BMP signaling restricts hemato-vascular development from lateral mesoderm during somitogenesis

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The bone morphogenetic protein (BMP) signaling pathway is essential during gastrulation for the generation of ventral mesoderm, which makes it a challenge to define functions for this pathway at later stages of development. We have established an approach to disrupt BMP signaling specifically in lateral mesoderm during somitogenesis, by targeting a dominant-negative BMP receptor to Lmo2+ cells in developing zebrafish embryos. This results in expansion of hematopoietic and endothelial cells, while restricting the expression domain of the pronephric marker pax2.1. Expression of a constitutively active receptor and transplantation experiments were used to confirm that BMP signaling in lateral mesoderm restricts subsequent hematopoietic development. The results show that the BMP signaling pathway continues to function after cells are committed to a lateral mesoderm fate, and influences subsequent lineage decisions by restricting hematopoietic development. 

KEY WORDS: Hematopoiesis, Endothelial, Pronephros, Transgenic, Zebrafish, Lmo2

INTRODUCTION

According to fate-mapping and explant studies, mesoderm is specified during gastrulation to contribute to tissues that are categorized broadly as being of ventral or dorsal character. This division is recognized by the expression of specific markers that define ventral (e.g. msx1 or vent1) or dorsal (e.g. chordin) fate. The signaling pathway associated with bone morphogenetic proteins (BMPs) is one key component that regulates this aspect of early mesoderm patterning (Dale and Jones, 1999), in association with other major developmental pathways regulated by WNT and FGF ligands (Munoz-Sanjuan and Hemmati-Brivanlou, 2001). Ventral mesoderm is subsequently patterned during post-gastrulation stages to distinguish lateral and intermediate derivatives. Lateral mesoderm gives rise to hematopoietic and vascular lineages, while intermediate mesoderm generates pronephric progenitors (Drummond, 2000; Thissle and Zon, 2002).

These secondary patterning events are less well defined, being characterized mainly by the expression of genes that are hallmarks for the differentiation of hematopoietic, vascular and pronephric progenitors from lateral and intermediate mesoderm (Davidson and Zon, 2000; Serluca and Fishman, 2001). By early somitogenesis, distinct stripes of cells can be distinguished in posterior mesoderm that express either the pronephric marker pax2.1 (pax2a – Zebrafish Information Network) or, immediately more medial, the hematovascular markers scl (tal1 – Zebrafish Information Network) and gata2 (Drummond, 2003). The developmental pathways that control these sub-divisions are also not well characterized, although it seems reasonable to consider that lateral and intermediate mesoderm continue to develop under the influence of the same predominant signaling pathways. It is, however, a challenge to test this, as disruption of the BMP, WNT or FGF pathways alters the initial specification events and this precludes analysis of subsequent developmental transitions. The fate of these cells has been considered to be not fully committed during somitogenesis, as forced expression of Scl is sufficient to convert the entire region to Gata1-expressing hematopoietic cells (Gering et al., 1998). However, this interpretation is complicated because it is not known at what stage of development Scl exerts this effect.

BMPs are members of the TGFβ super-family of secreted ligands and they bind type I and type II receptor complexes to initiate a signaling cascade that activates transcription of downstream ‘ventral’ target genes. This is a highly conserved pathway and loss of BMP signaling in fish (Mintzer et al., 2001; Schmid et al., 2000), frog (Graff et al., 1994) or mouse (Winnier et al., 1995) leads to expansion of dorsal mesoderm (for example trunk muscle) at the expense of ventral mesoderm (blood, vasculature and pronephros). The early requirement for BMP signaling was confirmed by the identification in zebrafish of numerous ‘dorsalized’ mutants that are defective for genes in the BMP pathway (for a review, see Hammerschmidt and Mullins, 2002), including lost-a-fin [alk8 (Bauer et al., 2001)], swirl [bmp2b (Kishimoto et al., 1997; Nguyen et al., 1998)], snailhouse [bmp7 (Dick et al., 2000)], mini fin [tolloid (Connors et al., 1999)] and somitabun [smad5 (Hild et al., 1999)]. Hyper-activation of the pathway leads to an opposite phenotype; Bmp4 overexpression expands the expression domain of hematopoietic-, vascular- and pronephric-specific markers, including scl, flk1 (kdr – Zebrafish Information Network) and pax2.1.

In contrast to the generation of early ventral mesoderm, it is not known if BMP signaling is directly involved in the further development of lateral mesoderm derivatives. For example, the cloche and bloodless mutants are characterized by defective differentiation of hematopoietic cells from lateral mesoderm, despite apparently normal patterning of ventral mesoderm. These mutants are rescued by forced expression of Scl but not Bmp4 (Liao et al., 1998). However, zygotic loss of the Smad5 gene product, which is a direct mediator of the BMP signaling pathway, causes defective differentiation in the blood and vasculature (Chang et al., 1999; Yang et al., 1999). This is consistent with a patterning role for BMP signaling post-gastrulation, e.g. to maintain, promote or restrict expression of the transcriptional regulatory programs that control the survival and differentiation of hematopoietic, vascular and pronephric progenitors from lateral mesoderm.
The receptors and the signaling components for the BMP pathway are expressed in the embryo post-gastrulation. However, manipulation of the pathway by overexpression of constitutively active or dominant-negative components, or loss of function through genetic and morpholino experiments cannot accurately interrogate later functions without disturbing early embryogenesis. To address function at subsequent stages, we developed an approach for conditional modulation of BMP signaling in lateral mesoderm of developing zebrafish embryos. We show that the BMP signaling pathway continues to function during somitogenesis to regulate the development of hematopoietic, vascular and pronephric lineages from lateral mesoderm. The data supports a model in which BMP signaling at this stage promotes pronephric fate while restricting the generation of hematopoietic derivatives.

MATERIALS AND METHODS

Zebrafish

Zebrafish embryos were maintained at 28°C and staged as described (Westerfield, 1995). Most experiments used embryos derived from parents of a hybrid strain, itself derived from crossing wild-type AB and TUB strains, obtained from the Zebrafish International Research Center (Eugene, OR). The gata1:gfp reporter strain (Long et al., 1997) was provided by Dr Shou Lin (UCLA, Los Angeles, CA). The fli1:gfp line (Lawson and Weinstein, 2002) was obtained from the Zebrafish International Research Center.

Plasmids

The parental I-SceI plasmid vector was assembled in two steps. First, oligonucleotides with the sequence containing the I-SceI sites were annealed and used to replace the pBluescript SK+ backbone previously digested with MluI and BssHII restriction enzymes. Next, the entire polyclinker of pBluescript SK+ was cloned back into the first vector (BssHII/BssHII). Each of the subsequent expression plasmids were then generated by subcloning into the parental I-SceI plasmid, using inserts derived from plasmids for lmo2:cre (Zhu et al., 2005), gata1:gfp (Long et al., 1997), CA-XBMPR (Candia et al., 1997) and pTurbo-Cre (Hug et al., 1996) inserts. The lmo2:ΔBR and gata1:ΔBR vectors were generated by PCR amplifying the ΔBR insert described previously (Zhang and Evans, 1996) in place of GFP. Likewise, the lmo2:carB and lmo2:cre vectors were generated by subcloning the respective cDNAs in place of GFP.

The sequence of the I-SceI oligomers are: FP, 5′-CCGGTATA- GGAGATTACAGGGTATAGCCCGAGGTCGATTGGAACAGGTTAATA-3′; RP, 5′-CCGGATTACCTGTTATCCTTGAGGCGGTCGATTGGCAGATTACGGCGCCATTACCGTGGTTCTCCT-TAA-3′; Primers used to amplify the ΔBR sequence are: FP, 5′-ATAGATTACCACCATGAGAGACAGCTTTCTT-3′; RP, 5′-ATAGATCCCC-TTTGTAATACCATATGATAAG-3′.

Microinjection

The I-SceI injection mixture was assembled into ice on just before injection. Plasmids were mixed with or without I-SceI meganuclease (1 µg/µl; New England Biolabs) in 0.5× I-SceI meganuclease restriction buffer. Each vector (40-80 pg) was injected in a volume of 2.3 pl at the one-cell stage. RNA encoding the dominant-negative BMP receptor (ΔBR) was generated in vitro from expression vector pSP64T using mMessage mMACHINE/H9004 RNA encoding the dominant-negative BMP receptor (vector (40-80 pg) was injected in a volume of 2.3 pl at the one-cell stage.

Whole mount in situ hybridization

Whole mount in situ hybridization was performed essentially as described (Alexander et al., 1998). Digoxigenin-labeled RNA antisense probes were described previously for gata1 (Detrich et al., 1995); xcl (Liao et al., 1998); pax2.1 (Krauss et al., 1991); lmo2 (Zhu et al., 2005); fli1 (Thompson et al., 1998); L-plastin (lpl; Zebrafish Information Network) (Herbomel et al., 1999); mpo (Bennett et al., 2001); and no tail (Schulte-Merker et al., 1992). The ΔBR digoxigenin-labeled antisense RNA probe was generated from the Xenopus ΔBR cDNA clone (Zhang and Evans, 1996). Two-color whole-mount in situ hybridization was performed essentially as described previously (Westerfield, 1995), although instead of Fast Red, INT Red (Sigma) was used to develop the red color. Fluorescein-12-UTP-labeled gata1 probe and digoxigenin-labeled pax2.1 probe were used sequentially.

Flow cytometry

Fluorescence activated cell sorting (FACS) was performed as described (Long et al., 1997). For each experiment, a clutch of embryos from the gata1:gfp transgenic line was divided into two sets, with one batch being injected and other uninjected batch serving as a control. Both groups were then dissociated at the 16- to 17-somite stage and cells analyzed by FACS under standard FITC conditions.

Cell transplantation and imaging

Donor embryos were microinjected at the one- to two-cell stage with a solution of 5% lysine fixable tetramethylrhodamine dextran (Molecular Probes) and 0.2 M KCl, either alone or including 100 pg RNA encoding Bmp4. Cell transplantation was performed as described (Ho and Kane, 1990). Donor cells (20-50) from high-stage embryos were transplanted into the margin of similarly staged hosts. Host embryos were derived from the gata1:gfp line. Chimeric embryos were cultured in embryo media + 0.2% penicilin-streptomycin until the 10- to 12-somite stage, fixed overnight in 4% paraformaldehyde and screened for localization of fluorescently labeled (red) donor to lateral mesoderm. Embryos with similar numbers of red cells were then stained with Hoechst, de-yolked, flattened and analyzed on a Zeiss Axiovert (200M) microscope. Criteria used to score the host embryo is as follows: an event is scored as positive when a green (GFP+) cell is in direct proximity with a red (donor) cell and by the absence of a GFP-negative but blue (Hoechst) cell between the two. Raw z-series of images were processed in Image-J to clearly analyze cell boundaries.

RESULTS

An approach to target conditionally gene expression to lateral mesoderm during zebrafish embryogenesis

We sought to deregulate with temporal and spatial specificity BMP signaling in lateral mesoderm, but to accomplish this it was necessary first to develop an effective transgenic targeting approach. For this purpose, we chose to use the promoter for the zebrafish lmo2 gene. The lmo2 gene is not expressed until after gastrulation; transcripts are first detected in two stripes of lateral mesoderm starting around the one-somite stage (Dooley et al., 2005). The pattern marks the hematopoietic, vascular and pronephric progenitors, of both rostral and caudal origin. A 2.5 kb promoter fragment derived from sequences upstream of the lmo2 transcriptional start site is sufficient to direct expression of GFP to recapitulate the endogenous expression pattern of lmo2 in lateral mesoderm (Zhu et al., 2005). This promoter therefore provides an appropriate tool for targeting gene expression to lateral mesoderm by transgenesis.

Transient transgenic expression in zebrafish is characterized typically by substantial mosaicism, which complicates interpreting any phenotypes caused by transgenic expression. To address this problem, we flanked transgenes with restriction sites for the I-SceI meganuclease. Co-injection of transgenes flanked with these restriction sites along with the I-SceI enzyme was shown previously in Medaka to decrease mosaicism and increase germline transmission (Thermes et al., 2002). We confirmed the utility of this approach in zebrafish by injecting a transgenic construct with the lmo2 promoter directing expression of GFP. This reporter transgene flanked by I-SceI sites [designated by I(transgene)] was co-injected either alone or with the meganuclease, and the resulting expression pattern of GFP was observed. In ~30% of the co-injected embryos an essentially uniform pattern of GFP expression is seen in lateral mesoderm, which effectively recapitulates the normal expression
pattern for the lmo2 gene (Fig. 1). This uniform pattern is never seen in embryos that are injected with the reporter in the absence of the meganuclease. The remaining co-injected embryos either express no GFP, or most often can still be characterized as displaying mosaic expression, similar to those that did not receive the meganuclease.

To determine if generation of this uniform expression pattern was unique to the lmo2 promoter, we tested another construct using, instead, the promoter for the gata1 gene. Gata1 is also expressed in lateral mesoderm, but labels at a later stage (five somites) more specifically the progenitors for the caudal embryonic hematopoietic program and subsequently the differentiated erythroid cells. When this reporter gene was co-injected with the I-SceI meganuclease the results were similar, in that again ~30% of the embryos showed uniform expression of GFP in the gata1 expression domain, similar to stably transgenic gata1:gfp lines (Fig. 1D). Compared with the GFP pattern observed using the lmo2 promoter, the gata1 promoter directs expression of GFP at a slightly later stage (observed first around the five-somite stage, compared with one somite for lmo2:gfp), which again is consistent with the normal expression patterns for these genes. In both cases, GFP expression is not observed outside of the normal domains for the endogenous genes. In summary, although the majority of the transient transgenic embryos still show mosaic expression, using the I-SceI approach, these promoters reliably target expression of transgenes throughout the normal expression domains in lateral mesoderm for about 30% of the injected embryos.

Inhibition of BMP signaling in lateral mesoderm alters hematopoietic and vascular development

An established approach to reduce specifically BMP signaling in developing embryos is by overexpression of a truncated BMP receptor. A BMP type I receptor that is deleted of the C-terminal kinase domain acts in a dominant-negative fashion by associating with BMP type II receptors and thereby blocking subsequent cellular signaling to the regulatory SMADs (Graff et al., 1994; Maeno et al., 1994). In previous studies, we used a truncated Xenopus BMP receptor to inhibit BMP signaling in developing Xenopus embryos, and showed that BMP signaling is required during gastrulation for establishment of the embryonic hematopoietic program (Zhang and Evans, 1996). This mutant receptor, called $\Delta BR$, is truncated of C-terminal sequences just after the transmembrane domain and therefore lacks the kinase domain entirely. In order to confirm that this mutant receptor is functional in zebrafish embryos, we injected purified RNA encoding $\Delta BR$ into fertilized zebrafish eggs. Embryos expressing $\Delta BR$ develop with substantial axial defects (not shown), including the lack of normal tail structures, and in this manner resembles the phenotype of swirl embryos, which are mutant for the bmp2 gene (Schmid et al., 2000). However, the phenotype, as also described previously (Hammerschmidt et al., 1996), is more severe than the bmp2 mutant, and is more similar to embryos depleted of both Smad1 and Smad5 (L. McReynolds and T.E., unpublished).

Therefore, forced expression of the $\Delta BR$ mutant receptor can be used to block BMP signaling during zebrafish development.

Thus, to investigate if there is a continued or later function for active BMP signaling in lateral mesoderm subsequent to ventral specification, our strategy was to use the lmo2 promoter to direct expression of $\Delta BR$ in developing embryos using transgenic constructs flanked with I-SceI restriction sites. To ensure the fidelity of the approach, we first injected the I(lmo2; $\Delta BR$)I transgene into wild-type embryos and analyzed the expression of the $\Delta BR$ cDNA by in situ hybridization. Because the transgene is derived from the Xenopus gene, the antisense probe is specific for the expressed transgene. As shown in Fig. 2A, there is no expression of the transgene detected at the tailbud stage, consistent with the expected activation of the transgene at around the one- to two-somite stage. Indeed by the three-somite stage, transgene expression is detected specifically in the emerging two stripes of lateral mesoderm (Fig. 2B). This pattern is extended by the eight-somite stage to recapitulate entirely the normal pattern of expression for lmo2 (Fig. 2C). The pattern appears essentially uniform in ~25% of the embryos, although even in these embryos there often appears some mosaicism (‘patchiness’) compared with what would be expected from a stable transgenic line. However, the approach is sufficient to delay expression of the mutant receptor until somitogenesis and to target it specifically to much of the lateral mesoderm.

We also did consider that the mutant receptor could be expressed at a low level, which might not be detected readily by in situ hybridization, but could still have functional consequences. In order to confirm by an independent assay that temporal expression of the mutant receptor is delayed until somitogenesis, we analyzed by in situ hybridization the expression pattern of the scl gene, which is an early BMP-dependent regulatory gene essential for hematopoiesis. The lmo2 and scl genes encode the earliest known markers for hematovascular progenitors in lateral mesoderm. Forced expression of RNA encoding $\Delta BR$ throughout early embryogenesis is sufficient to block completely the expression of scl (Zhang and Evans, 1996). However, activation of scl expression should not be altered if the lmo2 transgene delays expression of the mutant receptor. Indeed, as shown in Fig. 3 (summarized also in Table 1), embryos transgenic for I(lmo2; $\Delta BR$)I activate normally the initial pattern of scl expression. The transcripts are detected as early as the one- to two-somite stage in short parallel stripes of lateral mesoderm in bothFig. 1. The I-SceI meganuclease system can be used to reduce mosaicism effectively in transient transgenic animals. In a series of experiments, it was found that the full and normal pattern of expression can be recapitulated using either the lmo2 or gata1 promoters in ~30% of the injected embryos. Shown are representative examples of embryos derived from fertilized eggs that had been injected with transgenes and the meganuclease. (A, B) Two independent examples using the I(lmo2:gfp)I transgene examined at the 10-somite stage. GFP expression is present throughout the two stripes of lateral mesoderm (arrows). Views are dorsal, anterior towards the left. (C) A similar embryo analyzed at 20 hpf, with GFP expression throughout the ICM (arrow) and also anterior lateral mesoderm (arrowhead). The view is lateral, anterior towards the left. (D) A representative embryo, viewed as in C, derived from eggs injected with the I(gata1:gfp)I construct at 20 hpf. GFP expression is restricted to the ICM (arrow).
transgenic and control embryos. Therefore, we are confident that the transgene is not affecting initial stages of mesoderm patterning or the initial specification of hematopoietic progenitors.

In the next analysis, we injected the I(lmo2:ΔBR)I transgene into embryos that are transgenic for the gata1:gfp reporter. This transgenic line expresses GFP in primitive erythroid progenitors from the five–somite stage, and can be used to visualize the emergence of the committed embryonic hematopoietic program. If BMP signaling is required in Lmo2+ cells for erythroid development, we expected at least 30% of the injected embryos to show substantial defects in GFP expression. Instead the exact opposite result was observed. In multiple independent experiments, ~30% of the embryos derived from fertilized eggs injected with I(lmo2:ΔBR)I show markedly enhanced levels of GFP in the intermediate cell mass (ICM), the normal location of primitive hematopoiesis. Representative embryos are shown in Fig. 4, and the data are summarized in Table 1. In order to quantify the phenotype and to determine if the enhanced levels of GFP are due to increased transcript or protein levels or instead represent an increase in numbers of Gata1+ cells, batches of injected embryos were dissociated and GFP+ cells scored by FACS analysis. Embryos derived from fertilized eggs injected with I(lmo2:ΔBR)I show two- to threefold more GFP+ cells compared with equal numbers of control uninjected gata1:GFP embryos. This represents a conservative estimate of the expansion of the erythroid population originating from lateral mesoderm when BMP signaling is inhibited, as in this case GFP+ cells from the entire batch of injected embryos was scored, rather than selecting the 30% expected to generate the most significant phenotype. This result indicates that BMP signaling normally restricts the number of Gata1+ cells that develop from Lmo2+ lateral mesoderm progenitors.

Lateral mesoderm comprises both the posterior region, which generates erythroid cells, and an anterior region, which gives rise to head vasculature and cells of myeloid lineage (Lieschke et al., 2002). We extended our initial observations by characterizing gene expression patterns by in situ hybridization for various blood and vascular markers in both posterior and anterior regions. As expected from the analysis of the gata1:gfp transgenic fish, the expression pattern of the erythroid marker gata1 is expanded in the ICM of embryos injected with I(lmo2:ΔBR)I compared with controls (Fig. 5A–C). The expression pattern at the 12-somite stage is expanded laterally in the characteristic bilateral stripes, but also towards the most caudal region of the embryos, forming a complete loop. This loop pattern is never observed in the uninjected or control injected embryos. However, changes in gene expression were not limited to the posterior domain or to erythroid cells. In addition, the expression patterns for the macrophage marker l-plastin (Fig. 5D,E) and the granulocyte marker mpo (Fig. 5F,G) were each strikingly enhanced and expanded in the transient transgenic embryos with an observed frequency between 20-30% (summarized in Table 1). Other sets of control embryos that were injected with I(lmo2:cre)I transgene displayed normal patterns of gene expression, showing that the phenotypes are dependent on expression of the mutant BMP receptor (as shown, for example, in Fig. 5C).

The vascular endothelial cell marker flik1 is expressed in both anterior and posterior lateral mesoderm and blocking BMP signaling expands the pattern in both domains, although more markedly in tail and trunk regions (Fig. 5H,I). The effect on vascular endothelium was further investigated by injecting the I(lmo2:ΔBR)I transgene into embryos transgenic for the fli1:gfp reporter. Fli1 is a member of the ets family of transcription factors...
that is expressed throughout lateral mesoderm during early development, and exclusively in vascular endothelium as lateral mesoderm differentiates (Isogai et al., 2003). We find that inhibiting BMP signaling in Lmo2+ lateral mesoderm results in dilation of major vessels by 1.5 days post-fertilization (Fig. 5J,K). We conclude that blocking BMP signaling specifically in lateral mesoderm results in the expansion of markers representing both hematopoietic and vascular lineages.

Table 1. Scoring of injected embryos

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Marker</th>
<th>% uniform</th>
<th>n</th>
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<tbody>
<tr>
<td>I(lmo2:gfp)I</td>
<td>GFP</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>I(gata1:gfp)I</td>
<td>GFP</td>
<td>30</td>
<td>70</td>
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B Embryos that display markedly altered expression patterns (n)†

<table>
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<tr>
<th>Transgene</th>
<th>Marker</th>
<th>fli:gfp</th>
<th>gata1</th>
<th>scl</th>
<th>lmo2</th>
<th>l-plastin</th>
<th>mpo</th>
<th>flk1</th>
<th>pax2.1</th>
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<tr>
<td>I(lmo2:ΔBR)I (12-somite stage)</td>
<td>20 (100)</td>
<td>20 (55)</td>
<td>30 (41)</td>
<td>27 (37)</td>
<td>36 (57)</td>
<td>27 (27)</td>
<td>20 (54)</td>
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<tr>
<td>I(lmo2:cre)I</td>
<td>24 (52)</td>
<td>0 (50)</td>
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<table>
<thead>
<tr>
<th>Transgene</th>
<th>Marker</th>
<th>pax2.1 (up)</th>
<th>gata1 (down)</th>
<th>scl (down)</th>
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<tbody>
<tr>
<td>I(lmo2:caBR)I</td>
<td>25 (46)</td>
<td>16 (80)</td>
<td>18 (100)</td>
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*Embryos were scored that express GFP in a uniform pattern, similar to stably transgenic lines generated using the same transgenes. The number of embryos analyzed is indicated by n.

†Embryos were scored that displayed strikingly distinct changes in expression for each marker using the indicated transgene. For I(lmo2:ΔBR)I, the changes reflect increased expression levels, except for pax2.1 levels, which were decreased. When analyzed at the one- to two-somite stage, I(lmo2:ΔBR)I did not alter the initial expression pattern of scl. For I(gata1:ΔBR)I and I(lmo2:cre)I, the changes reflect increased expression levels. For I(lmo2:caBR)I, the numbers reflect increased expression levels for pax2.1 but decreased levels for gata1 and scl.

Fig. 4. Expression of a dominant-negative BMP receptor in lateral mesoderm results in enhanced hematopoiesis. Two representative embryos are shown in A and B; views are lateral, anterior towards the top, with embryos still in their chorions. (A) Control embryos from the gata1:gfp transgenic line demonstrate the normal expression pattern of GFP in the ICM. (B) Embryos from the gata1:gfp transgenic line that had been injected at the one-cell stage with the I(lmo2:ΔBR)I transgene and meganuclease. GFP expression is substantially increased in the ICM, compared with control embryos (arrowheads). (C) Dissociated cells were collected at the 16- to 17-somite stage from batches of control and transient transgenic embryos and analyzed by FACS to score quantitatively the numbers of GFP+ cells. Shown are results from one representative experiment, although the data were comparable in three independent experiments. Compared with control embryos (blue) the I(lmo2:ΔBR)I transgenic embryos (red) show more than double the normal amount of GFP+ hematopoietic cells.
BMP signaling regulates an early step of hematopoietic development in lateral mesoderm

Because both hematopoietic and vascular markers are expanded when BMP signaling is attenuated, it is possible that this results from alterations in the development of the hemangioblast, the common progenitor to both cell types. This should be reflected by effects on the expression of the early transcriptional program that regulates hemangioblast development. For example, co-expression of the transcription factors Lmo2 and Scl is thought to mark hemangioblast cells (Gering et al., 2003). When analyzed in situ hybridization at the 10- to 12-somite stage, the expression patterns for lmo2 and scl are both expanded in embryos injected with the lmo2:ΔBR transgene (Fig. 6A-D). This is in contrast to the initial pattern of scl earlier at the one-somite stage (Fig. 3). Expansion of a hemangioblast population is not expected to occur using a block that is more specific for the hematopoietic lineage. To test this, we analyzed embryos that had been injected instead with the I/gata1:ΔBR transgene. In this case the mutant receptor is expressed in the committed hematopoietic progenitors localized in lateral mesoderm at the five-somite stage, compared with when the lmo2 promoter is used to express the mutant receptor (throughout lateral mesoderm) starting around the one-somite stage. In this case we found that gata1 expression was still enhanced indicating that even in the Gata1+ cells the BMP signaling pathway is functioning to restrict hematopoiesis (Fig. 6E,F). However, in this case, the expansion of the endothelial marker flk1 does not occur, as we see no consistent difference in the flk1 expression pattern comparing the transgenic and control embryos (Fig. 6G,H). Therefore, the results are consistent with a function for the BMP signaling pathway to restrict the expansion of both blood and vascular lineages in lateral mesoderm from the earliest stages, including potentially through regulation of the common progenitor, the hemangioblast. However, the effect could just as well occur independently in both committed vascular and hematopoietic progenitors, as suggested by the fact that responsiveness to BMP signaling is still maintained in a subset of progenitor cells from which erythroid cells differentiate in lateral mesoderm.
Increased BMP signaling correspondingly restricts hematopoiesis in lateral mesoderm

Based on the results generated by inhibiting BMP signaling, excessive BMP signaling in lateral mesoderm should restrict the development of the primitive hematopoietic cells. In order to test this prediction, we used two independent assays. In the first approach, we performed transplant experiments to place embryonic cells that express exogenous Bmp4 in the lateral mesoderm. Donor embryos were generated by injecting fertilized eggs with RNA encoding Bmp4 and in addition a lysine-conjugated rhodamine fluorescent dextran, for lineage tracing purposes. At the shield stage, host embryos transgenic for the gata1:gfp reporter gene were transplanted with BMP-expressing donor cells. The donor cells were transplanted near the ventral margin in order to facilitate contribution to lateral mesoderm. Chimeric embryos were allowed to develop until 20 hours, and for those individual transplanted cells that end up in lateral mesoderm (identified by red fluorescence) we scored host cells that develop in immediate association for whether or not they express the gata1:gfp reporter. In other words, we examined whether a cell in lateral mesoderm that expresses exogenous Bmp4 enhances or suppresses the generation of erythroid cells from its immediate neighbors. As control for comparison, we performed the same transplantation protocol using donor cells that were injected only with the red lineage tracer. The transplanted embryos were also stained with Hoechst to identify all of the nuclei and to ensure that only cells in the immediate vicinity of the transplanted (red) cells were scored. Rationale for defining the scoring criterion is derived from studies showing that BMP signaling acts only on cells in the immediate vicinity (Nikaido et al., 1999). For this analysis we analyzed fully the numbers of associated GFP+ cells from six chimeras each, choosing those that had incorporated approximately equal numbers of donor cells in the lateral mesoderm. Representative results for one test and control embryo are shown in Fig. 7, and the data from the full analysis is tabulated in Table 2. The data show that cells within lateral mesoderm that express exogenous Bmp4 are surrounded by a significantly fewer number of GFP+ cells (on average 65%), compared with control transplanted cells that do not express exogenous Bmp4 ($P<0.002$). The data are fully consistent with the interpretation based on the effect of expressing the mutant receptor, and indicate that BMP signaling restricts the number of hematopoietic cells that develop from progenitors in the lateral mesoderm.

In a second independent test, we used a transgenic approach analogous to the strategy used for blocking BMP signaling. A constitutively active isoform of the type I BMP receptor (caBR) has been generated and tested previously (Candia et al., 1997). Therefore we used the I-Scel-mediated approach to generate transgenic embryos that express caBR in Lmo2+ lateral mesoderm. As shown in Fig. 8, embryos injected with the I(lmo2:caBR)I transgene develop a phenotype that is precisely the opposite of embryos injected with the I(lmo2:ΔBR)I transgene. In this case, there is a relative block of expression of hematopoietic markers, including gata1 and scl (data also summarized in Table 1). Therefore, the

**Table 2. Results of transplanting host cells expressing Bmp4 into host embryos**

<table>
<thead>
<tr>
<th>Treatment/outcome</th>
<th>Number of embryos</th>
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<tr>
<td>Transplants into gata1:gfp host</td>
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<tr>
<td>Host embryos where donor cells localized to lateral mesoderm</td>
<td>20</td>
</tr>
<tr>
<td>Host embryos analyzed for red to green cell ratio</td>
<td>6</td>
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**B Test transplantations with lineage tracer and Bmp4 mRNA**

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<tr>
<th>Treatment/outcome</th>
<th>Number of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplants into gata1:gfp host</td>
<td>300</td>
</tr>
<tr>
<td>Host embryos where donor cells localized to lateral mesoderm</td>
<td>35</td>
</tr>
<tr>
<td>Host embryos analyzed for red to green cell ratio</td>
<td>6</td>
</tr>
</tbody>
</table>

**C Hematopoietic (GFP+) cell to donor cell ratio**

<table>
<thead>
<tr>
<th>Test transplants (green cells/red cells)</th>
<th>Control transplants (green cells/red cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.82</td>
</tr>
<tr>
<td>2.</td>
<td>0.68</td>
</tr>
<tr>
<td>3.</td>
<td>0.85</td>
</tr>
<tr>
<td>4.</td>
<td>0.79</td>
</tr>
<tr>
<td>5.</td>
<td>0.74</td>
</tr>
<tr>
<td>6.</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Donor cells were collected from embryos derived either from eggs that were injected only with the lineage tracer (A) or had been co-injected with RNA encoding Bmp4 (B). The number of chimeras is indicated (about 10% had donor cells localized to lateral mesoderm). (C) The ratio of Gata1+ cells, as indicated by GFP expression, that develop in association with donor cells is shown for six independent chimeras. $P<0.002$, determined by comparing the six embryos from each set of transplants using the Mann-Whitney U test (non-parametric student t-test).
balance of BMP signaling in lateral mesoderm is necessary for normal hematopoietic development, such that repressed or hyperactive signaling leads to subsequent enhancement or restriction, respectively, in subsequent development of hematopoietic progenitors.

**Attenuating BMP signaling in lateral mesoderm restricts expression of the pronephric marker pax2.1**

In zebrafish embryos, the progenitors giving rise to the caudal region of the pronephros are derived from mesoderm that lies just ventrolateral to the hemato-vascular domain marked by scl or flk1 expression. As this is mesoderm that is also associated with hemato-vascular lineages, it is possible that the effects of manipulating BMP signaling could have either similar or opposite effects on pronephric development. To address this, we analyzed embryos derived from fertilized eggs that had been injected with the I(lmo2:Br) transgene for expression of the pronephric marker pax2.1. The pax2.1 expression domain overlaps with that for lmo2 and is immediately more ventral to the expression domain for scl in lateral mesoderm (note that in many species this is usually called ‘intermediate mesoderm’ and lies medial to the lateral hemato-vascular mesoderm). Transient transgenic embryos were analyzed at the 12 somite stage for pax2.1 transcripts by in situ hybridization of the pronephric marker pax2.1. The pax2.1 expression domain overlaps with that for lmo2 and is immediately more ventral to the expression domain for scl in lateral mesoderm (note that in many species this is usually called ‘intermediate mesoderm’ and lies medial to the lateral hemato-vascular mesoderm). Transient transgenic embryos were analyzed at the 12 somite stage for pax2.1 transcripts by in situ hybridization of the pronephric marker pax2.1. The pax2.1 expression domain overlaps with that for lmo2 and is immediately more ventral to the expression domain for scl in lateral mesoderm (note that in many species this is usually called ‘intermediate mesoderm’ and lies medial to the lateral hemato-vascular mesoderm). In contrast to the results obtained for hemato-vascular markers, pax2.1 expression levels are reduced by forced expression in lateral mesoderm of the mutant receptor, again observed most clearly with the expected frequency of ~30% of the embryos (Fig. 9A,B; Table 1). To confirm that BMP signaling actively promotes pronephric development, we tested the effect of expressing in lateral mesoderm the transgene that encodes a constitutively active BMP receptor (caBR). Approximately 30% of the embryos derived from eggs injected with the I(lmo2:caBR) transgene display an expansion of the pax2.1 expression domain (Fig. 9C).

**DISCUSSION**

It is well established that BMP signaling is required for the generation of ventral mesoderm during gastrulation, which results ultimately in the development of hematopoietic, vascular and
pronephric lineages (Davidson and Zon, 2000). Previous studies have addressed the temporal requirement for active BMP signaling, particularly in the *Xenopus* model system (Kikkawa et al., 2001; Kumano et al., 1999; Miyama et al., 1998; Xu et al., 1999; Zhang and Evans, 1996), and have clearly shown that the pathway functions late relative to the initial steps of mesoderm induction. By using a conditionally active form of Smad6, a negative regulator of the pathway, we were able to confirm that even post-gastrulation stages of mesoderm development require active signaling in order for the subsequent development of the primitive erythroid blood island (Schmerer and Evans, 2003). However, each of the approaches used previously was limited to blocking BMP signaling during the precommitment phase of hematovascular development, and thus could not interrogate accurately any subsequent requirement after progenitors are specified.

In order to probe functions for the pathway in the specified progenitors for these specific lineages, it was necessary to generate a conditional approach to block BMP signaling during somitogenesis within lateral mesoderm. Our results demonstrate that BMP signaling functions in lateral mesoderm to affect the decision of progenitors to commit either to a hematopoietic fate, or to a pronephric fate. Transgenesis facilitated by the I-sceI meganuclease provides an effective approach that is limited only by the availability of appropriate promoters. The *lmo2* promoter was ideal for our purpose as it is not expressed until around the one-somite stage, and therefore genes expressed using this promoter will not interfere with development prior to this point. However, the *lmo2* gene is subsequently activated throughout lateral mesoderm and so can be used to regulate simultaneously gene expression in the progenitors for hematopoietic, vascular and pronephric lineages. Our results indicate that BMP signaling does continue to regulate development of lateral mesoderm within *lmo2*+ cells and acts at this stage to restrict hematovascular development and promote pronephric mesoderm. Although the increased numbers of hematopoietic cells could be caused by changes in cell proliferation, immunohistochemistry using anti-phospho-histone H3 antibodies failed to show any significant increase in mitotic cell numbers in embryos expressing the mutant BMP receptor (data not shown).

Instead, the data support a model in which decreased levels of BMP signaling within lateral mesoderm results in changes of lineage fate, with increased numbers of hematopoietic and pronephric progenitors occurring at the expense of pronephric progenitors. This interpretation relies on the assumption that BMP ligands are normally expressed and active at these later stages of development. Although the expression patterns for a number of potential ligands are not fully described, there are clear candidates for the functional signal, including Bmp2b [in ventral mesoderm (Kishimoto et al., 1997)], Bmp4 [in posterior epidermis, tailbud and lateral mesoderm (Dick et al., 1999)] and, perhaps most strikingly, Bmp6, which is expressed in the two stripes of ventral mesendoderm during early stages leading into somitogenesis (Thiese and Thiese, 2005).

The effects caused by manipulating BMP signaling could be mediated by the direct regulation of key transcription factors such as *scl*. Much like the mutant BMP receptor, the forced expression of *scl* expands both blood and vascular lineages and at the same time is capable of suppressing the development of pronephric mesoderm (Gering et al., 2003). Moreover, expansion of the hematopoietic program caused by co-injection of RNA encoding *scl* and *lmo2* is restricted to pronephric mesoderm, consistent with an inherent plasticity of lateral mesoderm (Gering et al., 2003). Enhanced expression of *scl* could also explain the corresponding expansion in markers specific to differentiated blood and vascular cells, including *gata1* and *flk1*. In addition, myelopoiesis that occurs in the anterior part of the lateral mesoderm is also dependent on *scl*, as there is a strong reduction in *l-plastin* expression in the *scl* morphant (Dooley et al., 2005). Depletion of BMP signaling in our experiments also occurs in anterior lateral mesoderm and results in enhanced expression of both *l-plastin* and *myeloperoxidase*, markers for macrophage and granulocytes, respectively. The development of myeloid progenitors in the rostral domain has previously been shown to be resistant to alterations in BMP signaling prior to gastrulation, compared with the caudal erythroid domain (Lieschke et al., 2002). By contrast, the enhanced levels of the anterior hematopoietic markers caused by inhibiting BMP signaling during somitogenesis is particularly robust. This might be consistent with a lower level of endogenous signaling in the anterior region, corresponding with a gradient of BMP signaling from high posterior to low anterior, as proposed previously (Lieschke et al., 2002), so that the dominant-negative receptor most effectively decreases the levels below a particular threshold in the rostral domain. As *scl* expression is initially activated in ventral mesoderm by BMP signaling (Maeno et al., 1996; Zhang and Evans, 1996), if it remains a relevant target gene during somitogenesis, our data suggest that in defined progenitors the BMP pathway switches, instead, to downregulate or restrict *scl* levels.

According to our results, cells in the lateral mesoderm respond to different threshold levels of BMP signaling to determine cell fate. This is consistent with results in the chick embryo obtained by exposing presomitic regions with different levels of Bmp4 (Tonegawa et al., 1997). Somites exposed to the highest levels of signal are transformed entirely to lateral plate, whereas those cells exposed to a lower level express a distinct lateral somatic program. Similarly, in ectoderm the expression of a constitutively active BMP receptor is sufficient to convert in a cell-autonomous matter progenitors from a neural to an epidermal fate (Nikaido et al., 1999).

With respect to lateral mesoderm, the results are consistent with a threshold that influences specified but uncommitted lateral mesoderm to distinguish intermediate pronephric mesoderm, which below this threshold would otherwise commit to a hematopoietic fate. This is consistent with a gradient of BMP signaling that is highest in the most lateral regions and lower at more medial positions, being lowest in presomitic mesoderm owing to midline-derived inhibitors. We note that the *pax2.1* signal is never lost completely in the transgenic embryos expressing the dominant-negative BMP receptor in *lmo2*+ cells, but this probably reflects the lack of a complete overlap in *lmo2* and *pax2.1* expression, so that the most lateral pronephric progenitors avoid the transgene effect.

Although the level of BMP signaling may act to specify lineage at the level of the hemangioblast, for several reasons we suggest that it functions at a later stage to restrict the development of committed endothelial and hematopoietic cells. If the expression of *lmo2* defines the short-lived hemangioblast, then our experiments using the *lmo2* promoter are by definition manipulating expression at subsequent stages. Using the *gata1* promoter we show that the pathway continues to restrict the development of committed hematopoietic progenitors. It is perhaps relevant that the analysis of the mouse Smad5 knockout (Yang et al., 1999) showed a twofold increase in the numbers of myeloid progenitors on the yolk sac (although in this case erythroid progenitors were unchanged). The direct application of Bmp2 or Bmp7 to a highly enriched population of stem cells inhibits the proliferation of hematopoietic progenitors (Bhatia et al., 1999). Thus, although there is much evidence that BMPs support the development of hematopoietic mesoderm (e.g. Li et al., 2001), the pathway may then help to maintain a stem or early...
progenitor state and restrict the emergence of more differentiated hematopoietic cells. Although we did not test if the pathway is also restrictive to the development of committed endothelial progenitors, there is a large literature documenting both stimulatory and inhibitory effects of the TGFβ pathway on angiogenesis (Goumans et al., 2003). Notably, violet beurregarde embryos (vbg), carrying a mutation of the ALK1 (acvr1) gene, are characterized by vessel dilations caused by increased numbers of endothelial cells (Roman et al., 2002). Although ALK1 is a TGFβ/activin receptor, it signals via Smad1 and Smad5 (Goumans et al., 2002), and therefore this pathway could be influenced by the expression in angioblasts of the dominant-negative BMP receptor.

Recently, a distinct but related approach was developed to manipulate conditionally BMP signaling in zebrafish, using a heat shock-inducible promoter to express a dominant-negative receptor (Pyati et al., 2005). This approach was also successful at distinguishing specific roles for BMP signaling subsequent to early patterning and into early stages somitogenesis. Expression of the mutant receptor by heat-shock caused defects in ventral tail fin development and tail organizer development. However, in this study, specific alterations in hematopoietic-vascular development were not noted, in contrast to the results we present. The generation of phenotypes we describe may be facilitated by the targeted expression of the mutant receptor using the Imo2 promoter, compared with the relatively transient expression generated by a heat-shock. Combinations of these two independent approaches for conditional gene expression should help in dissecting the functions of important developmental signaling pathways in specific lineages and at defined embryonic transition states.

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References


truncated bone morphogenetic protein-4 receptor alters the fate of ventral mesoderm to dorsal mesoderm: Roles of animal pole tissue in the development of ventral mesoderm. Proc. Natl. Acad. Sci. USA 91, 10260-10264.


Roman, B. L., Pham, V. N., Lawson, N. D., Kutik, M., Childs, S., Lekven, A. C., Garrity, D. M., Moon, R. T., Fishman, M. C., Lechleider, R. J. et al. (2002). Disruption of acvl1 increases endothelial cell number in zebrafish cranial vessels. Development 129, 3009-3019.


