Bmp-4 acts as a morphogen in dorsoventral mesoderm patterning in *Xenopus*

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**Summary**

The marginal zone is a ring of tissue that gives rise to a characteristic dorsoventral pattern of mesoderm in amphibian embryos. *Bmp-4* is thought to play an important role in specifying ventral mesodermal fate. Here we show (1) that different doses of *Bmp-4* are sufficient to pattern four distinct mesodermal cell types and to pattern gene expression in the early gastrula marginal zone into three domains, (2) that there is a graded requirement for *Bmp* signal in mesodermal patterning, and (3) that *Bmp-4* has long-range activity which can become graded in the marginal zone by the antagonizing action of noggin. The results argue that *Bmp-4* acts as a morphogen in dorsoventral patterning of mesoderm.

Key words: *Bmp-4*, mesoderm, noggin, *Xenopus*, Xvent, XMyf-5

**Introduction**

Pattern formation of mesodermal tissues is a central process during vertebrate embryogenesis and is a determinant for the vertebrate body plan. In Amphibia, embryonic mesoderm arises from a ring of cells, the marginal zone, which is located between the animal and vegetal pole of the blastula/gastrula stage embryo. The marginal zone will give rise to a characteristic tissue pattern whose dorsoventral (d/v) sequence in the tadpole is notochord, muscle, pronephros and blood (Dale and Slack, 1987a,b).

Support for the possibility that a TGF-β-type growth factor acts as a morphogen in vertebrate mesoderm patterning comes from the observation that increasing concentrations of activin and Vg1 induce a ventrodorsal sequence of mesoderm from uninduced animal cap cells (Green and Smith, 1990; Green et al., 1992; Gurdon et al., 1994; Kessler and Melton, 1995). However, while this strongly supports the notion of a morphogen functioning in d/v patterning, there is neither evidence for a graded requirement nor a graded distribution of an activin-like molecule at present. Furthermore, there is recent evidence that both activin and Vg1 pattern mesoderm indirectly through a relay mechanism (Reilly and Melton, 1996a). This has left unresolved the question of the natural morphogen.

It has become clear that d/v axial patterning in *Drosophila* and *Xenopus* may involve conserved extracellular signals but inversed polarity (reviewed in Arendt and Nübler-Jung, 1994; Jones and Smith, 1995; De Robertis and Sasai, 1996; Ferguson, 1996). In *Drosophila*, *decapentaplegic* (*dpp*), a TGF-β-type growth factor, serves as a morphogen in the blastoderm that induces increasingly dorsal cell fate with increasing dose (Ferguson and Anderson, 1992; Wharton et al., 1993) and is antagonized by *short gastrulation* (*sog*) (Francois et al., 1994; Francois and Bier, 1995; Holley et al., 1995). Different concentrations of *dpp* have also been shown to play an important role in anteroposterior patterning of the *Drosophila* wing (Lecuit et al., 1996; Nellen et al., 1996).

A *Xenopus* homolog of *dpp*, bone morphogenetic protein 4 (*Bmp-4*) is expressed in the early gastrula marginal zone and is thought to play an important role in maintaining ventral mesoderm and antagonizing the organizer with the onset of zygotic transcription (Köster et al., 1991; Dale et al., 1992; Jones et al., 1992, 1996a; Suzuki et al., 1993, 1994; Fainsod et al., 1994; Graff et al., 1994; Maeno et al., 1994; Schmidt et al., 1995b, 1996; Steinbeisser et al., 1995). *Bmp-4* is antagonized by noggin (Smith and Harland, 1992; Smith et al., 1993; Harland, 1994; Reem-Kalma et al., 1995; Holley et al., 1996) and *chordin* (Sasai et al., 1994; Holley et al., 1996) and *chordin* (Sasai et al., 1994; Holley et al., 1996; Schmidt et al., 1995a), a gene related to *Drosophila sog* (Francois and Bier, 1995). Both noggin and *chordin* encode secreted proteins expressed in the gastrula dorsal lip, which bind with high affinity and thereby inactivate *Bmp-4* protein (Piccolo et al., 1996; Zimmerman et al., 1996). Since the counteracting activities of *dppBmp-4* and *sog/chordin* are conserved between insects and vertebrates these results raise the question of whether *Bmp-4* acts as a morphogen in d/v patterning of the gastrula marginal zone (Ferguson, 1996; Hogan, 1996; Holley et al., 1996; Piccolo et al., 1996; Zimmerman et al., 1996). If *Bmp-4* acts as a vertebrate morphogen for mesodermal patterning it should fulfill certain criteria in addition to being expressed at the right time and place. (i) It should elicit dose-dependent effects, (ii) there should be a graded requirement for *Bmp-4*, (iii) its activity should be graded in vivo and (iv) indirect (relay) effects should be excluded.

In this study, we have addressed these criteria to specifically investigate the role of *Bmp-4* on d/v patterning of mesoderm in the post-blastula embryo, when *Bmp-4* is active (Jones et al., 1996a), by investigating marginal zone patterning, not...
mesoderm induction in animal caps. We find (1) that different doses of Bmp-4 are sufficient to pattern four distinct mesodermal cell types and to pattern gene expression in the early gastrula marginal zone into three domains, (2) that there is a graded requirement for a Bmp signal in mesodermal patterning, and (3) that Bmp-4 has long-range activity which can become graded in the marginal zone by the antagonizing action of noggin.

MATERIALS AND METHODS

Embryos and explants
In vitro fertilization, embryo culture, staging, microinjection and culture of marginal zone explants and animal caps were carried out as described (Niehrs and De Robertis, 1991). To dorsalize embryos LiCl treatment was performed at the 32-cell stage by incubating embryos for 40 minutes in 0.12 M LiCl and subsequent washing. Explants were cultured in 0.5x MBS/0.2% gamma-globulin until sibling embryos reached stage 35. Explants were fixed and processed for histology as described (Niehrs et al., 1994).

cDNA library screening
The XMyf-5 clone (pXMyf-5) was isolated by screening a neurula stage plasmid library (cloned in pBS II KS) by in situ whole-mount hybridisation as described (Gawantka et al., 1995). Xsmad1 (Graff et al., 1996) was isolated from the same library by PCR (kind gift of Dr. D. Huylenbroeck). Chordin was isolated by PCR.

Whole-mount in situ hybridisation and β-Gal staining
Whole-mount in situ hybridisation was performed according to the protocol of Harland (Harland, 1991) with modifications (Gawantka et al., 1995). Staining for β-Gal with X-Gal was as in Sanes et al. (1986).

Microinjection experiments
pXsmad1 was cloned into pCS2 (Rupp et al., 1994), linearized with Asp718 and transcribed with SP6 RNA polymerase using the Megascript kit (Ambion) and a cap:GTP ratio of 5:1. pBmp-4 (Fainsod et al., 1994) was linearized with XhoI and transcribed with T3 RNA polymerase. ΔmTFR11 (Suzuki et al., 1994) was linearized with EcoRI and transcribed with SP6 RNA polymerase. nogginΔ5 (Smith and Harland, 1992) was linearized with NcoI and transcribed with SP6 RNA polymerase. β-galactosidase mRNA was made from pSp64TNLSGal (Lemaire et al., 1995) by linearization with XhoI and transcribing with SP6 RNA polymerase. Radial injection refers to microinjection of all four blastomeres of 4-cell-stage embryos into the equatorial region.

RT-PCR
Quantitative RT-PCR assays were carried out as described previously (Gawantka et al., 1995) with the primers described therein. The primers used for EF1-α were upstream: TGGCATTTGACATGATCCC; downstream: TACTATTTAATCTTGATGGCC; 285 bp.

RESULTS

Dose-dependent patterning of terminal differentiation by Bmp-4
Microinjection of Bmp-4 mRNA as well as treatment with Bmp-4 protein have been shown to induce ventral mesoderm from uninduced animal cap explants and microinjection of Bmp-4 mRNA into early embryos or dorsal marginal zones results in complete ventralization of mesodermal differentiation. This ventral mesoderm will differentiate into mesenchyme and blood (Köster et al., 1991; Dale et al., 1992; Jones et al., 1992; Suzuki et al., 1993; Schmidt et al., 1995b; Reem-Kalma et al., 1995; Steinbeisser et al., 1995). However, if the local concentration of Bmp-4 controls distinct mesodermal cell fates at gastrula stages in the marginal zone, increasing Bmp-4 doses should induce more than just ventral-most mesoderm from dorsal explants.

To test if Bmp-4 could induce multiple mesodermal tissues in a dose-dependent manner, Bmp-4 mRNA was radially microinjected at different doses into all blastomeres of 4-cell-stage embryos in the prospective marginal zone. At the early gastrula stage, dorsal marginal zones (DMZ) were explanted and cultured until late tadpole stage, when they were analyzed by histology. Fig. 1 shows that increasing Bmp-4 doses inhibited notochord formation and led to successive peaks of muscle, pronephros and blood differentiation. The results show that Bmp-4 ventralizes dorsal mesoderm in a dose-dependent manner. Thus, Bmp-4 not only induces ventral mesodermal cell fate but also muscle and pronephric cell fate at distinct concentrations, i.e. specifies multiple mesodermal cell types. Dose-dependent effects of Bmp-4 on DMZ were confirmed by using molecular markers specific for notochord, muscle and blood (data not shown).

We next aimed to reduce Bmp-4 signalling dose-dependently in order to investigate if there is a graded requirement for the cytokine in mesodermal differentiation. Two other Bmps of the TGF-β family are expressed in early Xenopus embryos. Bmp-7 is expressed in a pattern very similar to Bmp-4 and has comparable activities (Nishimatsu et al., 1992; Hawley et al., 1995). Maternally expressed Bmp-2 also has similar properties to Bmp-4 and thus could provide Bmp signalling before midblastula transition (MBT) (Shoda et al., 1994; Clement et al., 1995; Hemmati-Brivanlou and Thomsen, 1995). Due to possible redundant function of the Bmps (Winnier et al., 1995; Hogan, 1996), we used a dominant-negative Bmp receptor (ΔmTFR11), which permits to specifically abolish at least Bmp-2/4-type signalling (Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994).

ΔmTFR11 mRNA (Suzuki et al., 1994) was microinjected at different doses into all blastomeres of 4-cell-stage embryos and at the early gastrula stage ventral marginal zones were explanted and cultured until late tadpole stage and analyzed by histology. Fig. 1 shows that increasing doses of ΔmTFR11 inhibited blood differentiation and led to successive peaks of pronephros, muscle and notochord differentiation. Thus, removing Bmp signalling from ventral marginal zones leads to dose-dependent dorsalization of mesoderm. The series of tissues induced is in the opposite order of that observed for Bmp-4, as would be expected if distinct concentrations of Bmp-4 control mesoderm differentiation.

We conclude that different Bmp-4 doses are both necessary and sufficient for the specification of at least four tissue types in the marginal zone: notochord, muscle, pronephros and blood.

XMyf-5 as a molecular marker for the dorsolateral marginal zone
Given the dose-dependent effects of Bmp-4 on mesoderm differentiation, we wished to analyze marginal zone patterning by Bmp-4 at the gastrula stage, when the gene is normally
We therefore asked whether expression of gastrula marker genes is controlled by different doses of Bmp-4. If the local concentration of this cytokine controls d/v marker gene expression in the marginal zone, increasing Bmp-4 doses should lead to expansion of ventral gene expression and repression of more dorsal marker genes. As a marker for the dorsal
domain, we chose chordin, rather than noggin, because chordin unlike noggin, is induced by organizer genes (Sasai et al., 1994) and may thus better reflect organizer regulation.

Bmp-4 mRNA was microinjected into all blastomeres of 4-cell-stage embryos, in the prospective marginal zone. Although many doses were tested in two-fold titrations, only key doses are shown in this and the following experiments for simplicity. XMyl-5 and Xvent-1 each show two distinct responses to Bmp-4 (Fig. 3). At low dose XMyl-5 expression shifts from the lateral into the dorsal domain, suggesting that lack of XMyl-5 expression in the dorsal domain is due to absence of Bmp-4. However, at high dose XMyl-5 is repressed by Bmp-4. Xvent-1 expression also expands dorsally at low dose but is still excluded from the dorsal domain. At high dose, Xvent-1 becomes radially expressed. The expression of Xvent-2 becomes radial already at a low dose of Bmp-4 while the dorsal marker chordin is repressed by high dose of Bmp-4. Thus, at different concentrations of Bmp-4, a distinct expansion or repression of marker gene expression can be distinguished, as would be expected if local Bmp-4 concentration determines their expression boundaries.

We next aimed to reduce Bmp-4 signalling dose-dependently in order to investigate if there is a graded requirement for the cytokine for marginal zone patterning. 4-cell-stage embryos were microinjected with increasing doses of dominant-negative Bmp receptor ΔmTFR11 and analyzed for the expression of marker genes. Fig. 3 shows that two distinct threshold responses were observed. At low dose, injection leads to lateroventral expansion of XMyl-5 expression indicating that a Bmp is normally inhibiting XMyl-5 in this domain. At high mRNA dose, XMyl-5 is repressed. This suggests that XMyl-5 expression requires and occurs within a certain dose window of Bmp-4. Both Xvent-1 and Xvent-2 are repressed by ΔmTFR11, but Xvent-1 requires a low dose while Xvent-2 requires a high dose. Finally, chordin expression expands radially but requires a high dose of dominant-negative receptor mRNA, as would be expected for a dorsal marker.

Therefore, different concentrations of ΔmTFR11 elicit distinct responses of marker genes, as predicted if Bmp-4 regulates their expression dose-dependently. These threshold concentrations are closely correlated with the d/v expression boundaries that these genes normally exhibit: (a) The more ventral expressed Xvent-1 has a requirement for higher local Bmp-4 concentration than the more dorsal expressed Xvent-2. (b) The expression of XMyl-5 requires a lower Bmp-4 dose than its inhibition, providing an explanation for its stripe-like expression. (c) chordin, as the most dorsal marker does not require Bmp-4 signalling for expression and is repressed by it.

The results indicate that different Bmp-4 doses are both necessary and sufficient for patterning of three domains in the gastrula marginal zone.

**Dose-dependent modulation of Bmp-4 activity by noggin**

The results presented suggest that distinct doses of Bmp-4 control d/v mesoderm patterning in the marginal zone and that dorsalization may function via modulation of available Bmp-4. What, however, is the molecular form of graded Bmp-4 in vivo? Although Bmp-4 and Bmp-7 are expressed at the right time and place to pattern mesoderm in the early gastrula, their mRNAs do not appear to be distributed in a graded fashion in the marginal zone (Fainsod et al., 1994; Hawley et al., 1995; Schmidt et al., 1995b; our unpublished data). This raises the possibility, that Bmp activity, rather than protein distribution, may be graded (Ferguson, 1996; Holley et al., 1996; Piccolo et al., 1996; Zimmerman et al., 1996).

An important observation is that noggin and chordin, which are expressed in the dorsal domain (Sasai et al., 1994; Smith and Harland, 1992), are able to antagonize Bmp-4 signalling by direct binding and inactivation of Bmp-4 protein (Piccolo et al., 1996; Zimmerman et al., 1996). Therefore, dorsally expressed noggin and chordin may locally attenuate Bmp-4 protein by complex formation. It follows that, if Bmp-4 activity...
Mesodermal patterning by Bmp-4 is graded due to antagonizing action of noggin and chordin, then their relative ratio should determine mesodermal cell fate. This predicts that the effects of Bmp-4 should be reversible by noggin or chordin.

To test this for noggin, Bmp-4 mRNA was microinjected into all blastomeres of 4-cell-stage embryos, in the prospective marginal zone. Again, though only key doses are shown, many doses were tested in two-fold titrations (Fig. 4B). As with dominant-negative Bmp receptor, two thresholds in response to noggin mRNA injection are observed. Low doses of noggin activate XMMyf-5 on the lateroventral side and inhibit Xvent-1 expression. High doses are needed to repress XMMyf-5 and Xvent-2 and yield radial chordin expression. Thus, noggin is able to dorsalize marker gene expression in a dose-dependent manner as predicted if it functions to create graded Bmp-4 activity.
Taken together, the data suggest that Bmp activity rather than concentration is graded dorsoventrally in gastrula mesoderm due to the neutralizing action of noggin.

**Direct and long-range action of Bmp-4**

Bmp-4 is a secretory protein and it is relevant for the understanding of its patterning mechanism whether the Bmp-4 signal travels from the producing cells over a certain distance and thus can act at long range. To address this question, Bmp-4 mRNA was microinjected into blastomeres of 32-cell-stage embryos together with β-galactosidase containing a nuclear localization signal as lineage tracer. Embryos were subsequently dorsalized by incubation in LiCl to inhibit ventral gene expression in order to visualize induced Xvent-1. Embryos were fixed and processed for whole-mount in situ hybridisation at the gastrula stage using Xvent-1. Fig. 5A shows that Xvent-1 is induced by Bmp-4 and that the expression extends approximately 5-10 cell diameters beyond the injected cells containing light blue nuclei, indicating that the induction of Xvent-1 by Bmp-4 is non-cell-autonomous and long range.

However, the possibility remains that Bmp-4 acts by inducing some other factor that is ultimately responsible for long-range signalling. To address this question, we have made use of Xsmad1, an intracellular downstream target of Bmp-4 signalling that mediates its effects, presumably, by directly interacting with Bmp-4 target genes. Like Bmp-4, Xsmad1 induces ventral mesoderm and ventralizes dorsal mesoderm. It rescues the effects of dominant-negative Bmp receptor on mesoderm as well as ectoderm (Sekelsky et al., 1995; Graff et al., 1996; Hoodless et al., 1996; Liu et al., 1996; Niehrs, 1996; Thomsen, 1996). If Bmp-4 acts indirectly by inducing another signalling factor, then Xsmad1, faithfully reproducing all effects of Bmp-4, should also function at long range, despite being a transcription factor. If, however, Bmp-4 acts directly on mesodermal cells, only expression of the cytokine, but not that of its intracellular transducer should yield long-range effects.

To distinguish between these possibilities, Xsmad1 mRNA was microinjected into 32-cell-stage embryos together with β-galactosidase as lineage tracer. Embryos were again subsequently dorsalized by incubation in LiCl. Fig. 5A shows that Xvent-1 is induced by Xsmad1.

**Fig. 5.** Direct and long-range action of Bmp-4 and noggin. (A) (Top) 32-cell-stage embryos were microinjected with 0.2 ng β-galactosidase lineage tracer mRNA containing a nuclear localization signal and either without (lacZ) or with 30 pg Bmp-4 or 500 pg Xsmad1 (XMad-1) mRNA as indicated into two marginal zone blastomeres, followed by treatment with LiCl. Embryos were processed for whole-mount in situ hybridisation at stage 10+ with Xvent-1. Note that Xvent-1 expression extends beyond the injected cells (light blue nuclei) in the Bmp-4-injected embryo, while it is restricted to the β-galactosidase-positive cells in Xsmad1-injected embryos. Embryos are shown in lateral view with the animal side up. The insets show a magnification of the highlighted area. (Bottom) 4-cell-stage embryos were uninjected (control) or microinjected into all blastomeres with Bmp-4 or Xsmad1 mRNA. Note that the phenotype of both Bmp-4- as well as Xsmad1-microinjected embryos is complete Bauchstück-type ventralization. (B) 32-cell-stage embryos were microinjected with 0.2 ng β-galactosidase lineage tracer mRNA containing a nuclear localization signal and either without (lacZ) or with 25 pg (low) or 100 pg (high) noggin mRNA, as indicated, into two marginal zone blastomeres. Embryos were processed for whole-mount in situ hybridisation at stage 10+ with Xvent-1 or Xvent-2 as indicated. Note that the Xvent-1 and Xvent-2 repression halos extend beyond the injected cells (light blue nuclei) in the noggin-injected embryos and that the size of the halo is dose dependent. Embryos are shown in lateral view with the animal side up.
However, expression of Xvent-1 is restricted to the β-galactosidase-positive cells, indicating that the induction of Xvent-1 by Xsmad1 is cell autonomous. We cannot exclude the possibility, however, that Bmp-4 signalling involves a relay that is independent of Xsmad1 function, e.g. by some novel receptor.

This difference between Xsmad1 and Bmp-4 action is not due to a weaker phenotypic effect of the former on mesodermal cells since embryos radially injected into the equatorial region with the same concentration of mRNA as used above, showed comparable phenotypes, namely Bauchstöck-type ventralization (Fig. 5A, bottom). We conclude that Bmp-4 can signal long range to mesodermal cells and does not involve secondary signalling molecules.

To investigate if also noggin has long-range effects, low or high doses of noggin mRNA were co-injected with β-galactosidase and the expression of Xvent genes was analyzed. Fig. 5B shows that repression of Xvent gene expression occurs in a halo around the β-galactosidase-positive cells at low noggin dose. At high dose, Xvent-1 expression was suppressed in the whole embryo with exception of a small region outside the field of view. Xvent-2 is less sensitive to high doses of noggin and the increased halo is clearly visible. We conclude that noggin acts long range and that the expression level determines the size of Xvent gene expression as would be expected if the local Bmp-4 activity is modulated by noggin.

**DISCUSSION**

**d/v patterning by Bmp-4**

The conservation of d/v patterning in arthropods and vertebrates (reviewed in Arendt and Nübler-Jung, 1994; Jones and Smith, 1995; De Robertis and Sasai, 1996; Ferguson, 1996) has raised the possibility that, like dpp in *Drosophila* (Ferguson and Anderson, 1992; Wharton et al., 1993), Bmp-4 acts as a morphogen in vertebrates.

Here we have investigated the possibility that Bmp-4 functions dose-dependently in d/v patterning of *Xenopus* mesoderm at the gastrula stage. We find that (1) progressively higher levels of Bmp-4 signalling are sufficient and required for differentiation of notochord, muscle, pronephros and blood.

- **Fig. 6.** Model of mesodermal patterning at the gastrula stage by a Bmp-4 morphogen gradient. Bmp-4 mRNA is uniformly expressed in the marginal zone except in the dorsal domain. Noggin is expressed in the dorsal domain. Their protein products (red and blue continuous lines) overlap dorsolaterally leading to attenuated Bmp-4 activity (dashed red line), resulting in dorsal (future notochord), dorsolateral (future muscle) and lateroventral (future blood, lateral plate, pronephros) positional values. Xvent-1 has a high dose requirement for Bmp-4, XMyf-5 has a requirement for low dose and is inhibited by high Bmp-4 dose. Xvent-2 has a requirement for low Bmp-4 dose.

- (2) It is demonstrated by both gain- and loss-of-function experiments that three marginal zone domains are patterned by different Bmp-4 concentrations in the gastrula stage. The more ventral the boundary of a gene expressed in the marginal zone, the higher its requirement for Bmp signalling. (3) Evidence is presented that Bmp-4 activity becomes graded due to the antagonizing action of noggin. (4) Bmp-4 is shown to act directly on mesodermal cells in a long-range fashion, excluding secondary signalling factors.

These results argue for a model (Fig. 6), in which positional information in the gastrula marginal zone is provided by graded Bmp-4 activity, which is high ventrally and low dorsally. Graded Bmp-4 activity is the result of superimposition of antagonizing Bmp-4 and noggin proteins. In the dorsolateral domain, Bmp-4 protein is attenuated, allowing for XMyf-5 expression and muscle differentiation, both of which require Bmp-4 signalling. Yet, both muscle differentiation and XMyf-5 expression are repressed by high Bmp-4 levels, which induce blood differentiation and Xvent-1 expression. Thus, dorsal, dorsolateral and lateroventral fate are determined by distinct Bmp-4 concentrations. Although the Bmp-4 activity gradient remains to be visualized by some direct means, both noggin and Bmp-4 can signal long range in the marginal zone as would be expected in this model. Interestingly, in the animal cap Bmp-4 appears to have short-range action (Jones et al., 1996b). This suggests regional differences in diffusibility of Bmp-4. The shape of the Bmp-4 activity gradient may be therefore influenced by these differences, which may be mediated by the extracellular matrix.

Noggin is formally and functionally equivalent to the dorsalizing signal proposed by Smith and Slack (1983), i.e. has all the properties of a bona fide inducer of dorsolateral fate (our results and Smith et al., 1993). Given the observation, however, that noggin functions by sequestering and neutralizing Bmp proteins (Zimmerman et al., 1996), it may mechanistically not be considered an instructive inducer unless a separate receptor is identified. Rather, it modifies the positional information that is provided by Bmp-4 signalling, thus generating graded activity of the Bmp-4 morphogen. In this mechanistic view, the organiser may have a passive role in signalling, because positional information read by cells in the marginal zone corresponds to the local Bmp-4 activity, which becomes graded by organizer signals. Put differently, what appears macroscopically to be induction (dorsalization), is microscopically modification of ventral morphogen read-out.

Is Bmp-4 signalling sufficient to specify all mesoderm? This is unlikely. For example, the secondary axes induced by dominant-negative Bmp receptor injections are incomplete, they do not form heads (Graff et al., 1994; Suzuki et al., 1994), suggesting that no head mesoderm is present. For head mesoderm formation then, other factors, probably emanating...
from the Nieuwkoop center will be required and these factors may not be equivalent to absence of Bmp-4 signalling.

Lateroventral mesoderm gives rise to three tissues, pronephros, lateral plate and blood. Lateral plate and blood have a common embryonic origin and a late inductive signal from ventral endoderm is thought to induce blood from lateral plate (Zon, 1995), hence their initial specification may involve identical patterning by Bmp-4. Indeed, we find blood and lateral plate differentiation at the same doses of Bmp-4 (R. D. and C. N., unpublished observation). In contrast, pronephros and blood do not have a common embryonic origin. Pronephros arises mostly from the lateral marginal zone, while blood and lateral plate are both fated and specified predominantly in the ventral marginal zone (Dale and Slack, 1987a,b). Consistent with this, we have shown that distinct Bmp-4 doses can induce pronephros and blood. Yet, at the gastrula stage, only one common lateroventral domain was observed in the marginal zone corresponding to the high positional value of Bmp-4, where Xvent-1 is expressed, and which contains the progenitors of both tissues, lateral plate/blood and pronephros. When earlier pronephros markers become available, we might therefore expect a molecularly distinct ventrolateral domain, which may correspond to a third Bmp-4 threshold response in the gastrula marginal zone.

**The role of other signalling factors**

While we have focused on Bmp-4 in this study, there are at least two other TGF-β-type Bmps expressed in early *Xenopus* embryos. Bmp-2 (Clement et al., 1995; Hemmati-Brivanlou and Thomesen, 1995; Nishimatsu et al., 1992, 1993) and Bmp-7 (Nishimatsu et al., 1993; Hawley et al., 1995). While maternally expressed Bmp-2 may be involved in mesoderm induction, the low levels of protein present at gastrulation (Clement et al., 1995) and the fact that antisense injection of Bmp-2 mRNA does not show neuralizing effects (in contrast to antisense Bmp-4 mRNA; Sasai et al., 1995) makes a function during gastrulation less likely. Bmp-7 is expressed in the marginal zone during gastrula stages and shows activities similar to Bmp-4 (Hawley et al., 1995), suggesting that it may act in concert with Bmp-4. In addition, it can form functional heterodimers with Bmp-4 (Aono et al., 1995). Disruption of the mouse Bmp-4 gene is embryonic lethal, with embryos dying around gastrulation, but penetrance is variable, suggesting that other Bmps may indeed act redundantly during gastrulation (Winnier et al., 1995).

Activin and Vg1 are TGF-β-type signalling molecules that have been proposed to play a role in d/v patterning already at mesoderm induction. Both are sufficient for dorsoventral patterning of mesoderm in animal cap cells (Green et al., 1992; Gudrow et al., 1994; Kessler and Melton, 1995). However, activins seem to be unlikely candidates for natural patterning agents of *Xenopus* mesoderm. First, loss of activin-β A and B in mice does not disrupt early development (Matzuk et al., 1995; Vassalli et al., 1994). Second, overexpression of the activin-binding protein follistatin at doses that inhibit ectopic activin action has no axis-inhibiting effect (Schulte-Merker et al., 1994). Third, it was recently shown that activin, Vg1, TGF-β and Xnr2 elicit short-range responses and act via a relay mechanism in *Xenopus* animal caps by inducing secondary signals (Reilly and Melton, 1996a, Jones et al., 1996b), although the results are conflicting for activin (Jones et al., 1996b). Good candidates for the signals relayed by activin and Vg1 are Bmp-4 and noggin.

Xwnt-8 is a weakly ventralizing signalling molecule expressed in the lateroventral domain of the gastrula (Christian and Moon, 1993). It may be part of the Bmp-4 pathway, since it is induced by Bmp-4 in animal caps (Graff et al., 1994).

While we have not tested this, it is likely that the vertebrate sog homolog chordin (Piccolo et al., 1996), as well as follistatin (Hemmati-Brivanlou et al., 1994; Sasai et al., 1995; Yamashita et al., 1995), may act similar to noggin, since both are able to sequester TGF-β proteins. Possibility, they have a different range of action within the marginal zone and thus control the exact size of mesodermal domains by attenuating Bmp-4 in spatially complex manner. Since Bmp-2, Bmp-4 and Bmp-7 are expressed in later stages in *Xenopus* (Fainsod et al., 1994; Clement et al., 1995; Hawley et al., 1995; Hemmati-Brivanlou and Thomesen, 1995; Schmidt et al., 1995b), they may be attenuated in various tissues by noggin (Smith and Harland, 1992), chordin (Sasai et al., 1994) and possibly follistatin (Hemmati-Brivanlou et al., 1994), all of which have distinct expression patterns at later stages. It will therefore be interesting to investigate whether Bmp-4 elicits dose-dependent effects on patterning of other tissues as well. Another case in which Bmp-4 (and Bmp-7) is antagonized is early neurogenesis in *Xenopus*, where Bmp-4 inhibits neuralization and chordin, noggin and follistatin promote neuralization (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Hawley et al., 1995; Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). Possibly then, there is an intermediate threshold of Bmp-4 that promotes neural crest formation (Li et al., 1995).

In addition to these modulators of Bmp-4 activity with a likely permissive signalling function, the organizer expresses signalling molecules of the TGF-β class that could have an instructive signalling role. Nodal-related proteins 1-3 are transiently expressed in the blastopore lip and have dorsalizing activity in ectopic expression (Jones et al., 1995; Smith et al., 1995). Given the observation that at least two organizer signals function permissively, it will be interesting to investigate if nodal-related proteins also act permissively by attenuating Bmp-4, e.g. by complex formation. Finally, Admp (Moos et al., 1995), a Bmp-type signalling molecule expressed in the organizer has, puzzlingly, ventralizing activity if expressed before MBT. Its role as well as that of retinoic acid in mesoderm patterning (Ruiz i Altaba and Jessel, 1991) remain unclear at present.

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