Transgenic zebrafish reveal stage-specific roles for Bmp signaling in ventral and posterior mesoderm development

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Summary

Bone morphogenetic protein (Bmp) signaling is crucial for the formation and patterning of zebrafish ventral and posterior mesoderm. Mutants defective in the Bmp pathway have expanded trunk muscle, abnormal tails and severely impaired development of ventral mesodermal derivatives such as vasculature, blood and pronephros. As Bmps continue to be expressed in the ventral and posterior mesoderm after gastrulation, it is likely that Bmp signaling continues to play an important developmental role during outgrowth of the posterior body. However, because Bmp signaling plays an essential role during the gastrula stages, it has not been possible with mutants or standard disruption techniques to determine the later functions of the Bmp pathway. To study the role of Bmp signaling in the ventral and posterior mesoderm during trunk and tail outgrowth, we generated a transgenic zebrafish line containing a heatshock-inducible dominant-negative Bmp receptor-GFP fusion. Our data show that Bmps are important for tail organizer formation and for patterning the ventral mesoderm during early gastrulation. However, from mid-gastrulation to the early somitogenesis stages, Bmp signaling is important for ventral tail fin development and for preventing secondary tail formation. We conclude that the role of Bmp signaling in the ventral and posterior mesoderm changes as gastrulation proceeds.

Key words: Bmp signaling, Transgenic zebrafish, Ventral mesoderm, Tail

Introduction

The formation of the vertebrate trunk and tail is a complex process involving carefully regulated cell differentiation and morphogenesis (reviewed by Griffith et al., 1992). The tailbud is a specialized domain arising from the ventrolateral regions of gastrula-stage fish and frog embryos, which contain precursors for all the tail tissues except the notochord (Gont et al., 1993; Kanki and Ho, 1997; Davis, 2000). A variety of studies in both fish and frogs have suggested that the molecular signaling pathways important for development of the tail could exert their influence beginning at the early gastrula stages (Gont et al., 1993; Mullins et al., 1996). Furthermore, genes that will mark the tailbud during trunk and tail morphogenesis, such as *eve1/Xhox3*, are already expressed in early gastrula embryos (Joly et al., 1993), suggesting that the patterning of the posterior body begins very early in development, well before a morphologically apparent tailbud has formed. Whether or not the tailbud cells continue to be under the influence of patterning signals during later gastrulation and elongation of the tailbud has been less clear.

One crucial signaling pathway for vertebrate trunk and tail mesoderm formation is the Bone morphogenetic protein (Bmp) pathway (reviewed by Dale and Jones, 1999). Bmps are members of the TGFβ superfamily of secreted proteins, and they bind TypeI/TypeII receptor complexes to initiate a signaling cascade that activates transcription of downstream targets such as the homeobox genes *vox* and *vent* (Onichtchouk et al., 1996; Melby et al., 2000; von Bubnoff and Cho, 2001; Ramel and Lekven, 2004). During gastrulation in *Xenopus* and zebrafish, Bmp signaling is highest on the ventral side of the embryo, where it patterns the mesoderm to adopt fates including blood, vasculature, pronephros and tail muscle. Loss of Bmp signaling in both animals leads to expansion of dorsal structures, such as trunk muscle, at the expense of ventral structures (Re'em-Kalma et al., 1995; Mullins et al., 1996; Kishimoto et al., 1997). Zebrafish mutants support the model that a gradient of Bmp signaling patterns various tissue types during gastrulation in the developing embryo (reviewed by Dale and Wardle, 1999). For example, the *swirl* mutant, which lacks *bmp2b*, has radialized trunk somites, reduced blood, vasculature and pronephros, and no tail tissue (Kishimoto et al., 1997). Other mutants in the Bmp pathway, including *snailhouse* hypomorphs (Dick et al., 2000), *piggytail* (Kramer et al., 2002), *lost-a-fin* (Mintzer et al., 2001) and *minifin* (Connors et al., 1999), have progressively less severe phenotypes than *swirl* mutants. For instance, in zygotic *lost-a-fin* embryos, which are mutant for the Bmp receptor *alk8*, the patterning of the ventral and posterior mesoderm is normal, but the ventral tail fin is absent (Mintzer et al., 2001). Such data suggest that the highest levels of Bmp signaling are required for formation of the ventral tail fin, while lower levels of Bmp signaling are required for other ventral and posterior tissues.

The role of Bmps in tail development has been further elucidated by experiments involving overexpression of *bmp* RNA in the early zebrafish embryo. When injected in combination with RNA encoding Wnt8 and the Nodal ligand

Cyclops (Cyc), Bmp induces the formation of ectopic tails (Agathon et al., 2003). Moreover, whereas tails can form at lower efficiency when Bmp is expressed with either Wnt8 or Cyc alone, Bmp is indispensable for tail formation. A second set of experiments suggests that Bmp signaling is important for reserving a population of tailbud cells for somitogenesis in the tail. When dorsal determinants, which activate Bmp antagonists such as *chordin* (Sasai et al., 1994) and *noggin* (Zimmerman et al., 1996), are removed before gastrulation, embryos do not develop trunk somites and instead only develop tail somites (Ober and Schulte-Merker, 1999). If, however, dorsal determinants are removed in a *swirl* background, which lacks zygotic Bmp signaling, the embryos recover trunk somite formation and lose the tail somites. These experiments indicate that Bmp signaling negatively regulates trunk somitogenesis while promoting tail somitogenesis, potentially by setting aside a population of cells to form tail somites rather than trunk somites.

Such studies show a requirement for Bmp signaling in formation of the zebrafish tail organizer. However, they do not determine whether Bmps exert their influence on tailbud cells only during gastrulation, or if Bmps continue to be required in the post-gastrula tailbud. Bmps are expressed in the tailbud and surrounding tissues throughout somitogenesis (Martinez-Barbera et al., 1997; Dick et al., 2000), making it seem likely that they are playing important roles during trunk and tail formation. As mutants are defective in Bmp signaling from the onset of zygotic transcription, and overexpression, dominant-negative and morpholino experiments have been performed only before the gastrula stages, it is unclear what the role of Bmp is in the ventral and posterior mesoderm after the end of gastrulation. To address this question, we generated a zebrafish transgenic line for conditionally reducing Bmp signaling. We found that Bmp signaling plays an important role in primary tail formation during early gastrulation, and that it prevents the formation of ectopic tails from the mid-gastrula stages through early somitogenesis. High levels of Bmp signaling appear to be important for the development of most ventral mesodermal derivatives during early gastrulation, whereas the ventral fin requires Bmp signaling from early gastrulation through the early somitogenesis stages.

Materials and methods

Dominant-negative Bmp receptor cloning

The *Xenopus* type Ia Bmp receptor (Graff et al., 1994) was amplified with the primers GAGATCTAAAATGAGAAAACGACTTTTCATTGC and CTCTAGACTGGATCTGCTTGGCTATAGTACG, truncating the receptor just before the kinase domain. This fragment was inserted into the *Bam*HI and *Xba*I sites of CS XLT GFP (kind gift of J. Miller), fusing GFP in frame to the C-terminus of the truncated receptor downstream of the *hsp70* promoter. *IsceI* meganuclease sites flank the transgene.

Generation of a stable transgenic line

Uncut DNA (1 nl) was injected into 1-cell stage WIK/AB embryos. *IsceI* meganuclease was co-injected with the DNA to maximize the number of integration events. The injection mix was: 100 pg/nl DNA, 0.5× *IsceI* Buffer, 10% *IsceI* enzyme. The F_1 generation was screened for heatshock-inducible GFP fluorescence, and a stable line was generated.

Heatshock conditions

Embryos from an F_1 out-cross were typically heatshocked for 1 hour at desired stages by placing them for 1 hour in a 37°C air incubator, and then screened for GFP fluorescence after 1.5 hours at 28.5°C. Embryos were either fixed at 2 hours post-heatshock for in-situ hybridization analysis or left in a 28.5°C air incubator for later visualization. For PCR machine heatshocks, single embryos were separated into PCR tubes with 20 μl of embryo medium. The PCR block was ramped to 37°C for 1 hour, then held at 28.5°C.

Confocal microscopy

Truncated Bmp receptor-GFP/*tbx6-gfp* embryos were heatshocked from shield to bud stage in a 37°C air incubator. For late tail growth, embryos with ectopic tails and GFP fluorescence in the tail were identified at the 22-24-somite stage. Embryos were mounted in embryo medium containing 1% low-melt agarose. For early tail growth, 20 embryos were mounted at the 16-17-somite stage in 1% low-melt agarose with their tails exposed to the medium. Images were acquired using a Zeiss LSM Pascal confocal microscope and a 40× lens. Slices (1.6 μm) were taken once per hour.

Transplants

For wild-type transplants (Moens and Fritz, 1999), 1-cell stage WIK/AB embryos were injected with fluorescein-dextran (M_r 10,000). At the dome stage, cells were transplanted from labeled donor embryos to the ventral side of shield-stage host embryos from a transgenic in-cross. Host embryos were immediately heatshocked in an air incubator at 37°C through bud stage to maximize ectopic tail formation. Embryos with ectopic tails at 24 hours were scored for the presence or absence of fluorescein-labeled cells within the ectopic tails. Control transplants from transgenic donors were performed in the same manner, but donor embryos were collected from in-crosses of transgenic fish. PCR analysis of genomic DNA was used to determine whether donor embryos were wild type or transgenic.

Results

Generation of a zebrafish transgenic line to conditionally attenuate Bmp signaling

Overexpression of truncated Bmp receptors is a powerful method for specifically reducing Bmp signaling during development in both fish and frogs (Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994; Schmidt et al., 1995; Neave et al., 1997). The truncated receptor acts as a dominant-negative by associating with endogenous receptor complexes and preventing intracellular phosphorylation of receptor SMADs upon ligand binding (Fig. 1) (Graff et al., 1994). The advantage to using a dominant-negative receptor as an inhibitor rather than a secreted Bmp inhibitor such as Noggin (Sasai et al., 1994) or Chordin (Zimmerman et al., 1996) is that it allows for cell-autonomous inhibition of Bmp signaling, which is very useful in transplant studies.

A previous study showed that a truncated *Xenopus* Bmp receptor is a very effective inhibitor of Bmp signaling in zebrafish embryos (Neave et al., 1997). This receptor is truncated just after the transmembrane domain, so it lacks the kinase domain and adjacent sequences. In order to trace the cells expressing the dominant-negative Bmp receptor, we generated a construct with GFP fused to the C-terminus of the truncated receptor. However, this fusion protein no longer inhibited Bmp signaling when overexpressed (data not shown). Thus, we generated a second construct with GFP replacing the kinase domain, but we left the sequences between the

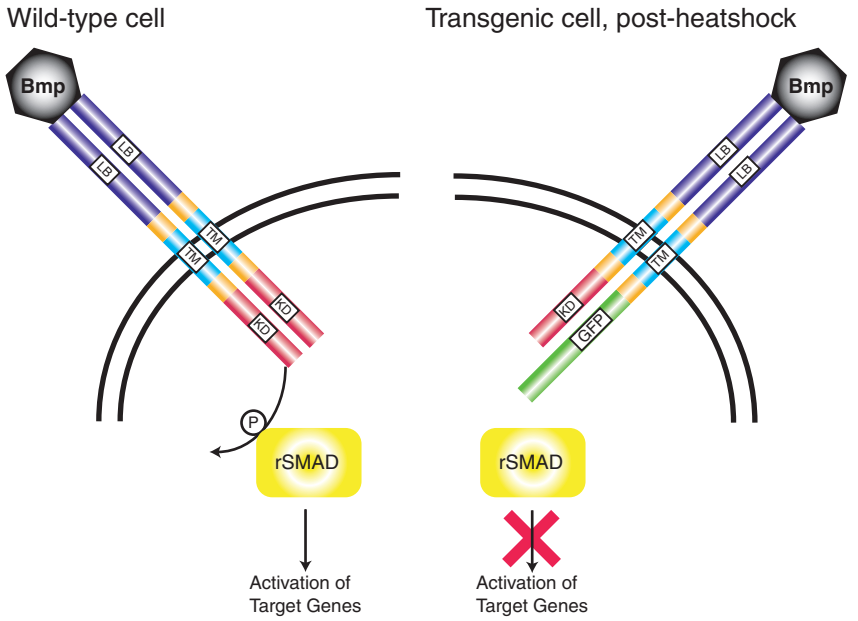

Fig. 1. A method for attenuating Bmp signaling in transgenic zebrafish. Diagram of a wild-type cell receiving a Bmp signal (left) and a transgenic cell receiving a Bmp signal after heatshock (right). In a wild-type cell, the Bmp ligand binds to a Type I/Type II receptor complex, which then phosphorylates and activates a receptor SMAD (rSMAD), leading to target gene activation. After heatshock of the transgenic cell, the truncated Type I Bmp receptor containing GFP in place of the kinase domain displaces the endogenous Type I receptor, preventing the receptor complex from activating rSMADs. As a result, downstream target genes are not activated, and the cell is reduced in its capacity to respond to Bmp signals. KD, kinase domain; LB, ligand-binding domain; P, phosphate; TM, transmembrane domain.

transmembrane domain and the original kinase domain intact. To test the efficacy of the new construct, we injected 1-cell zebrafish embryos with RNA encoding this truncated receptor-GFP fusion. We found that the new truncated receptor-GFP protein was as effective at inhibiting Bmp signaling as the original truncated Bmp receptor (data not shown).

To express the truncated Bmp receptor-GFP fusion at different times of development, we placed this construct under the control of the *hsp70* promoter (Halloran, 2000) and generated a zebrafish transgenic line. To determine if the induction of the transgene would recapitulate phenotypes of known Bmp pathway mutants, we heatshocked embryos before the onset of gastrulation and observed their phenotypes during the development of the posterior body. Transgenic embryos were sorted by GFP fluorescence 1 hour after the completion of the heatshock and scored at both the 14-somite stage and 30 hours post-fertilization (hpf) for the degree of dorsalization using the scale developed by Mullins et al. (Mullins et al., 1996). In this scale, C5 represents an embryo completely lacking Bmp signaling (*swirl* phenotype), whereas C1 is a mildly dorsalized embryo. Heatshocking embryos at 3 hpf produced severely dorsalized phenotypes in transgenic embryos (Fig. 2B,C). By contrast, sibling non-transgenic embryos that received the same heatshock treatment had no discernible defects (Fig. 2A), demonstrating that the effects we saw were not due to the heat treatment. The majority of transgenic embryos were in the C4 dorsalization class, with severe expansion of somites and curling of the trunk and tail (Fig. 2B; Table 1). Embryos in this class resembled hypomorphic *snailhouse (bmp7)* mutants (Dick et al., 2000). Some embryos were severely dorsalized at the end of gastrulation (10 hpf, data not shown), but they did not survive beyond the end of somitogenesis. These embryos were scored as a C5 phenotype, resembling *swirl (bmp2b)* mutant embryos (Kishimoto et al., 1997). A third class of embryos had C3 phenotypes, with expansion of posterior somites and curling at the end of the tail (Fig. 2C). Embryos in the C3 class resemble *piggytail (smad5)* mutants (Kramer et al., 2002). Interestingly, heatshocking embryos at 3 hpf in a PCR machine rather than an air incubator produced a more severe dorsalization, with all the embryos having a C5 phenotype (Table 2). These data demonstrate that induction of the transgene before gastrulation phenocopies zebrafish mutants with severe reductions in Bmp signaling.

As the truncated receptor is fused to GFP, we could readily examine the kinetics of induction. We observed the peak levels of GFP expression (and thus, truncated Bmp receptor expression) in embryos at 2 hours post-heatshock. By 6 hours post-heatshock, GFP fluorescence had begun to fade. We hypothesize that this rapid turnover is due to recycling of the truncated receptors within cells. Such a model would fit with

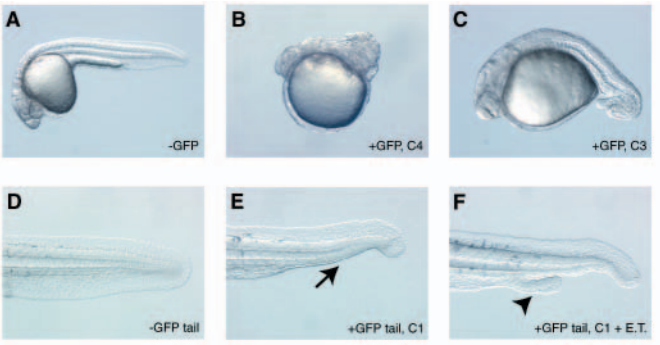

Fig. 2. Phenotypes caused by reducing Bmp signaling at various developmental stages. Embryos from a transgenic outcross were heatshocked in an air incubator at 3 hpf (A-C) or shield stage (D-F), and the GFP-positive embryos were sorted and scored for dorsalized phenotypes at 30 hpf. Note the fully formed tail in the wild-type sibling (whole embryo in A, tail view in D). Transgenic embryos heatshocked at 3 hpf display both C4 (B) and C3 dorsalized (C) phenotypes. Note the curled trunk and tail in B and the curled tail in C. Transgenic embryos heatshocked at shield stage display either a C1 phenotype (E; arrow denotes loss of the ventral tail fin) or a C1 phenotype coupled with the formation of an ectopic tail (F; arrowhead denotes second tail). ET, ectopic tail.

Table 1. Scoring of transgenic embryos after incubator heatshock at various stages

HS stage (*n*)	C5	C4	C3	C2	C1	C1+2° tail	No effect
3 hpf (20)	15%	70%	15%	0%	0%	0%	0%
Shield (27)	0%	0%	0%	0%	100%	18%	0%
Bud (39)	0%	0%	0%	0%	90%	15%	10%
3-somite (23)	0%	0%	0%	0%	100%	4%	0%
6-somite (70)	0%	0%	0%	0%	62%	3%	38%
10-somite (48)	0%	0%	0%	0%	0%	0%	100%

Table 2. Scoring of transgenic embryos after PCR machine heatshock at various stages

HS stage (*n*)	C5	C4	C3	C2	C1	C1+2° tail	No effect
3 hpf (20)	100%	0%	0%	0%	0%	0%	0%
Shield (22)	0%	0%	41%	0%	59%	18%	0%
70% (23)	0%	0%	0%	0%	100%	17%	0%
Bud (21)	0%	0%	0%	0%	100%	14%	0%
3-somite (25)	0%	0%	0%	0%	100%	8%	0%
6-somite (24)	0%	0%	0%	0%	54%	4%	46%
10-somite (25)	0%	0%	0%	0%	0%	0%	100%

previous reports of signaling receptors being rapidly degraded to allow for transient responses to extracellular signals (Rosenfeld et al., 2002; Strous and van Kerkhof, 2002; Teis and Huber, 2003). Therefore, our transgenic system provides a rapid, short-lived method for reducing Bmp signaling in the zebrafish embryo. As a result, we could study the role of Bmp in patterning tissues during brief windows of time.

Effects of reducing Bmp signaling on ventral and posterior mesoderm at different stages of development

Having developed a system for inducibly interfering with Bmp signaling, we wished to examine the effects of reducing Bmp signaling at different stages of development. Heatshocking embryos at shield stage caused phenotypes that were far less severe than those resulting from 3 hpf heatshocks. At 30 hpf, all transgenic embryos had the C1 dorsalized phenotype, with normal tails lacking ventral tail fins (Fig. 2E; Table 1). The ventral tail fin is thought to be the tissue that is most sensitive to losses of Bmp signaling, revealed by *minifin (tolloid)* and *lost-a-fin (alk8)* mutants (Connors et al., 1999; Mintzer et al., 2001). In addition to the C1 phenotype, typically one-fifth of transgenic embryos formed ectopic tail-like structures at their posterior ends (Fig. 2F; Table 1), although in one clutch as many as 45% of the embryos had these structures, which we henceforth refer to as ectopic tails. Heatshocking at the end of gastrulation (bud stage), and during somitogenesis (3-somite and 6-somite stages) also caused loss of the ventral tail fin (Table 1), indicating that ventral tail fin tissue is dependent upon continued Bmp signaling after gastrulation and through the early somitogenesis stages. Ectopic tail phenotypes were also induced by heatshocking at bud through 6-somite stages, but with lower penetrance than heatshocking at shield stage (Table 1). Heatshocking after the 6-somite stage had no effect on posterior development.

We reasoned that heatshocking individual embryos in a PCR machine might decrease the lag time of transgene expression, since this method caused a C5 phenotype in 100% of embryos heatshocked at 3 hpf. Indeed, this method resulted in GFP fluorescence within half an hour of heatshock. As shown in Table 2, heatshocking at shield (early gastrulation) using the PCR machine caused a more severe effect than similar heatshocks in an air incubator, with 41% of embryos having a C3 phenotype. By 70% epiboly (mid-gastrulation), however, heatshocking in the PCR machine caused only a C1 phenotype in transgenic embryos. Percentages of ectopic tails caused by heatshocking at various stages matched closely with similar heatshocks in the air incubator (compare Tables 1 and 2). Thus, Bmp signaling is important for ventral tail fin formation and

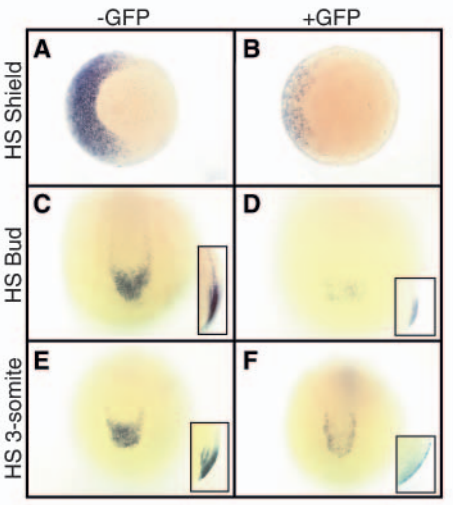

Fig. 3. *eve1* expression is dependent on Bmp signaling both during and after gastrulation. Embryos were heatshocked in an air incubator at the indicated stages, and the embryos containing the transgene were sorted by GFP fluorescence. (A,B) Vegetal views of embryos heatshocked at shield stage and assayed for *eve1* expression at 70% epiboly. Note the light staining on the left (ventral) side of the transgenic embryo (B) compared with the wild-type sibling (A). (C,D) Posterior views of embryos heatshocked at bud stage and assayed for *eve1* expression at the 7-somite stage. Note the darker staining of *eve1* in the wild-type embryo (C), with expression extending farther anteriorly than in the transgenic embryo (D). (E,F) Posterior views of embryos heatshocked at the 3-somite stage, then assayed for *eve1* expression at the 9-somite stage. Note the darker staining of *eve1* in the wild-type embryo (E) than in the transgenic embryo (F). Insets in C-F show lateral views of the tailbud. HS, heatshock.

suppression of ectopic tails from the mid-gastrula stage through early somitogenesis.

eve1 is a zebrafish homeobox gene expressed in the ventral mesoderm during gastrulation and in the tailbud during somitogenesis, thus serving as a good marker of ventroposterior fates within the embryo (Joly et al., 1993; Mullins et al., 1996). As *eve1* expression is reduced in *bmp* mutants (Joly et al., 1993; Mullins et al., 1996), we wished to examine whether *eve1* expression is dependent on Bmp signaling only during gastrulation, or whether it continues to be affected by reduction of Bmp signaling after gastrulation. We heatshocked embryos at shield stage, bud stage and 3-somite stage, then assayed for *eve1* expression by whole-mount in-situ hybridization. As predicted from mutant analyses, heatshocking at shield stage reduced *eve1* expression at the ventral margin in transgenic embryos compared with wild-type siblings (compare Fig. 3B with 3A). Interestingly, heatshocking after the end of gastrulation (bud and 3-somite stages) still caused reduced *eve1* levels in the tailbuds of transgenic embryos (compare Fig. 3C,E with 3D,F). The size of the tailbud was not decreased, as the homeobox gene *vent1* (Kawahara et al., 2000; Melby et al., 2000) was expressed at normal levels in the tailbuds of transgenic embryos after heatshock (data not shown). These data show that *eve1* expression is dependent upon Bmp signaling during both gastrulation and early somitogenesis.

High levels of Bmp signaling are necessary for the formation of ventral mesodermal derivates, including blood, vasculature and pronephros. In *bmp* mutant embryos, markers of these cell types are severely reduced or absent (Mullins et al., 1996). Additionally, Bmp signaling is important for the development of blood precursors after gastrulation in *Xenopus* embryos (Schmerer and Evans, 2003). We sought to examine whether high levels of Bmp signaling are similarly important for development of ventral mesodermal derivatives after gastrulation in zebrafish. We heatshocked one clutch of embryos at 3 hpf to inhibit Bmp signaling throughout gastrulation, and then heatshocked a second clutch continuously from bud to 12-somites. We examined the expression of *flk-1* (*kdr* – Zebrafish Information Network) (Sumoy et al., 1997), *gata1* (Detrich et al., 1995) and *pax2.1* (Pfeffer et al., 1998) to determine whether vascular, hematopoietic and pronephric cells, respectively, were affected. After heatshocking at 3 hpf, *flk-1* expression was dramatically reduced in transgenic embryos compared with controls (compare Fig. 4A and 4C). *flk-1*-expressing cells were limited to the very anterior and posterior ends of transgenic embryos, while these cells were spread throughout the trunk of control embryos. However, embryos heatshocked from bud to 12-somites had normal *flk-1* expression (compare Fig. 4B with 4C). Like *flk-1, gata1* expression was decreased in transgenic embryos heatshocked at 3 hpf (compare Fig. 4D with 4F). While control embryos had bilateral stripes of lateral plate mesoderm that strongly expressed *gata1*, this expression was limited to a few cells in the blood islands of transgenic embryos. Blood progenitors were apparently not affected by reductions in Bmp signaling after gastrulation, however, as *gata1* expression was normal in transgenic embryos heatshocked from bud to 12-somites (compare Fig. 4E with 4F). Finally, *pax2.1* expression was dramatically reduced in the pronephric ducts and tubules of transgenic embryos

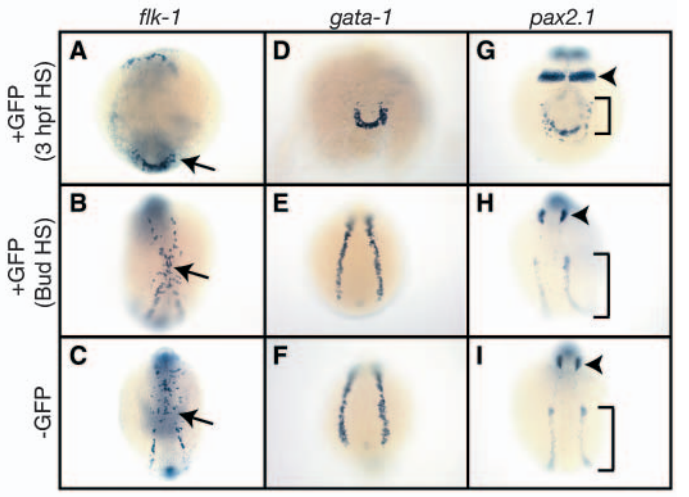

Fig. 4. High levels of Bmp signaling are important for ventral mesoderm patterning during gastrulation but not afterward. Embryos were heatshocked in an air incubator at 3 hpf for 1 hour, or heatshocked continuously from bud stage, sorted for GFP fluorescence and fixed at the 12-somite stage. Expression of *flk-1* (A-C), *gata1* (D-F) or *pax2.1* (G-I) was then examined by in-situ hybridization. Non-transgenic embryos heatshocked under either protocol were identical, so only a single example is shown for the –GFP samples (C,F,I). Expression of all three markers was reduced in transgenic embryos (A,D,G) compared with wild-type siblings (C,F,I) after 3 hpf heatshocks. Gene expression was unaltered following post-gastrula heatshocks (B,E,H). Arrows in A-C denote *flk-1*-expressing cells. Arrowheads in G-I denote *pax2.1* expression in the otic placode, while brackets indicate *pax2.1* expression in the pronephric ducts and tubules. Views in A-C and G-I are dorsal, with anterior to the top. (D-F) Posterior views of embryos.

heatshocked at 3 hpf compared with controls (compare Fig. 4G with 4I). A few disorganized cells could be seen in the posterior of the transgenic embryo, while pronephric ducts and tubules were easily identified in wild-type siblings. Anteriorly, *pax2.1* expression marking the otic placode was wider in transgenic embryos, indicating that these embryos, like some of the *bmp* mutants (Mullins et al., 1996), had expanded anterior tissues. In embryos heatshocked from bud to 12-somites, there was no difference in either the pronephric or anterior *pax2.1* expression (compare Fig. 4H with 4I). Taken together, these data suggest that high levels of Bmp signaling are not required after gastrulation for development of vascular, blood or pronephric cells in zebrafish.

The ectopic tails develop only mesoderm and fin tissue

As loss of Bmp signaling had previously been shown to cause a reduction in tail organizer formation (Mullins et al., 1996), we were intrigued by the formation of ectopic tail-like structures when we reduced Bmp signaling after early gastrulation. We examined the tissue types within the ectopic tails to understand the extent of tail formation in these structures. Fin and pigment were clearly apparent at 3 days post-fertilization (dpf) in the ectopic tails (Fig. 5A). This initially suggested that the ectopic tails could organize epidermal and neural crest cells. To determine whether the ectopic tail, like the primary tail, forms a tail-organizing

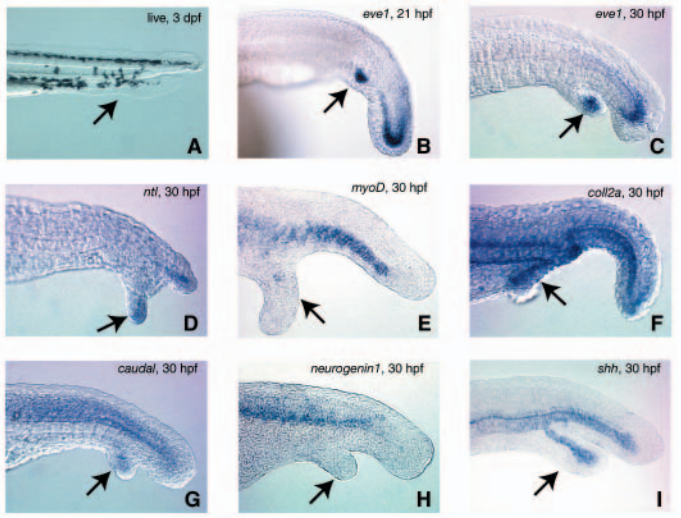

Fig. 5. Analysis of gene expression in the ectopic tails. Embryos were heatshocked from shield to bud stage to maximize ectopic tail formation, then photographed live at 3 dpf (A), or fixed at either 21 hpf (B) or 30 hpf (C-I). Expression of *eve1* (B,C), *no tail* (*ntl*; D), *myod* (E), *collagen 2a* (*coll2a*; F), *caudal* (G), *neurogenin1* (H), or *sonic hedgehog* (*shh*; I). Arrows in each panel indicate the location of the ectopic tail. In A note the presence of both fin and pigment tissue in the secondary tail. In all other panels except H note the expression of each corresponding gene in the ectopic tails. *ntl* expression in D was localized only to the tailbud of the ectopic tail. All images except I are representative of the majority of ectopic tails examined. We observed *sonic hedgehog*-expressing cells in 44% (n=16) of ectopic tails.

center, we examined *eve1* expression at various time points after heatshock. As discussed earlier, Bmp signaling is crucial for normal levels of *eve1* in the tailbud even after gastrulation. However, when we heatshocked at bud stage and examined *eve1* expression at 21 hpf (before ectopic tails were obvious under a dissecting microscope), we saw a new patch of *eve1* expression in the ventral portion of the tail (Fig. 5B). This result suggests that secondary tailbuds form before ectopic tails begin growing. *eve1* expression was maintained in the secondary tailbud at 30 hpf, after the ectopic tail grew to a significant length (Fig. 5C). Another tailbud marker, *no tail*, which is critical for primary tail formation (Schulte-Merker et al., 1994), was also expressed in the ectopic tail (Fig. 5D). Additionally, we saw expression of *myod* (Weinberg et al., 1996) in cells of the ectopic tail, demonstrating that the tails have developing muscle tissue (Fig. 5E). Hypochord cells also migrated into ectopic tails, as marked by *coll2a* (Yan et al., 1995) expression (Fig. 5F). We next wished to analyze whether the ectopic tails had neural tissue. We examined *caudal* (Joly et al., 1992) expression (Fig. 5G) and *neurogenin* (Korzh et al., 1998) expression (Fig. 5H), which are both expressed in the neural tube, but only *caudal* was expressed in the ectopic tails. As *caudal* is also expressed in the primary tailbud at this stage, we believe that the cells expressing this gene in the ectopic tails are actually tailbud cells. We examined other markers of neural cell types, including *islet1* (Inoue et al., 1994), which marks primary motoneurons, the pan-neuronal marker *hu* (Marusich et al., 1994) and the neural crest marker *crestin* (Luo et al., 2001), but we did not observe expression of any of these markers in secondary tails (data not shown). Thus, the ectopic tails we examined lacked neural tissue at 30 hpf, and subsequent pigment formation was probably derived from neural crest cells migrating ventrally out of the primary tail. Surprisingly, when we examined *sonic hedgehog* (Strahle et al., 1996) expression to assess whether floorplate cells were present, we only saw expression in cells that looked like notochord in some of the ectopic tails (Fig. 5I). These cells were columnar in shape, as opposed to the round floorplate cells, and they branched off the posterior part of the primary notochord. Thus, we conclude that secondary tails can have many of the same tissue types as primary tails, including tailbud cells, somitic tissue, notochord, hypochord, epidermis and pigment (which probably migrates into the secondary tails later than 30 hpf). The ectopic tails are mostly mesodermal, however, and they do not actively develop neural tissue.

Visualizing growth of the ectopic tails in living embryos

To examine the growth of the ectopic tails in a more dynamic fashion, we utilized a transgenic zebrafish line previously developed in our laboratory. This line, which expresses GFP under control of the *tbx6* promoter, marks cells of the tailbud, presomitic mesoderm and newly formed somites (Szeto and Kimelman, 2004). As the truncated Bmp receptor-GFP transgenic fish are only fluorescent for about 6 hours after heatshock, we crossed these fish to *tbx6-gfp* fish, heatshocked at shield stage to induce ectopic tail formation, and visualized *tbx6* promoter-driven GFP during late somitogenesis stages. Visualizing ectopic tail growth by confocal microscopy from the 25-somite stage, we easily observed GFP expression in the ventral region of the embryo (Fig. 6A). Interestingly, the GFP fluorescence demonstrated that while only a small portion of the ectopic tail separated from the main body, this structure could run a considerable distance along the body axis (arrows in Fig. 6A). We also observed the maturation of the presomitic mesoderm into small somites (the first ectopic somite seen at the 25-somite stage is marked by an asterisk). This closely mirrored the maturation of somitic cells in the primary tail. Rather than being a simple outgrowth containing various cell types from the primary tail, these data show that the secondary tail is active in elongating and forming mature somites.

We next wished to examine whether the ectopic tails arose from the primary tail splitting into two tails, or from a completely distinct tail-forming region. We examined truncated Bmp receptor-GFP/*tbx6-gfp* transgenic embryos by confocal microscopy beginning at the 17-18-somite stage, just after primary tail formation begins. At this stage, approximately 20% of the transgenic embryos formed small ectopic patches of GFP ventral to the primary tailbud (Fig. 6Ba,c). These ectopic patches, which later produced ectopic tails (Fig. 6Bb,d), started to grow out just after the primary tail had extended beyond the 17-18-somite position. We also examined embryos from earlier stages (12-13 somites), but we did not detect ectopic tail formation until at least the 16-17-somite stage (data not shown). Based on the time and position where the ectopic tails first began forming, we suggest that a population of mesodermal tail progenitors is left behind as the primary tail grows out, and these progenitors form the ectopic tail.

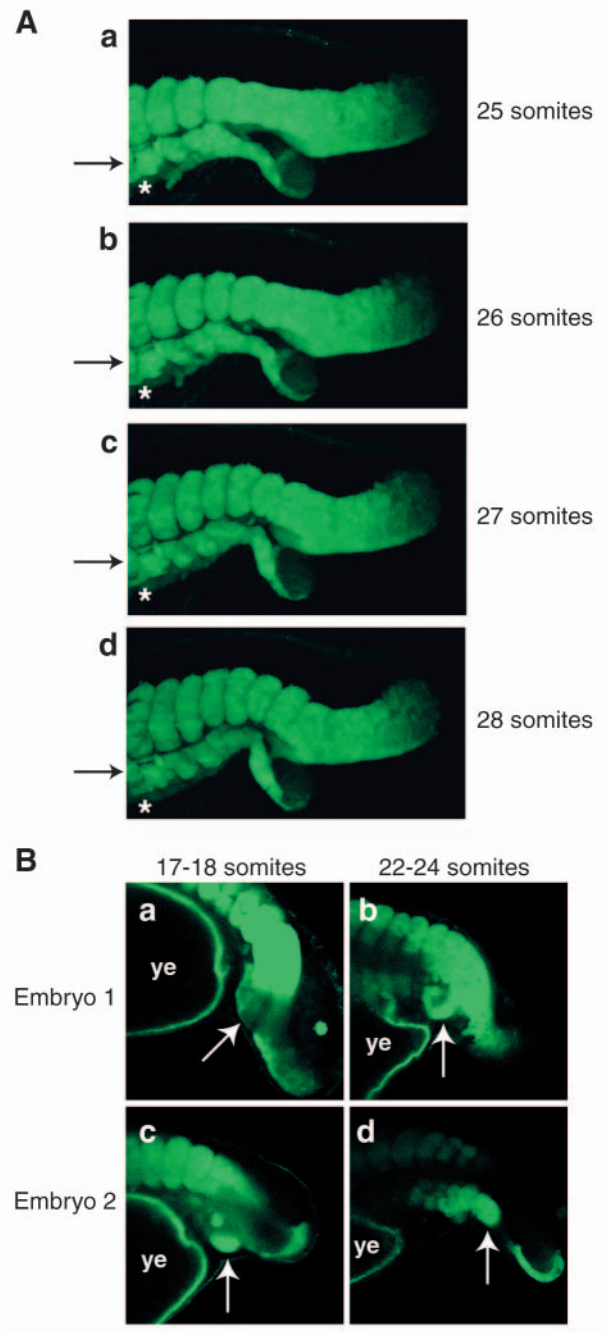

Fig. 6. Dynamic growth of secondary tails. Confocal microscopic examination of live truncated Bmp receptor-GFP/*tbx6-gfp* transgenic embryos during secondary tail growth. (A) Time points were started at the 25-somite stage, then taken every hour afterwards, corresponding to approximately one somite per time point at room temperature. Note the growth of the GFP-expressing secondary tail and the maturation of the secondary tail's presomitic mesoderm into somitic blocks. Arrows indicate the second tail, which grows along the primary axis. The asterisk indicates the first fully formed ectopic tail somite at the 25-somite stage. (B) In a separate experiment, truncated Bmp receptor-GFP/ *tbx6-gfp* embryos were mounted at the 16-17-somite stage, then monitored by confocal microscopy to detect the emergence of a second tail. Confocal slices from two representative embryos are shown. Arrows denote the tip of the second tailbud, and 'ye' indicates the yolk extension. In a and c, note the ventral patch of GFP expression corresponding to an emerging second tail. (b,d) The extending second tail about 4 hours later. The ectopic tail is growing into the plane of the picture in b and out of the plane in d.

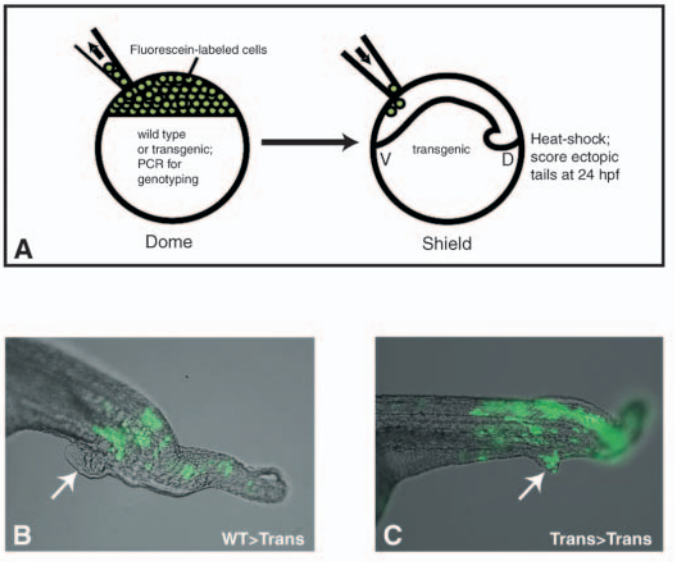

Fig. 7. Only cells with reduced Bmp signaling can populate ectopic tails. (A) Diagram of the transplant scheme used. Fluorescein-labeled donor cells (wild type or transgenic) were transplanted at dome stage to the ventral side of shield-stage transgenic hosts. Hosts were immediately heatshocked, then analyzed at 24 hpf (after the transgenic GFP faded) for presence of fluorescent cells in the ectopic tails. (B) Wild-type donor cells in a transgenic host. Note the absence of fluorescein-labeled donor cells in the ectopic tail (denoted by an arrow). (C) Transgenic donor cells in a transgenic host. Note the presence of fluorescein-labeled donor cells in the ectopic tail (denoted by an arrow) as well as in the primary tail.

Transplantation of cells with reduced Bmp signaling

We next asked whether cells with normal Bmp signaling capabilities could populate ectopic tails. We reasoned that trunk and/or tail cells are mis-specified to form ectopic tails when Bmp signaling levels are reduced at mid-gastrulation or early somitogenesis. To test this hypothesis, we performed transplants of fluorescein-labeled wild-type donor cells into ventral regions of transgenic hosts and heatshocked the hosts at shield stage (Fig. 7A). We then scored for the presence of donor cells in the ectopic tails at 24 hpf. As controls, we performed the same experiment with transgenic donor cells to be sure that cells with reduced Bmp signaling would move into ectopic tails. As shown in Fig. 7, wild-type transplanted cells did not populate ectopic tails (Fig. 7B; 0%, n=12), while transgenic cells did (Fig. 7C; 57%, n=14). The transgenic cells could also populate the primary tail (Fig. 7C). By contrast, the transgenic cells transplanted into wild-type embryos never produced an ectopic tail (data not shown), potentially because we could not get a large enough group of transgenic cells in one location to form an ectopic tail. These data indicate that cells capable of receiving Bmp signals are actively recruited into primary trunk or tail tissue, while cells that have reduced Bmp signaling capacity can go into either the primary trunk and tail or the ectopic tail.

Discussion

Our results demonstrate the dynamic temporal roles of Bmp signaling during development of ventral and posterior mesoderm in zebrafish. While other groups have revealed the importance of the Bmp pathway in ventroposterior patterning, they could not address the timing of these patterning events during embryogenesis. Data presented here suggest that Bmps are critical for primary tail formation and ventral mesoderm development during the early gastrula stages, but the development of these tissues is not dependent on the same levels of Bmp signaling from the mid-gastrula stages onward. Instead, Bmps are important for preventing the formation of ectopic tail structures and for promoting ventral fin formation through early somitogenesis.

Combinatorial signaling in trunk and tail somite formation

Patterning of trunk and tail mesoderm involves the input of signals as diverse as Wnts (Lekven et al., 2001), Fgfs (Griffin et al., 1995) and Nodals (Thisse et al., 2000) in addition to Bmps. The importance of each of these pathways in forming the posterior body has been revealed by mutant studies, but their temporal-specific roles are less clear. A pharmacological Fgf inhibitor revealed the necessity of Fgf signaling for somite formation during posterior body outgrowth (Griffin and Kimelman, 2003), and we have observed that the Wnt pathway is needed continuously during the somitogenesis stages for trunk and tail development (E. Herbig, U.J.P. and D.K., unpublished). As all four signaling molecules are expressed in the ventral region during gastrulation and in the posterior body during somitogenesis, it is likely that they are not only playing individual roles in forming the trunk and tail, but combinatorial roles as well. Indeed, combinatorial signaling between Bmps and Wnts has been recently shown to be important for maximal expression of the zebrafish tailbud gene *tbx6* (Szeto and Kimelman, 2004), and the zebrafish organizer-repressing genes *vox* and *vent* (Ramel and Lekven, 2004). Our data suggest that Bmps are individually important for formation of the tail organizer during early gastrulation, but they are not crucial for trunk or tail formation after this stage. One reason that the role of Bmp in tail development may shift so dramatically is that signaling pathways such as those for Wnt and Fgf can compensate for the loss of Bmp signaling beyond the early gastrula stages. The ventroposterior gene *eve1*, for example, is regulated by Bmp and Fgf signals (Joly et al., 1993; Griffin et al., 1995). While Bmp is needed for initiation and maintenance of maximal *eve1* expression, Fgf may be sufficient to maintain enough *eve1* expression after early gastrulation for proper tail organizer function. Another possibility is that Bmp signaling initially establishes the ventroposterior domain of mesodermal cells during early gastrulation, but it has no role at later times other than to regulate the formation of the ventral fin. While this is a relatively small domain within the embryo, it is important for normal development and would provide a reason for the embryo to maintain Bmp signals in the tailbud during somitogenesis. With the ability to conditionally reduce Bmp activity in zebrafish embryos it will now be possible to examine the interactions with other signaling pathways to determine if Bmp acts combinatorially with other pathways after gastrulation, or whether Bmp has a much more circumscribed role during this time.

Bmp signaling and ventral mesoderm development

In the gradient model for Bmp signaling, ventral mesodermal derivatives require the highest levels of Bmp activity during gastrulation. Bmps in the most ventral part of the margin specify a population of cells to form multipotent hemangioblast cells (Walmsley et al., 2002) as well as cardiac precursors (Kishimoto et al., 1997) and pronephros cells (Mullins et al., 1996). These cells are subsequently patterned to give rise to different subpopulations of the cardiovascular, blood and pronephric systems. Data in *Xenopus* using an inducible *smad6* Bmp inhibitor construct suggest that Bmp signaling is important for maintenance of blood precursors even after gastrulation (Schmerer and Evans, 2003). However, our data indicate that in zebrafish, neither blood, vascular nor pronephric precursors are affected by reduction of Bmp activity after the gastrula stages. Like the tail mesodermal cells, it seems likely that Bmp induces a group of cells in the margin to adopt the most ventral fates, while other signals maintain and pattern these cells. Such a model is supported by mutant analyses, in which zygotic mutants for *somitabun* and *lost-a-fin* have normal ventral mesoderm, but maternal zygotic mutants for these genes have deficient ventral mesoderm (Mintzer et al., 2001; Kramer et al., 2002). Therefore, the early acting maternal supplies of *smad5* and *alk8* are sufficient for ventral mesoderm formation in the absence of functional zygotic protein.

We cannot exclude a later role for Bmps in regulating downstream patterning or morphogenesis of the ventral mesodermal derivatives. Indeed, the analysis of *radar* morphants suggests that reducing the post-gastrula activity of this Bmp family member compromises vascular integrity in the trunk and tail (Hall et al., 2002). While we did not observe such a phenotype in later heatshocks (data not shown), it is possible that the dominant-negative Bmp receptor does not inhibit Radar signaling or that we did not heatshock at the appropriate stage. However, we can conclude that Bmps are required for early patterning of ventral mesodermal derivatives during gastrulation, but not afterward.

Roles of Bmp signaling after early gastrula stages

Our data show that Bmp signaling plays very different roles in posterior development after the early gastrula stages (Fig. 8). Rather than maintaining tail formation during somitogenesis, Bmp is important for preventing the formation of ectopic tails and for promoting the formation of the ventral fin. Heatshocks between shield stage and early somitogenesis stages cause phenotypes closely resembling those of severe *minifin* mutants (Connors et al., 1999). In *minifin* mutants, the extracellular metalloprotease Tolloid is mutated, leading to reductions in Bmp signaling levels when zygotic Tolloid has been depleted. As discussed earlier, these mutants do not develop ventral tail fins. Our transgenic analysis allows us to postulate that the zygotic *minifin* mutant phenotype is due to reduced Bmp signaling after the early gastrula stages, when specification of the primary tail has already occurred. This model is in agreement with data from Connors and Mullins (S. A. Connors and M. C. Mullins, personal communication), in which transgenic rescue of *minifin* mutant embryos is most efficiently achieved from the late gastrula stages onward. Similar to our results, they find that rescue of the *minifin* phenotype can only be effectively achieved through the early

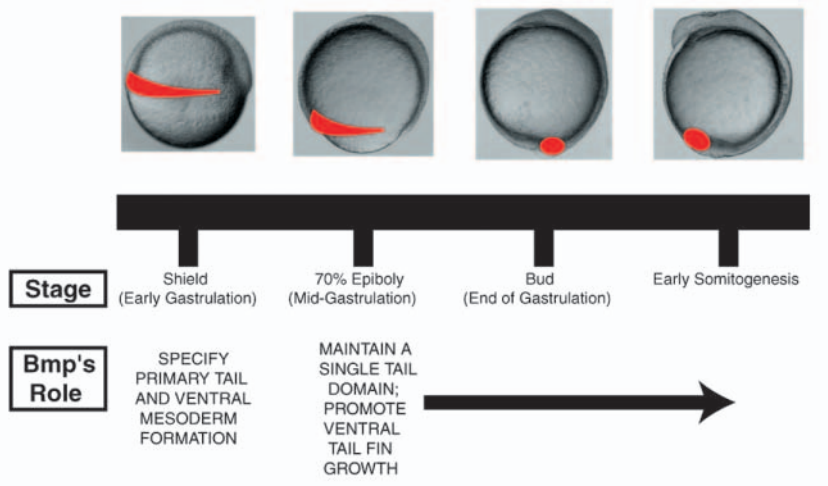

Fig. 8. A model for the distinct temporal roles of Bmp signaling during posterior mesoderm development. Embryos at different stages of development are shown, with posterior mesodermal Bmp signaling denoted in red. Below is a timeline of development and the roles of Bmp signaling in ventral mesoderm and tail tissues at various stages.

somitogenesis stages. Using two different approaches, these results demonstrate a role for Bmp signaling during the late gastrula and early somitogenesis stages in ventral tail fin formation.

Lowering Bmp signaling after early gastrulation causes the formation of secondary tails. We can detect, in live embryos or by in-situ hybridization, multiple tissue types, including fin, pigment, somitic cells, hypochord and occasionally notochord in secondary tails. Aside from pigment cells (which probably migrate into the ectopic tails after 30 hpf), we do not detect the presence of neural tissue in ectopic tails. Our results differ from those of Agathon et al. (Agathon et al., 2003), who showed that *extra* Bmp signaling, in combination with Wnt and/or Nodal, induces ectopic tail formation. The ectopic tails they observed, however, were quite different from the ones we observed, and could have both trunk and tail somites. Whereas their ectopic tails often extended out from the trunk region, we observed ectopic tails forming only within the tail region, and only at the time the primary tail began to grow. Additionally, whereas the ectopic tails they observed had both neural and mesodermal tissue, the ectopic tails formed in our embryos were solely mesodermal (with a surrounding fin). The difference between our results and theirs is likely to be due to the experimental approaches. Whereas Agathon et al. (Agathon et al., 2003) overexpressed a combination of ligands beginning at the 128-256-cell stage, we inhibited only Bmp signaling after the early gastrula stage. These results support the model that the role of Bmp signaling changes during the early period of development.

As *bmps* are strongly expressed in the ventral portion of the primary tail, it is likely that this signaling normally prevents the organization of secondary tail structures. In line with this, our transplant data indicate that only posterior cells lacking Bmp signaling can contribute to ectopic tail tissues. The formation of ectopic tails is, therefore, a cell-autonomous effect. Wild-type cells in a background of cells with low Bmp signaling behaved like wild-type cells in a wild-type background, whereas only cells with a lowered capacity to respond to Bmp signaling became part of the ectopic tail. Thus, Bmp signaling may be important for limiting the number of tail precursors during the mid-gastrula through early somitogenesis stages.

Two different morphogenetic mechanisms could explain the emergence and growth of secondary tails. In the first, loss of Bmp signaling through the early somitogenesis stages could lead to a split in the tail later in development. In this view, the ventral portion of the primary tailbud splits away from the remainder of the tailbud, pulling away tissue such as the hypochord and notochord from the primary tail during outgrowth. A second mechanism is that the most ventral tail mesoderm could be converted to a new tail organizer when Bmp signaling is disrupted. As this organizer grows out, it forms primarily somites, and it also recruits the hypochord and notochord away from the primary axis. These two models are not mutually exclusive, and we favor a combination of both. Our experiments examining the emergence of the ectopic tail at the 17-18-somite stage suggest that the ventral portion of the primary tailbud dissociates from the main tailbud to form an ectopic tail. This is not, however, a simple split of the tail into equal parts, as is commonly seen in *Xenopus* embryos with disrupted convergence extension movements (reviewed by Ueno and Greene, 2003). Rather, a very specific domain of the embryo gives rise to mesodermal tail tissues independent of the primary tail. We suggest that the ectopic tail forms from a population of tail progenitor cells that detach from the primary tailbud at the onset of tail formation and are left behind in the ventral region of the embryo. Furthermore, we propose that these are the cells that would otherwise form ventral tail mesoderm, but their fate changes due to an early disruption in Bmp signaling. Future studies will be needed to determine why in some cases these cells are left behind to form an ectopic tail, whereas in most cases the cells integrate into the primary tailbud.

New avenues of research

Bmp signaling is used at many times in development to affect a wide variety of cell types. Although its role has been extensively studied during early times in the lower vertebrates, the conditional dominant-negative fish described here will allow investigators to study Bmp signaling throughout development and in adult fish. Because the dominant-negative Bmp receptor acts cell-autonomously, it is well suited for transplant studies. Moreover, as the receptor is GFP-tagged, in crosses of heterozygous fish it is easy to identify the embryos expressing the transgene. Finally, because the dominant-negative Bmp receptor turns over fairly rapidly, it is possible to do timecourse experiments to determine when Bmp signaling is needed for particular developmental events. We expect that these fish will be a useful resource for analysis of Bmp signaling in zebrafish.

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