Hedgehog induction of murine vasculogenesis is mediated by Foxf1 and Bmp4

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The first vasculature of the developing vertebrate embryo forms by assembly of endothelial cells into simple tubes from clusters of mesodermal angioblasts. Maturation of this vasculature involves remodeling, pruning and investment with mural cells. Hedgehog proteins are part of the instructive endodermal signal that triggers the assembly of the first primitive vessels in the mesoderm. We used a combination of genetic and in vitro culture methods to investigate the role of hedgehogs and their targets in murine extraembryonic vasculogenesis. We show that Bmps, in particular Bmp4, are crucial for vascular tube formation, that Bmp4 expression in extraembryonic tissues requires the forkhead transcription factor Foxf1 and that the role of hedgehog proteins in this process is to activate Foxf1 expression in the mesoderm. We show in the allantois that genetic disruption of hedgehog signaling (Smo–/–) has no effect on Foxf1 expression, and neither Bmp4 expression nor vasculogenesis are disturbed. By contrast, targeted inactivation of Foxf1 leads to loss of allantoic Bmp4 and vasculature. In vitro, Bmp4 induces formation of angioblasts express Flk1 and require Vegf for proliferation, and genetic ablation of either receptor or ligand leads to a completely avascular phenotype [for a review of Vegf in vascular development, see Coultas et al. (Coultas et al., 2005)].

Vascular endothelial growth factor (Vegf) and its receptor Flk1 (Kdr – Mouse Genome Informatics) are essential in all blood vessel development – angiogenesis as well as vasculogenesis – and genetic ablation of either receptor or ligand leads to a completely avascular phenotype [for a review of Vegf in vascular development, see Coultas et al. (Coultas et al., 2005)]. Angioblasts express Flk1 and require Vegf for proliferation, differentiation and survival. Vegf production is induced by hypoxia and concentration gradients of Vegf guide vascular sprouts into poorly oxygenated areas (Gerhardt et al., 2003). In vitro models and mutant phenotypes in several vertebrate species have illustrated the involvement of additional growth factor pathways in vascular development, but the relationships between them are not fully understood.

INTRODUCTION

Our vasculature is a highly dynamic organ, constantly reorganized and modified to adjust to local changes in the need for oxygen and nutrients. The dominating mechanism of neovascularization in the adult is angiogenesis, i.e., extension or remodeling of existing vessels, usually by outgrowth of vascular sprouts. By contrast, the first embryonic vasculature is created through vasculogenesis, i.e., the formation of tubes of endothelial cells directly from mesodermal progenitors. Endothelial cells fuse into a primitive capillary plexus, from which the mature vasculature develops through remodeling, pruning and investment with pericytes and smooth muscle cells (SMCs). At the molecular level, adult neovascularization recapitulates many of the steps of embryonic vascular development (Bikfalvi and Bicknell, 2002; Jain, 2003).

Bone morphogenetic proteins (Bmps), in particular Bmp4, are required for mesoderm formation and consequently for development of all mesodermally derived tissues, including blood vessels (Mishina et al., 1995; Winnier et al., 1995). Bmp4 ventralizes mesoderm and is antagonized by dorsal/midline Bmp inhibitors such as noggin (Harland, 1994). In vitro, Bmp4 induces formation of Flk1+/Tal1+ cells, which require Vegf for proliferation and further differentiation (Park et al., 2004). In quail, ectopic Bmp4 has been shown to induce vascularization at the midline and expression of Quek1 (the Flk1 homolog) in the lateral plate (Reese et al., 2004). Interpretation of the phenotypes of loss-of-function mutants in mouse is complicated by the importance of Bmp signaling in gastrulation. However, Bmp4-null embryos that survive into the somite stage display a paucity of blood islands and extraembryonic mesoderm in the yolk sac (Winnier et al., 1995). Furthermore, targeted inactivation of either of the Bmp signal transducers Smad1 (Lechleider et al., 2001; Tremblay et al., 2001) or Smad5 (Chang et al., 1999; Yang et al., 1999) leads to embryonic lethality owing to a defective vasculature.

Several lines of evidence implicate the hedgehog pathway in vertebrate blood vessel formation. Inactivation of sonic hedgehog (Shh) is associated with decreased or defective vascular development (Brown et al., 2000; Pepicelli et al., 1998), whereas its overexpression in neuroectoderm causes hypervascularization (Rowitch et al., 1999). In adult mice, Shh stimulates neovascularization (Pola et al., 2001). In contrast to ectopic Vegf, which mainly induces endothelial sprouts, Shh promotes the entire angiogenesis program, including recruitment of mural cells, remodeling and vascular maturation (Pola et al., 2001). Extraembryonic vasculogenesis requires signals from the visceral endoderm and hedgehog proteins have been shown to be part of this signal. Indian hedgehog (Ihh) is highly expressed in the murine visceral endoderm (Becker et al., 1997; Farrington et al., 1997) and is the primary hedgehog family member responsible for induction of yolk sac vasculogenesis. Targeted inactivation of Ihh leads to...
poor development of the yolk sac vasculature (Byrd et al., 2002), which results in the death of approximately 50% of Ihh-null embryos at mid-gestation (St-Jacques et al., 1999). Ihh–/– embryo bodies are unable to form blood islands (Byrd et al., 2002) and recombinant Ihh can substitute for visceral endoderm as the inducer of vasculogenesis and activator of Bmp4 in tissue recombination experiments (Dyer et al., 2001). In chicken embryos, Shh can substitute for endoderm as the inducer of vascular tube formation (Vokes et al., 2004).

Different models have been put forward regarding the mechanism through which hedgehogs induce vascular development. In adult mice, Shh has been reported not to act directly on the endothelial cells, but rather through interstitial mesenchymal cells that respond by producing angiopoietins and Vegf (Pola et al., 2001). Vokes et al. (Vokes et al., 2004) suggested that Shh induces embryonic vascular tube formation by directly influencing the morphological properties of endothelial cells independently of Vegf.

Here, we use a combination of genetics and in vitro explant culture techniques to determine the mechanisms by which the hedgehog and Bmp signaling pathways regulate extraembryonic vascular tube formation. We show that Foxf1 (also known as Foxf1α – Mouse Genome Informatics), which encodes a forkhead transcription factor, is a mesodermal target for endodermal hedgehog signaling. Foxf1 activates the expression of Bmp4 in mesodermal cells, which in turn induces vascular tube formation. In one tissue undergoing extensive vasculogenesis – the murine allantois – expression of Foxf1 is independent of hedgehog. Allantoic Bmp4 expression and vasculogenesis are unaffected by abrogation of hedgehog signaling through deletion of smoothened (Smo). Genetic inactivation of Foxf1, on the other hand, leads to loss of both Bmp4 expression and vasculogenesis in this tissue, a defect that can be rescued in vitro by exogenous Bmp4. That the same pathway also operates in a tissue where vasculogenesis normally requires hedgehog was shown by the ability of exogenous Bmp4 to rescue vascular plexus formation in Smo–/– yolk sacs.

**MATERIALS AND METHODS**

**Mouse strains**

The Foxf1– (Foxf1mulvo) has been described elsewhere (Mahlapuu et al., 2001b). Shhmut (Chiang et al., 1996) was obtained from The Jackson Laboratory (Bar Harbor, ME). Ihh and Smo strains (St-Jacques et al., 1999; Zhang et al., 2001) were kindly provided by Dr A. P. McMahon and a strain carrying the Bmp4foxz knock-in allele (Lawson et al., 1999) was kindly provided by Dr B. L. M. Hogan. Wild-type mice were C57Bl/6 (Charles River) and all mutants were maintained by breeding with this strain. Genotyping was performed by PCR as previously described (Lawson et al., 1999; Mahlapuu et al., 2001b; St-Jacques et al., 1999; Zhang et al., 2001).

**In situ hybridization, immunohistochemistry and histological staining**

Embryos homozygous for the Bmp4foxz allele were stained with X-Gal (Hogan et al., 1994), embedded in paraffin and sectioned. Whole-mount in situ hybridization was performed as described (Blixt et al., 2000) with an antisense RNA probe for Foxf1 (Mahlapuu et al., 2001a). Rat monoclonal antibodies against Pecam (also known as Pecam1 – Mouse Genome Informatics) and Flik1 as well as a FITC-conjugated anti-CD41 (Igta2b – Mouse Genome Informatics) were purchased from BD Biosciences Pharmingen, and the FITC-conjugated anti-SMA (Acta2 – Mouse Genome Informatics) mouse monoclonal from Sigma. Detection of rat monoclonal antibodies was by biotinylated secondary antibodies and either HRP-streptavidin amplification with the TSA TM Biotin System (NEN Life Science Products) and DAB, or Alexa Fluor-streptavidin (Molecular Probes) immunofluorescence. Haematoxylin-Eosin (H&E) staining of paraffin sections was used for general histology.

**Explant cultures**

Allantoic buds were dissected from E8.25 embryos and cultured on cover slips for 24 hours in DMEM medium (Gibco) supplemented with 10% fetal calf serum, l-glutamine (2 mM) and penicillin-streptomycin (10 U/ml) in 5% CO2, 100% humidity. Recombinant Bmp2, 4, 6 and 7 (0.1 ng/ml) and noggin (1 or 10 ng/ml) (all from R&D Systems) were added to the culture medium when the explant cells had adhered to the substrate (approximately 3 hours after dissection). Yolk sacs were dissected and spread, mesoderm side up, on MF filters (Millipore), supported by stainless steel grids in 30 mm culture dishes. Approximately 3 ml of BGJb medium (Life Technologies) supplemented with 0.2 mg/ml ascorbic acid, 10 U/ml penicillin-streptomycin and 0.1% BSA was added to establish an air-fluid interface at the level of the explants and the cultures were kept at 37°C in 5% CO2, 100% relative humidity for 24 hours. All results were verified by repeating the explant culture experiment at least three times.

**RESULTS**

**Foxf1 expression and vasculogenesis are activated by Ihh and Shh**

During organogenesis, Foxf1 is activated in mesenchyme of the lung and gut in response to endodermal hedgehog signaling (Mahlapuu et al., 2001a; Ormestad et al., 2006). To investigate whether the same relationship exists in the early mouse embryo, we analyzed Foxf1 expression in Ihh–/– embryos. Ihh is the dominating hedgehog in the yolk sac endoderm at the stage when the extraembryonic vasculature develops. At E9.5, Ihh–/– yolk sacs had fewer and thinner blood vessels, compared with wild-type (wt) (Fig. 1A,B) (Byrd et al., 2002). The reduced vasculature causes resorption of approximately 50% of Ihh-null embryos before establishment of a placental circulation, whereas the rest develop to term (St-Jacques et al., 1999). This contrasts with Foxf1–/– embryos, which have a fully penetrant avascular yolk sac phenotype (Fig. 1C) and are invariably resorbed by E10.5 (Mahlapuu et al., 2001b). Judged by whole-mount in situ hybridization, Foxf1 expression was reduced in Ihh–/– blood islands, which at E8.5 appeared as a freckled staining pattern on the wt yolk sac surface (Fig. 1F,G). No alterations in expression pattern were observed in allantoic or lateral mesoderm (Fig. 1D,E).

The persistence of Foxf1 mRNA in Ihh–/– embryos raised questions about whether, at this stage, Foxf1 expression is independent of hedgehogs, or if other hedgehogs might compensate for the loss of Ihh. The severe phenotype of Shh–/–, Ihh–/– embryos, as compared with either single mutant, indicates a widespread redundancy and a role for Ihh in processes previously thought to require only Shh (Zhang et al., 2001). Similarly, Shh might play a minor, but – in the absence of Ihh – significant, role in yolk sac development. We therefore analyzed Shh–/–; Ihh–/– embryos and found their yolk sacs to be thin, transparent and essentially avascular (Fig. 2B,D). This indicates that Shh and Ihh both contribute to induction of yolk sac vasculogenesis. Closer inspection revealed adhesion between amnion and yolk sac in the hedgehog double mutants (arrowheads in Fig. 2B,D), which is reminiscent of the extensive adhesions observed in extraembryonic mesoderm of Foxf1 mutant embryos (Mahlapuu et al., 2001b). No Foxf1 mRNA could be detected in yolk sac or lateral mesoderm of hedgehog double-null embryos. However, the allantois and the most-posterior embryonic mesoderm, exiting from the posterior primitive streak, expressed Foxf1 (Fig. 2F).

The wrinkles on the E9.5 Foxf1–/– yolk sac surface (Fig. 1C) could be mistaken for blood vessels, but actually consist of folds in the visceral endoderm that are prevented from expanding by the constricted mesodermal layer (Fig. 3E,G) (Mahlapuu et al., 2001b). The yolk sac mesoderm in this mutant has abnormal adhesion properties, presumably owing to ectopic co-expression of Vcam1
with its ligand, α4-integrin, throughout the extraembryonic mesoderm (Mahlapuu et al., 2001b). The visceral endoderm was seen to be lined on the inner surface with a thin layer of endothelial (Pecam+ and Flk1+; Fig. 3) cells, but the bulk of the yolk sac mesoderm was detached and formed a thick layer between the amnion and the yolk sac endoderm. The separation started in the mesometrial pole at E8.5 and spread to the entire yolk sac by E9.5 (Fig. 3E,G,I,J). The prolific and adhesive mesoderm of yolk sac and amnion sets the Foxf1–/– mutant apart from the Shh–/–; Ihh–/– mutant, in which the amnion appeared normal and the yolk sac mesoderm was hypoplastic and reduced to a thin lining of the endoderm. Hedgehog-independent Foxf1 expression and vasculogenesis in the allantois
The residual Foxf1 expression in Shh–/–; Ihh–/– double knockouts could potentially be due to the third hedgehog family member, desert hedgehog (Dhh), or to hedgehog-independent mechanisms. In the case of the allantois, which at the analyzed stages had fused with the chorion to form the placenta, there is also the possibility of influence from maternal factors. Whole-mount in situ hybridization showed Dhh expression associated with the yolk sac vasculature at E10.5, but failed to detect any Dhh mRNA in the embryo proper or extraembryonic structures at E8.5 (see Fig. S1 in the supplementary material). This suggested that Dhh is not responsible for the activation of Foxf1 in allantois and primitive streak, although we could not exclude the potential importance of an expression level below the detection limit. We therefore analyzed Foxf1 expression in Smo–/– embryos. Smo encodes the signal-transducing component of the hedgehog receptor and its inactivation abrogates all hedgehog signaling (Zhang et al., 2001). The Smo–/– embryos were indistinguishable from Shh–/–; Ihh–/– embryos and showed robust Foxf1 expression in the allantois (Fig. 4C,D), as well as in the primitive streak region (Fig. 4D). Hence, Foxf1 transcription in these
In the Pecam staining of E8.5 wt (A left, B) and is well developed in the wt, but absent from the mutant. (sacs of the embryos in A. A primitive vascular plexus of endothelial tubes mouse embryos. B and C are higher magnifications of parts of the yolk staining of paraffin sections of E9.5 wt (D,F) and endoderm (arrowhead in E). (H) Flk1 staining of cryosections of E8.5 wt (H) and Foxf1+/- (I) embryos. The separation of the yolk sac mesoderm has started in the mesometrial pole of the yolk sac (I), but not in the antimesometrial (I). al, allantois; am, amnion; post, posterior part of the embryo proper; yse, yolk sac endoderm; ysm, yolk sac mesoderm.

Fig. 3. Defective vasculogenesis in Foxf1-/- yolk sac. (A-C) Whole-mount Pecam staining of E8.5 wt (A left, B) and Foxf1-/- (A right, C) mouse embryos. B and C are higher magnifications of parts of the yolk sacs of the embryos in A. A primitive vascular plexus of endothelial tubes is well developed in the wt, but absent from the mutant. (D-G) Pecam staining of paraffin sections of E9.5 wt (D,F) and Foxf1+/- (E,G) embryos. In the Foxf1 mutant, the yolk sac mesoderm is divided into a thin Pecam+ layer that lines the inner surface of the yolk sac endoderm, and a detached thick layer that only makes sporadic contact with the yolk sac endoderm (arrowhead in E). (H-J) Flk1 staining of cryosections of E8.5 wt (H) and Foxf1+/- (I) embryos. The separation of the yolk sac mesoderm has started in the mesometrial pole of the yolk sac (J), but not in the antimesometrial (I). al, allantois; am, amnion; post, posterior part of the embryo proper; yse, yolk sac endoderm; ysm, yolk sac mesoderm.

tissues differs from that in lateral and yolk sac mesoderm by being independent of hedgehog signaling. Cryptic Dhh expression, as well as maternal hedgehogs, can be dismissed as reasons for the persistent expression of Foxf1 in Shh-/-, Ihh-/- double knockouts.

The rudimentary yolk sac vasculature in Smo-/- and Shh-/-; Ihh-/- mutants is consistent with an important role of hedgehogs in vascular development, as suggested in several studies (reviewed by Byrd and Grabel, 2004). The loss of yolk sac Foxf1 expression in these mutants, together with the avascular extraembrionic phenotype of Foxf1-/- embryos, fit a model in which Foxf1 is a mesodermal target that mediates the role of hedgehogs in blood vessel formation. However, the allantois is a highly vascularized tissue in which Foxf1 expression is independent of Smo. We therefore asked whether vasculogenesis would proceed normally in the absence of hedgehog signaling in a tissue that expresses Foxf1, by analyzing allantoic vascular development. Explants of wt allantoic buds spontaneously formed a simple, but prolific, vascular plexus within 24 hours of culture, readily identified by immunostaining for the endothelial marker Pecam (Fig. 4E). Allantoic buds from Smo-/- embryos formed extensive networks of endothelial tubes, indistinguishable from those of wt explants (Fig. 4F). This demonstrated that formation of a vascular plexus can occur independently of hedgehog signaling. Allantoic bud explants from Foxf1-/- embryos contained Pecam+ cells, but these did not form vascular tube networks (Fig. 4E). Taken together, these results show that Foxf1 is an important component of the vasculogenesis pathway and that Foxf1 expression requires hedgehog signaling in some, but not all, tissues.

The vasculogenic activity of Foxf1 is mediated by Bmp4

Which target genes of Foxf1 mediate its vasculogenic activity? Several observations suggest Bmp4 as a good candidate. Expression of Bmp4 has been shown to be activated by Foxf1 proteins in some tissues (Mahlapuu et al., 2001b; Ormestad et al., 2006). Furthermore, Bmp4 is known to be associated with blood vessel formation in several systems (reviewed by Moser and Patterson, 2005). Several predictions follow from the hypothesis that Bmp4 is a key target of Foxf1 that induces vasculogenesis: Bmp4 should be expressed at the sites and stages of active vasculogenesis; it should exhibit reduced expression in avascular tissues of the hedgehog and Foxf1 mutants; and the distinct vascular phenotypes of Smo-/- and Foxf1-/- embryos should be reflected in a corresponding difference in Bmp4 expression. A Bmp4lacZ knock-in allele (Lawson et al., 1999) allowed visualization of Bmp4 transcription in thin tissues such as the yolk sac and amnion. Embryos heterozygous for this allele had lacZ staining in all extraembryonic mesoderm (yolk sac, amnion and allantois), the primitive streak, the lateral plate mesoderm and the heart (Fig. 4G,H and Fig. 5A-E). In the Smo-/- background, Bmp4lacZ expression in yolk sac and lateral plate was reduced dramatically, but no significant change was observed in the primitive streak or allantois (Fig. 4I,J). In the Foxf1-/- background, Bmp4lacZ expression was similarly reduced in yolk sac and lateral plate and remained high in the primitive streak. However, in contrast to Smo-/-, the Foxf1 mutant embryos exhibited a dramatic reduction of Bmp4lacZ expression in the allantois (Fig. 5F-J). These results suggest that Bmp4 expression during gastrulation is independent of Foxf1, but as the cells move out of the primitive streak to form the lateral or extraembryonic mesoderm, maintenance of Bmp4 transcription requires Foxf1. A similar relationship appears to exist between Foxf1 and hedgehogs, because expression of Foxf1 in the primitive streak is hedgehog-independent, but its persistence in lateral and yolk sac mesoderm requires Smo and at least one of the
ligands, Shh or Ihh. The exception is the allantois, which retains Foxf1 and Bmp4 expression even in the absence of hedgehog signaling.

If Bmp4 is a key target of Foxf1 in vasculogenesis, inhibition of Bmp signaling should block vascular tube formation. To test this, we used the allantoic bud explant culture system. Inhibition of Bmp signaling in wt explants by the addition of noggin blocked formation of endothelial tubes (Fig. 6A-C), showing that Bmps are essential for vasculogenesis in this tissue. Next, we reasoned that if the failure of vasculogenesis in Foxf1+/− embryos was due mainly to the reduction in Bmp4 expression, then exogenous Bmp4 might be sufficient to overcome this block. Foxf1+/− explants contained Pecam+ cells, but they failed to organize into a defined network of tubes (Fig. 6E). Supplementing the culture medium of Foxf1+/− explants with Bmp4 to a final concentration of 0.1 ng/ml restored their ability to form vessels (Fig. 6F), demonstrating that a crucial function of Foxf1 in vasculogenesis is to drive expression of Bmp4.

Similar results were obtained with Bmp2, 6 and 7 (see Fig. S2 in the supplementary material), suggesting that vascular tube formation is not dependent on a specific Bmp ligand. Concentrations of exogenous Bmp4 one order of magnitude higher, or lower, were less efficient at supporting vasculogenesis.

The results presented here show that hedgehog signaling is dispensable for vascular tube formation in the allantois and suggest that this is accomplished by uncoupling Foxf1—and thereby Bmp4—expression from the hedgehog pathway. We next asked whether the same downstream mechanisms operate in a tissue where hedgehog signaling is essential for vascular development. In other words, can the requirement for hedgehogs be bypassed by the addition of Bmp4? We cultured yolk sac explants from Smo−/− embryos in vitro and supplemented the medium with Bmp4. Yolk sacs from E9.5 Smo−/− embryos were cut in half and the left- and right-hand sides cultured on separate permeable membranes. Bmp4 was added to one of the halves and after 24 hours of culture the explants were stained with the Pecam antibody. As shown in Fig. 6G,H, addition of Bmp4 led to formation of a well-developed plexus of endothelial tubes in spite of the absence of functional hedgehog signal transduction. This demonstrates that hedgehog signaling is
dispensable for vasculogenesis in the yolk sac, as long as Bmp4 is present. It also proves that progenitors of endothelial cells are present, i.e. the Smo–/– yolk sac phenotype is not caused by a failure of progenitor cells to migrate and colonize the extraembryonic mesoderm.

**Foxf1 and Bmp4 restrict allantoic smooth muscle cell differentiation**

In addition to endothelial cells, the extraembryonic mesoderm gives rise to mesothelial, hematopoietic and smooth muscle cells. Previous results suggested that altered or mixed cell fates in Foxf1–/– extraembryonic mesoderm might contribute to the failure of vasculogenesis (Mahlapuu et al., 2001b). Primitive hematopoietic cells (γ-globin) were aberrantly present in the amnion and abundant in the mesometrial pole of the yolk sac, but absent in the antimesometrial pole and in the allantois. Widespread misexpression of the SMC marker α-actin (SMA) was observed in yolk sac, amnion and allantois (Mahlapuu et al., 2001b). To investigate whether the excess of SMC progenitors was accompanied by a decrease in angioblasts, and whether this ratio is controlled by Bmp, we cultured Foxf1–/– allantoic explants in the presence and absence of Bmp4 and stained for SMA and Flk1 (Fig. 7). Wild-type explants had a moderate number of SMA+ cells loosely associated with the Flk1+ endothelial tubes (Fig. 7A). Foxf1–/– explants contained Flk1+ cells, but SMA+ cells were much more abundant and widespread in Foxf1–/– explants than in wt (Fig. 7B). Consistent with the presence of definitive hematopoietic progenitors in allatoic buds (Zeigler et al., 2006), a few CD41+ cells (Mikkola et al., 2003) were observed, but did not differ significantly in number between the genotypes (see Fig. S3 in the supplementary material). Inclusion of Bmp4 in the culture medium not only induced vascular formation, but also significantly reduced the number of SMA+ cells (Fig. 7C). Thus, Bmp can reverse the abnormal accumulation of SMA+ cells that results from inactivation of Foxf1 and normalize the SMC/angioblast ratio. This might reflect an ability of Bmp signaling to influence fate decisions in the extraembryonic mesoderm, or to inhibit proliferation of SMC progenitors.

**DISCUSSION**

We present evidence for a pathway in which endodermal hedgehog ligands (Ihh and Shh) induce expression of Foxf1 in the mesoderm; Foxf1 in turn activates Bmp4 transcription and Bmp signaling promotes the assembly of vascular tubes from mesodermal progenitors (Fig. 8). In the presence of other sources of Bmp, neither hedgehog nor Foxf1 was required for vasculogenesis. For example, Bmp4 expression in the heart was not affected by targeted inactivation of Foxf1 and the endocardium developed normally in Foxf1–/– embryos. The allantoic vascular plexus developed normally in Smo–/– embryos. In both of these examples, endothelial tube formation was associated with robust Bmp4 expression. However, apart from the primary assembly of endothelial tubes discussed here, development of a mature vasculature involves many additional steps and we do not exclude the possibility that hedgehogs and Foxf1 might have important functions in these later stages.
The described pathway simplifies the interpretation of several observations that have troubled previously suggested models of direct, cell-autonomous involvement of hedgehog in vascular tube formation. For example, Smo–/– mutants have absent or severely defective dorsal aortae along much of the embryo, but towards the posterior end the morphology of these blood vessels improves to near normal (Vokes et al., 2004; Zhang et al., 2001). Inactivation of Smo abrogates all hedgehog signaling, which makes this gradient in vascular development difficult to reconcile with hedgehog being required for endothelial tube formation by acting directly on angioblasts. This prompted speculation of alternative pathways, both hedgehog-dependent and -independent, acting in different parts of the embryo (Byrd and Grabel, 2004; Vokes et al., 2004). The high level of hedgehog-independent Bmp4 expression in the primitive streak area generates a localized, posterior source of Bmp4 in Smo–/– embryos, which readily explains the observed gradient in dorsal aorta development. In contrast to hedgehog signaling, which is completely blocked in Smo–/– mutants, Bmp4 expression is only reduced. Variation, stochastic or genetic, in the residual amounts of Bmp will inevitably give rise to interindividual phenotypic variation. This is seen, for example, in the development of Smo–/– embryonic vasculature (Vokes et al., 2004) and in the yolk sac of Smo–/– embryos, which often have patches of rudimentary plexus formation in the area closest to the embryo (Byrd et al., 2002).

Proliferation of primitive streak mesoderm is reduced in Foxf1–/– mutants, but no significant decrease in proliferation or survival was detected in the extraembryonic mesoderm (Mahlapuu et al., 2001b). This is consistent with the robust growth of Foxf1–/– allantoic explants in vitro and implies that the failure to form a vascular plexus is not a consequence of a general hypoplasia. In other systems, Bmps have been shown to increase Vegf expression and stimulate proliferation of Flk1+ cells (Deckers et al., 2002; He and Chen, 2005; Nimmagadda et al., 2005; Reese et al., 2004). It is therefore likely that Bmp acts through the Vegf pathway also in extraembryonic vasculogenesis.

Why does the allantois differ from other extraembryonic mesodermal structures with regard to activation of Foxf1? In fish and amphibians, vasculogenesis and hematopoiesis are initiated in ventral mesoderm. A vascular bed encloses yolk contained in the primitive gut and both hedgehog, expressed in the endoderm, and Bmp4, expressed in the ventral mesoderm, are important for its formation (Brown et al., 2000; Harland, 1994). As an adaptation to terrestrial development, the endoderm-mesoderm bilayer of oviparous amniotes folds into three distinct compartments – the gut, the yolk sac and the allantois – all with the same basic organization and with vasculogenesis occurring throughout. The selective forces that shaped these extraembryonic structures are absent in placental mammals. Owing to phylogenetic constraints, the structures are still part of mammalian embryology, but have lost their original functions and in some cases acquired novel ones. In consequence, they display a remarkable morphological diversity across mammalian species. The murine allantois represents an evolutionary oddity that has lost the endodermal component and thus the source of hedgehog. This would arrest vasculogenesis, unless replaced by autocrine hedgehog signaling in the mesoderm, or by other means of activating the key.
mesodermal targets. In mouse, this problem appears to have been solved by hedgehog-independent transcriptional activation of the Foxf1 gene.

Speculation regarding redundancy between hedgehog paralogs as the explanation for the residual vasculature in Ihh mutants has focused on Dhh. Dhh mRNA is present in the yolk sac endoderm, but has not been detected earlier than E10.5 (Farrington et al., 1997). Shh mRNA is present in the yolk sac endoderm, but at a low level that has been assumed to be without physiological relevance (Farrington et al., 1997). The phenotypes of Shh−/−; Ihh−/− and Smo−/− mutants were indistinguishable and differed strikingly from both hedgehog single mutants. This demonstrates a widespread redundancy between Shh and Ihh. Specifically, our results show that Shh contributes to yolk sac vascular development, but does not support the notion of a role for Dhh in this process. It should be emphasized that the main argument for dismissal of Dhh is not the absence of detectable expression, but the identical phenotypes (including allantoic vasculogenesis) of Shh−/−; Ihh−/− and Ihh−/− Smo−/−; Ihh−/−.

Bmp4 expression during gastrulation does not require Foxf1. However, once mesodermal cells exit from the primitive streak and reach the lateral plate or extraembryonic structures, Foxf1 becomes essential for maintaining high-level Bmp4 transcription. Injection of BMP4 mRNA in 8-cell Xenopus embryos induced transcription of Foxf1 in gastrula stage animal caps (Tseng et al., 2004). This is consistent with the onset of expression being earlier for Bmp4 than Foxf1. Hence, during gastrulation, Foxf1 expression in nascent mesoderm appears to require Bmp, whereas later on the relationship is reversed. Interestingly, a similar relationship has been described in Drosophila where Dpp (the Bmp homolog) is required for activation of binouc (the Foxf homolog) early in development in the trunk visceral mesoderm primordium, but Binouc activates dpp at later stages in the visceral mesoderm (Zaffran et al., 2001).

The activation of Foxf1 by Bmp4 in early Xenopus embryos (Tseng et al., 2004) contrasts with the inhibition of Foxf1 in murine lung mesenchyme by Bmp4 from the distal lung bud epithelium (Mahlapuu et al., 2001a). The epithelio-mesenchymal cross-talk during lung branching morphogenesis is complex and it is unclear whether the effect on Foxf1 is direct or indirect. In the early embryo, where Foxf1 and Bmp4 are co-expressed in the mesoderm, there is no evidence for feedback inhibition.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/20/4432/DC1

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