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BMP Signaling Is Required for Heart Formation in Vertebrates

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In these studies, we have taken advantage of a transient transgenic strategy in *Xenopus* embryos to demonstrate that BMP signaling is required *in vivo* for heart formation in vertebrates. Ectopic expression of dominant negative Type I (tALK3) or Type II (tBMPRII) BMP receptors in developing *Xenopus* embryos results in reduction or absence of heart formation. Additionally, blocking BMP signaling in this manner downregulates expression of XNkx2-5, a homeobox gene required for cardiac specification, prior to differentiation. Notably, however, initial expression of XNkx2-5 is not affected. Mutant phenotypes can be rescued by co-injection of mutant with wild-type receptors or co-injection of mutant receptors with XSmad1, a downstream mediator of BMP signaling. Whole-mount *in situ* analyses indicate that ALK3 and XSmad1 are coexpressed in cardiogenic regions. Together, our results demonstrate that BMP signaling is required for maintenance of XNkx2-5 expression and heart formation and suggest that ALK3, BMPRII, and XSmad1 may mediate this signaling.

Key Words: **BMP; truncated BMPR; cardiogenesis; tinman homologues; ALK3; BMPRII; Smad1.**

INTRODUCTION

Experiments performed *in vitro* in chick explants suggest that bone morphogenetic protein (BMP) signaling may be required for cardiac specification and heart formation during late gastrulation or early neurula stages (Lough *et al.*, 1996; Schultheiss *et al.*, 1997). In vertebrates, mRNAs encoding BMPs are expressed in cardiogenic regions during embryogenesis (Hemmati-Brivanlou and Thomsen, 1995; Schultheiss *et al.*, 1997). However, direct *in vivo* evidence for the requirement of BMP signaling in vertebrate heart formation has been lacking. Accordingly, we initiated studies in developing *Xenopus* embryos to examine the requirement for BMP signaling in cardiac specification and heart formation.

BMPs are members of the transforming growth factor β (TGF-β) superfamily. TGF-β family ligands bind to heterodimeric receptors comprising Type I and Type II serine–threonine kinase receptors. Ligand binding induces transphosphorylation of Type I receptors by constitutively active Type II receptors. The activated Type I receptor then phosphorylates an interacting effector Smad, which heterodimerizes with a co-Smad (Smad4) and is translocated to the nucleus. This Smad complex can then bind either independently or in concert with other transcription factors to effect gene transcription. The specificity of this response is thought to be mediated by selective ligand binding by specific receptor complexes and selective interactions of Type I receptors with effector Smads which then bind specific targets (Chen *et al.*, 1998; Howell *et al.*, 1999).

Currently, there are a number of BMP ligands (e.g., BMPs 2, 4, 5, 7, 10; GDF5), four Type I receptors (ALK1, 2, 3, and 6), three Type II receptors (BMPRII, ActRIIA, and ActRIIB), three effector Smads (Smad1, 5, and 8), two co-Smads (Smad4α and β), and two inhibitory Smads (Smad6 and 7) which have been shown to be involved in BMP signaling (Chen *et al.*, 1998; LeSueur and Graff, 1999; Masuyama *et al.*, 1999).

A number of *in vivo* studies have been performed in mice to ablate specific members of BMP signaling pathways. The phenotypes of many of these knockouts are extremely early embryonic lethal (BMP4, ALK3, ALK2, Smad 4), precluding analyses of effects on heart development (Gu *et al.*, 1999; Mishina *et al.*, 1995; Sirard *et al.*, 1998; Winnier *et al.*, 1995). Other knockouts display a range of cardiac phenotypes, including defects in critical left–right morphogenesis of the heart (ActRIIb), abnormal placement and delayed

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fusion of cardiac primordia (BMP2 or Smad5), and defective chamber maturation (septation, trabeculation, cushion formation) (double knockout of BMP7 and BMP5) (Oh and Li, 1997; Solloway and Robertson, 1999; Yang *et al.*, 1999; Zhang and Bradley, 1996). Each of these is a critical aspect of heart development. However, perhaps owing to early lethality or genetic redundancy, the critical role of BMP signaling in cardiac mesoderm specification and heart formation has not been addressed by the mouse ablation studies.

In the studies described here, we have utilized two well-characterized dominant negative BMP receptors, tALK3 and tBMPRII (Frisch and Wright, 1998; Suzuki *et al.*, 1994), to block BMP signaling in developing *Xenopus* embryos. We have taken advantage of a transgenic strategy recently developed in our laboratory which results in efficient, relatively stable, yet mosaic expression of transgenes following direct DNA injection into early embryos (Fu *et al.*, 1998a). This approach enables us to bypass the early lethality and general effects on mesodermal induction of blocking BMP signaling, allowing the detection of specific effects on cardiogenesis. We earlier used the same approach to demonstrate a requirement for tinman homologue action during vertebrate cardiogenesis (Fu *et al.*, 1998b).

MATERIALS AND METHODS

Plasmid DNAs for Microinjection

DNAs encoding tXALK3 (Suzuki *et al.*, 1994), wild-type XALK3 (Graff *et al.*, 1994), tXBMPRII, wild-type XBMPRII (Frisch and Wright, 1998), and XMad1 (Graff *et al.*, 1996) were subcloned into a CS2 expression vector (Turner and Weintraub, 1994) which had been modified by the addition of AAV-ITR sequences (Fu *et al.*, 1998a). The control CS2 n-βgal ITR plasmid has been described previously (Fu *et al.*, 1998a).

Embryo Microinjection

Xenopus laevis were purchased from *Xenopus* I and Nasco companies. Ovulation was induced by injecting 300–500 U each of human chorionic gonadotropin. Fertilized eggs were dejellied with 2% cysteine-HCl (pH 7.8) immediately following cortical rotation. Plasmid DNAs were linearized prior to injection and injected with 5 μg/μl neutral tetramethylrhodamine dextran (MW 70,000) as a lineage tracer (Cleaver *et al.*, 1996) Embryos were injected into one cell at the two-cell stage in 0.1× MMR solution containing 3% Ficoll 400, and sodium penicillin and streptomycin sulfate, each at 10 μg/ml. After several cell cycles (about 3–4 h), the embryos were transferred to 0.1× MMR solution and maintained at 15°C. Developmental stages of injected embryos were determined by comparison to stages of uninjected control embryos (Nieuwkoop and Faber, 1967). Only morphologically normal embryos were scored, and each experiment was performed a minimum of three times.

DNAs for the dominant negative receptor and control constructs were linearized prior to injection.

TABLE 1

Differentiated Cardiac Phenotype of Embryos Injected with Dominant Negative BMP Receptors

DNA	Amt inj (pg)	No. scored	Mutant (%)	*P* value[a]
CS2nβ-gal	200	60	2 (3.0)	
tALK3	200	112	29 (26)	0.0039*
tBMPRII	200	143	38 (27)	0.0024*

Note. Injections were performed into one cell at the two-cell stage, and embryos were harvested postcardiac differentiation at stages 28–42 and examined by whole-mount *in situ* analyses for expression of *XMLC2* or *XcTnI*. Embryos which exhibited asymmetric reduction (20% or more) or absent staining for either of the two differentiation markers, or with clearly abnormal cardiac morphology (see Fig. 1 for examples), were scored as mutant.

[a] *P* values were generated by χ^2 analyses and refer to the null hypothesis that experimental and control embryos are equivalent.

* The null hypothesis is rejected at a significance level of 0.01.

Whole-Mount in Situ Hybridization and Histology

Riboprobes were prepared by *in vitro* transcription of linearized DNA templates in the presence of digoxigenin-11–UTP (Boehringer Mannheim). The cDNA plasmid encoding XMLC2 has been described previously (Evans *et al.*, 1995) and was linearized with *Bam*HI and transcribed with T7 RNA polymerase to generate an antisense riboprobe. The cDNA plasmid encoding XcTnI was obtained by a PCR containing degenerate oligonucleotide primers complementary to conserved regions of chick and mouse cTnI and cDNA from adult *Xenopus* heart. The resulting cDNA cloned into vector pCRII encoded sequences in agreement with those reported for *XcTnI* (Drysdale *et al.*, 1994). pCRcTnI was linearized with *Eco*RI and transcribed with T7 RNA polymerase to generate an antisense riboprobe for *XcTnI*. Riboprobes for *XNkx2-5* were obtained by linearizing plasmid *XCsx2* (courtesy of Seigo Izumo) with *Xba*I and transcribing with T7 RNA polymerase. Probes were purified by an RNase-free Chroma Spin-100 column (Clontech) prior to use and were not hydrolyzed.

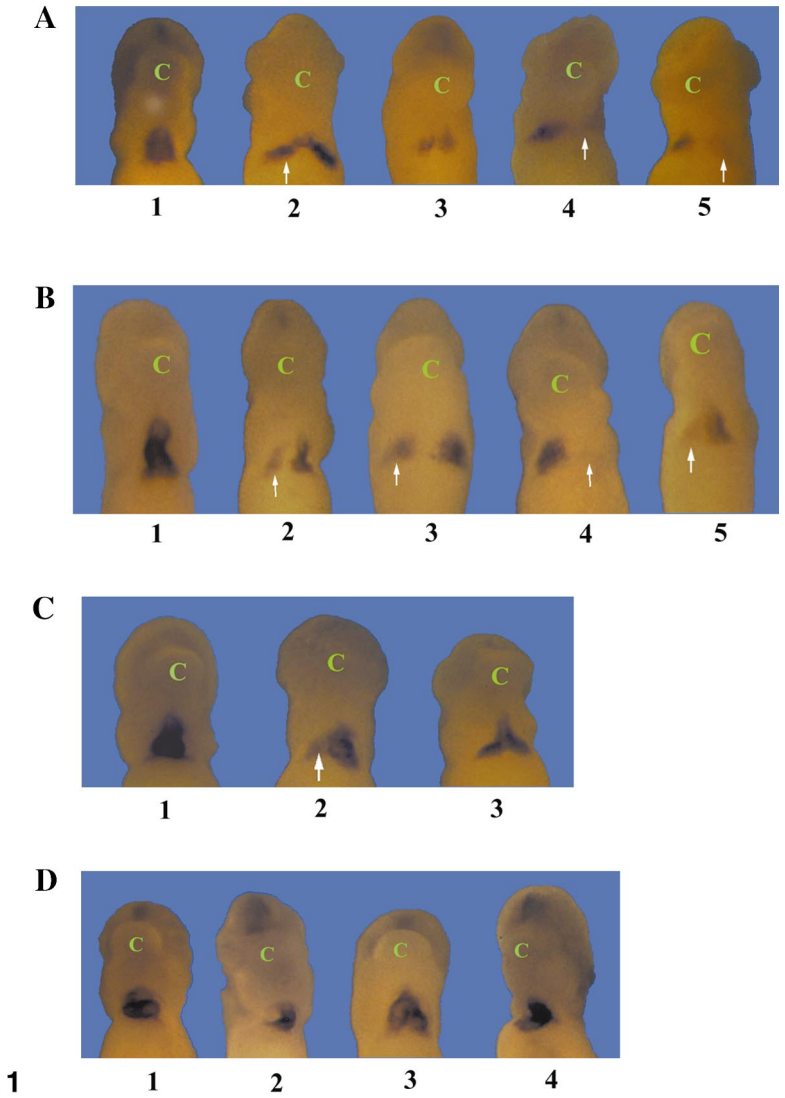
Embryos were fixed and processed as described (Harland, 1991), being stained with BCIP/NBT. The staining reaction was allowed to proceed from 2 h to overnight. Stained embryos were fixed and dehydrated prior to photographing.

Some specimens were sectioned after staining and were fixed 1–2 h, dehydrated in ethanol, permeabilized briefly in Histoclear followed by 2 × 20 min changes in 1:1 xylene:paraffin wax at 60°C, and embedded in paraffin wax. Sections (10 μm) were cut, dried, dewaxed by passing through Histoclear 2 × 10 min, mounted in Permount, and photographed.

RESULTS

Ectopic Expression of Dominant Negative BMP Receptors Adversely Affects the Expression of Differentiated Cardiac Markers and Results in Absence of Morphologically Recognizable Heart

To examine the requirement for BMP signaling in cardiac specification, our strategy was to ectopically express dominant negative BMP receptors by injecting DNA plasmids



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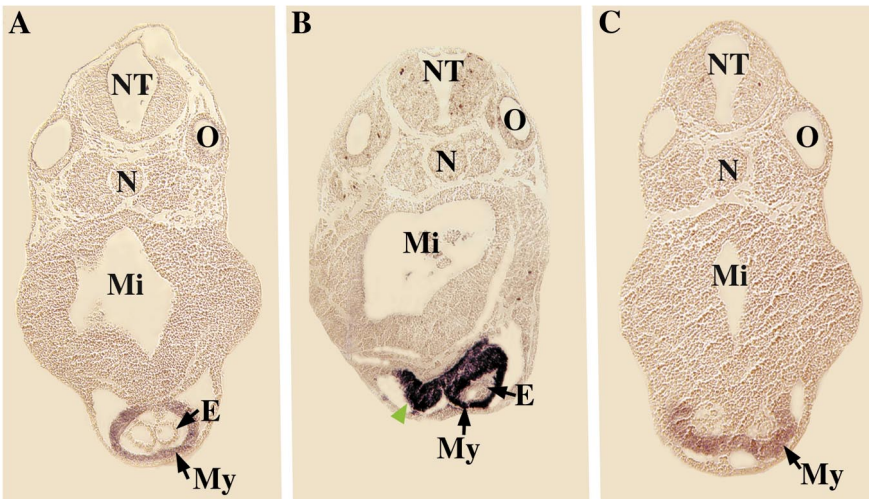

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encoding the receptor into *Xenopus* embryos. Control injections were performed with β-galactosidase expression vectors. To improve the efficiency of transgene expression, expression cassettes within each vector were bracketed by one copy of the inverted terminal repeat sequences (ITRs) from adeno-associated virus (AAV) (Fu *et al.*, 1998a).

The dominant negative BMP receptors which we utilized were well-characterized C-terminal truncations of the cytoplasmic domain (Frisch and Wright, 1998; Graff *et al.*, 1994; Suzuki *et al.*, 1994). Truncated receptors retain the ability to heterodimerize with wild-type partners, yet are unable to trigger a signaling cascade. Both Type I and Type II dominant negative BMP receptors have previously been used to block BMP signaling in *Xenopus* embryos.

In *Drosophila*, loss-of-function mutants of a Dpp receptor, *thickveins* (*tkv*), exhibit no dorsal vessel formation (Frasch, 1999). Tkv is a Type I serine/threonine kinase receptor and XALK3 is the *Xenopus* homologue of Tkv. Therefore, we thought it important to explore the effect of expressing truncated XALK3 (tXALK3) on heart formation in *Xenopus* embryos. However, evidence that Type I BMP receptors can interact with either BMP Type II or activin Type II receptors (Liu *et al.*, 1995; Rosenzweig *et al.*, 1995) raises the question as to the specificity of Type I dominant negative receptors. In addition, a truncated Type II receptor (XActRIIb) has been shown to block both activin and BMP signaling *in vivo* (Chang *et al.*, 1997). Recently, a BMP Type II receptor, *BMPRII*, has been cloned from *Xenopus*. Previous results in mammals identified BMPRII as the only Type II receptor which exclusively bound BMPs, but not activins or TGFβs (Liu *et al.*, 1995; Rosenzweig *et al.*, 1995). Consistent with this observation, dominant negative XBMPRII (tXBMPRII) was demonstrated to selectively block BMP, but not activin, signaling in *Xenopus* embryos (Frisch and Wright, 1998). Moreover, *XBMPRII* mRNA is expressed in cardiogenic regions (Frisch and Wright, 1998). Consequently, to ensure that we were specifically blocking BMP signaling with our dominant negative receptors, we performed injections with DNAs encoding tXBMPRII as well as tALK3.

Following injection into one cell at the two-cell stage, embryos were allowed to develop and subsequently scored by whole-mount *in situ* hybridization for the expression of

TABLE 2

Rescue of tBMPR Mutant Phenotypes by Co-injection with Wild-Type BMPRs and XSmad1

DNA	Amt inj (pg)	No. scored	Mutant (%)	*P* value[a]
CS2n-βgal	200	60	2 (3.0)	
CS2n-βgal	400	172	15 (8.8)	
CS2n-βgal	500	212	20 (9.4)	
tALK3	200	22	7 (32)	0.0156*
tALK3 + wtALK3	200 + 200	31	5 (16)	0.3973
tALK3 + wtALK3	200 + 300	27	3 (11)	0.8469
tBMPRII	200	143	38 (27)	0.0024**
tBMPRII + wtBMPRII	200 + 200	68	14 (21)	0.0850
tBMPRII + wtBMPRII	200 + 300	88	5 (5.7)	0.4334
tALK3	200	112	29 (26)	0.0039**
tALK3 + XSmad1	200 + 200	74	10 (14)	0.4305
tALK3 + XSmad1	200 + 300	75	7 (9.0)	0.9870

Note. Rescue experiments were performed by co-injection of dominant negative receptors and the wild-type constructs indicated, into one cell at the two-cell stage. Embryos were harvested at stages postdifferentiation and scored for the presence of mutant phenotypes by whole-mount *in situ* analyses for *XMLC2*. Increasing the amount of co-injected wild-type results in a corresponding decrease in observed mutant phenotypes, indicating that the observed phenotypes do not result from a nonspecific effect of DNA injection on cardiogenesis.

[a] *P* values were generated by χ^2 analyses and refer to the null hypothesis that experimental and control embryos are equivalent.

* The null hypothesis is rejected at a significance level of 0.05.

** The null hypothesis is rejected at a significance level of 0.01.

FIG. 1. Whole-mount RNA *in situ* analysis of heart differentiation markers following injection of dominant negative BMP receptor constructs. Embryos were injected into one cell at the two-cell stage with CS2n-βgal, tBMPRII, or tALK3, harvested at various stages postdifferentiation (St. 27/28), and stained for expression of mRNAs encoding *XMLC2* or *XcTnI*. Embryos shown are anterior ventral views. In each series, the leftmost embryo is a control injected with CS2n-βgal, illustrating normal heart development. In embryos injected with DNAs encoding tBMPRs, note abnormal fusion and reduced or absent staining on one side of the embryo. Later stage embryos (C and D) exhibit distinctly abnormal heart morphology. Embryos shown were stained for *XMLC2* mRNA, with the exception of the embryos shown in A, 5 and B, 5, which were stained for *XcTnI* mRNA. Comparable results were obtained with either marker. C, cement gland. White arrows indicate asymmetric staining. (A) St. 29/30: (1) CS2n-βgal injected, (2) tBMPRII injected, (3) tALK3 injected, (4) tALK3 injected, (5) tALK3 injected. (B) St. 31/32: (1) CS2n-βgal injected, (2) tBMPRII injected, (3) tALK3 injected, (4) tALK3 injected, (5) tALK3 injected. (C) St. 33/35: (1) CS2n-βgal injected, (2) tBMPRII injected, (3) tALK3 injected. (D) St. 38: (1) CS2n-βgal injected, (2) tBMPRII injected, (3) tBMPRII injected, (4) tALK3 injected.

FIG. 2. Section analyses of embryos stained by whole-mount *in situ* for expression of *XMLC2* mRNA. Sections shown are from the stage 35 embryos shown in Fig. 1C. (A) Control-injected embryo, showing normal heart formation. (B) tBMPRII-injected embryo, exhibiting abnormal cardiac development, with rather than a single uniform heart tube, a bipartite structure, in which one side exhibits both reduced heart tissue and absence of a lumen (green arrowhead). (C) tALK3-injected embryo, in which staining for expression of *XMLC2* mRNA reveals partially fused heart tubes. E, endocardium; Mi, midgut; My, myocardium; N, notochord; NT, neural tube; O, otic vesicle.

TABLE 3
Asymmetric *XNkx2-5* Expression in *Xenopus* Embryos Injected with tBMPRs

		St. 14–20			St. 21–30		
DNA	pg	No. scored	Mut (%)[a]	*P* value[b]	No. scored	Mut (%)	*P* value
CS2nβ-gal	200	119	2 (2.0)		81	4 (5.0)	
tALK3	200	104	2 (2.0)	0.3234	79	16 (20)	0.0330*
tBMPRII	200	101	1 (1.0)	0.7573	150	34 (23)	0.0079**

Note. Embryos were injected with either dominant negative BMP receptor into one cell of the two-cell stage, harvested at stages 14 through 30, and scored for the expression of *XNkx2-5* by whole-mount *in situ* analyses.

[a] Mut, mutant. Embryos which exhibited asymmetric reduction (20% or more) in staining for *XNkx2-5* expression were scored as mutant.

[b] *P* values were generated by χ^2 analyses and refer to the null hypothesis that experimental and control embryos are equivalent.

* The null hypothesis is rejected at a significance level of 0.05.

** The null hypothesis is rejected at a significance level of 0.01.

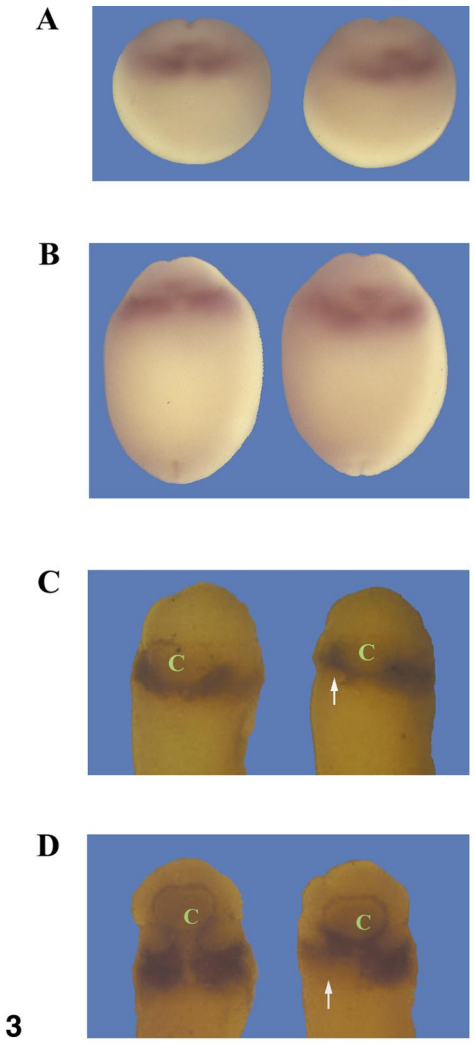
genes which are specifically expressed in differentiated heart, *XMLC2* or *XcTnI* (Drysdale *et al.*, 1994; Logan and Mohun, 1993). For each dominant negative receptor, we performed preliminary studies injecting from 100 to 400 pg of DNA to determine the dose of receptor which would give us a significant frequency of mutant phenotypes with the experimental cDNA constructs, but relatively low frequency of abnormal phenotypes with control β-galactosidase injections. We found that 200 pg appeared to be an optimal dose for either dominant negative receptor, and most of our experiments were performed utilizing this dose, unless otherwise indicated.

Cardiac mutant phenotypes were scored in embryos which appeared relatively normal phenotypically. In this manner we examined the relatively specific effects of blocking BMP signaling on cardiogenesis. Grossly abnormal embryos were not scored. Injection of AAV-ITR DNA plasmids into *Xenopus* embryos results in a distribution of embryos exhibiting varying amounts of transgene expression, in which some embryos express the transgene in virtually all descendants of the injected cell and others express in subsets of the descendants (Fu *et al.*, 1998a). In the case of the dominant negative receptor injections, it is likely that the grossly abnormal embryos were those in which the transgene was expressed early on in a large percentage of the descendents of the injected cell. Numerous studies have shown that BMP signaling is critical for early mesodermal patterning in *Xenopus* (Dale and Jones, 1999). In mice, ablation of several members of the BMP signaling pathway (*BMP4*, *ALK3*, *ALK2*, *Smad 4*) results in early embryonic lethality, confirming an early requirement for BMP signaling in order for normal development to proceed (Gu *et al.*, 1999; Mishina *et al.*, 1995; Sirard *et al.*, 1998; Winnier *et al.*, 1995).

Our results with injection of constructs encoding either tALK3 or tBMPRII were comparable. Injection of either receptor construct resulted in a statistically significantly increased frequency (26–27%) of mutant hearts relative to control embryos injected with the β-galactosidase expression vector (3.0%) (Table 1). Similar phenotypes were observed upon injection with either truncated BMP receptor and with scoring for either *XMLC2* or *XcTnI* expression.

FIG. 3. Whole-mount RNA *in situ* analysis of *XNkx2-5* expression following injection of dominant negative BMP receptor constructs. Embryos were injected into one cell at the two-cell stage with CS2n-βgal, tBMPRII, or tALK3 harvested from stages 14 through 30, and stained for expression of *XNkx2-5* mRNA. Embryos in A are anterior views. The remaining images show anterior ventral views. In each, the leftmost embryo is a control injected with CS2n-βgal, illustrating normal *XNkx2-5* expression. Following injection with either tBMPR, embryos harvested from stages 14 through 20 exhibited normal *XNkx2-5* expression, as represented in A and B. Embryos from later stages, however, exhibited asymmetric *XNkx2-5* staining, at both pre- and postdifferentiation stages, as represented in C and D. Embryos shown were injected with DNA encoding tBMPRII. Comparable results were obtained with tALK3. (A) St. 15, (B) St. 19, (C) St. 25, (D) St. 28.

FIG. 4. Section analyses of embryos stained by whole-mount *in situ* for expression of *XNkx2-5* mRNA. Green arrowheads indicate regions where *XNkx2-5* staining is reduced in response to ectopic expression of tBMPRs. (A) Control St. 25 (predifferentiation) embryo, demonstrating *XNkx2-5*-positive staining in both cardiogenic mesoderm and pharyngeal endoderm. (B) St. 25 embryo which was injected with tALK3, exhibiting downregulation of *XNkx2-5* in both pharyngeal endoderm and cardiogenic mesoderm. (C) St. 29/30 embryo (postdifferentiation) which was injected with tALK3, exhibiting asymmetric downregulation of *XNkx2-5* in cardiac mesoderm. CM, cardiogenic mesoderm; NT, neural tube; Mi, midgut; N, notochord; PE, pharyngeal endoderm.



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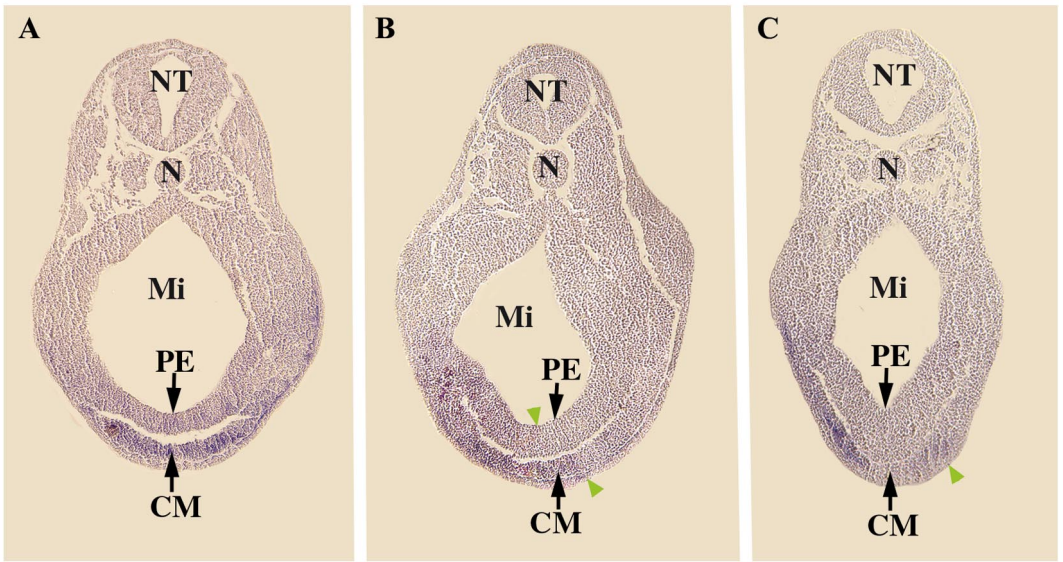

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Consequently, the summarized data represent embryos scored for either marker of cardiac differentiation. Results following co-injection of both dominant negative receptors were comparable to those obtained with each alone, suggesting that the two tBMPRs are blocking the same pathway (data not shown).

A range of mutant phenotypes was observed. Embryos scored as mutant were those in which *MLC2* or *cTnI* staining was asymmetric, either reduced or absent on one side (reflecting the asymmetric injection of the dominant negative receptor construct into one cell at the two-cell stage), or which exhibited delayed or abnormal fusion of cardiac primordia (Fig. 1). Notably, some embryos exhibited complete absence of expression of differentiated markers on one side. Embryos of later stages, when the heart has fully looped, were scored as mutant if hearts exhibited clearly abnormal morphology (Fig. 1D).

Section analysis was performed for some embryos following whole-mount RNA *in situ*, as shown by representative sections in Fig, 2. Examination of sections confirmed the impressions from whole-mount staining, demonstrating greatly reduced or absent cardiac mesoderm on one side (Fig. 2B) and incomplete fusion of heart tubes (Fig. 2C). As can be seen from Fig. 2B, the reduction in staining for *XMLC2* mRNA reflects a reduction in cardiac mesoderm per se.

Rescue of Cardiac Mutant Phenotypes by Co-injection of Dominant Negative Receptors with Their Wild-Type Counterparts or XSmad1

To confirm the specificity of action of the dominant negative receptors, rescue experiments were performed by co-injecting 200 pg of cDNA encoding a truncated receptor with 200 or 300 pg of its wild-type counterpart. Control injections were performed by injection of 400 or 500 pg of the β-galactosidase expression plasmid. As can be seen from Table 2, results of these experiments demonstrated that inclusion of wild-type receptor rescued the mutant phenotypes, in a dose-responsive manner. Co-injection of 200 pg of tBMPR plasmid and either 200 or 300 pg of the corresponding wild-type receptor plasmid resulted in a frequency of mutant phenotypes which was not significantly different from that observed in control injected embryos (see *P* values, Table 2).

Smads are downstream mediators of TGFβ family signaling, and Smads 1, 5, and 8 are considered specific for BMP signaling (Chen *et al.*, 1998). As an additional test of the specificity of action of tALK3, we performed rescue experiments co-injecting expression constructs for tALK3 and XSmad1. As for the wild-type receptor, expression of XSmad1 resulted in a dose-dependent rescue of the mutant cardiac phenotypes (Table 2), reinforcing the idea that the observed phenotypes were a result of blocking BMP signaling pathways.

Expression of Truncated BMP Receptors Results in the Downregulation of a Marker for Cardiogenic Mesoderm, XNkx2-5

To determine whether blocking BMP signaling affected earlier stages of cardiac mesoderm specification than examined by scoring for expression of differentiated markers, we harvested embryos from stages 14–30 following injection with either tALK3 or tBMPRII and scored for expression of *XNkx2-5* mRNA. *XNkx2-5* mRNA is first detectable by *in situ* hybridization at midgastrulation (approximately stage 14), when it is expressed in both cardiogenic mesoderm and pharyngeal endoderm (Tonissen *et al.*, 1994). Previous studies have demonstrated that XNkx2-5 is required for cardiac specification and heart formation (Fu *et al.*, 1998b; Grow and Krieg, 1998).

Results of our analysis of *XNkx2-5* expression in injected embryos are summarized in Table 3. We were unable to observe any effects of ectopic expression of the dominant negative BMPRs on *XNkx2-5* expression in embryos from stages 14–20. However, at later stages (21–30), we observed a statistically significant increase in the number of embryos exhibiting asymmetric staining for *XNkx2-5:* 20–23% versus 5% in control embryos (Table 3). Embryos stained for *XNkx2-5* mRNA and representative of observed phenotypes are shown in Fig. 3, including earlier stages with no apparent alteration in *XNkx2-5* staining following injection of tBMPR constructs (Figs. 3A and 3B) and later predifferentiated (Fig. 3C) and postdifferentiated stages (Fig. 3D) which exhibited asymmetric staining in response to ectopic expression of tBMPRs.

Section analysis of control and mutant embryos following whole-mount RNA *in situ* analyses demonstrated that *XNkx2-5* expression was asymmetrically downregulated in both pharyngeal endoderm and cardiogenic mesoderm, in pre- and postdifferentiation embryos, as shown in Fig. 4.

ALK3 and Smad1 Are Expressed in Cardiogenic Regions during Xenopus Development

Previous data have indicated that *XBMPRII* mRNA is expressed in cardiogenic regions during *Xenopus* development (Frisch and Wright, 1998). However, there are no published data on the expression of *ALK3* mRNA during *Xenopus* development. Some data have been published on the expression of *XSmad1* (Meersseman *et al.*, 1997; Thomsen, 1996); however, cardiogenic regions were not examined. Therefore, to investigate whether *ALK3* or *Smad1* is expressed in a manner consistent with their being involved in cardiogenesis, we performed whole-mount *in situ* analysis on embryos of various stages. Results of this analysis are shown in Fig. 5.

Strong staining for *ALK3* mRNA (Figs. 5A–5D) was observed in otic vesicle, in eye, in dorsoanterior neural tube, and in pharyngeal structures. Lower levels of staining were observed in anterior ventral and posterior ventral regions of the embryo and along the entire dorsolateral region. This staining was not observed with a sense control probe,

demonstrating its specificity (data not shown). The staining in anterior ventral regions overlaps with cardiogenic regions (indicated by arrowheads), suggesting that low levels of receptor are expressed in cardiogenic regions at all stages examined. The *ALK3* mRNA expression pattern overlaps, but is distinct from, that previously observed for BMP4 (Fainsod *et al.*, 1994). *BMP4* mRNA is strongly expressed in otic vesicle, eye, pharyngeal regions, and posterior and anterior ventral regions, the latter including cardiogenic regions. In contrast to *BMP4,* however, *ALK3* is not expressed in the midventral region (corresponding to the ventral blood islands).

Examination of *XSmad1* expression revealed a generally more restricted, yet partially overlapping pattern of expression compared to that observed for *XALK3* (Figs. 5E–5H). As observed previously, *XSmad1* was highly expressed in otic vesicle and cephalic neural crest (in migrating neural crest and pharyngeal crest derivatives) (Thomsen, 1996). At early stages (Figs. 5E and 5F), low levels of specific staining in anterior ventral regions coincide with cardiogenic regions; however, at later stages (Figs. 5Gand 5H), *XSmad1* does not appear to be expressed in cardiogenic regions.

DISCUSSION

BMP Signaling Is Required for Heart Formation and Expression of Differentiated Markers and Complete Fusion of Heart Primordia

Our results with whole-mount *in situ* analysis indicate that ectopic expression of either dominant negative ALK3 or BMPRII resulted in a significant number of mutant embryos with reduced or abrogated expression of two markers of the differentiated heart, *XMLC2* and *XcTnI* (Table 1 and Fig. 1). Sectional analyses indicated that the reduced or absent staining on the injected side reflected an absence of morphologically recognizable heart (Fig. 2). Another frequently observed mutant phenotype was incompletely fused heart primordia. Embryos harvested at various stages from the time of appearance of differentiated markers (stage 27/28) to a time when the heart is beating and looped (stage 38) revealed that blocking BMP signaling prevented normal fusion at all stages (Fig. 1). This confirms results previously observed in mouse embryos in which abnormal fusion of heart primordia was observed in embryos null for either *BMP2* or *Smad5* (Oh and Li, 1997; Zhang and Bradley, 1996).

The cardiac mutant phenotypes we observed could be rescued in a dose-responsive manner by increasing amounts of counterpart wild-type receptor, affirming that the phenotypes were not arising from a nonspecific effect of DNA injection (Table 2). In addition, rescue of mutant phenotypes by XMad1, a downstream mediator of BMP signaling, confirmed that the dominant negative Type I receptor, ALK3, is specifically interfering with BMP signaling.

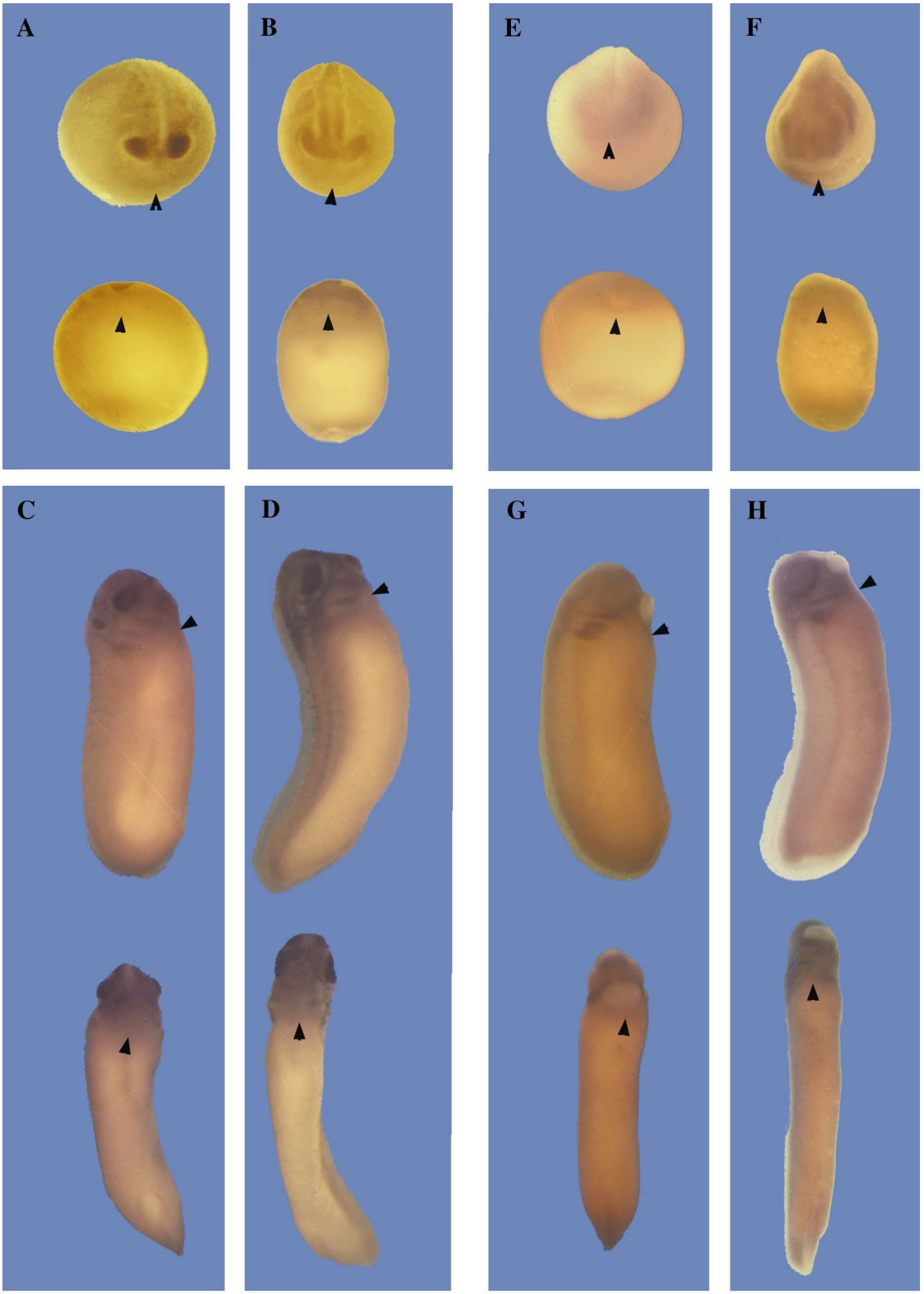
Together, our data prove that BMP signaling is required *in vivo* for heart formation and expression of differentiated markers.

BMP Signaling Is Required to Maintain Nkx2-5 Expression Prior to Differentiation

XNkx2-5 has been shown to be required for cardiac specification in *Xenopus* (Fu *et al.*, 1998b; Grow and Krieg, 1998). The observed decrease or absence of heart tissue in a significant proportion of mutant embryos following injection with dominant negative BMP receptors was reminiscent of phenotypes we had previously observed by injecting a dominant negative *XNkx2-5* construct into developing embryos (Fu *et al.*, 1998a). Accordingly, we examined *XNkx2-5* expression following ectopic expression of tALK3 or tBMPRII.

XNkx2-5 transcripts can be observed by RNase protection in very early gastrula, but appear to be strongly upregulated between stages 12 and 14 (Evans *et al.*, 1995). By whole-mount *in situ* analyses, *XNkx2-5* transcripts are first readily observed at stage 14 (late gastrula/early neurula). Early *XNkx2-5* expression occurs in both cardiogenic mesoderm and adjacent endoderm (Cleaver *et al.*, 1996; Evans *et al.*, 1995). Examination of *XNkx2-5* expression in embryos injected into one cell at the two-cell stage with tALK3 or tBMPRII indicated that *XNkx2-5* expression in embryos from stages 14–20 was unaffected. Bilateral heart primordia in anterior, lateroventral mesoderm at stage 15 begin to fuse at the anterior midline between stages 16 and 19. This fusion is accompanied by a ventralward migration so that by stage 20, the single heart anlage is located ventrally, caudal to the cement gland (Nieuwkoop, 1967). At these stages, *XNkx2-5* expression persists in both presumptive cardiac mesoderm and juxtaposed presumptive pharyngeal endoderm. Subsequently the *XNkx2-5*-positive endoderm migrates anteriorward relative to the cardiogenic mesoderm. Following the arrival of the heart primordium to its final ventral location, ectopic expression of tALK3 or tBMPRII resulted in a reduction or absence of *XNkx2-5* mRNA on the injected side of the embryo (Fig. 3). This downregulation of *XNkx2-5* expression occurs prior to differentiation and in differentiated heart and occurs in both pharyngeal endoderm and cardiogenic mesoderm (Fig. 4). The frequency of mutant phenotypes observed for *XNkx2-5* expression (20–23%) was comparable to the frequency of mutant phenotypes observed for the expression of differentiated markers (26–27%), suggesting that downregulation of *XNkx2-5* expression is correlated with reduction or absence of heart formation.

Our results suggest that initial *XNkx2-5* expression is not as sensitive to inhibition of BMP signaling following ectopic expression of tALK3 or tBMPRII as later, postgastrula expression. In *Drosophila* during early gastrulation, *tinman* is initially expressed throughout the mesoderm, then, postgastrulation, becomes restricted to dorsal mesoderm and is subsequently selectively maintained in cardiac mesoderm



(Bodmer *et al.*, 1990; Frasch, 1999). The early panmesodermal expression is dependent on regulation by an E-box transcription factor, Twist. However, restricted dorsal mesodermal expression is dependent on signaling by Decapentaplegic (Dpp), the *Drosophila* homologue of BMP. This regulation occurs through a Dpp-responsive element in the *tinman* promoter, which binds to Mad, Medea (homologues of Smads), and Tinman itself (Frasch, 1999). Intriguingly, the cardiac-specific element in the *tinman* promoter also appears to be dependent on Dpp signaling (Rolf Bodmer, personal communication), and the promoter element driving cardiac-specific expression contains essential CRE sites that may serve as Dpp-response elements (Venkatesh *et al.*, 2000). Although the initially observed expression of *XNkx2-5* does not occur in a panmesodermal manner as for *tinman*, our results suggest that initial expression of *XNkx2-5* during gastrulation, as with initial expression of *tinman*, may be independent of BMP signaling, or at least BMP signaling which is blocked by the tBMPRs we have utilized in these studies.

In chick, *cNkx2-5* expression is first observed in cardiac primordia and adjacent endoderm at stage 5 (Schultheiss *et al.*, 1995), comparable to the time at which *Xenopus Nkx2-5* is first observed. Exposure of chick precardiac mesendoderm to noggin, a BMP inhibitor, either by culturing explants in noggin-containing medium (Schultheiss *et al.*, 1995; Schlange *et al.*, 2000) or by implantation of noggin-expressing CHO cells *in vivo* (Schlange *et al.*, 2000), downregulates expression of *cNkx2-5* and other cardiac restricted genes and prevents heart development. This effect is observed when noggin is added between stages 4 and 8, but not following stage 8, suggesting that cardiac cells no longer require BMP signaling by the latter stage. In these experiments, embryos and explants were assayed 24–48 h following noggin addition, therefore an early independence of Nkx2-5 expression on BMP signaling, as seen in our study, would not have been observed. In our experiments, dominant negative BMP receptor expression is driven by the CMV enhancer, which is expressed in *Xenopus* during the earliest stages of gastrulation (Kroll and Amaya, 1995). Our results provide the first evidence that maintenance, not initiation, of *Nkx2-5* expression is dependent on BMP signaling postgastrulation, at least in frog embryos.

In vitro experiments performed with chick embryo explants have suggested that at least two successive inductive events occur to specify cardiogenic mesoderm (Ladd *et al.*, 1998). In the first, during early gastrulation (Hamilton Hamburger (HH) stages 2–3), the hypoblast can induce beating tissue in posterior epiblast (Yatskievych *et al.*, 1997) and in a second event, at late gastrulation (HH stage 5), anterior lateral endoderm (ALE) can induce the overlying mesoderm to activate cardiogenic markers and form beating tissue (Schultheiss *et al.*, 1995; Sugi and Lough, 1995; Yatskievych *et al.*, 1997). The inducing activities of hypoblast and ALE appear to be distinct. Activin can substitute for the former activity, whereas a combination of BMP and fibroblast growth factor can substitute for the latter. In *Xenopus* embryos, a combination of explant and ablation studies suggests that two tissues are required for induction of cardiogenic mesoderm during early gastrulation (Nieuwkoop–Faber stage 10): the dorsal organizer and deep anterior endoderm (Nascone and Mercola, 1995; Sater and Jacobson, 1990). Our results suggest that *Xenopus* cardiac specification, in a manner comparable to that seen in chick, may also involve a later, BMP-dependent event, to maintain *Nkx2-5* expression and perhaps expression of other cardiac-specification genes.

Expression of ALK3, BMPRII, and Smad1 mRNAs Is Consistent with Their Mediating BMP Signaling Which Is Required for Cardiogenesis

To investigate whether endogenous *ALK3* is expressed at the appropriate time and place to be mediating the observed effects of its dominant negative mutant on cardiogenesis, we performed whole-mount *in situ* analysis of *ALK3* mRNA expression during *Xenopus* development. To the best of our knowledge, these results represent the first published study of *XALK3* expression and indicate that *XALK3* is expressed at low levels in cardiogenic regions (Fig. 5). In concert with our results following ectopic expression of tALK3, our data suggest that *ALK3* is a candidate to be an endogenous mediator of BMP signals for critical aspects of cardiogenesis, including specification and heart formation. It is interesting to note that *BMPRII* expression at early neurula stages is observed in posterior

FIG. 5. Whole-mount *in situ* analysis of ALK3 and Smad 1 mRNA expression during *Xenopus* development. To examine expression of *ALK3* and *Smad1* in cardiogenic regions during development, we performed whole-mount RNA *in situ* analyses. Overall, expression of both mRNAs was relatively weak, yet specific staining was observed. Cardiogenic regions are indicated by black arrowheads (for reference, compare to staining for *XNkx2-5* as seen in Fig. 3). (A–D) Embryos stained for expression of *ALK3* mRNA. (E–H) Embryos stained for expression of *Smad1* mRNA. The staining patterns of the two genes are overlapping, but distinct. In stage 21/22 and early tailbud embryos, staining is observed in both anterior and posterior regions. This pattern overlaps with that previously observed for *BMP4* and *BMPRII* (Fainsod *et al.*, 1994; Frisch and Wright, 1998). *ALK3* is strongly expressed in eye and is also expressed in neural tube, head, and pharyngeal structures. *Smad1*, as previously published (Thomsen, 1996), is relatively strongly expressed in migrating cephalic neural crest and its derivatives, neural tube, head, and eye. For A, B, E, and F, upper embryos are shown in anterior view, oriented with dorsal at the top; lower embryos are ventral views, oriented with anterior at the top. For C, D, G, and H, upper embryos are lateral views, oriented with anterior at the top; lower embryos are ventral views, oriented with anterior at the top. (A and E) St. 17/18, (B and F) St. 21/22, (C and G) St. 25/26, (D and H) St. 28/29.

involuting mesoderm, but not in anterior, cardiogenic mesoderm, yet at late neurula/early tailbud stages appears to be strongly expressed in cardiogenic regions (Frisch and Wright, 1998). The appearance of *BMPRII* mRNA in cardiogenic regions coincides with the time at which we observe a dependence of *XNkx2-5* expression on BMP signaling, suggesting that availability of BMPRII may regulate the activation of BMP signaling to maintain *Nkx2-5* expression. Co-injection of DNA encoding tALK3 and tBMPRII did not increase the frequency of mutant phenotypes observed, suggesting that both receptors are blocking the same pathway to result in the observed phenotypes.

To confirm that tALK3 was targeting BMP signaling, we performed rescue experiments with XSmad1. We found that XSmad1 could rescue the cardiac mutant phenotypes resulting from ectopic expression of tALK3, in a dose-dependent manner. Our results suggest that XSmad1 may mediate heart formation downstream of BMP signaling. Accordingly, we investigated whether *XSmad1* is expressed in cardiogenic regions. Although some *in situ* results have been previously published for XSmad1 (Meersseman *et al.*, 1997; Thomsen, 1996), no data were presented on cardiogenic regions. Results of our whole-mount *in situ* analyses suggest that *XSmad1* is expressed at low levels in cardiogenic regions, at earlier stages examined, but not at later stages (Fig. 5). In concert with the rescue data, our data indicate that *XSmad1* may be involved in critical aspects of early cardiogenesis. In this regard, it is interesting to note that recent studies in developing chick embryos demonstrate that *cSmad1* is expressed at low levels in early cardiogenic regions between stages 6 and 7, and *cSmad5* is expressed weakly in cardiac cells at stage 8 (Yamada *et al.*, 1999).

Previous investigators have observed that individual truncated ALK receptors can be specific in their mode of action. In particular, it has recently been demonstrated that targeted ectopic expression of tALK2, but not tALK3 or tALK4, can affect heart looping (Ramsdell and Yost, 1999). Our results indicate that expression of tALK3 can affect heart formation, expression of *XNkx2-5* and differentiated markers, and fusion of heart primordia. Together with the previous results on heart looping, our results suggest that distinct BMP signaling pathways may mediate distinct aspects of heart formation.

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