Inhibition of FGF signaling converts dorsal mesoderm to ventral mesoderm in early Xenopus embryos

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Abstract

In early vertebrate development, mesoderm induction is a crucial event regulated by several factors including the activin, BMP and FGF signaling pathways. While the requirement of FGF in Nodal/activin-induced mesoderm formation has been reported, the fate of the tissue modulated by these signals is not fully understood. Here, we examined the fate of tissues when exogenous activin was added and FGF signaling was inhibited in animal cap explants of Xenopus embryos. Activin-induced dorsal mesoderm was converted to ventral mesoderm by inhibition of FGF signaling. We also found that inhibiting FGF signaling in the dorsal marginal zone, in vegetal-animal cap conjugates or in the presence of the activin signaling component Smad2, converted dorsal mesoderm to ventral mesoderm. The expression and promoter activities of a BMP responsive molecule, PV.1, and a Spemann organizer, noggin, were investigated while FGF signaling was inhibited. PV.1 expression increased, while noggin decreased. In addition, inhibiting BMP-4 signaling abolished ventral mesoderm formation induced by exogenous activin and FGF inhibition. Taken together, these results suggest that the formation of dorso-ventral mesoderm in early Xenopus embryos is regulated by a combination of FGF, activin and BMP signaling.

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1. Introduction

An important event during early vertebrate development is the formation of three germ layers (ectoderm, mesoderm and endoderm). Mesoderm formation is induced in the marginal zones of Xenopus blastula embryos by inductive signals originating in the vegetal cells. Subtypes of mesoderm are regionally specified into both dorsal and ventral mesoderms during or after mesoderm formation (Kessler and Melton, 1994; Smith, 1993, 1995).

Several mesoderm-inducing factors have been identified in Xenopus, belonging to the fibroblast growth factor (FGF) and transforming growth factor-\(\beta\) (TGF-\(\beta\)) families (Kessler and Melton, 1994; Kimelman and Kirschner, 1987). Bone morphogenetic protein (BMP) and activin, members of the TGF-\(\beta\) family and FGF can induce a wide variety of mesodermal response genes, including Xbra (Smith et al., 1991; Kimelman and Kirschner, 1987), Xhox3 (Ruiz i Altaba and Melton, 1989), Xwnt8 (Christian et al., 1991), and Xnot (von Dassow et al., 1993); these inducers have important differences in terms of mesoderm induction. Analyses of mesoderm inducer activity reveal that BMP induces ventral mesoderm (Dale et al., 1992; Dale and Wardle, 1999; Fainsod et al., 1994), and high concentrations of activin tend to induce dorsal and axial structures, such as the notochord and muscle, whereas FGF induces lateral mesoderm, including mesenchyme and mesothelium (Green and Smith, 1990). Moreover, in animal pole explants, high concentrations of activin induce dorsal mesoderm markers (Spemann organizer genes; chordin, noggin and goosecoid) and some neural markers (N-CAM and Otx2), while any concentration of basic fibroblast growth factors (bFGF) fails to induce organizer genes or neural markers (Green et al., 1990; Green and Smith, 1990; Smith, 1993).

There is evidence that FGF is not required for initial mesoderm induction but that it is required to maintain mesodermal fate through a regulatory loop involving FGF and Xbra (Saxas et al., 1994; Schulte-Merker and Smith, 1995). Xbra is an immediate early gene activated by mesoderm inducers, including members of the FGF and TGF-\(\beta\) families (Smith et al., 1991). Moreover, the FGF/Ras/Raf/MAPK cascade is required for both activin- and FGF-mediated mesoderm induction (Hartley et al., 1994; MacNicol et al., 1993; Whitman and Melton, 1992). Notably, FGF signaling is required for the full induction of

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mesoderm by activin; expression of dominant-negative FGF receptor (DNFR) prevents exogenous activin-induced dorsal mesoderm formation (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). There is little evidence, however, describing the effects of combined activin and FGF signaling on the induction of dorsal mesoderm formation, and the effect of FGF inhibition in the presence of activin is not clearly understood. Additionally the role of BMP signaling in this process is unknown.

In the present study, we demonstrate that dorsal mesoderm, induced by activin, was converted to ventral mesoderm when FGF signaling was inhibited. Moreover, we show that endogenous BMP signaling has a role in this process. These results suggest that mesodermal patterning in early Xenopus embryos requires the correct combination of signals.

2. Results

2.1. Inhibition of FGF signaling leads to activin-mediated dorsal to ventral mesoderm transformation

Previously, it was reported that DNFR blocks activin-mediated induction of mesodermal marker genes (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). DNFR reduces the expression of mesodermal genes (Xbra, Xnot, Xwnt8 and actin), which can be induced by either activin or FGF signaling. Here, we confirm that activin-mediated dorsal mesoderm formation is dependent on FGF signaling, and we have characterized the cell fate of the tissues investigated.

To characterize specific mesoderm types induced by various doses of activin, we performed animal cap assays after activin treatment. A low dose (0.05 ng/ml) of activin did not induce either ventral mesoderm (as shown by α-globin expression) or dorsal mesoderm (chordin and α-actin expression) but did induce the pan-mesoderm marker, Xbra, in ectoderm cells (Fig. 1A and B).

To confirm the role of FGF signaling in activin-mediated mesoderm induction, the expression of molecular markers was examined in activin treated (25 ng/ml) animal cap explants isolated from embryos injected with 0.25–4 ng of DNFR mRNA (lacking the intracellular tyrosine kinase domain). In early stage explants, the pan-mesoderm marker Xbra and dorsal mesoderm markers chordin and noggin, which are induced by activin, were notably inhibited by DNFR (Fig. 1C). In contrast, the ventral specific marker PV.1, repressed by activin, was recovered by DNFR addition (Fig. 1C). In later stage explants, the expression of the
dorsal mesoderm gene $\alpha$-actin was decreased by DNFR, which led to a strong induction of the ventral mesoderm marker $\alpha$-globin (Fig. 1D). However, the expression of endodermal genes was not significantly changed (Fig. 1D). Therefore these results show that inhibition of FGF signaling converts activin-mediated dorsal mesoderm to ventral mesoderm, indicating that FGF signaling may be able to modify the mesoderm-inducing capacity of activin.

2.2. Dorsal mesoderm induced by Smad2 is converted to ventral mesoderm by inhibition of an intracellular mediator of FGF (Ras)

Next, we investigated whether the determination of mesoderm fate, induced by the interaction of activin and FGF signaling, also occurred intracellularly at the molecular level via Smad2 (an intracellular mediator of activin signaling) and Ras (an intracellular mediator of FGF signaling). RT-PCR analysis was performed with both DNFR mRNA- (2 ng) and activin-treated animal cap cells. Notably, the expression of Smad2 mediated dorsal mesoderm genes, including a pan-mesoderm marker, were abolished by DNFR injection (Fig. 2A and B). Also, we found that expression of ventral specific genes repressed by Smad2 (GATA2 and PV.1) were restored to control levels by DNFR (Fig. 2A). In later stage explants, the expression of the dorsal mesoderm marker $\alpha$-actin (induced by Smad2), was repressed by DNFR. The ventral mesoderm marker $\alpha$-globin, however, was induced by Smad2/DNFR (Fig. 2B). Ras is an intracellular mediator of FGF signaling. To test whether dominant-negative Ras (DN-Ras) might affect ventral mesoderm formation in the presence of activin signaling, we examined the expression of $\alpha$-actin and $\alpha$-globin in the presence of either activin or Smad2 with DN-Ras. The expression of $\alpha$-actin induced by either activin or Smad2 was significantly reduced by DN-Ras, whereas $\alpha$-globin expression was notably increased under the same conditions (Fig. 2C and D).

Therefore, our results suggest that FGF signaling determines the fate of activin-mediated mesoderm and it requires the interaction of the intracellular signaling molecules Ras and Smad2.

2.3. Activin signaling, when FGF signaling is inhibited, mimics the ventral signaling molecules in animal cap explants

In this study, we have shown that both activin and Smad2-induced noggin expression was abolished by inhibition of FGF signaling, while PV.1 expression was increased (Figs. 1C and 2A). PV.1 belongs to the Xvent1 subfamily of the Xvent homeobox multigene family (Rastegar et al., 1999). To determine the activity of PV.1 and noggin genes promoter regions, embryos were co-injected with PV.1- or Noggin-luciferase reporter genes and DNFR. Animal caps dissected from embryos were incubated with activin

Fig. 2. Smad2, a activin signal mediator, induces ventral mesoderm by inhibition of FGF signaling. RT-PCR analysis of mRNA injected (2 ng) or activin treated animal cap cells. (A) Xbra (pan-mesoderm marker), Chordin/Noggin (dorsal mesoderm marker) and PV.1/GATA2 (ventral specific marker) PCRs at stage 11. (B, C and D) $\alpha$-Globin (later ventral mesoderm and blood marker) and $\alpha$-Actin (later dorsal mesoderm marker) PCR reactions at stage 24. EF-1$\alpha$, loading control; -rt, control reaction without reverse transcriptase; cont, animal cap samples obtained from non-injected embryos; we, whole embryo positive control.
(25 ng/ml) until stage 13. The animal cap explants were then harvested, and luciferase activity was measured.

PV.1-luciferase activity was inhibited in both activin-treated and Smad2-injected samples. As expected, the promoter activity of PV.1 was enhanced by co-expression of DNFR (Fig. 3A and C). However, Noggin-luciferase activity was increased by activin but downregulated by co-expression of DNFR (Fig. 3B). Therefore, the promoter assay indicates that activin/Smad signaling affects dorsal and ventral gene expression by inhibition of FGF signaling. The results are consistent with RT-PCR data indicating that activin signaling, when FGF signaling is inhibited, mimics the activity of ventral signaling molecules in animal cap explants.

2.4. The mesoderm-inducing activity of activin-like signals originating from the endoderm is modulated by FGF signaling during mesoderm formation

To verify whether activin-like signaling is also affected by FGF signaling in vivo, we established an artificial mesoderm development system, using recombination of animal cap explants and vegetal explants before mesoderm determination stages (Fig. 4A). Because several activin-like signals are localized in the vegetal cells of the embryos, vegetal explants recombined with an animal cap explant induce differentiation of the animal cap cells into mesoderm and epidermis rather than epidermal ones (Nieuwkoop, 1969; Woodland and Jones, 1987).

FGF signaling is found in the marginal zone and animal hemisphere but not in the vegetal hemisphere (Cornell et al., 1995). Therefore, animal cap explants were obtained from embryos injected with DNFR mRNA. The animal–vegetal conjugates were cultured until either stage 11 or 24 and the expression of several markers was then assessed. Control animal–vegetal fusion tissue expressed the pan-mesodermal marker Xbra and displayed low-level expression of α-globin and α-actin (Fig. 4B, lane 2 and C, lane 3). These results indicate that the mesoderm induction elicited under normal endogenous conditions was not specified into dorsal or ventral mesoderm in the absence of other growth factors. However, conjugates of un-injected animal caps and eFGF-injected vegetal poles abolished α-globin expression and increased α-actin expression (Fig. 4C, lane 2), suggesting that complete dorsal mesoderm induction requires FGF signaling as well as an activin-like signal (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). However, conjugates of DNFR expressing animal cap explants and vegetal pole inhibited the expression of α-actin (Fig. 4C, lane 1) while enhancing the expression of both GATA2 (Fig. 4B, lane 1) and α-globin (Fig. 4C, lane 1) compared to control samples (Fig. 4C).

Therefore, these observations indicate that activin-like signal originating from the endoderm may induce multipotent meso-endoderm, and FGF signaling may mediate the determination of these cells to either dorsal- or ventral-fate.
2.5. Inhibition of FGF signaling in the dorsal marginal zone converts dorsal mesoderm to ventral mesoderm

Based on the hypothesis that FGF signaling determines the dorso-ventral fate of mesoderm cells in the endoderm–ectoderm conjugation system, we further examined whether FGF signaling had a similar function in the dorsal marginal zone where the dorsal mesoderm is generated. To determine whether the dorsal marginal zone was also affected by FGF signaling in vivo, we dissected the dorsal marginal zone (DMZ) at stage 11 and incubated it in the presence of either SU5402, an inhibitor of the FGF receptor, or U0126, an inhibitor of Erk, a key component of FGF signaling (Fig. 5).

The dissected DMZs were cultured until either stage 11 or 24. The control DMZ tissue expressed the pan-mesodermal marker, Xbra, and dorsal specific markers, chordin and noggin at stage 11, and α-actin at stage 24 (Fig. 5A, lane 1 and B, lane 1). When DMZ tissues were treated with FGF signaling inhibitors, expression of dorsal specific genes was diminished, while that ventral specific genes (GATA2 at stage 11, and α-globin at stage 24) significantly increased (Fig. 5A, lane 1 and 2, and B, lane 1 and 2).

Additionally, we tested the effect of FGF in the ventral marginal zone (VMZ), where the ventral mesoderm is specified (Kumano et al., 1999). The mRNA of eFGF was injected into the VMZ of 4-cell stage embryos and VMZ tissues were dissected at stage 11. Isolated VMZ tissues were cultured until stage 24 and the expression of α-actin and α-globin was analyzed by RT-PCR. As expected, injection of eFGF into VMZ induced α-actin and inhibited α-globin (Fig. 5C).

These results confirm that complete dorsal mesoderm formation requires FGF signaling (Cornell and Kimelman, 1994; Labonne and Whitman, 1994). In addition, our observations indicate that dorso-ventral patterning of mesoderm cells is affected by the presence and absence of FGF signaling. Taken together, these results suggest that inhibition of FGF signaling in the dorsal marginal zone converts the dorsal mesoderm to ventral mesoderm.

2.6. Inhibition of BMP-4 signaling abolishes ventral mesoderm formation induced by activin and DNFR

The BMP signaling pathway is essential for epidermal differentiation and ventral mesoderm formation (Dale et al., 1992; Dale and Wardle, 1999; Fainsod et al., 1994). We have shown that the inhibition of FGF signaling converts activin-mediated dorsal mesoderm to ventral mesoderm in animal cap explants and that the activity of activin mimics that of ventral signaling molecules. To determine the role of BMP signaling in activin/DNFR-mediated
Fig. 6. The expression of dominant-negative BMP-4 receptor blocks globin expression induced by activin and DNFR, and the inhibition of Smad1 (via activin), was recovered by inhibition of FGF signaling. Animal cap explants injected with 2 ng of DNFR and/or 2 ng of dominant-negative BMP-4 receptor (DNBR) were dissected and incubated in media with activin (25 ng/ml) until stage 24 (A). (B and C) Animal cap cells expressing mRNA (1 ng) or activin (25 ng/ml) were used for western blot (B and C) or RT-PCR. (D) Chordin (dorsal mesoderm marker) and PV.1/GATA2 (ventral mesoderm marker) PCRs at stage 11. α-Globin (late ventral mesoderm and blood marker) and α-Actin (late dorsal mesoderm marker) RT-PCRs at stage 24. EF-1α, loading control; -rt, control reaction without reverse transcriptase; cont, animal cap samples obtained from non-injected embryos; we, whole embryo positive control.

dorso-ventral patterning, animal cap explants were injected with 2 ng of DNFR and/or 2 ng of dominant-negative BMP receptor (DN-BR), dissected and incubated in media with activin (25 ng/ml) until stage 24. Interestingly, though inhibition of BMP signaling completely blocked the induction of α-globin induced by activin and DNFR (Fig. 6A), α-actin was recovered by co-injection with DNBR. These results indicate that inhibition of BMP-4 signaling abolishes activin/DNFR-mediated ventral mesoderm formation.

BMP-4 signaling, via the phosphorylation of Smad1, is essential for ventral mesoderm formation (Faure et al., 2000). To determine the role of phospho-Smad1 in the ventral mesoderm formation processes, we analyzed phospho-Smad1 levels in the animal cap system when exogenous activin was added and FGF signaling was inhibited. Animal caps isolated from embryos injected with DNFR mRNA were cultured in the presence of 25 ng/ml of activin until stage 12. Phosphorylation of Smad1 was detected by western blot with an phospho-Smad1 antibody, and total protein levels in the samples were normalized. Endogenous Smad1 phosphorylation was abolished in activin/DNFR RNA injected embryos; we, whole embryo positive control.

3. Discussion

In the present study, we have shown that FGF signaling is required for dorsal mesoderm formation and that the inhibition of FGF signaling converts dorsal mesoderm to ventral mesoderm. A previous study showed that in mammalian cells, phosphorylation of a linker region of Smad2, mediated by Ras via Erk/MAP kinases, causes (Kretzschmar et al., 1999) cytoplasmic retention of Smad2 and therefore inhibition of TGF-β-induced transcriptional responses (Kretzschmar et al., 1999). However, in Xenopus, FGF/Ras/Erk signaling is required for activin/Smad2 mediated dorsal mesoderm formation and transcriptional responses. Therefore, we tested the effect of Smad2-EPSM (Erk/Pro-directed kinase site mutant construct) with serine to alanine or threonine to valine mutations in the Smad2 linker region. Interestingly, when we assayed a Smad2 specific reporter gene, Smad2-EPSM showed almost the same activity compared with that of wild type Smad2 (data not shown). The expression of Xbra and actin was also retained in animal cap explants obtained from embryos injected with Smad2-EPSM (Supplementary data 2). Additionally, co-treatment of Smad2-EPSM and DNFR converted dorsal mesoderm to ventral mesoderm at levels similar to that of wild type Smad2 (Supplementary data 2). Together, the results indicate that dorsal mesoderm formation activity of Smad2 in Xenopus is not significantly affected by EPSM mutations. Therefore, the results exclude the involvement of phosphorylation event of the linker region of Smad2 by FGF/Ras/Erk signaling in the dorsal to ventral mesoderm transformation processes.

Cornell et al. (1995) hypothesized that an activin-like signal gradient is present from a high concentration near the vegetal hemisphere to a lower concentration in the equatorial region. At the same point in development, FGF signaling is present in the marginal zone and animal hemisphere. Thus, mesoderm is induced in the marginal zone, where activin and FGF signals overlap.
Key regulators of activin (phospho-Smad2) and FGF (phospho-MAPK) are co-localized in the marginal zone and originate dorsally during early developmental stages (Schohl and Fagotto, 2002; Christen and Slack, 1999). Specifically, MAPK is activated in the marginal zone, at first stronger dorsally than ventrally. These studies suggest that FGF signaling is involved in the specification of dorsal and ventral mesoderm. Recent data also suggest that FGF signaling plays a role in dorso-ventral patterning of the mesoderm (Branney et al., 2009; Fletcher and Harland, 2008).

Other studies have shown that ventral injection of eFGF suppresses ventral blood island (VBI) formation, whereas expression of DNFR results in a dramatic expansion of the VBI (Kumano and Smith, 2000; Xu et al., 1999). Additionally, it has been reported that FGF4 is required for the restriction of the ventral blood island by activating the myogenic program (Isaacs et al., 2007). Our present study provides the functional role of FGF signaling in dorso-ventral specification, in which the gradient of FGF signaling from dorsal to ventral is crucial. If a gradient of FGF concentration is not formed dorsally to ventrally, that of FGF activity may be generated in the marginal zone through the action of BMP-4 signaling. Because BMP-4 signaling prevents MAPK activity in ventral ectoderm, inhibition of BMP-4 signaling by extracellular BMP antagonists leads to an increase of MAPK activity in dorsal neuroectoderm (Goswami et al., 2001).

Based on the hypothesis that BMP-4 signaling is known to be essential for ventral mesoderm and ectoderm formation, we investigated its role in ventral mesoderm formation induced by activin and DNFR. Inhibition of BMP-4 signaling abolished ventral mesoderm formation (Fig. 6A, lane3). Additionally, when FGF signaling was inhibited and activin signaling was activated in animal cap explants, the levels of BMP-4 expression and phospho-Smad1, an intracellular mediator of BMP-4, were recovered to levels consistent with that of the control (Fig. 6B and Supplementary data 1). Interestingly, co-injection of DNFR dorsalized the mesoderm ventralized by addition of DNFR and exogenous activin (Fig. 6A, lane 3). Because FGF signaling inhibits BMP signaling via phosphorylation of the linker region of Smad1 (Pera et al., 2003), it is possible that inhibition of FGF signaling may lead to the recovery of BMP-4 signaling and the ventralization of the mesoderm (Fig. 6A, lane2). Therefore, it is possible that FGF inhibition ventralizes the mesoderm, at least partially, through BMP activation. This may explain why DNFR can dorsalize the mesoderm ventralized by DNFR. However, both the levels of BMP-4 expression and phospho-Smad1 were not increased compared to untreated control animal cap explants, suggesting that ventral mesoderm formation caused by activin and DNFR may not be dependent on BMP-4 signaling alone.

A dorsal to ventral FGF gradient may be crucial for dorso-ventral patterning of both ectoderm and mesoderm. FGF signaling is required for dorsal mesoderm formation as well as for neuroectoderm (dorsal ectoderm). Inhibition of both FGF and BMP signaling caused a loss of neuroectoderm in animal cap explants. Therefore, gradients of BMP and FGF signaling from ventral to dorsal and from dorsal to ventral, respectively, may be crucial for the dorso-ventral patterning of the ectoderm as well as the mesoderm.

In this study, strong inhibition of FGF signaling frequently led to loss of the pan-mesoderm marker Xbra, but the expression of ventral specific markers GATA2 and PV.1 and z-globin expression were retained. To limit the role of FGF signaling in dorso-ventral patterning of mesoderm, we used vegetal-animal conjugates and marginal explants in which the mesoderm is actually formed. We also used low doses of FGF inhibitors (DNFR, SU5402) to maintain Xbra expression (Fig. 1C, lane 2 and 3 and Supplementary data 3). Inhibition of FGF signaling in the DMZ induced z-globin, SCL (hematopoietic marker) and FoxF1 (lateral plate marker), indicating that inhibition of FGF signaling in the DMZ induces not only ventral mesoderm but also lateral mesoderm (Supplementary data 3). This result further supports the hypothesis that a gradient of FGF signaling may be important for the dorso-ventral patterning of the mesoderm. Under the same conditions, the expression of ventral specific genes GATA2 and PV.1 was increased in DMZs treated with SU5402 at early developmental stage (Supplementary data 3). Although GATA2 and PV.1 are not specific markers for ventral mesoderm, these results suggest that the presence or absence of FGF signaling affects the patterning of the mesoderm. Additionally, conjugates of animal cap and dorsal vegetal regions (DVRs) (used as an inducer of an activin-like signal) treated with SU5402 (50 mM/ml), promoted z-globin expression and inhibited z-actin expression. However, conjugates of animal cap and ventral vegetal regions (VVrs) treated with bFGF (20 ng), promoted z-actin expression and inhibited z-globin expression (Supplementary data 4).

FGF signaling affected dorsal and ventral fates of mesodermal cells in the DMZ and VMZ (Fig. 5). Taken together, these results suggest that active FGF signaling leads to dorsal mesoderm formation, while inhibition of FGF signaling leads to ventral mesoderm formation.

In this report, we have demonstrated that active FGF signaling leads to dorsal mesoderm, while inhibition of FGF, together with activin-like signaling, leads to ventral mesoderm. Here, we propose that the dorso-ventral patterning of the mesoderm is determined by mesoderm modifying factors such as BMP-4, BMP antagonists (Spemann organizer molecules) and FGF. BMP-4 and FGF are mesoderm inducers as shown by expression of the marker Xbra. They may also act as mesoderm modifiers of pan-mesoderm induced by the activin-like signal originating from the endoderm. The dorso-ventral specification of the pan-mesoderm may not be possible without a combination of mesoderm modifiers. Spemann organizer molecules inhibit BMP signaling and contribute to dorsal mesoderm formation induced by endogenous FGF in the dorsal marginal zone. In the ventral marginal zone, FGF signaling may be reduced by BMP signaling and is therefore too low to induce the dorsal mesoderm in collaboration with activin signaling. Therefore, this region may be differentiated into ventral mesoderm with a high level of BMP-4 in the ventral marginal zone. In this model, the dorsal to ventral gradient of FGF is crucial. The activity of FGF and BMP signaling in the marginal zone may be regulated by reciprocal signal inhibition. In addition, the expression of various FGF molecules, receptors and inhibitors may also be regulated by activin-like, BMP-4 and other signaling pathways through unknown mechanisms.

4. Materials and methods

4.1. Embryo injection and animal cap assay

_Xenopus laevis_ embryos were obtained by artificial fertilization (Smith and Slack, 1983). Developmental stages were designated as described by Nieuwkoop and Faber (1967). Vitelline membranes were removed by immersing embryos in a 2% cysteine solution (pH 8). Embryos at the one- or two-cell stage were injected with mRNA or DNA into the animal pole as described in the figure legends. Animal caps, the area around the animal (pigmented) pole of the blastula embryos, were dissected from the injected embryos at stage 8–9 and cultured to various stages in 67% Leibovitzs L-15 medium (Invitrogen, Carlsbad, CA) containing BSA (1 mg/ml), 7 mM Tris–HCl (pH 7.5) and gentamicin (50 µg/ml) for 1 or 2 days. When used, activin was added to L-15 medium.
4.2. In vitro transcription

All synthetic mRNAs used for microinjection were produced by in vitro transcription. Dominant-negative FGF receptor (DNFR; Amaya et al., 1991), dominant-negative ras (DN-ras; Asn17 JH4-Ras; Whitman and Melton, 1992) and dominant-negative BMP receptor (DNBR; Suzuki et al., 1994) were inserted into the pSp64T vector. Smad2 and Smad2-EPSPM were subcloned into the CS2(+) vector (Kretzschmar et al., 1999). Each of the cDNAs was linearized and used for in vitro transcription of capped mRNA using an in vitro transcription kit (Ambion) according to the manufacturer’s instructions. The resulting RNA was quantified by ethidium bromide staining and compared to an RNA standard. All data shown in this paper are from single experiments; however, in all cases the results were confirmed in multiple independent trials.

4.3. Total RNA isolation

Total RNA was extracted from whole embryos or cultured explants using TRIzol reagent (Tel-Test Inc.) following the manufacturer’s instructions. Briefly, the homogenate (500 μl) was added to 100 μl of chloroform, shaken vigorously by hand for 15 s and incubated for 5 min at room temperature. The homogenate was centrifuged at 12,000 × g for 15 min at 4 °C, and the RNA-containing aqueous phase was transferred to a fresh tube and an equal volume of isopropanol was added. The RNA was purified by ethanol precipitation and collected in DEPC-treated water.

4.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription was performed using the Superscript pre-amplification system (Invitrogen). Reverse transcription was performed in the presence of oligo dT-adapter primers with reverse transcriptase and the supplied buffer. The cDNA reaction was performed in the presence of oligo dT-adapter primers with reverse transcriptase and the supplied buffer. The cDNA reaction was performed in the presence of oligo dT-adapter primers with reverse transcriptase and the supplied buffer. The cDNA reaction was performed in the presence of oligo dT-adapter primers with reverse transcriptase and the supplied buffer. The cDNA reaction was performed in the presence of oligo dT-adapter primers with reverse transcriptase and the supplied buffer.

4.5. Luciferase assay

Luciferase activity was measured using a luciferase assay system according to the manufacturer’s instructions (Promega). A 2525 bp 5’ flanking region of PV.1 was cloned into pG2Luciferase plasmid DNA using Xhol and HindIII restriction enzymes. A 1556 bp 5’ flanking region of noggin was cloned into pG3Luciferase plasmid DNA with KpnI and BamHI restriction enzymes (Tao et al., 1999). PV.1 or noggin-luciferase plasmid DNA was injected either alone or together with synthetic mRNAs into one-cell stage embryos or two blastomeres of two cell stage embryos. After injection, animal caps were removed at stage 8.5–9 and cultured in the presence of activin until stage 13 when they were harvested. Four to five animal caps per group were pooled and homogenized in 10 μl of lysis buffer per explant. Luciferase activity was measured using a luminometer with 50 μl luciferase substrate and 10 μl animal cap lysate. All experiments were repeated at least three times using independent samples.

4.6. Western blot analysis

Animal caps were dissected from the injected embryos or un-injected embryos at stage 8–9 and cultured either in the presence or absence of activin and SU5402 containing L-15 medium until stage 12. Twenty to thirty animal cap explants were lysed in 20–30 μl of lysis buffer (20 mM Tris (pH8), 137 mM NaCl, 1% NP-40, 10% Glycerol) containing 1 mM PSMF, 4 mM Na3VO4, 1.5 mM β-glycerophosphate and 1 × protease inhibitor cocktail (Calbiochem). 40–60 μl of Freon (1,1,2-Trichlorotrifluoroethane, HPLC Grade, Sigma-Aldrich) was added and mixed for 5 s. Embryo extracts were cleared by centrifugation at 12,000 rpm for 15 min. Between 5 and 6 μg of protein was separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore). The membranes were incubated with antibodies against actin (AC-40, Sigma) and phospho-Smad1 (Cell signaling) followed by reaction with horseradish peroxidase-linked anti-mouse IgG (NEB) or anti-rabbit IgG. Immunoreactive bands were detected by the ECL western blotting detection system (Amersham) following the manufacturer’s instruction.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.diff.2011.05.009.

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