominant negative inhibitors of neural development (Kretzschmar et al., 1997a; Pera et al., 2003; Sater et al., 2003). These experiments suggest that cross-regulation of BMP and FGF signaling pathways, as well as independent action by each, may be an important component of neural induction.

Much of the experimental confirmation for the BMP gradient model in frogs relies on two methods: BMP inhibitors injected as mRNA into early cleavage-stage embryos and explanted ectodermal tissue (“animal caps”). However, injection of inhibitors may affect early patterning, as their consequent BMP antagonism begins by the 16–32 cell stage. While “animal caps” provide an elegant method for examining early ectoderm patterning, they also isolate the ectoderm from adjacent tissues, potentially removing some of the signals that modulate ectodermal identity. In addition, the process of animal cap isolation, particularly if it is coupled to partial dissociation of cells to allow exposure to extracellular factors, introduces a set of perturbations that may not reflect endogenous physiology. Therefore, to more accurately characterize the function of BMP signaling during Xenopus neural determination, BMP antagonism should be carried out in a stage-specific manner in intact embryos.

Conditional regulation of transcription factor function can be achieved in Xenopus by generating fusion proteins to the hormone-binding domain of the glucocorticoid receptor (GR) (Kolm and Sive, 1995). Such proteins are held inactive until the addition of the hormone dexamethasone (DEX). We reasoned that fusion of the GR hormone-binding domain to a cytoplasmic antagonist of BMP signaling might allow similar conditional regulation of the BMP pathway. Among the regulators of BMP signaling, the inhibitory Smads, Smad6 and Smad7, both act in the cytoplasm (Bhushan et al., 1998; Casellas and Brivanlou, 1998; Hata et al., 1998). Smad6 specifically blocks signaling by the BMP/GDF pathway, at least in part by binding to phosphorylated Smad1 and competing with Smad4 (Hata et al., 1998). Smad7 antagonizes both BMP and nodal-like signaling by binding to type I receptors and inducing their degradation (Kavsak et al., 2000; Suzuki et al., 2002). However, misexpression of the C-terminal half of Smad7 elicits phenotypes suggesting BMP-specific inhibition (Nakayama et al., 2001); its action at the level of the receptor furthermore suggests that it should be possible to track the effectiveness of ΔSmad7 as an inhibitor of endogenous signals by examining receptor-dependent Smad phosphorylation.

We report here the construction of a tightly controlled conditional BMP inhibitor, combining a GR regulated ΔSmad7 with a novel regulated form of the tobacco etch virus (TEV) protease. We have confirmed the effectiveness, specificity, and regulation of this inhibitor by examination of endogenous Smad phosphorylation using phosphoSmad antibodies (Faure et al., 2000). Using this inhibitor, we have examined the consequences of stage-specific BMP antagonism on neural and neural crest induction. Consistent with previous studies, BMP inhibition induces expression of neural markers in explanted gastrula-stage Xenopus ectoderm (Hawley et al., 1995; Knecht et al., 1995; Lamb and Harland, 1995; Lamb et al., 1993; Wilson and Hemmati-Brivanlou, 1995). In intact embryos, early BMP inhibition also induces ectopic neural tissue, yet this capacity is lost prior to gastrulation, as BMP inhibition after mid-blastula stage (stage 8) fails to cause ectopic neural induction. While later inhibition does not induce neural identity, it does induce ectopic neural crest, suggesting that BMP-dependent neural and neural crest specification occur at different times. We also find that co-activation of FGF signaling greatly enhances BMP antagonist-mediated neural induction. Furthermore, unlike BMP antagonism alone, the combination of BMP inhibition and FGF activation can drive neural induction both before and after the onset of gastrulation in whole embryos and in stage 11 animal caps. These results
indicate that in the intact frog embryo, as in chick, BMP inhibition by itself is insufficient for neural induction. This argues against a BMP antagonism as the sole determinant of early ectodermal cell identity, suggesting a more complex model where extra-ectodermal signals modulate neural induction through interactions between the BMP and FGF signaling pathways.

Materials and methods

Cloning of expression constructs

DNA encoding ΔSmad7 was amplified from Xenopus Smad7 (a gift of A. Hemmati-Brivanlou) by PCR using the following primers: ΔSm7-up 5'-TTC GAA TTC CAT GGA CTT TCT CAA ACC C-3'; ΔSm7-dn 5'-CAC TAT AGT TCT AGA GGC TCG AGT CCG CGG-3'. These primers contain an EcoRI site in the upstream primer and an XhoI site in the downstream one, and these restriction sites were used to clone ΔSmad7 into pCS2-based expression vectors.

To insert three tobacco etch virus (TEV) protease cleavage sites between ΔSmad7 and the C-terminal glucocorticoid receptor (GR) domain, two complimentary oligos were synthesized: 3TEVsense 5'-TCA GAC CTT GGA AAT ACA GAT and 3TEVantisense 5'-GTG AGA ATC TGT ATT TCC AAG GT-3'. These were mixed at 500 μg/μl (each oligo) in 100 mM NaCl, heated to 85°C, allowed to cool gradually to room temperature. The annealed oligos create sticky ends compatible with an RI site upstream and an NheI site downstream, and these restriction sites were used to ligate the oligo in between the ΔSmad7 and GR coding regions of the ΔSmad7GR DNA.

The TEV2GR construct was generated using PCR to insert an EcoRI site upstream and an XhoI site downstream of the DNA encoding the TEV protease (Lucast et al., 2001) (Primers: TEV-up 5'-AAG AGG AGG AAT TCC ATG GCT GAA AGC-3'; TEV-dn 5'-TGG TGA TGG TGC TCG AGC AAT TCC GAG-3'). These were used to clone the protease into pCS2-based expression vectors.

Western blot analysis

Embryos were homogenized (20 μl/embryo) in a modified RIPA buffer (150 mM Tris–HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 2 mM EDTA, 25 mM β-glycerophosphate, 100 mM NaF, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM calyculin A, 2× complete protease inhibitor cocktail (Roche), 4 μg/ml pepstatin A, 1 mM PMSF) and centrifuged for 10 min at 14,000 × g (4°C). The supernatant was removed and suspended in an equal volume of 2× Laemmli loading buffer. Lysates were separated by 7.5% SDS-PAGE and transferred to nitrocellulose (Protran). Membranes were blocked with 1% polyvinyl pyrrolidone (PVP) (Sigma) in TBST (except for anti-pSmad2, which was blocked in 5% PVP + 0.01% thimerosal overnight at room temperature), then incubated overnight at 4°C with primary antibody under the following conditions: anti-pSmad1 (Faure et al., 2000) 1:200 in TBST + 0.1% BSA, anti-Smad2 (Faure et al., 2000) 1:500 in TBST, anti-actin (Sigma) 1:1000 in TBST, anti-myc (Santa Cruz) 1:1000 in TBST + 5% nonfat dry milk. After several washes in TBST, blots were incubated with HRP conjugated secondary antibody for 1–2 h at RT, washed several times in TBST, and HRP activity was detected by chemiluminescence (Amersham).

Embryo manipulations

Embryos were collected from Xenopus females and artificially fertilized as previously described (Watanabe and Whitman, 1999). Staging of embryos was according to Nieuwkoop and Faber (1967). Prior to first cleavage, embryos were dejellied in 2% cysteine (pH 7.5). To aid visualization of anatomical features, albino embryos were stained in 0.5% Nile Blue in 0.1× MMR, followed by several washes in 0.1× MMR. Embryos were placed in 3% ficoll, 1× MMR and injected at 2-, 4-, or 8-cell stage with 10 nl RNA. Following injection, embryos were kept in ficoll at 14°C for 1–2 h before being placed in 0.1× MMR. Animal cap dissections were performed in 0.7× MMR and caps were kept in 0.7× MMR until they were collected. Where noted, Dexamethasone (DEX) (Sigma) was added to culture medium at a concentration of 10 μM (Stock: 10 mM in EtOH). AP20187 (ARIAD Pharmaceuticals) was added at a concentration of 1.25 μM (Stock: 1.25 mM in EtOH). SUS402 (Calbiochem) was added to embryo culture medium at a concentration of 20 μM (Stock: 2 mM in DMSO).

Whole mount immunostaining

Embryos were collected and fixed for 1 h at room temp in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, pH 8, 1 mM MgSO4, 3.7% formaldehyde) then incubated for 10 min in 100 mM lysine in PBS + 0.1% Tween (PBS-Tween) at RT. Embryos were washed several times in PBT (PBS, 2 mg/ml BSA, 0.1% Triton X-100) and stored in PBT at 4°C for no longer than 24 h prior to immunostaining. Prior to immunostaining, embryos were blocked in 10% normal goat serum (NGS) (Jackson Immunoresearch) in PBT. Embryos were incubated in 1:20 dilution of anti-pSmad1 antibody in 10% NGS overnight at 4°C, washed six times for 15 min each in PBT, then incubated with HRP conjugated secondary antibody (1:250). Staining was developed using an ImmunoPure Metal-enhanced DAB Kit (Pierce), as per manufacturer’s instructions for 15–20 min.
In situ hybridization

Embryos were fixed in MEMFA as described above. Where β-galactosidase staining was performed, embryos were incubated in 1 mg/ml magenta-gal (ICN) in 20 mM K₃Fe(CN)₆, 20 mM K₄Fe(CN)₆, 3H₂O, 2 mM MgCl₂, 0.01% sodium deoxycholate and 0.02% NP-40 at room temperature. In situ hybridizations were performed as previously described (Wawersik et al., 1999), with the following modifications: embryos were permeabilized for 30 min in RIPA (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 1 mM EDTA, 50 mM Tris–HCl pH 8.0), followed by 20 min fixation in MEMFA. Hybridization and washes were done at 60°C. Alkaline phosphate staining carried out overnight at 4°C in the dark in BM Purple (Roche) supplemented with 2 mM levamisole. After color development, embryos were fixed for 2 h in MEMFA at RT and stored in PBS.

RT-PCR analysis

Collection of RNA and generation of cDNA was performed as previously described (Watanabe and Whitman, 1999). The following primers were used: NCAM forward 5'-CACATCTCCACCAAAATGC-3'; NCAM reverse 5'-GGA ATC AAG CGG TAC AGA-3'; EF-1α forward 5'-CAG ATT GGT CGT GGA TAT GC-3'; EF-1α reverse 5'-ACT GCC TTG ATG ACT CCT AG-3'; Sox2 forward 5'-GAG GAT GGA CAC TTA TGC CCA C-3'; Sox2 reverse 5'-GGA CAT GCT GTA GGT AGG CGA-3'. PCR conditions: 22 cycles, 95°C 30 s, 55°C 30 s, 72°C 45 s.

Imaging

Embryos were photographed using a Leica MZFLII stereomicroscope and a MagnaFire SP digital camera (Optronics). In some cases, after image capture, image brightness and color balance were slightly modified using Photoshop 7 (Adobe).

Results

The C-terminal fragment of Smad7, ∆Smad7, inhibits phosphorylation of Smad1, but not of Smad2

Misexpression of the C-terminal half of Smad7 (∆Smad7, Fig. 1A) in Xenopus embryos produces phenotypes similar to those observed upon ectopic BMP inhibition, suggesting that ∆Smad7 may specifically inhibit BMP signaling (Nakayama et al., 2001). To formally test this hypothesis, we injected RNA encoding ∆Smad7 into embryos and examined the phosphorylation states of R-Smads in stage 10+ embryos using phosphorylation-specific antibodies to the BMP- and nodal-dependent Smads (Faure et al., 2000). Note that the BMP receptors phosphorylate identical amino acid sequences at the C-termini of Smads 1, 5, and 8 (Hoodless et al., 1996; Kretzschmar et al., 1997b; Macias-Silva et al., 1998). Consequently, our phospho-specific antibody cannot distinguish between these Smads, and we will therefore refer to the BMP-regulated R-Smads in frog simply as ‘Smad1.’ Injection of RNA encoding a constitutively active type I BMP receptor (ALK2*) induces Smad1 phosphorylation but does not affect phosphorylation of the nodal-regulated R-Smad, Smad2 (Fig. 1B). Injection of RNA encoding the BMP antagonist noggin also does not affect Smad2 phosphorylation but specifically inhibits Smad1 phosphorylation. Injecting increasing amounts of ∆Smad7 RNA inhibits Smad1 phosphorylation in a concentration-dependent manner without affecting Smad2 phosphorylation.

Generation of a hormone-inducible inhibitor of BMP signaling

In Xenopus, transcription factor function can be conditionally regulated by fusion to the hormone-binding domain of the glucocorticoid receptor (GR) (Kolm and Sive, 1995). Such proteins are held inactive until the addition of the hormone dexamethasone (DEX). While we find that addition of a GR domain to the C-terminus of ΔSmad7 (ΔSmad7GR, Fig. 1A) confers some conditional regulation (data not shown), DEX-induced phospho-Smad1 inhibition is only observed at high ∆Smad7GR concentrations, suggesting that the GR domain reduces ΔSmad7 activity in the presence of DEX. However, in the absence of DEX, these same ΔSmad7GR concentrations weakly inhibit Smad1 phosphorylation and produce significant phenotypic abnormalities (data not shown). Thus, a usable conditional inhibitor requires either tighter ΔSmad7 regulation in the absence of DEX or increased ΔSmad7 activity upon DEX addition.

To alleviate interference with activated ΔSmad7GR function, we devised a means to remove the GR domain in vivo. We generated a hormone inducible tobacco etch virus (TEV) Nla protease by fusing GR domains onto both the N- and C-termini (TEV2GR, Fig. 1A). The TEV Nla protease
specifically recognizes the heptapeptide sequence E-X-X-Y-X-Q/S/G (Kapust et al., 2001; Lucast et al., 2001), and we inserted three of these cleavage sites in tandem between the D\text{Smad7} and the GR coding domains (D\text{Smad7tevGR}). We hypothesized that upon DEX addition, TEV2GR would cleave the GR domain from D\text{Smad7tevGR}, removing interference by the GR domain on D\text{Smad7} activity. Such increased activity might require injection of less D\text{Smad7tevGR} RNA, reducing the leaky activation in the absence of DEX.

To test this, we injected 300 pg of RNA encoding D\text{Smad7tevGR} into embryos, together with several concentrations of TEV2GR. A myc tag was added to the N-termini of D\text{Smad7} and of D\text{Smad7tevGR}, and Western blots using an antibody to this tag show increased accumulation of the cleaved form of D\text{Smad7tevGR} in the presence of DEX (Fig. 1D). Ten picograms of TEV2GR RNA cause little accumulation of cleaved D\text{Smad7tevGR} in the absence of DEX, but is sufficient to induce both cleavage of D\text{Smad7tevGR} and inhibition of Smad1 phosphorylation upon DEX addition.

We also functionally tested whether D\text{Smad7tevGR}/TEV2GR exhibited leaky activation by examining their effect on embryonic development. Two hundred, 300, or 400 pg of D\text{Smad7tevGR} RNA were injected with 10 pg TEV2GR RNA, and the resulting embryos were allowed to develop to tailbud stage (Fig. 2). In the absence of DEX, embryos injected with 200 or 300 pg D\text{Smad7tevGR} and 10 pg TEV2GR are phenotypically comparable to uninjected controls (compare Figs. 2B and C with A), while increasing the D\text{Smad7tevGR} RNA concentration to 400 pg causes head defects, weak dorsalization, and occasional axis
duplication (arrows, Fig. 2D). Addition of DEX dorsalizes embryos at all concentrations tested (Figs. 2F–H), with effects ranging from moderate at 200 pg DSmad7tevGR to severe at 400 pg. Though DEX-induced embryos at 300 pg DSmad7tevGR/10 pg TEV2GR are not as severely affected as those at higher concentrations (compare Figs. 2G with H), 300 pg is the highest concentration examined at which uninduced embryos show no unusual phenotypes. Therefore, all subsequent experiments were carried out using 300 pg DSmad7tevGR and 10 pg TEV2GR, which function together as a potent, tightly regulated conditional inhibitor of BMP signaling through Smad1.

Inhibition by DSmad7tevGR/TEV2GR can be rapidly activated at multiple stages of development

To examine whether DSmad7tevGR/TEV2GR-mediated phosphoSmad1 inhibition can be activated at multiple stages of development, we injected RNA into both cells of two-cell stage embryos. From these embryos, animal cap explants were isolated at stage 8 and DEX was added when sibling embryos were at either stage 9 or stage 11. The caps were then allowed to develop overnight at 14°C, until sibling embryos had reached stage 16, when they were harvested and phosphoSmad1 levels were analyzed by Western blot (Fig. 3A). Phospho-Smad1 inhibition was not observed in caps in the absence of DEX. Addition of DEX at either stage 9 or stage 11 resulted in pSmad1 inhibition comparable to that in caps from embryos where DEX was added at stage 5. These results indicate that BMP antagonism by DSmad7tevGR/TEV2GR is equally efficient when DEX is added to ectoderm before or after the onset of gastrulation.

The delay between DEX addition and maximal BMP inhibition was tested by injecting DSmad7tevGR/TEV2GR RNA into both cells of two-cell stage embryos. Animal caps were isolated from these embryos at stage 9 and allowed to develop at 14°C for 6 h. DEX was added 1, 2, 3, or 6 h before harvesting, and both phosphoSmad1 levels and accumulation of cleaved DSmad7tevGR were analyzed by Western blot (Fig. 3B). One hour after the addition of DEX, some pSmad1 inhibition is observed. Interestingly, little accumulation of cleaved DSmad7tevGR is observed in these samples, suggesting that the uncleaved form may mediate initial pSmad1 inhibition. Significant accumulation of
cleaved ΔSmad7tevGR and pSmad1 inhibition comparable to that in caps from embryos to which DEX was added at stage 5 is evident by 3 h after DEX addition. At 14°C, stages 8, 9, and 10 are separated by roughly 6 h each (Nieuwkoop and Faber, 1967), suggesting that at this temperature, the delay in BMP inhibition is short enough to allow dissection of stage-specific requirements for BMP signaling. Therefore, all subsequent experiments were carried out in embryos or animal caps incubated at 14°C.

Regulated ΔSmad7 induces neural markers in explanted ectoderm

Addition of noggin protein, a secreted BMP antagonist, induces neural marker expression in explanted ectoderm when added before stage 10.5 (Knecht et al., 1995; Lamb et al., 1993). We therefore tested the ability of regulated ΔSmad7 to similarly induce neural expression in animal caps. RNA encoding ΔSmad7tevGR and TEV2GR was injected into both cells of two-cell stage embryos. Animal caps were cut at stage 8, and DEX was added to caps when sibling embryos reached stage 9 or stage 11. Caps to which DEX was not added and caps cut from embryos to which DEX was added at stage 4 served as negative and positive controls, respectively. When sibling embryos reached stage 16, RNA was collected and assayed by RT-PCR for the neural markers Sox2 and neural cell adhesion molecule (NCAM) (Balak et al., 1987; Kishi et al., 2000) (Fig. 4A). No induction of either marker is seen in animal caps from uninjected embryos or in caps to which DEX was not added. NCAM and Sox2 expression are also absent in caps where DEX was added at stage 11, consistent with the observation that neural competence is lost prior to that stage (Knecht et al., 1995; Lamb et al., 1993; Servetnick and Grainger, 1991a). Also consistent with previous work, BMP antagonism beginning at stage 4 or stage 9 induces both Sox2 and NCAM, indicating neural induction in those animal caps (Knecht et al., 1995; Lamb et al., 1993). In multiple experiments, however, we consistently noted weaker neural induction when DEX was added at stage 9 than at stage 4. To further examine this, stage 8 animal cap ectoderm was isolated from ΔSmad7tevGR/TEV2GR-injected albino embryos. DEX was added at these cultures at stage 9 or stage 11, and neural induction was examined by in situ hybridization for Sox2. We observed no Sox2 induction in uninjected caps (Fig. 4B), minimal Sox2 expression in ΔSmad7tevGR/TEV2GR-injected caps that were not treated with DEX (Fig. 4C), and expression comparable to unininduced controls when DEX is added at stage 11 (compare Figs. 4F with C). A large increase in Sox2 levels is evident in caps from embryos where DEX was added at stage 4 (Fig. 4D). As suggested by RT-PCR, Sox2 is induced upon DEX addition at stage 9 (Fig. 4E), but the extent of induction is decreased relative to stage 4-induced controls, with the overall area of the cap expressing Sox2 reduced (compare Figs. 4E with D). Similar results were observed using NCAM as a neural marker (data not shown). These results suggest that while BMP antagonism can induce neural fate in pre-gastrula ectoderm, some restriction in the ectoderm’s neural competence occurs between stages 4 and 9 of Xenopus development.

The ability of BMP inhibition to induce ectopic neural tissue in intact embryos is stage dependent

Next, we examined the ability of BMP inhibition to induce ectopic neural tissue in intact embryos at specific stages. To ensure that ΔSmad7tevGR and TEV2GR efficiently and conditionally antagonize BMP signaling in the intact embryo, RNA encoding both proteins was injected into one cell of two-cell stage embryos, along with RNA encoding green fluorescent protein (GFP) to mark the injected side (Figs. 5E–H). DEX was added either at stage 5 (16 cells), stage 10 (early gastrula), or stage 14 (early neurula). The embryos were then collected at stage 16 and...
immunostained for phospho-Smad1. In the absence of DEX, no phospho-Smad1 alteration is observed (arrow, Fig. 5A), but addition of DEX at stage 5 abolishes Smad1 phosphorylation on the injected side (red arrow, Fig. 5B). DEX addition at later stages also causes BMP inhibition, as phospho-Smad1 signal is absent on the injected side upon addition of DEX at either stage 10 (Fig. 5C) or stage 14 (Fig. 5D).

Next, to confirm that the observed phospho-Smad1 antagonism reflects inhibition of BMP function, we examined the expression of Msx1, a transcriptional target of BMP signaling, by in situ hybridization (Suzuki et al., 1997). ΔSmad7tevGR/TEV2GR RNA was injected into one cell at the two-cell stage, with co-injection of RNA encoding h-galactosidase (h-gal) marking the injected side. In the absence of DEX, Msx1 RNA is present on the injected side (arrow, Fig. 5I), but expression is absent or reduced upon addition of DEX at either stage 10 (Fig. 5J) or stage 14 (Fig. 5K). Little or no effect is observed on either of these markers in controls where no DEX is added (A, I).

To assess the effect of stage-dependent BMP inhibition on neural induction in intact embryos, ΔSmad7tevGR and TEV2GR mRNAs were injected into one cell of two-cell stage embryos, along with h-gal RNA as a marker of the injected side. DEX was added either at stage 6 (32-cell), stage 8 (mid-blastula), stage 9 (late blastula), or stage 11 (mid-gastrula). Embryos were allowed to develop at 14°C until stage 16 (mid-neurula), when they were harvested and neural development assessed by in situ hybridization for the neural markers Sox2 and neural cell adhesion molecule (NCAM) (Balak et al., 1987; Kishi et al., 2000) (Fig. 6). In the absence of DEX, no neural plate expansion is observed on the injected side with either marker (Figs. 6A, F). DEX addition at stage 6 or stage 8, laterally expands the expression domains of both Sox2 and NCAM (arrows, Figs. 6B, C, G, H), indicating that BMP inhibition at these stages induces neural development. However, when DEX is added at stage 9 or later (and BMP signaling is inhibited by stage 9 + 3 h), ectopic neural tissue is decreased or absent compared to earlier DEX addition (arrows, Figs. 6D, E, I, J). Thus, while BMP inhibition induces neural identity in animal caps and at early stages, this response is lost in intact embryos between stages 8 and 9, prior to the onset of gastrulation.

Neural crest exhibits stage-dependent responses to BMP inhibition that are temporally distinct from that of neural tissue

To further investigate the stage-dependent effects of BMP inhibition on neural induction, we examined the expression of several neural plate border markers. ΔSmad7tevGR- and TEV2GR-injected embryos were examined for expression of N-tubulin, a marker of differentiated neurons along the neural plate border (arrow, Fig. 7A) (Chitnis et al., 1995; Woda et al., 2003), and Hairy2a, a bHLH transcription factor expressed along the neural plate border and in the neural crest (arrows, Fig. 6E) (Glavic et al., 2004; Jen et al., 1997). Inhibition of BMP
signaling at stage 5 causes loss of both N-tubulin and Hairy2a expression on the injected side (Figs. 7B, F). In the case of Hairy2a, patchy expression can be seen in the ventral ectoderm (arrows, Fig. 7F), suggesting that in addition to being shifted laterally, the neural border is disorganized in these embryos. In accordance with our previous results, these changes in N-tubulin and Hairy2a expression are not observed when BMP signaling is...
inhibited at stage 9 or at stage 11 (Figs. 7C, G). In both cases, N-tubulin expression is present on the injected side, though this expression is shifted slightly laterally in stage 9 inhibited embryos (Figs. 7C, D). In embryos to which DEX was added at stage 9, the position of the neural border as marked by Hairy2a is almost identical to that on the uninjected side (white arrow, Fig. 7G). However, the domain of Hairy2a marking neural crest cells is substantially enlarged (red arrow, Fig. 7G). Similar results are observed on DEX addition at stage 11: Hairy2a expression along the neural border remains intact, but expression in the neural crest region is expanded (Fig. 7H).

To ask whether the effect of stage-specific BMP inhibition on Hairy2a expression in presumptive neural crest is indicative of changes in neural crest development, we examined expression of additional early neural crest-specific markers. FoxD3 encodes a transcription factor expressed in premyriatd neural crest and essential for normal neural crest development (Sasai et al., 2001). In uninjected embryos, no FoxD3 alteration is observed on the injected side (arrow, Fig. 7I). DEX addition at stage 5, expands FoxD3 expression ventrally (Fig. 7J), with the domain divided into two regions: a broad dorsal area with weak expression (asterisk) and a narrow band of high expression along the ventral edge (arrow). The weak domain likely represents neural plate expansion, with the region of high expression corresponding to neural crest formation at the neural plate border. DEX addition at stage 9 or stage 11 also expands FoxD3 expression, though in a manner distinct from that caused by BMP inhibition at stage 5. FoxD3 expression is expanded ventrally in stage 9- and stage 11-treated embryos, with high expression levels throughout the enlarged domain (red arrows, Figs. 7K, L). However, in contrast to stage 5-induced embryos, the dorsal limit of FoxD3 expression is unchanged when DEX is added at stage 9 or stage 11 (white arrows).

We also examined expression of PDGFRα, a marker of late neural crest development. (Ho et al., 1994; Soriano, 1997; Tallquist and Soriano, 2003). Like FoxD3 and Hairy2a, PDGFRα expression is unaffected in the absence of DEX (Fig. 7M), but is expanded laterally by DEX addition at stages 5, 9, or 11 (red arrows, Fig. 7N–P). In addition, the medial edge of PDGFRα expression, which lies along the neural plate border, is shifted laterally by stage 5 DEX addition, but not by addition at later stages (white arrows, Figs. 7N–P). Thus, the changes in PDGFRα expression are consistent with those of FoxD3 and Hairy2a, indicating that BMP inhibition affects neural crest development as late as stage 11. This is in contrast to the effect of BMP inhibition on neural induction, which is limited to the period before stage 9. A comparison of the frequency of ectopic neural and neural crest induction is summarized in Table 1. These observations confirm that regulated ΔSmad7 can affect changes in development after stage 8 and suggest temporally distinct roles for BMP inhibition in neural and neural crest specification.

Table 1

<table>
<thead>
<tr>
<th>Tabulation of ectopic neural and neural crest markers by stage-specific BMP inhibition (# embryos with ectopic expression/total # examined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neural markers (Sox2, NCAM)</td>
</tr>
<tr>
<td>Neural markers (FoxD3, PDGFR, Snail, Hairy2a)</td>
</tr>
<tr>
<td>Neural crest markers (FoxD3, PDGFR, Snail, Hairy2a)</td>
</tr>
</tbody>
</table>

FGF activation and BMP antagonism induce ectopic neural expression

One possible explanation for the stage specificity of ectopic neural induction by ΔSmad7evGR/TEV2GR in intact embryos is that during gastrulation, BMP antagonism is modified by additional signals in specifying neural fate. Members of the FGF family are attractive candidates for such signals, as FGFRs and BMPs interact to influence the development of a variety of organs (Beil and Maas, 1998; Chen et al., 2000; Zuniga et al., 1999). To ask whether stage-specific FGF signaling acts in conjunction with BMP antagonism, we used an inducible form of FGFR1 (iFGFR1) (Pownall et al., 2003). Rather than relying on physiological ligand binding, iFGFR1 is activated by the synthetic dimerizing agent AP20187, which induces iFGFR dimerization and activates its downstream signaling pathway. In the absence of AP 20187, iFGFR1 expression in frog embryos does not induce ectopic Sox2 expression (Fig. 8A). Sox2 is also unaffected upon iFGFR activation at stage 5 or stage 9 (arrows, Figs. 8B, C). However, iFGFR1 enhanced the induction of ectopic neural tissue by regulated ΔSmad7 when the two are activated at stage 5 (compare Figs. 8E to H). Furthermore, while ΔSmad7evGR activation at stage 9 is insufficient for neural induction, activating ΔSmad7evGR and iFGFR1 together at this stage strongly induces neural development (compare Figs. 8F to H). Stage-specific neural induction by BMP inhibition and FGF activation is summarized in Table 2.

FGF signaling has been implicated in Xenopus neural induction (Hongo et al., 1999; Launay et al., 1996; Pera et al., 2003). To ask whether this requirement is stage-specific, we added SU5402, a pharmacological inhibitor of FGF signaling, to embryos at either stage 9 or stage 11 (Mohammadi et al., 1997; Monsoro-Burq et al., 2003; Wilson et al., 2000). These embryos were allowed to develop to stage 16 and the consequences of drug treatment on neural development were assessed by in situ hybridization for Sox2 (Fig. 9). Little effect on neural induction was observed in DMSO-treated control embryos or in embryos to which SU5402 is added at stage 11 (Figs. 9A, C). Addition of SU5402 at stage 5 prevents gastrulation, and the effects of this treatment on neural induction are therefore uninterpre-
able (data not shown). 83% of DMSO-treated and 80% of stage 11-treated embryos develop normal neural plates. In embryos treated at stage 9 with SU5402, spina bifida is apparent in the posterior spinal cord (red arrow, Fig. 9B), reflecting the role of FGF signaling in gastrulation (Amaya et al., 1991, 1993). However, a substantial reduction in the size of the neural plate is also observed (white arrow, Fig. 9B), with only 28% of these embryos developing normal neural plates. Thus, together with the synergistic effect of FGF activation and BMP antagonism, these data suggest a stage-specific role for FGF signaling in neural induction.

In addition to proposed roles in both the induction and patterning of neural ectoderm (Holowacz and Sokol, 1999; Hongo et al., 1999; Ishimura et al., 2000; Koshida et al., 2002; Launay et al., 1996; Pera et al., 2003; Ribisi et al., 2000), FGF signals are involved in mesodermal patterning (Amaya et al., 1991, 1993; Cornell and Kimelman, 1994; Gotoh et al., 1995; Hartley et al., 1994; Isaacs et al., 1994; Kumano and Smith, 2000; LaBonne and Whitman, 1994, 1997; LaBonne et al., 1995; Slack et al., 1996; Smith et al., 1993; Weinstein et al., 1998). FGF signaling is also required for ectodermal patterning by the dorsal and paraxial mesoderm (Ishimura et al., 2000; Monsoro-Burq et al., 2003). Therefore, the activation of FGF signaling may modify BMP-antagonist-mediated neural induction and development by altering the adjacent mesoderm. To examine this possibility, we tested the ability of conditional BMP inhibition and FGF activation to induce neural identity in explanted ectoderm. RNA was injected into both cells of albino embryos at the two-cell stage and inducers (DEX and AP20187) were added either at stage 5 or stage 11. Animal caps were cut at stage 8 and harvested at stage 16, and neural induction was assessed by in situ hybridization for Sox2 (Fig. 10). Uninjected controls showed no Sox2 expression (Fig. 10A). Injected caps without inducers showed minimal Sox2 induction, indicating a low level of leakiness in the regulation of iFGFR and ΔSmad7TEVGR (Fig. 10B). Neural induction is markedly increased upon addition of inducers at stage 5 (Fig. 10C). In contrast to caps in which BMP is inhibited alone, FGF activation and BMP inhibition at stage 11 also induce Sox2 expression (compare Fig. 10D with Fig. 4F), indicating that, as in the intact embryo, FGF activation potentiates BMP-antagonist mediated neural induction.

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No inducer</th>
<th>Inducer, St. 5</th>
<th>Inducer, St. 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>iFGFR1</td>
<td>2/12 (16%)</td>
<td>2/18 (11%)</td>
<td>0/16 (0%)</td>
</tr>
<tr>
<td>ΔSmad7TEVGR/TEV2GR</td>
<td>0/22 (0%)</td>
<td>20/24 (83%)</td>
<td>6/28 (21%)</td>
</tr>
<tr>
<td>iFGFR1 + ΔSmad7TEVGR/TEV2GR</td>
<td>6/16 (38%)</td>
<td>28/30 (93%)</td>
<td>40/40 (100%)</td>
</tr>
</tbody>
</table>

Fig. 8. FGF activation must be combined with BMP inhibition to induce ectopic neural expression during gastrulation. Embryos were injected with 20 pg iFGFR1 RNA (A–C), 300 pg ΔSmad7TEVGR and 10 pg TEV2GR RNA (D–F), or 20 pg iFGFR1 + 300 pg ΔSmad7TEVGR/10 pg TEV2GR RNA (G–I). Inducer (10 μM DEX and/or 1.25 μM AP20187) was added at stage 5 (B, E, H) or stage 9 (C, F, I). In the absence of inducer, ectopic neural induction is not observed in injected embryos (A, D, G). No ectopic neural induction is observed on the injected side (arrows) in iFGFR1-injected embryos when AP20187 is added at stage 5 (B) or stage 9 (C). Ectopic neural induction is observed in ΔSmad7TEVGR/TEV2GR-injected embryos upon addition of DEX at stage 5 (arrow, E), but induction is minimal when DEX is added at stage 9 (arrow, F). FGF activation and BMP inhibition appear to act synergistically, as strong ectopic neural induction is observed when iFGFR and ΔSmad7TEVGR/TEV2GR are combined, both when inducer is added at stage 5 (H) or at stage 9 (I).
Induction of neural-specific gene expression by stage 11 activation of FGF and inhibition of BMPs, but not by BMP inhibition alone is further confirmed by RT-PCR detection of NCAM expression (Fig. 10E). We also note that the activation of FGF signaling in conjunction with ΔSmad7-mediated BMP inhibition leads to elongation of animals caps, suggesting the induction of mesoderm by FGF signaling (Isaacs et al., 1994; Smith, 1995; Smith et al., 1993). Our data are generally consistent with previous observations on synergy between FGF signaling and BMP induction on neural induction (Lamb and Harland, 1995), although in contrast to these authors, we do see brachyury expression when FGF signaling is activated at stage 11; this difference may reflect different methods used to activate FGF signaling in the two studies.

To confirm the induction of mesoderm, we examined gene expression in injected animal caps by RT-PCR for Xbra, an immediate early marker of mesoderm induction (Cunliffe and Smith, 1992; Smith et al., 1991). Xbra expression is present in caps where FGF signaling is present, but not in uninduced controls or in caps where ΔSmad7 alone has been activated (Fig. 10E). Thus, while the combination of BMP antagonism and FGF activation can alter the timing of neural competence in ectodermal explants, we cannot distinguish between direct neural induction by FGFs or a mechanism where FGFs induce and pattern mesoderm that subsequently modifies the ectoderm’s response to BMP inhibition.

Discussion

The BMP gradient model would suggest that, because neural-inducing BMP antagonists are expressed in the organizer, BMP-dependent neural/epidermal decisions should take place during gastrulation, after the organizer has formed. However, the experiments underlying the gradient model do not directly address this point, as the BMP antagonists used are unregulated and active well before gastrulation, and so cannot answer questions regarding the timing of signals (Hawley et al., 1995; Marchant et al., 1998; Wilson and Hemmati-Brivanlou, 1995). To test the hypothesis that BMP inhibition during gastrulation controls neural induction, we have developed an inducible inhibitor of BMP signaling. The foundation of this inhibitor is the C-terminal half of Smad7, ΔSmad7, which we confirm here to be a BMP-specific antagonist, blocking Smad1 phosphorylation without affecting that of Smad2. We also show that when combined with an inducible tobacco etch virus (TEV) protease (TEV2GR) ΔSmad7tevGR, a hormone-inducible form of ΔSmad7, acts upstream of Smad1 phosphorylation to inhibit BMP signaling. This inhibition is rapidly induced, is cell autonomous, and can be activated at multiple developmental stages in explanted tissue and in intact embryos, indicating that ΔSmad7tevGR and TEV2GR can be used to functionally examine stage-specific predictions based on the BMP gradient model.

In intact embryos, BMP inhibition alone is not sufficient for neural induction during gastrulation

As with unregulated BMP inhibitors, ΔSmad7tevGR induces neural markers in animal caps, confirming the ability of BMP antagonists to drive neurulation in isolated ectoderm before stage 10.5 (Hawley et al., 1995; Knecht et al., 1995; Lamb et al., 1993; Wilson and Hemmati-Brivanlou, 1995). Yet we also show that ΔSmad7tevGR activation at stage 9 does not induce neural identity in intact embryos. This is inconsistent with the BMP gradient model’s prediction that BMP antagonists alone induce neural identity during gastrulation, suggesting a more complex mechanism mediating the neural/epidermal decision.

Two trivial explanations for the loss of BMP-dependent neural responsiveness in whole embryos are a delay between DEX addition and maximal activation of ΔSmad7tevGR activity and/or an inability of regulated ΔSmad7 to completely block Smad1 phosphorylation at post-blastula stages. If either of these were the case, late-stage DEX addition might be insufficient to drive neural induction, as BMP signaling would be only partly inhibited at the time...
Fig. 10. FGF activation lengthens the period of neural competence in animal caps and induces expression of Xbra. Embryos were injected with 20 pg iFGFR1 and 300 pg ΔSmad7tevGR/10 pg TEV2GR RNA. Inducers (10 μM DEX and/or 1.25 μM AP20187) were added at stage 5 (C) or stage 11 (D). Animal caps were cut at stage 9 and collected at stage 14, when they were subjected to either in situ hybridization for Sox2 (E) or RT-PCR. No Sox2 expression is detected in caps from uninjected embryos (A), and minimal Sox2 is present in caps from injected embryos to which no inducer was added (B). Strong up-regulation of Sox2 is observed in caps to which inducers were added at stage 5 (C). Addition of inducers at stage 11 also strongly activates Sox2 expression (D). This is in contrast to caps from embryos injected with ΔSmad7tevGR and TEV2GR and activated at stage 11 (compare with Fig. 4F). Note the elongation in caps induced at both stage 5 and stage 11 (arrows, C, D), suggesting mesoderm induction due to the activation of FGF. (F) RT-PCR for NCAM confirms the ability of iFGFR and ΔSmad7tevGR, but not ΔSmad7tevGR alone, to induce neural development when activated at stage 11. RT-PCR for Xbra further supports the induction of mesoderm in iFGFR- and ΔSmad7tevGR/TEV2GR-injected caps, but not in caps injected with ΔSmad7tevGR and TEV2GR alone.

when cell fate decisions are made. However, several of our experiments argue against this. First, ΔSmad7tevGR activation as late as stage 14 completely inhibits anti-phospho-Smad1 immunostaining in both stage 16 animal caps and whole embryos. Second, activation of ΔSmad7-mediated BMP antagonism at stage 9 is sufficient to activate neural-specific gene expression in animal caps. While this neural induction is somewhat weaker than when BMP inhibition commences at stage 5, it nevertheless argues that ΔSmad7 activation at late blastula/early gastrula-stages is capable of inducing neurulation in some contexts. Third, while full BMP inhibition abolishes expression of the BMP target gene Msx1, partial inhibition has been reported to have the opposite effect at the neural plate border (Tribulo et al., 2003). We show that Msx1 expression is absent in intact embryos following early- and late-stage ΔSmad7tevGR/TEV2GR activation, suggesting complete BMP antagonism at both stages. Finally, in animal caps cultured at 14°C, ΔSmad7tevGR-mediated phospho-Smad1 inhibition is complete by 3 h after DEX addition. At this same temperature, stages 9 and 10 are separated by approximately 6 h, and stages 9 and 10.5 are separated by more than 8 h (Nieuwkoop and Faber, 1967). Thus, full ΔSmad7tevGR activity is achieved well before both the onset of gastrulation and the loss of neural competence, arguing against delayed BMP inhibition as an explanation for our results.

A second explanation for the stage-dependence of ΔSmad7tevGR-mediated neural induction is that neural specification by BMP antagonists may occur before gastrulation. In support of this hypothesis, several neural-inducing genes are expressed in pre-gastrulation embryos (Kroll et al., 1998; Mizuseki et al., 1998; Penzel et al., 1997). In addition, some early neural marker expression is unaffected by inhibition of organizer formation, suggesting that the organizer is not absolutely required for neural induction (Wessely et al., 2001). Dorsal–ventral asymmetry of endogenous Smad1 phosphorylation has been reported as early as stage 9 (Schohl and Fagotto, 2002), indicating that BMP signaling is modulated by earlier dorsal determinants. Chief among these are components of the Wnt signaling pathway, and ectopic Wnt activation in animal caps is sufficient to induce neural identity and to repress BMP4 transcription (Baker et al., 1999; Gomez-Skarmeta et al., 2001). This finding likely reflects a role for β-catenin in establishing the dorsal–ventral axis, as recent experiments suggest the β-catenin-dependent formation of a “blastula Chordin and Noggin expressing” (BCNE) region in the dorsal ectoderm by stage 9 (Kuroda et al., 2004). These cells give rise to a large part of the anterior nervous system, the floor plate, and the notochord, and explants from this region taken prior to gastrulation give rise to neural tissue. Taken together, these observations point towards specification of neural identity prior to the onset of gastrulation.

While our experiments do not rule out a role for pre-organizer neural induction, we suggest an additional possibility: in the intact embryo, signals in addition to BMP antagonists influence neural induction. In chick embryos, neural fate acquisition in vivo requires FGF signaling, and the FGF-inducible neural marker ERNI is expressed prior to gastrulation, suggesting that FGFs’ role in neural induction may be quite early in development (Streit et al., 2000; Wilson et al., 2000). When cultured ectoderm is
exposed to low concentrations of FGF inhibitors, BMP inhibition can rescue neural induction, indicating an interaction between the two pathways. (Streit et al., 1998; Wilson et al., 2001). By itself, BMP inhibition in chick does not induce neural character, but Chordin, a BMP antagonist, can induce neural identity in ectoderm that has been co-cultured with the chick organizer, Hensen’s node (Streit et al., 1998). This capacity correlates with expression of Churchill (ChCh), a target of the FGF signaling pathway (Sheng et al., 2003). ChCh is expressed prior to gastrulation and appears to sensitize early chick ectoderm to neural inducing signals, supporting the hypothesis that FGF signaling is important for the initiation of neural competence before gastrulation. Additional signals, possibly including BMP antagonists, are then required to maintain neural character, as FGF signaling alone does not efficiently drive neural induction in explants (Streit et al., 2000; Wilson et al., 2000).

In Xenopus, the role for FGFs in neural induction is less clear. Several studies suggest a requirement for FGF signaling in posteriorizing neural tissue, but not for its induction (Holowacz and Sokol, 1999; Ribisi et al., 2000). On the other hand, dominant negative FGFR-1 blocks BMP antagonist-mediated neutralization of animal caps, FGF8 overexpression induces ectopic neural tissue, and dominant negative FGFR-4 inhibits expression of early neural markers in embryos (Hongo et al., 1999; Launay et al., 1996; Pera et al., 2003). Here, we present evidence for a stage-specific requirement for FGFs in neural development, as pharmacological inhibition of FGF signaling has a greater inhibitory effect on neural induction at stage 9 than at stage 11. Moreover, we demonstrate that FGF activation enhances the neural inductive capacity of BMP inhibition. We also show that FGF induction removes stage-dependent restrictions on BMP antagonist-dependent neural formation in both animal caps and in intact embryos, suggesting that pre-gastrulation neural fate specification is determined synergistically by interactions between the BMP and FGF signaling pathways.

Consistent with a role for the combination of FGF signaling and BMP antagonism in neural induction, the Smad1 linker domain contains several MAPK phosphorylation sites (Kretzschmar et al., 1997a). Phosphorylation of these sites can alter Smad1 transcriptional activity and is functionally important for neural induction, as mutations that block MAPK phosphorylation act as dominant negative regulators of neural development (Pera et al., 2003; Sater et al., 2003). In stage 10.25 Xenopus embryos, high levels of phosphorylated MAPK are present in the organizer, with slightly lower levels in the adjacent ectoderm (Schohl and Fagotto, 2002), suggesting that in vivo, FGF signaling acts both on dorsal mesoderm and dorsal ectoderm. While we find that FGF signaling in animal caps induces mesoderm, and therefore cannot determine whether FGFs’ synergistic effects with BMP inhibition result from direct signaling to the ectoderm or indirect action on the mesoderm, both the interaction between MAPK and Smad1 and the endogenous MAPK signaling pattern suggest that FGFs’ effects on neural induction in whole embryos may be direct.

Regardless of the molecular mechanism, interaction between the mesoderm and ectoderm presents an explanation for the difference in neural competence in explanted tissue and intact embryos. Signals from dorsal and paraxial mesoderm have been shown to influence ectodermal identity in a variety of contexts (Doniach, 1993; Ishimura et al., 2000; LaBonne and Bronner-Fraser, 1998; Mancilla and Mayor, 1996; Marchant et al., 1998; Monsoro-Burg et al., 2003; Penzel et al., 1997; Poznanski and Keller, 1997; Servetnick and Grainger, 1991b). Furthermore, Ishimura et al. (2000) present evidence that in explanted ectoderm, neural induction by dorsal mesoderm is not blocked by overexpression of BMP4. Induction was blocked, however, by the combination of BMP4 and a dominant negative FGF receptor, suggesting that both signals are involved in interactions between mesoderm and ectoderm leading to neural specification. Thus, previous work elucidating the effects of BMP antagonism in animal caps may have uncovered a basic mechanism for neural and neural crest induction, which, in the intact embryo, is refined by positive and negative regulatory signals from adjacent tissues.

**The gradient hypothesis and timing of neural vs. neural crest induction**

Substantial evidence supports the ability of BMP signaling to induce distinct ectodermal identities in a concentration-dependent manner (Barth et al., 1999; Hawley et al., 1995; Knecht and Harland, 1997; Marchant et al., 1998; Piccolo et al., 1996; Sasai et al., 1995; Suzuki et al., 1997; Tribulo et al., 2003; Wilson and Hemmati-Brivanlou, 1995; Wilson et al., 1997; Woda et al., 2003; Zimmerman et al., 1996). In addition, in situ examination reveals that endogenous Smad1 phosphorylation has its lowest levels near the Spemann organizer and highest levels in more ventral cells, consistent with a signaling gradient (Faure et al., 2000; Schohl and Fagotto, 2002). Thus, it seems likely that a BMP gradient has a role in dorsoventral patterning. However, mounting evidence, including the work presented here, suggests that additional signals affect early neural and neural crest patterning (Domingos et al., 2001; Holowacz and Sokol, 1999; Hongo et al., 1999; LaBonne and Bronner-Fraser, 1998; Launay et al., 1996; Monsoro-Burg et al., 2003; Pera et al., 2003; Streit and Stern, 1999; Streit et al., 2000; Wilson et al., 2000, 2001). These signals likely modify the BMP gradient, giving rise to more temporal and spatial specificity than has previously been appreciated.

It is in this context that we must consider the temporally distinct specification of neural and neural crest identities. While temporal differences in neural and neural crest
specification have been previously noted, the molecular mechanism underlying such discontinuities was unclear (Glavic et al., 2004; Servetnick and Grainger, 1991a; Tribulo et al., 2003; Woda et al., 2003). We show here that this difference includes the response to BMP antagonism, which induces neural fate in intact embryos only until stage 8, but ectopic neural crest induction by BMP inhibition extends to stage 11. As is the case with stage-specific changes in neural competence, this result could reflect a lag between DEX addition and complete inhibition of BMP activity. As outlined above, however, the effects on phospho-Smad and Msx1 expression, and the time course of \( \Delta \text{Smad7} \text{evGR} \) activation argue against partial BMP inhibition as an explanation for this phenomenon.

While our data do not rule out the function of a single gradient with distinct action timing in neural and neural crest cells, they suggest that BMP signals influence cell fate during multiple, temporally distinct periods. As the neural plate is specified first, signals from neural cells may influence the development of adjacent, unspecified neural crest. Analysis of gene expression points to changes in the signaling microenvironment at the neural plate border, as expression of elements of the Notch signaling pathway, as well as both Bmp4 expression and Smad1 phosphorylation can be detected at the neural plate border during, but not before, early neurulation (stage 12–13) (Glavic et al., 2004; Kurata et al., 2001). In addition, Dlx3, a transcriptional target of BMP signaling, is required in the non-neural ectoderm for normal neural crest and neural border development, and Notch signaling from neural cells affects Bmp4 expression at the neural plate border (Glavic et al., 2004; Kurata et al., 2001; Woda et al., 2003). Thus, given the temporal discontinuity of neural and neural crest specification, we must consider a model in which the earlier induced tissue influences the development of the latter. Rather than patterning the ectoderm as a single gradient, early BMP signaling may distinguish broadly between neural and epidermal identities, while during gastrulation, BMP activity at the neural–epidermal interface refines this distinction, leading to specialization of cell types at the neural plate border.

**Are FGF activation and BMP inhibition required for neural induction?**

We show here that BMP antagonist-dependent neural induction in intact embryos is temporally limited and that during gastrulation, BMP inhibition induces neural identity only in combination with FGF activation. While these data suggest a developmentally important interaction between BMPs and FGFs, they are essentially gain of function, and therefore do not formally prove the necessity of either signal in neural induction. In frog, neural formation is inhibited by ectopic BMP activation or FGF inhibition, suggesting that both signals are required for normal neural induction (Hawley et al., 1995; Holowacz and Sokol, 1999; Hongo et al., 1999; Launay et al., 1996; Pera et al., 2003; Wilson and Hemmati-Brivanlou, 1995). However, these experiments rely on unregulated FGF antagonists and BMP activators, which, as our data demonstrate, may not accurately reflect endogenous development. We observe stage-specific effects on neural induction upon application of SU5402, a pharmacological antagonist of FGF signaling. It should be noted that while SU5402 does affect neural plate formation in embryos, indicating a role for FGF signaling in endogenous neural induction, we do not know whether SU5402 causes a complete block to FGF signaling in the intact embryo. An even more dramatic effect on neural plate formation might be possible if such a complete block were to be achieved. An additional complication in interpreting the effects of SU5402 in intact embryos stems from the fact that FGF signaling is involved in mesodermal development during gastrulation (Amaya et al., 1991, 1993; Kroll and Amaya, 1996) as well as neural induction, and therefore some portion of the effects of SU5402 result from indirect action on mesodermal specification. In neurula stage embryos, transgenic Bmp4 expression inhibits anterior neural development, but this strategy activates ectopic Bmp4 only after gastrulation, precluding conclusions regarding its effect on neural induction (Hartley et al., 2001). Even transplantation of beads or cells secreting BMPs or BMP antagonists must be viewed with caution, as embryonic wounding rapidly induces MAPK activity, which may interact with BMP signaling (LaBonne and Whitman, 1997). Thus, while the available evidence suggests that BMP inhibition and FGF activation are critical in neural induction, definitive proof requires the generation of cell autonomous, inducible activators of BMP signaling and inhibitors of the FGF pathway.

Given the range of events in which BMPs and FGFs are implicated, understanding morphogenesis requires a knowledge of not only where but when these signals act. Elucidation of the temporally distinct developmental functions they perform will ultimately yield insights into the molecular mechanisms that modify and interpret signals and interacting pathways. The generation of conditional cell signaling inhibitors and activators represents a significant step towards this understanding, as it affords temporal control over the manipulation of signaling pathways, allowing functional analysis of their timing during development.

**Acknowledgments**

The authors acknowledge Drs. Kristin Artinger, Harold Isaacs, Dan Kessler, Kelly McLaughlin, Mark Mercola, and Yoshiki Sasai for generously providing DNA constructs. SU5402 was a kind gift of Dr. Minoru Watanabe (Gifu University, Japan). We also thank the members of the Whitman Lab for helpful comments on the manuscript. This work was supported by a Ruth L. Kirschstein National Research Service Award (S.W.), and by grants from the NICHD (M.W.).
References


Bei, M., Maas, R., 1998. FGFs and BMP4 induce both Mxl1-independent and Mxl1-dependent signaling pathways in early tooth development. Development 125, 4325–4333.


neuralizing molecule that demarcates the future neural plate at the onset of gastrulation. Development 125, 3247–3258.


induced neural cells, but is not sufficient for neural induction in the chick embryo. Development 125, 507–519.


