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BMP-3 is a novel inhibitor of both activin and BMP-4 signaling in *Xenopus* embryos

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Abstract

In *Xenopus*, the biological effects of BMP-3 oppose those of ventralizing BMPs, but the mechanism for this antagonism remains unclear. Here, we demonstrate that BMP-3 is a dorso-anteriorizing factor in *Xenopus* embryos that interferes with both activin and BMP signaling. BMP-3 acts by binding to ActRIIB, the common type II receptor for these proteins. Once BMP-3 binds to ActRIIB, it cannot be competed off by excess ligand making a receptor complex that is unable to activate R-Smads and transduce signal. Consistent with a model where BMP-3 interferes with activin and BMPs through a shared receptor, we show that overexpression of *BMP-3* can only be rescued by co-injection of *xActRIIB*. Our results identify BMP-3 as a novel antagonist of both activin and BMPs and uncover how some of the diverse developmental processes that are regulated by both activin and BMP signaling can be modulated during embryogenesis.

Keywords: BMP-3; Activin; BMP; ActRIIB; Antagonist; *Xenopus*

Introduction

Members of the TGF-β superfamily regulate crucial aspects of vertebrate development. This is particularly evident in *Xenopus* where the interplay between BMP and activin/nodal signaling is required for patterning during gastrulation. Activin/nodal-like ligands direct both the induction and specification of mesoderm and endoderm along the dorsal–ventral and anterior–posterior axes, while at the same time BMPs suppress dorsal and trunk mesoderm and neural fates (Harland and Gerhart, 1997; Kimelman and Griffin, 2000; Schier, 2003; Whitman, 1998). Later in development, activins and nodals are key determinants in the establishment of left–right asymmetry and patterning of the nervous system while BMPs play a critical role in the formation of many tissues and organs including neural crest, heart, limb and skeleton (Hogan, 1996; Schier, 2003).

BMPs and activin exert their effects through a signaling cascade in which ligand binding brings together type I and type II receptor serine/threonine kinases on the cell surface (Massague and Chen, 2000). Once activated, the type II receptor phosphorylates the type I receptor, which in turn phosphorylates receptor regulated Smad proteins (R-Smads). These phosphorylated R-Smads then complex with Smad4, translocate to the nucleus and together with other proteins regulate transcription of target genes. R-Smads 2,3 are phosphorylated to initiate signaling by activin-like ligands while R-Smads1,5,8 are phosphorylated during BMP signal transduction (Massague and Chen, 2000; Miyazawa et al., 2002). While BMPs and activins are able to bind to the same type II receptors (activin type II and IIB), they have separate type I receptors and it is through the type I receptor/R-Smad pairings that a specific BMP or activin signal is propagated. In addition, BMPs signal through BMP-specific receptor complexes that utilize BMPRII, a receptor not used by activin-like ligands (Massague and Chen, 2000; Miyazawa et al., 2002). The flexible nature of these BMP and activin ligand–receptor

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interactions allows them to serve as multifunctional regulators during development and organogenesis, but at the same time signaling by these factors must be tightly controlled both spatially and temporally to ensure specificity. The most common level of control over TGF-β-like signals takes place in the extracellular space where antagonists like noggin, chordin, follistatin, gremlin and cerberus prevent ligands from binding to their receptors (Balemans and Van Hul, 2002; Miyazono, 2000). Control of signaling also occurs at the cell membrane, as well as within the cytoplasm and nucleus of the target cell (Miyazono, 2000; von Bubnoff and Cho, 2001).

BMP-3, a member of the TGF-β superfamily, has biological effects that oppose those of osteogenic BMPs and TGF-β1 in a variety of mammalian systems (Daluiski et al., 2001; Faucheux et al., 1997). In *Xenopus* embryos, we and others have shown that unlike *BMP-2*, *BMP-4* and *BMP-7*, which are potent ventralizing agents, *BMP-3* induces dorsalization (Daluiski et al., 2001; Hino et al., 2003). These effects of *BMP-3* are most similar to the BMP antagonists noggin, chordin and follistatin, inhibiting the mesoderm-inducing activities of *BMP-2* and *ADMP* in animal cap assays (Hino et al., 2003). xBMP-3b, a molecule 83% homologous to xBMP-3, can also antagonize BMP-like ligands as well as nodal-like proteins (Hino et al., 2003). While these data identify BMP-3 and BMP-3b as novel antagonists in the BMP/TGF-β-signaling cascade, they do not give any clues as to how they might exert their effects.

In this study, we examine the mechanism by which BMP-3 antagonizes TGF-β family ligand signaling in *Xenopus* embryos. We find that BMP-3 inhibits the mesoderm-inducing activities of both activin and BMP-4 by reducing the phosphorylation of R-Smads and that *BMP-3* by itself is unable to activate BMP or activin R-Smads and thus does not signal using a TGF-β pathway. Inhibition by *BMP-3* can be overcome by activated BMP or activin type I receptors or by increased levels of ActRIIB. Finally, we find that BMP-3 binds to ActRIIB and that this binding cannot be competed by addition of excess activin. Our data support the hypothesis that BMP-3 is a novel TGF-β family antagonist that acts by interfering with activin and BMP binding to ActRIIB, a type II receptor common to both signaling pathways. By using this mechanism, BMP-3 is able to modulate diverse developmental processes that are controlled by both activin and BMP signaling at the level of the individual target cell, providing the embryo with additional means to regulate these signaling molecules.

Materials and methods

Embryo assays and microinjection

Xenopus embryos were generated, cultured and staged using standard methods (Sive et al., 2000). RNAs were injected into the animal poles of 2- to 4-cell embryos or the ventral side of 4- to 8-cell embryos. The ventral side of embryos was determined based on animal pole pigmentation and blastomere size. Animal cap assays were performed by injection of mRNA at the 2-cell stage into the animal pole followed by explantation at stages 8–9. Explants were cultured in 0.5× MMR until sibling embryos reached the appropriate stage and then collected for RT-PCR or Western blot analysis. In experiments using recombinant proteins, explants were incubated in control or hBMP-3-conditioned media that were diluted 1:5 in 0.5× MMR. Serum-free-conditioned media containing homodimeric BMP-3 were made in stably transfected CHO cells following previously described methods (Israel et al., 1992). Injected and control explants treated with activin-A (R&D Systems, 3 ng/ml) and bFGF (R&D Systems, 100 ng/ml) were cultured in 0.5× MMR with 0.5% BSA.

Plasmids and RNA preparation

For the preparation of capped mRNA, pCS2-hBMP3 (Daluiski et al., 2001), pSP64T-BMP4 (Jones et al., 1992), pSP64T-activinβb (Sokol et al., 1991), pCS2-Xnr1 (Jones et al., 1995), pCS2-actBMPR (Hansen et al., 1997), pSP64T-ALK4-T206E (Chang et al., 1997), pSP64T-ActRIIB (XAR) (Hemmati-Brivanlou and Melton, 1992), pSP64T-ALK4 (Chang et al., 1997), pCS2-xBMPRII (Frisch and Wright, 1998), pCS2-ActRII(KR)/Myc (Yeo and Whitman, 2001), pcDNA3-activin/HA (Cheng et al., 2004), pCS2-ALK4(KR)/Flag (Cheng et al., 2004) and p64TXβ-globin (Hemmati-Brivanlou and Melton, 1992) plasmids were linearized and transcribed using the mMessage Machine SP6 and T7 Kits (Ambion).

Histological analysis

Animal caps were fixed in 4% paraformaldehyde in PBS and processed for paraffin embedding as described in Sive et al. (2000). Sections were stained with hematoxylin and eosin using standard methods.

RT-PCR analysis

RNA was extracted and cDNA synthesized as described previously (Chang et al., 1997; Wilson and Melton, 1994). Non-radioactive RT-PCR assays on animal caps were carried out as described previously (Dosch et al., 1997; Gawantka et al., 1995). All RT-PCR experiments were performed at least twice and PCR products analyzed on 3% TBE gels. The PCR conditions and primer set sequences for *nrp-1*, *EF1-α*, *Krox20*, *HoxB9*, *En2*, *Xbra*, *muscle-actin*, *epidermal keratin*, *XAG*, *Xhox3*, *endodermin*, *noggin*, *chordin*, *follistatin* and *msx-1* were as reported (Hemmati-Brivanlou and Melton, 1994; Lamb and Harland, 1995; Wilson and Hemmati-Brivanlou, 1995) (http://hhmi.ucla.edu/derobertis). *EF1-α* was used as a loading control.

Reverse transcriptase-negative (−RT) reactions were performed to show the absence of contaminating genomic DNA.

Phospho-Smad Western blot analysis

Ectodermal explants were isolated at stages 8–9 and collected along with BMP-3-injected whole embryos when siblings reached stage 10.5. The explants and whole embryos were lysed and Western blot analysis performed as described by Yeo and Whitman (2001). The following antibodies were used: anti-phospho Smad1,5,8 polyclonal (Cell Signalling Technology), anti-phospho-Smad2 polyclonal (Faure et al., 2000) and anti-actin monoclonal (AC-40, Sigma).

Co-immunoprecipitation analysis

Co-immunoprecipitation with *Xenopus* embryo protein extract was performed as described (Harms and Chang, 2003; Yeo and Whitman, 2001). RNAs encoding *hBMP3*, *ActRII (KR)/Myc*, *activin/HA* and *ALK4 (KR)/Flag* were injected into 2-cell *Xenopus* embryos. Animal halves were isolated at stage 8/9, collected when sibling embryos reached stages 10.5 and cross-linked with 10 mM DTSSP (Pierce) for 2 h at 4°C when necessary. Protein extracts were immunoprecipitated with the appropriate antibodies and when necessary treated with PNGase F (New England Biolabs) to deglycosylate receptors. Samples were then subject to SDS–PAGE and Western blot analysis. ECL (Amersham) was used to visualize proteins of interest. The following antibodies were used for immunoprecipitation and Western blot analysis: anti-recombinant hBMP-3 polyclonal antibody (H-73, Santa Cruz), anti-c-*myc* mouse monoclonal antibody (A-6, Covance), anti-HA polyclonal antibody (Covance) and anti-flag polyclonal antibody (Sigma).

Results

BMP-3 dorso-anteriorizes embryos

In previous studies, we showed that *BMP-3* mRNA injected on the dorsal side of *Xenopus* embryos had mild dorsalizing activity (Daluiski et al., 2001), producing a phenotype opposite to that of ventralizing BMPs. To investigate whether BMP-3 might antagonize BMP or other TGF-β-like ligand activity, we microinjected ventral vegetal blastomeres of 8-cell embryos with *BMP-3* mRNA and looked for secondary axis formation. Strong dorsalizing factors like noggin, chordin, follistatin or dominant-negative BMP receptors (tBR) mimic the activity of Spemann's organizer and cause complete axis duplication in this assay (Harland and Gerhart, 1997). Fig. 1 (panels A–C) shows that no secondary axes were observed with *BMP-3* at all doses tested (250 pg, 500 pg, 1 ng and 1.5 ng). However, embryos injected ventrally with 500 pg of *BMP-3* mRNA (a dose chosen because it gave the most consistent results) displayed a unique range of phenotypes that did not seem to mimic those induced by either BMP or activin/nodal

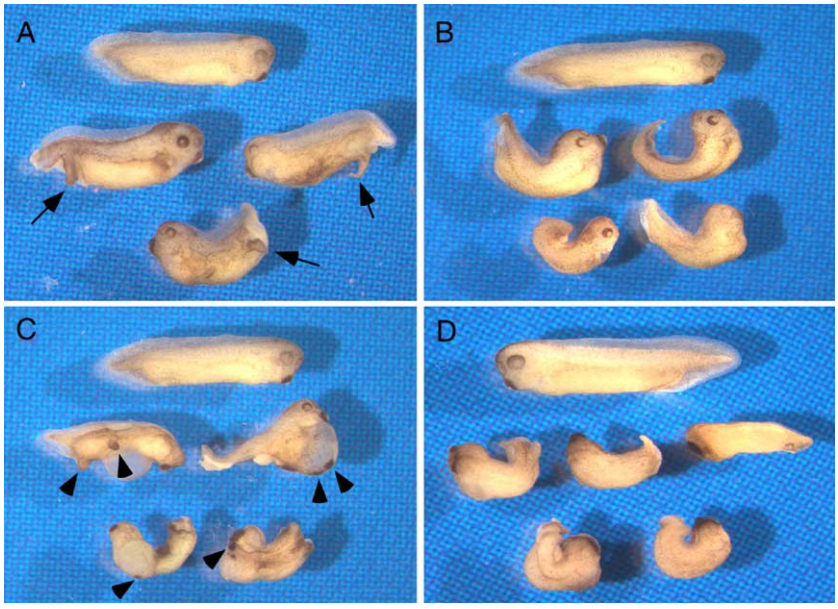

Fig. 1. Overexpression of *BMP-3* produces dorso-anteriorized embryos. Embryos at the top of each panel are uninjected controls; embryos at the bottom of each panel are injected with *BMP-3*. (A–C) Phenotypes of tadpole stage embryos injected with 500 pg of *BMP-3* mRNA into a single ventral vegetal blastomere at the 4- to 8-cell stage. (A) Aberrant tail formation caused by injection of *BMP-3* (21%, $n = 180$). Arrows indicate abnormal tail protrusions. (B) Shortened and curved body axes caused by *BMP-3* injection (38%, $n = 180$). (C) Ectopic cement glands (arrowheads) induced by injection of *BMP-3* (41%, $n = 180$). (D) Phenotype of tadpole stage embryos injected with 500 pg *BMP-3* mRNA into both animal blastomeres at the 2-cell stage. Embryos have truncated body axes, enlarged cement glands, reduced eyes and head structures and spina bifida (80%, $n = 200$).

antagonist. These included aberrant tail formation (Fig. 1A), shortened, curved axes (Fig. 1B) and ectopic cement glands (Fig. 1C). Many of these embryos exhibited a delay in blastopore closure at gastrula stages that contributed to the observed defects (data not shown). We also microinjected *BMP-3* mRNA (500 pg) into both animal blastomeres of 2-cell *Xenopus* embryos to look at the effects of general overexpression. These embryos were anteriorized with shortened axes, enlarged cement glands, reduced eyes and in some cases gastrulation defects that led to spina bifida (Fig. 1D). The gain of function phenotypes we see are consistent with the expression of xBMP-3 in chordal mesoderm, notochord and cement gland in the *Xenopus* embryo (Hino et al., 2003). Together these gain of function studies suggested that *BMP-3* might play a role in dorso-anterior patterning by effecting gastrulation movements during *Xenopus* development.

BMP-3 induces cement gland and neural tissue in animal cap explants

Next we tested the biological activity of *BMP-3* in animal cap assays. When excised from blastula stage embryos, animal cap explants differentiate into epidermis. If the explants are treated with activin or nodal, they form mesodermal and endodermal tissues, while if exposed to BMP antagonists, they form neural tissue and cement gland (Harland and Gerhart, 1997). mRNA encoding *BMP-3* was injected into both animal blastomeres of 2-cell embryos. The animal caps were excised at blastula stages and cultured until controls reached early and late tadpole stages when they were analyzed by histology and RT-PCR for tissue-specific molecular markers. Control animal caps formed rounded balls of atypical epidermis (Figs. 2A and E). *BMP-3*-injected animal caps underwent morphological changes, developing pigmented cement glands and neural tissue (Figs. 2B and F). RT-PCR analysis confirmed these results. *BMP-3* mRNA strongly induced the cement gland marker, *XAG* and the pan-neural marker, *nrp-1*, while downregulating the epidermal marker, *epidermal keratin* (Fig. 2I). When we characterized the neural tissue induced by *BMP-3*, we found expression of the forebrain–midbrain marker *Otx2* but not any of the more posterior markers such as *Engrailed 2* (midbrain–hindbrain), *Krox-20* (hindbrain) or *HoxB9* (spinal cord)

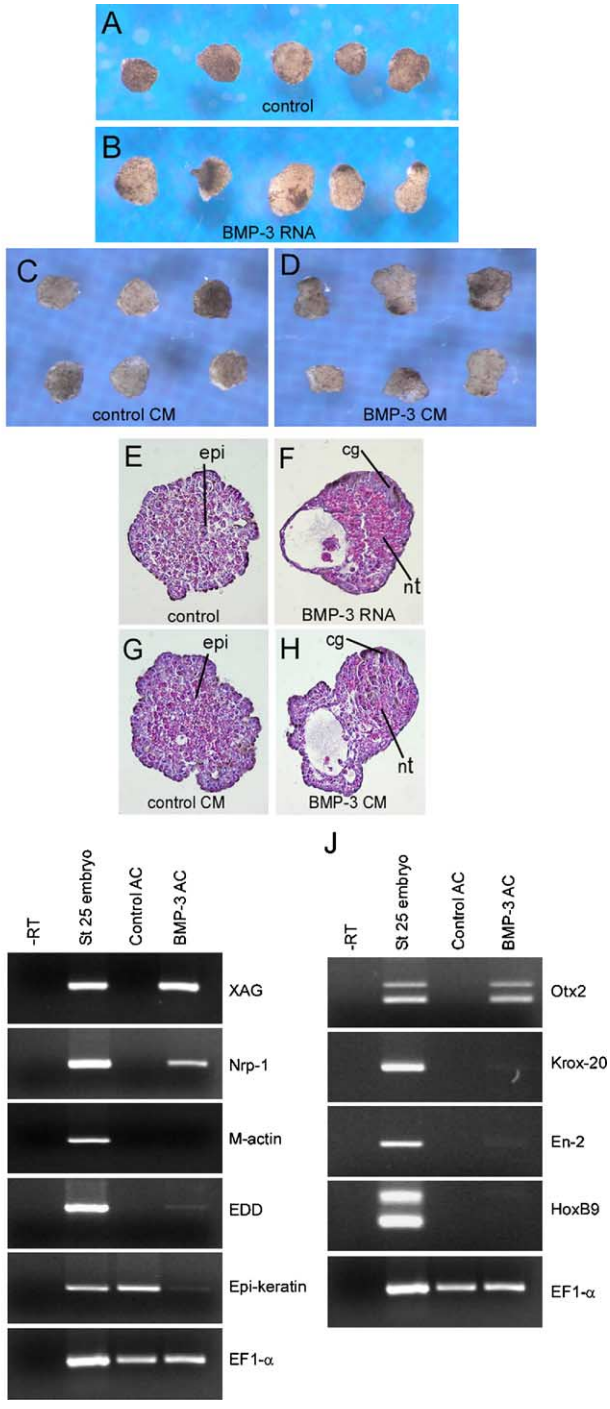

Fig. 2. *BMP-3* induces cement gland and neural tissue in animal cap explants. (A–H) Morphology of animal caps treated with BMP-3. 2-cell embryos were injected with 500 pg of *BMP-3* mRNA and animal caps cut at stage 8/9 or uninjected animal caps were incubated in control or BMP-3-conditioned media (CM) diluted 1:5 and then all explants were cultured until sibling control embryos reached late tadpole stages. (A) Control animal caps formed rounded balls of epidermis. (B) *BMP-3*-injected animal caps formed pigmented cement gland tissue. (C) Animal caps incubated in control CM from CHO cells formed epidermis like control caps in panel A. (D) Animal caps treated with BMP-3-conditioned media formed pigmented cement gland tissue as well. Histological sections of control (E) and control CM (G) animal caps showing epidermal tissue (epi). Histological sections of a *BMP-3*-injected animal cap (F) and an animal cap incubated in BMP-3 CM (H) showing the presence of cement gland (cg), neural tissue (nt) and a vesicle. (I–J) RT-PCR analysis of animal cap explants for expression of epidermal, mesodermal, neural and endodermal markers. *EF1-α* was used as an RNA loading control. *BMP-3* mRNA (500 pg) was injected into each animal blastomere at the 2-cell stage and animal caps prepared at tailbud stage 25. (I) *BMP-3* injection induced the general neural marker, *nrp-1*, as well as the cement gland marker, *XAG*. (J) *BMP-3*-injected animal caps analyzed for region-specific neural markers. Only the anterior marker *Otx-2* is induced. Animal cap (AC); *endodermin* (EDD); *engrailed 2* (En-2); *epidermal keratin* (Epi-keratin); *muscle actin* (M-actin); no reverse transcriptase control (−RT).

(Fig. 2J). Induction of only anterior neural tissue in animal caps is a characteristic activity of BMP inhibitors (Harland, 2000; Weinstein and Hemmati-Brivanlou, 1999). Interestingly, *BMP-3* appeared to induce this neural tissue directly, as we did not detect any expression of *muscle actin*, a mesodermal marker, even at high doses (1.5 ng) (Fig. 2I). Additionally, we saw that *BMP-3* did not turn on the expression of molecular markers of endoderm, such as *endodermin* (Fig. 2I) or *Xsox17-β* (data not shown). Using animal cap assays, we also found that incubation of explants in BMP-3 protein resulted in the same phenotype as injecting BMP-3 mRNA. Cement gland and neural tissue were consistently induced when animal caps were cultured in hBMP-3-conditioned media (Figs. 2D and H). Our results further indicate that BMP-3 has biological activities in *Xenopus* embryos that are similar to factors that inhibit both BMP and activin signaling, but that BMP-3 differs from previously described antagonists in that it induces cement gland and neural tissue, but not secondary axis formation or endoderm.

BMP-3 blocks activin and BMP-4 mesoderm-inducing activity in animal cap explants

Using animal cap assays we tested the ability of BMP-3 to directly antagonize BMP and activin activity. When co-injected with RNA encoding *BMP-4*, *BMP-3* was able to partially block the ability of *BMP-4* to induce the expression of the early mesodermal marker, *Xbra*, as well as the ventral mesoderm marker, *Xhox3* (Fig. 3A). However, *BMP-3* was not able to block the pro-epidermal activity of *BMP-4*. Figs. 3A–B show that expression of *msx-1*, a direct downstream target of BMP and a marker of ventro-lateral ectoderm, and *epidermal keratin* remained elevated in co-injected caps, indicating that *BMP-3* interferes with the ventralizing activity of *BMP-4* only in the mesoderm. In contrast to *noggin*, *chordin* or *tBR* which can rescue the *BMP-4* ventralized phenotype in whole embryos (reviewed in Harland and Gerhart, 1997), we could not rescue ventralized *BMP-4*-injected embryos with co-injected *BMP-3* (see Fig. 7G and data not shown). These results show that in *Xenopus*, BMP-3 is a weak inhibitor of BMP when compared to other antagonists and suggest that BMP-3 may be blocking BMP activity using a novel mechanism.

We next investigated whether BMP-3 could antagonize activin signaling. When *BMP-3*-injected animal caps were incubated in activin protein, the morphogenic movements indicative of mesoderm induction were blocked (Fig. 3C). RT-PCR assays showed that *BMP-3* strongly inhibited activin mRNA and protein-induced expression of *muscle actin* (Fig. 3D), indicating BMP-3 can antagonize activin signaling as well as BMP signaling. Since our injected BMP-3 mRNA blocks activin protein activity, this suggests that BMP-3/activin heterodimers are not causing the effects we see in our assays and is agreement with previous data showing that activin does not form heterodimers with *BMP-4* (Wittbrodt and Rosa, 1994).

Finally, we wanted to determine if *BMP-3* was acting as a general inhibitor of mesoderm induction in *Xenopus*. To do this, we tested the ability of *BMP-3* to block mesodermal marker gene expression activated by *Xnr1* (*Xenopus* nodal related 1), a TGF-β-like ligand shown to play important roles in mesoderm formation in early embryos (Schier, 2003) and by bFGF, a protein implicated in inducing and maintaining mesodermal fates in *Xenopus* (LaBonne and Whitman, 1994; LaBonne et al., 1995). We found that injection of *BMP-3* could not block the induction of *Xbra* by *Xnr1* supporting the previous findings of Hino et al. (2003) (Fig. 3E). In addition, we saw that *BMP-3* did not effect the induction of *muscle actin* by bFGF protein (Fig. 3F). Our results confirm that in *Xenopus*, *BMP-3* can only inhibit a subset of TGF-β family signals, including ventralizing BMP signals (BMP-2, -4 and ADMP) and now reveal that BMP-3 can also antagonize dorsalizing, activin signals.

BMP-3 antagonizes activin and BMP signals upstream of receptor activation

BMP and activin signaling are tightly regulated during embryonic development by potent extracellular antagonists that bind directly to these ligands, preventing them from interacting with their receptors. Extracellular antagonists can also inhibit signaling in an indirect manner, as has been shown for BMPs and noggin, where the ligand upregulates antagonist expression creating a negative feedback loop (Amthor et al., 1999; Gazzerro et al., 1998; Nifuji and Noda, 1999). Existing data from our lab ruled out the possibility that BMP-3 itself was an extracellular ligand antagonist because we were unable to show direct binding of BMP-3 to other BMPs or activin (Daluiski et al., 2001 and L.W. Gamer and V. Rosen, unpublished). To investigate the possibility that BMP-3 acts by regulating levels of known extracellular antagonists, we assayed *BMP-3*-injected animal caps for the upregulation of *noggin*, *chordin* or *follistatin*. *BMP-3* was unable to induce expression of any of these antagonists in late blastula stage animal caps, even when injected at high doses (1.5 ng) (Fig. 4A).

We next investigated whether BMP-3 could block the effects of constitutively activated type I activin or BMP receptors in the animal cap assay by looking for induction of mesodermal marker genes by these receptors. If BMP-3 acts intracellularly by interfering with receptor activation, we should observe a downregulation of mesodermal marker expression. Conversely, if BMP-3 inhibits signaling upstream of the receptor, marker gene expression should be unaffected. For our experiments, we co-injected the constitutively activated activin type I receptor (*CA-ALK4*) or the constitutively activated BMP type I receptor (*CA-ALK3*) with *BMP-3* into both animal blastomeres of 2-cell embryos. We then isolated animal caps and looked at the

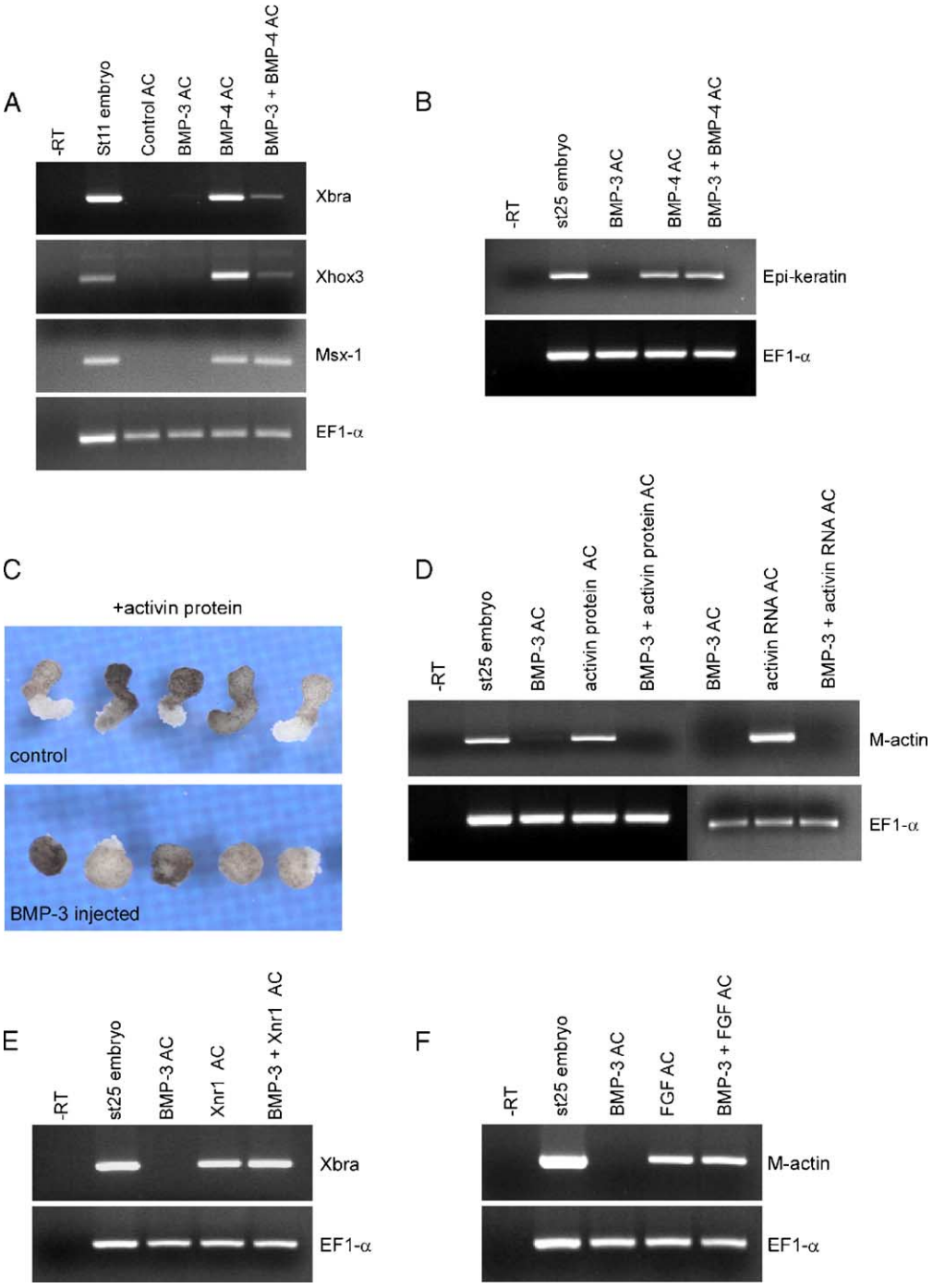

Fig. 3. Inhibitory effects of *BMP-3* on *BMP-4* and activin. (A–B) *BMP-3* (500 pg) and *BMP-4* (500 pg) were injected separately, and together, into embryos at the 2-cell stage. Animal caps were analyzed by RT-PCR. (A) In gastrula stage animal caps, *BMP-3* blocked the induction of the early mesodermal markers *Xbra* and *Xhox3* by *BMP-4* but did not block the induction of *msx-1*, an epidermal marker and direct target gene of *BMP-4*. (B) In tailbud stage animal caps, *BMP-3* did not block the induction of *epidermal keratin* by *BMP-4*. (C) Control and *BMP-3* (500 pg) mRNA-injected animal caps were treated with activin protein (3 ng/ml) and cultured until tailbud stages. *BMP-3* suppressed the morphogenic movements of activin-treated animal caps. (D) RT-PCR analysis of control and *BMP-3*-injected animal caps treated with activin protein or co-injected with *activin* mRNA (200 pg). *BMP-3* blocked the induction of *muscle actin* by activin protein and mRNA. (E) RT-PCR analysis of gastrula stage animal caps injected with *BMP-3* (500 pg) and *Xnr1* (50 pg). *BMP-3* did not block the induction of *Xbra* by *Xnr1*. (F) RT-PCR analysis of tailbud stage control and *BMP-3*-injected animal caps treated with bFGF (100 ng/ml). *BMP-3* did not inhibit the induction of *muscle actin* by bFGF protein. Animal cap (AC); no reverse transcriptase control (−RT).

effects on mesodermal marker expression by RT-PCR. We found that *BMP-3* was unable to block *CA-ALK3* or *CA-ALK4* from inducing the expression of *Xbra* or *muscle actin* (Figs. 4B and C). These data indicate that BMP-3 acts upstream of receptor activation before R-Smad phosphorylation by type I receptor.

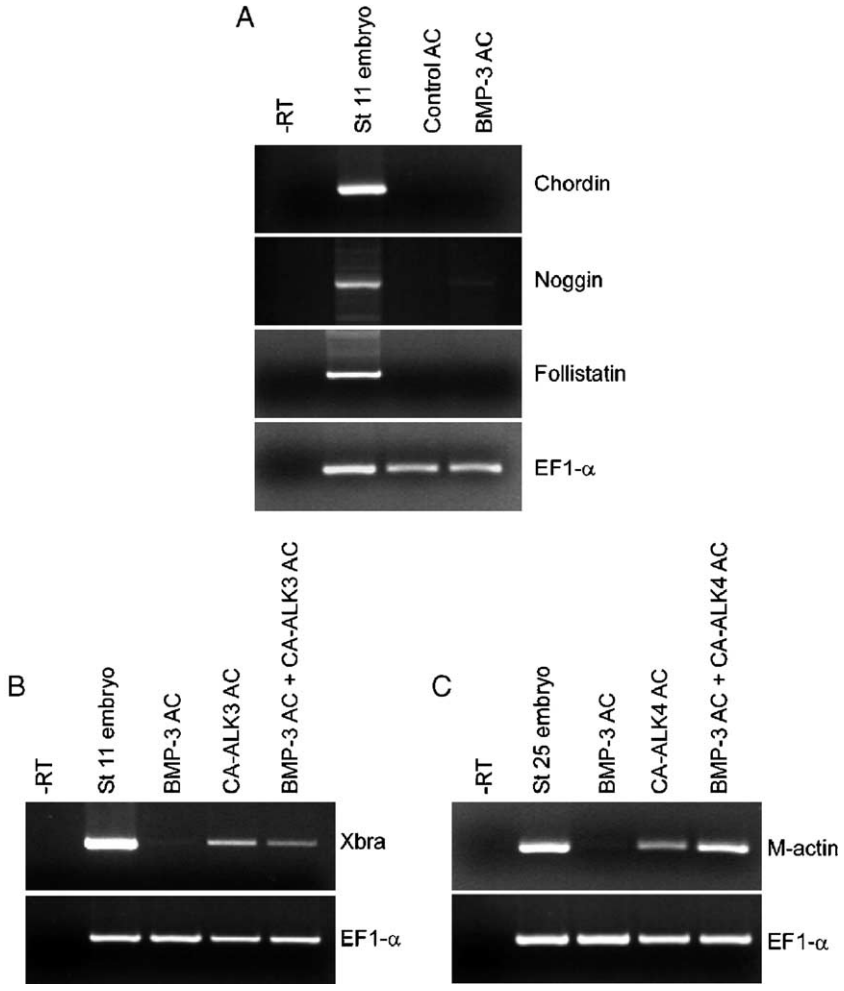

Fig. 4. BMP-3 acts upstream of receptor activation to block BMP and activin signaling. (A) *BMP-3* does not induce BMP and activin antagonists. 2-cell embryos were injected with 500 pg of *BMP-3* mRNA and animal caps prepared at gastrula stage 11 for RT-PCR analysis. *BMP-3* does not upregulate the expression of *chordin*, *noggin* or *follistatin*. (B–C) BMP-3 is acting at the receptor level. (B) *BMP-3* (500 pg) and constitutively active BMP type I (*CA-ALK3*) receptor (2 ng) were injected separately and together at the 2-cell stage. Animal caps were prepared at stage 11. *BMP-3* could not block the induction of *Xbra* by *CA-ALK3*. (C) *BMP-3* (500 pg) and constitutively active activin type I (*CA-ALK4*) receptor (2 ng) were injected separately and together at the 2-cell stage. Animal caps were prepared at stage 25. *BMP-3* could block the induction of *muscle actin* by *CA-ALK4*. Animal cap (AC); no reverse transcriptase control (−RT).

We then considered what receptor pathway BMP-3 could use to antagonize both activin and BMP signaling. We injected 4-cell embryos with *BMP-3*, *BMP-4* or *activin* mRNA and examined R-Smad phosphorylation in isolated animal cap explants at stage 10.5. As expected, Western blot analysis showed strong phosphorylation of Smad1,5,8 by *BMP-4* and phosphorylation of Smad2 by *activin* (Figs. 5A and B). Surprisingly, we did not detect phosphorylation of any R-Smads by *BMP-3* alone (Figs. 5A and B, lane 3). When we co-injected *BMP-3* together with *BMP-4* or *activin*, we found that *BMP-3* decreased the level of phosphorylation of Smad1,5,8 and strongly reduced the phosphorylation level of Smad2 (Figs. 5A and B, lane 4). These results confirm that BMP-3 antagonizes signaling by activin and BMP and identifies BMP-3 as an inhibitor of signal transmission at the level of ligand binding to receptor. Based on these findings, we also examined the effects of BMP-3 overexpression on endogenous Smad phosphorylation in whole embryos. Here we found that BMP-3 strongly reduced the level of Smad2 phosphorylation (Fig. 5D) but did not seem to effect Smad1,5,8 activation in gastrula stage 10.5 embryos (Fig. 5C). These data fit well with recent antisense morpholino experiments by Piepenburg et al. (2004), reinforcing the importance of activin signaling through Smad2 for early mesodermal patterning. Our results show that BMP-3 efficiently blocks endogenous activin signaling by decreasing Smad2 activation but is not as effective at blocking endogenous Smad1,5,8 phosphorylation. This may be due to the fact that BMPs can use both ActRII/B and BMPR-II to transmit signals while activin utilizes only ActRII/B. Therefore, if BMP-3 blocks signaling at ActRII/B then BMPs may still be able to induce Smad1,5,8 phosphorylation through BMPR-II. These results led us to examine if BMP-3 interacted with ActRII/B.

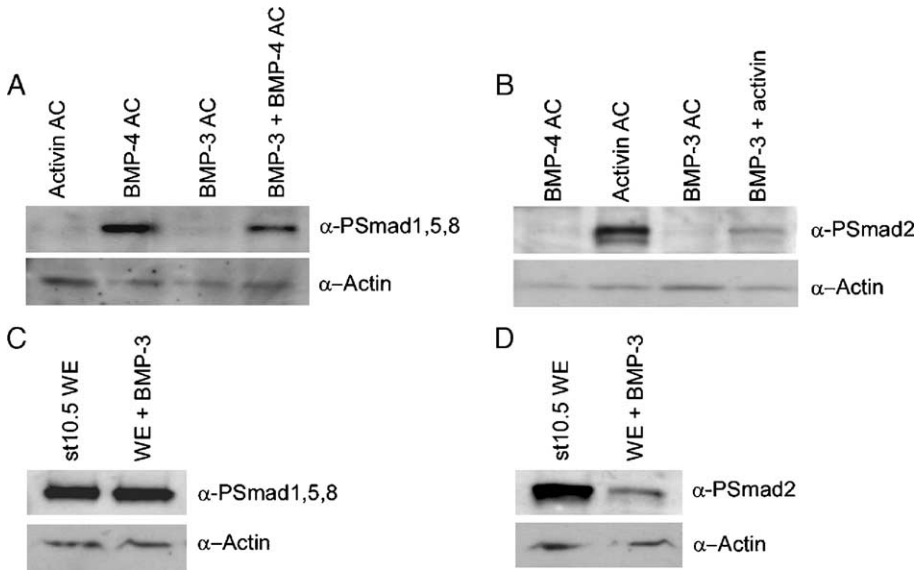

Fig. 5. BMP-3 does not activate R-Smads but interferes with R-Smad phosphorylation by BMP-4 and activin. *BMP-3* (500 pg), *BMP-4* (500 pg) and *activin* (200 pg) mRNAs were injected separately and together, into all animal blastomeres of 4-cell embryos. Activation of Smad1,5,8 and Smad2 in stage 10.5 animal caps and whole embryos was measured by Western blot analysis using anti-phospho-Smad1,5,8 antibody (α-PSmad1,5,8) and anti-phospho-Smad2 antibody (α-PSmad2). Cytoskeletal actin (α-Actin) was used a loading control for total protein. (A) *BMP-4* induced strong phosphorylation of Smad1,5,8 while *BMP-3* and *activin* did not. Co-expression of *BMP-4* and *BMP-3* decreased the level of Smad1,5,8 activation by *BMP-4* (lane 4). (B) *Activin* induced strong phosphorylation of Smad2 while *BMP-4* and *BMP-3* did not. Co-expression of *activin* and *BMP-3* significantly reduced the level of Smad2 activation by *activin* (lane 4). (C) *BMP-3* injection did not effect endogenous phosphorylation of Smad1,5,8 in whole embryos. (D) *BMP-3* injection greatly decreased the phosphorylation of Smad2 in whole embryos compared to controls. The two bands recognized by the anti-phospho-Smad2 antibody are Smad2 and a splicing variant of Smad2 (Faure et al., 2000). Animal cap (AC); whole embryo (WE).

BMP-3 binds ActRIIB

Having established that BMP-3 blocks both activin and BMP at the receptor level, the receptor targeted by BMP-3 would have to be one common to both the activin and BMP-signaling pathways, most likely the type II activin receptor. ActRII/B has been reported to bind multiple ligands including BMP-2, BMP-7, GDF11 and activin (Massague and Chen, 2000). To look for an interaction of BMP-3 with ActRIIB, we co-expressed *BMP-3* with epitope tagged *ActRIIB* (*ActRIIB/Myc*) in early *Xenopus* embryos and used the extracts of early gastrula stage animal halves for co-immunoprecipitation assays. When we did this, we found that BMP-3 was co-precipitated with ActRIIB by the anti-myc antibody (Fig. 6A, lane 3).

Since BMP-3 and activin can both bind ActRIIB, it is possible these two proteins compete for binding to their common receptor leading to the inhibition of activin signaling. To test this hypothesis, we performed binding competition assays in early *Xenopus* embryos. *BMP-3* was co-expressed with *ActRIIB/Myc* in the absence or presence of increasing amounts (2 ng, 4 ng, 6 ng) of *activin/HA* and protein extracts of gastrula stage animal halves were immunoprecipitated with anti-myc antibody. Fig. 6B shows that increasing amounts of *activin/HA* did not significantly decrease the amount of BMP-3 immunoprecipitated by ActRIIB. The reverse experiment gave similar results with increasing doses of *BMP-3* unable to significantly reduce the levels of activin immunoprecipitated by ActRIIB (Fig. 6C). Our data suggest that once BMP-3 binds the type II receptor, it cannot be competed off by excess activin. These results agree with our observation that animal caps preloaded with BMP-3 cannot respond to activin protein added later (see Figs. 3C and D). The interaction we observe may reflect the ability of BMP-3 and activin to bind to different sites on ActRIIB. Even if the two ligands do not compete for the same site on the receptor, binding of BMP-3 precludes signal transduction by the activin–ActRIIB complex. We did not perform these same competition experiments using BMP-4 as BMP-4 is known to prefer the type I receptor, and is unlikely to bind to type II receptor in the absence of type I receptor in vivo (Shi and Massague, 2003).

We next looked to see how BMP-3 binding to type II receptor affected the recruitment of type I receptor to the receptor complex. To do this, *BMP-3* was injected into *Xenopus* embryos with *ActRIIB/Myc* alone or with *ActRIIB/Myc* and the type I activin receptor, *Alk4/Flag* and protein extracts were immunoprecipitated with anti-myc antibody. We found that BMP-3 was able to make a complex containing both ActRIIB and Alk4 (Fig. 6D). Our data suggest this complex is inactive and prevents activin signal transduction.

To further evaluate and confirm our binding assay results, we performed rescue experiments using whole embryos in which *BMP-3* mRNA was co-injected with

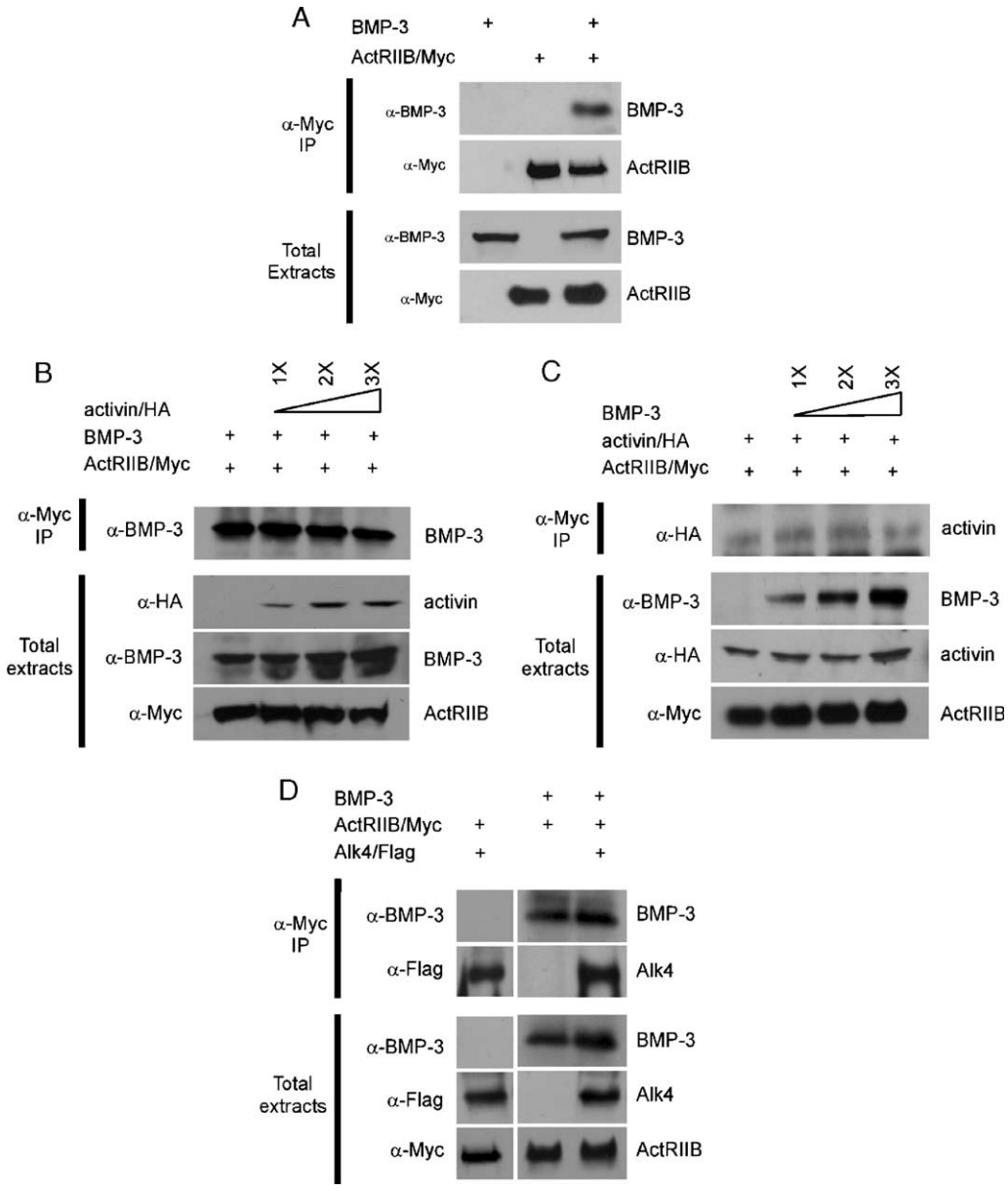

Fig. 6. BMP-3 binds ActRIIB but does not compete with activin and can form a receptor complex with ActRIIB and Alk4. (A) BMP-3 binds to ActRIIB. RNAs encoding *BMP-3* (2 ng) and *ActRIIB/Myc* (2 ng) were injected into the animal poles of 2-cell stage *Xenopus* embryos. Protein extracts made from gastrula stage animal halves were immunoprecipitated with anti-myc antibody. (B) Increasing doses of activin do not compete off BMP-3 bound to ActRIIB. RNAs encoding *BMP-3* (2 ng) and *ActRIIB/Myc* (2 ng) were co-injected with increasing amounts of *activin/HA* (2 ng, 4 ng, 6 ng). Extracts were immunoprecipitated with anti-myc antibodies. (C) Increasing doses of BMP-3 do not compete off activin bound to *ActRIIB*. RNAs encoding *activin/HA* (2 ng) and *ActRIIB/Myc* (2 ng) were co-injected with increasing amounts of *BMP-3* (2 ng, 4 ng, 6 ng). Extracts were immunoprecipitated with anti-myc antibodies. (D) BMP-3 can make a receptor complex containing ActRIIB and Alk4. RNAs encoding *BMP-3* (2 ng), *ActRIIB/Myc* (2 ng) and *Alk4/Flag* (2 ng) were injected into embryos. Extracts were immunoprecipitated with anti-myc antibodies. Proteins in co-immunoprecipitates and total extracts were probed in Western blot analysis with the indicated antibodies: hBMP-3 (mature ligand ~30 kDa, α-BMP-3), ActRIIB/Myc (~120 kDa, α-Myc), activin/HA (mature ligand ~16 kDa, α-HA) and Alk4/Flag (~70 kDa, α-Flag). ActRIIB/Myc and Alk4/Flag are kinase-defective activin receptor mutants that were used to prevent receptor internalization. Immunoprecipitation, (IP).

RNAs for various wild-type activin and BMP receptors. At the late tadpole stage, embryos were scored for rescue to normal development. In these experiments, only *xActRIIB* rescued the *BMP-3* phenotype back to proper body axis and head formation in a dose-dependent manner (Figs. 7B–D). Co-injection of *xBMPRII*, a BMP-specific type II receptor (Frisch and Wright, 1998) (Fig. 7E), *xALK4*, a type I activin receptor (Chang et al., 1997) (Fig. 7F), *BMP-4* (Fig. 7G) or *activin* (Fig. 7H) as a ligand controls did not rescue the *BMP-3* phenotype at the doses tested. The discovery that BMP-3 associates with ActRIIB along with the finding that ActRIIB rescues BMP-3-injected embryos allows us to conclude that BMP-3 blocks activin and BMP signaling by binding to this common type II receptor. These results are

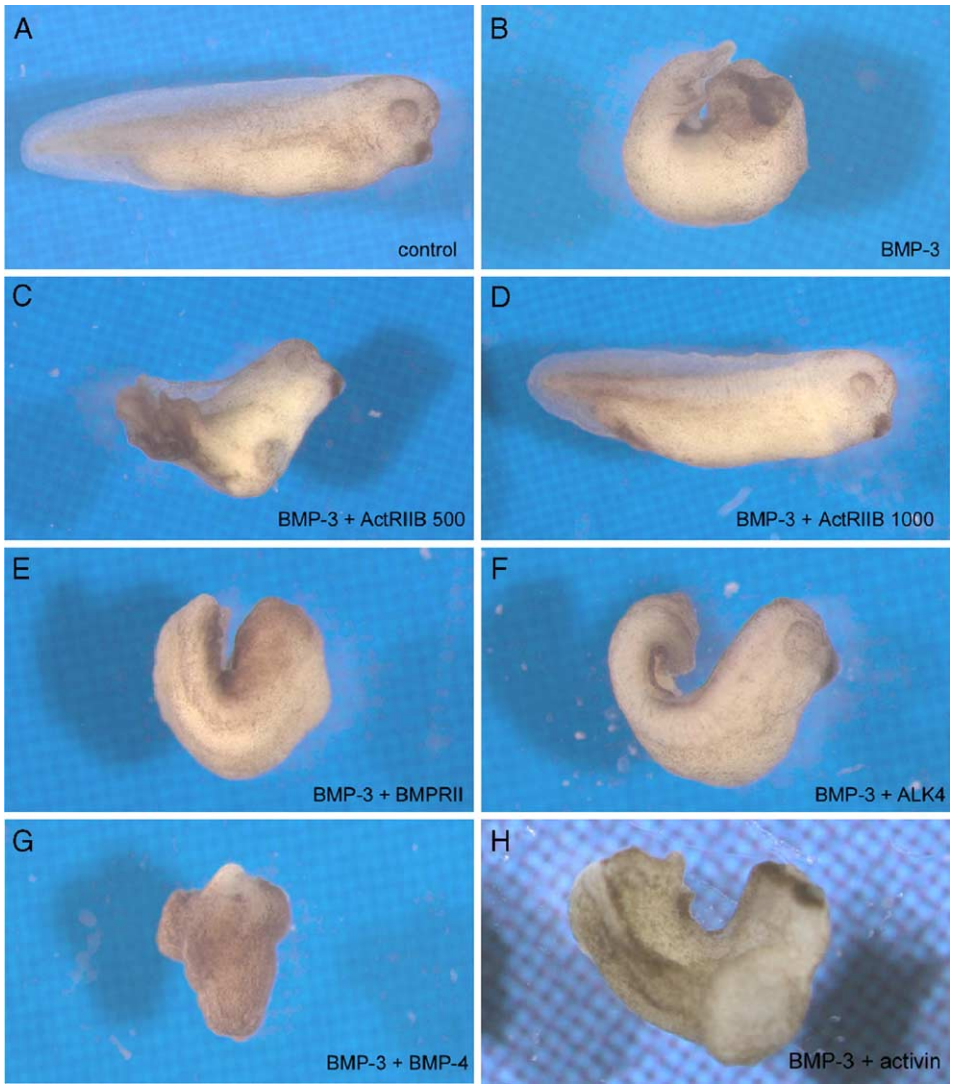

Fig. 7. *ActRIIB* rescues the phenotype of *BMP-3*-injected embryos. The phenotype produced by injection of *BMP-3* into the embryo can be rescued only by co-injection of wild-type *xActRIIB*. 4-cell stage embryos were injected into a single ventral vegetal blastomere with the indicated mRNAs and scored for rescue to normal at late tadpole stages. (A) Control uninjected tadpole. (B) Dorso-anteriorized phenotype of *BMP-3* (500 pg)-injected embryo. (C) Co-injection of *BMP-3* (500 pg) and *xActRIIB* (500 pg) partially rescued the anterior of the embryo. (D) Co-injection of *BMP-3* (500 pg) and *xActRIIB* (1 ng) completely rescued the phenotype to normal body axis (76%, $n = 85$). (E) Co-injection of *BMP-3* (500 pg) and *xBMPRII* (1.5 ng) did not rescue the embryo. (F) Co-injection of *BMP-3* (500 pg) and *xALK4* (1.5 ng) did not rescue the embryo. (G) Co-injection of *BMP-3* (500 pg) and *BMP-4* (250 pg) did not rescue the embryo. (H) Co-injection of *BMP-3* (500 pg) and *activin* (200 pg) did not rescue the embryo.

consistent with the observation that BMP-3 is a more potent inhibitor of activin signaling (which uses only ActRII) than BMP signaling (which uses both ActRII and BMPRII).

Discussion

In this report, we use the wealth of information about the activities of BMP and activin-like molecules in *Xenopus* to explore the mechanism used by BMP-3 to modulate these signals in the context of *Xenopus* development. Our data show that BMP-3 is a dorso-anteriorizing factor that antagonizes both activin and BMP-4 by binding to ActRII, their common receptor, and interfering with signal transduction. The interactions of BMP-3, activin and BMP-4 are outlined in Fig. 8. When activin and BMP-3 are both present, if BMP-3 occupies ActRII, activin is excluded, and an inactive receptor complex forms that blocks Smad2 phosphorylation. As a result, activin signaling in target tissues is diminished (Fig. 8C). This was shown in biochemical binding and competition assays as well as in our animal cap experiments, where *BMP-3* prevented mesoderm formation by blocking activin phosphorylation of Smad2. When BMP-4 and BMP-3 are both present, if BMP-3 occupies ActRII, Smad1,5,8 phosphorylation cannot occur through receptor complexes that contain ActRII (Fig. 8D). However, since BMP-4 is not bound by BMP-3, BMP-4 is able to activate BMP signaling through BMPR-II, allowing

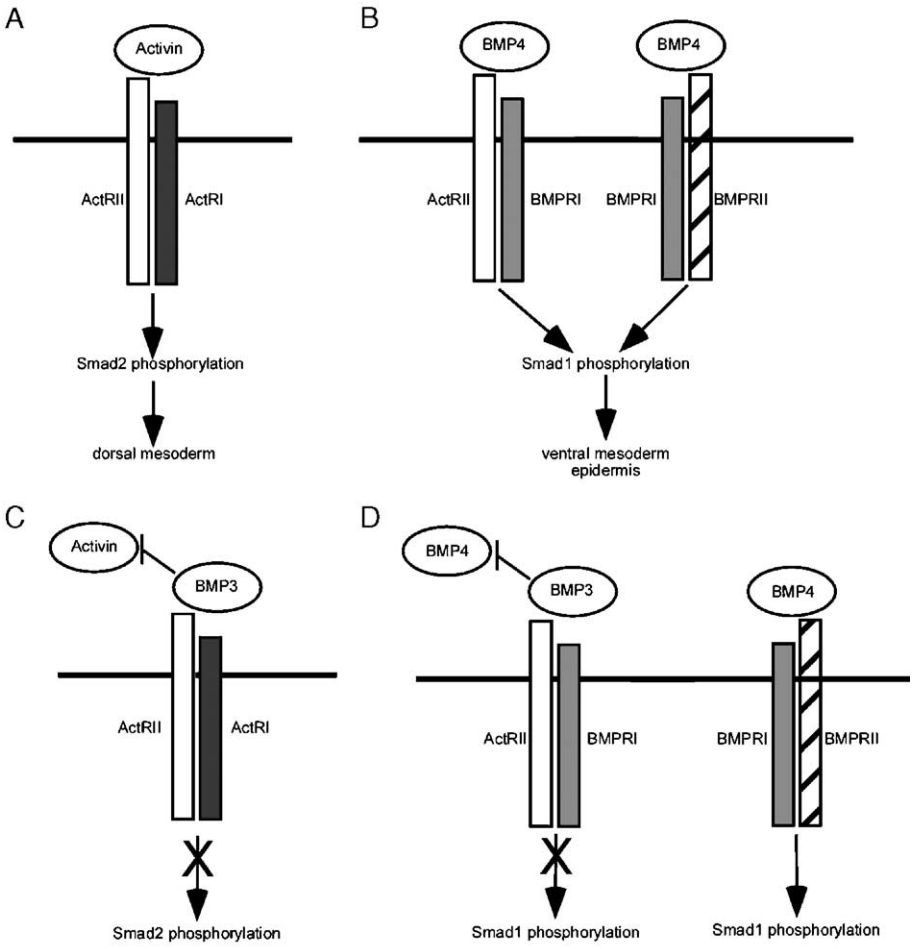

Fig. 8. Model of BMP-3 interaction with activin and BMP-4 in *Xenopus* embryos. (A) Activin signals through ActRII–ActRI complexes to cause Smad2 phosphorylation and pattern dorsal mesodermal tissues. (B) BMPs signal through ActRII–BMPRI and BMPRII–BMPRI complexes to cause Smad1 phosphorylation and to pattern ventral mesoderm and epidermal tissues. (C) When activin and BMP-3 are both present, BMP-3 interferes with activin binding to ActRII, Smad2 activation and activin signal transduction are blocked. (D) When BMP-4 and BMP-3 are both present, BMP-3 interferes with BMP-4 binding to ActRII, the level of Smad1 phosphorylation and BMP signaling through ActRII is decreased. Since BMP-3 does not bind BMPRII, BMP-4 is still available to signal through the BMPRII–BMPRI complex to activate Smad1.

BMP signaling in cells that express this BMP-specific type II receptor (Fig. 8D). Evidence to support this idea is provided by the induction of the epidermal markers *msx-1* and *epidermal keratin* when *BMP-3* and *BMP-4* are co-injected into animal caps and by our observation that there is Smad1,5,8 signaling in whole embryos overexpressing BMP-3. Interestingly, our animal cap assays also showed that *BMP-3* could not block the activity *Xnr1* (Hino et al., 2003), a ligand that signals through ActRIIB/Alk4 and requires an EGF-CFC co-receptor (Cheng et al., 2003; Schier, 2003). Based on our results, we conclude the potency of BMP-3 as an antagonist of TGF-β superfamily signaling is greatest for ligands that act directly through ActRII.

While our data identify BMP-3 as a receptor antagonist, there are additional ways in which BMP-3 might act to inhibit activin and BMP signaling. One intriguing possibility is that BMP-3 forms heterodimers with BMPs or activin, creating dominant-negative ligands. This is consistent with reports that injected BMP-3 can form heterodimers with BMP-2 in *Xenopus* embryos (Hino et al., 2003). Our animal cap assays show that BMP-3-conditioned media (which contains only BMP-3 homodimers) have the same biological activity as injected BMP-3 mRNA. We also observed that injected activin mRNA cannot rescue the BMP-3 whole embryo phenotype. These results suggest that the BMP-3-antagonistic activity in our experiments is unlikely to be due to heterodimerization between BMP-3 and activin. It is still possible that BMP-3/BMP-2/4 or BMP-3/activin heterodimers could form in *Xenopus* embryos and have their own receptor affinities that would allow for selective inhibition of a subset of TGF-β members. This is something we plan to investigate in future studies.

Our gain of function studies with BMP-3 in *Xenopus* embryos support the idea that the biological actions of BMP-3 are due to its ability to modulate both activin and BMP signaling through ActRII. Selectively blocking ActRII may be an important way to affect specific developmental

processes. For example, overexpression of *BMP-3* in whole embryos causes the formation of enlarged and ectopic cement glands. In recent years, a model has been put forth suggesting that intermediate levels of BMP signaling in the ectoderm lead to cement gland formation, while high levels of BMP signaling promote epidermis and low levels of BMP signaling result in neural tissue (Wardle and Sive, 2003). Our data lend support to this hypothesis, since BMP-3 does not completely block BMP signaling, cement gland tissue may be induced in embryos and animal caps because of moderate levels of BMP signaling that may be occurring through BMPRII. Additional support for this idea comes from the fact that *BMP-3* and *BMP-4* are both expressed in the developing cement gland (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Hino et al., 2003) where this regulatory interaction could take place. In gastrula stage embryos, *BMP-3* transcripts are found throughout the marginal zone mesoderm where they appear to overlap with the expression of *activin* (Dohrmann et al., 1993; Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Hino et al., 2003). This co-localization may allow BMP-3 to play a role in restricting activin in a cell-specific manner, so that once BMP-3 binds to ActRII it is unavailable to transmit activin signals, ensuring proper dorso-anterior mesodermal patterning. In addition, *BMP-3* expression overlaps with *BMP-4* expression in gastrula stage ectoderm (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Hino et al., 2003) where BMP-3 could act to decrease BMP signaling, allowing other BMP antagonists that are expressed in Spemann's organizer to successfully induce the full range of nervous system tissues. The ability of BMP-3 to induce anterior neural tissue and inhibit the mesoderm-inducing activities of both activin and BMP support these ideas.

Although our gain of function studies indicate BMP-3 has a role patterning the *Xenopus* embryo, previously reported antisense morpholino oligonucleotide experiments for BMP-3 did not show any phenotype (Hino et al., 2003). Since BMP-3 acts as an antagonist, it may need to be ablated in conjunction with other activin/BMP inhibitors in order to determine its effects on embryonic development as has been recently shown in triple morpholino knockdown experiments for noggin, chordin and follistatin (Khokha et al., 2005). It is also possible that another highly related BMP family member (like BMP-3b/GDF-10) compensates for the loss of BMP-3 in early embryos, or that the role of BMP-3 in *Xenopus* development may be temporally restricted to later stages. These ideas are supported by data from BMP-3 null mice in which the bone phenotype is first evident postnatally (Daluiski et al., 2001) and from GDF-10 (BMP-3b) knockout mice that do not have any obvious phenotype (Zhao et al., 1999). Future studies that focus on the effects of loss of BMP-3 in combination with other activin/BMP antagonists may allow us to determine its full role during *Xenopus* development.

Our current data regarding BMP-3 signaling in *Xenopus* embryos agree with the work of Hino et al., 2003 who reported that BMP-3 antagonized ventralizing BMPs but not nodal-like ligands. However, our findings on how this antagonism occurs in *Xenopus* need to be reconciled with previously published observations in mammalian cell lines where BMP-3 activated a TGF-β/activin-responsive reporter and also inhibited BMP signaling downstream of the BMP type I receptor (Daluiski et al., 2001). The most likely explanation for these differences is that BMP-3 acts in cell-context-dependent manner, similar to that observed for inhibin which is an effective blocker of activin signaling but only antagonizes BMP signaling in cell types that express the co-receptor betaglyan (Wiater and Vale, 2003). Another possibility is that the methodology employed in the mammalian cell assays in which ligand and receptor plasmids are transfected into unresponsive cells that are then used for reporter assays is not as reliable as utilizing *Xenopus* embryos that possess all of the signal transduction machinery. This is certainly the true for assessing endogenous Smad activation (Fig. 5), a more direct measure of TGF-β-like ligand activity.

In conclusion, our data best fit a model in which BMP-3 interferes with activin and BMP-4 binding to the type II activin receptor without activating R-Smads. As a consequence, signaling through activin and BMP receptor complexes that utilize ActRII is diminished. Thus, BMP-3 joins a growing family of TGF-β-like molecules including inhibin (Wiater and Vale, 2003) and lefty (Chen and Shen, 2004; Cheng et al., 2004), which by virtue of their ability bind to TGF-β family receptors and co-receptors but not activate signal transduction, serve as modulators of growth factor activity during embryonic development.

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