Patterning of angiogenesis in the zebrafish embryo

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SUMMARY

Little is known about how vascular patterns are generated in the embryo. The vasculature of the zebrafish trunk has an extremely regular pattern. One intersegmental vessel (ISV) sprouts from the aorta, runs between each pair of somites, and connects to the dorsal longitudinal anastomotic vessel (DLAV). We now define the cellular origins, migratory paths and cell fates that generate these metameric vessels of the trunk. Additionally, by a genetic screen we define one gene, *out of bounds* (*obd*), that constrains this angiogenic growth to a specific path.

We have performed lineage analysis, using laser activation of a caged dye and mosaic construction to determine the origin of cells that constitute the ISV. Individual angioblasts destined for the ISVs arise from the lateral posterior mesoderm (LPM), and migrate to the dorsal aorta, from where they migrate between somites to their final position in the ISVs and dorsal longitudinal anastomotic vessel (DLAV). Cells of each ISV leave the aorta only between the ventral regions of two adjacent somites, and migrate dorsally to assume one of three ISV cell fates. Most dorsal is a T-shaped cell, based in the DLAV and branching ventrally; the second constitutes a connecting cell; and the third an inverted T-shaped cell, based in the aorta and branching dorsally. The ISV remains between somites during its ventral course, but changes to run mid-somite dorsally. This suggests that the pattern of ISV growth ventrally and dorsally is guided by different cues.

We have also performed an ENU mutagenesis screen of 750 mutagenized genomes and identified one mutation, *obd* that disrupts this pattern. In *obd* mutant embryos, ISVs sprout precociously at abnormal sites and migrate anomalously in the vicinity of ventral somite. The dorsal extent of the ISV is less perturbed. Precocious sprouting can be inhibited in a VEGF morphant, but the anomalous site of origin of *obd* ISVs remains. In mosaic embryos, *obd* somite causes adjacent wild-type endothelial cells to assume the anomalous ISV pattern of *obd* embryos.

Thus, the launching position of the new sprout and its initial trajectory are directed by inhibitory signals from ventral somites. Zebrafish ISVs are a tractable system for defining the origins and fates of vessels, and for dissecting elements that govern patterns of vessel growth.

Key words: Zebrafish, Angiogenesis, Vascular pattern

INTRODUCTION

Although a high degree of reproducibility to vessel patterning has been noted since 1543 (Vesalius, 1543), it is not understood how that order is established. The original embryonic vessels of the vertebrate trunk are a single large artery and vein, generated by the differentiation and coalescence of angioblasts from the lateral mesoderm, a process termed vasculogenesis (Risau and Flamme, 1995). Most subsequent vessel formation in the embryo is by sprouting from pre-existing vessels, a process known as angiogenesis. It is also the mechanism of vascularization of tumors. At a cellular level, angiogenesis involves localized endothelial cell proliferation and migration, followed by remodeling of the nascent vessel, the latter including the removal, growth or subdivision of the new channels (Risau, 1997). How particular regions are marked to serve as the site of the sprout is not known, nor are mechanisms for pathway guidance of the sprout.

Several growth factors have been shown to be crucial for normal angiogenesis in the embryo. Vascular endothelial growth factor, VEGF, is expressed in the vicinity of sprouting vessels, and its receptor (VEGF-R2/Flk-1/kdr) on the angioblasts and new vessels, and both are required for vasculogenesis and angiogenesis (Leung, 1989; Keck, 1989; Shalaby, 1995; Carmeliet, 1996; Ferrara, 1996). Angiopoietin 1 and angiopoietin 2, and their receptor Tie2 are reciprocally expressed in surrounding mesenchyme and early vessels, and appear to function in vascular remodeling and stabilization (Dumont, 1994; Maisonpierre and Radziejewski, 1997; Sato, 1995; Suri et al., 1996). Several EphB receptors and ephrinB ligands are expressed, some with arterial versus venous specificity, both on vessels and in surrounding tissues, and have

been shown to be required for remodeling of angiogenesis (Adams, 1999; Wang, 1998).

In the zebrafish, cells in the lateral posterior mesoderm express endothelial and hematopoietic markers, suggesting they include bipotential precursors for both the hematopoietic and angioblastic lineages, termed hemangioblasts (Fouquet, 1997; Stainier et al., 1995). By lineage tracking we have previously shown that the LPM includes precursors for the dorsal aorta and posterior cardinal vein (Zhong et al., 2001).

We have focused on the origin and pathway guidance of the ISVs, vessels that connect the dorsal aorta to the DLAVs. The ISVs are particularly interesting because their pattern appears to be established primarily, unlike other vessels such as those of the yolk sac, brain or retina, which first form in a honeycomb-like plexus to be remodeled subsequently into more organized regularly branching larger and smaller vessels (Suri et al., 1996; Benjamin, 1998). Thus, it appears that for ISVs there might be designated sites for branching and initial pathway guidance. By *flk-1* labeling, ISVs appear to sprout from the aorta, beginning at the 24 somite stage (Fouquet, 1997). In mutants that lack the dorsal aorta, such as the zebrafish mutant *floating head*, intersegmental arteries fail to form. No sprouts are observed in mutants that fail to undergo tubulogenesis of the aorta, such as *sonic-you*. However, sprouts form normally in the *one eyed pinhead* mutant, which has an artery but lacks a vein (Brown et al., 2000). Thus, a well-formed aorta appears to be a prerequisite for ISV formation. It is not clear what guides the initial positioning of the sprouts. VEGF is needed for the outgrowth to occur: zebrafish exposed to low-dose VEGF morpholino antisense establish an aorta but not ISVs (Nasevicius et al., 2000). The source of angioblasts for the ISVs has been examined in chick, where tissue transplants have suggested derivation from both the splanchnopleural mesoderm and the roof of the dorsal aorta (which is somitically derived) (Pardanaud, 1996).

We have examined by lineage analysis, the source, cell fates and migratory trajectory of angioblasts that constitute the ISVs. Additionally, we have performed a genetic screen to identify mutations perturbing primary vascular pattern formation.

We find that the angioblasts for zebrafish ISVs arise in the lateral posterior mesoderm. Each sprout is fashioned from three cell types, all of which originate in the aorta and are deployed in a carefully choreographed manner. One cell remains ventrally in the aorta and sprouts a dorsally directed process, resembling a plumber's T junction; a second cell is a mirror image of this, based dorsally in the DLAV; and a third cell generates the tube in between. At no time do cells or sprouts of the sprout extend beyond the intersegmental boundaries, suggesting that there are strong repulsive guidance signals. One of these signals controls the site of take-off of the sprout, as revealed by the mutation, *out of bounds*, in which all angiogenic cell fates are generated, but sprout launching and ISV growth are not restricted to the intersegmental region.

MATERIALS AND METHODS

Lineage and histology
Endogenous AP activity was assayed after fixation and permeabilization of 72 hpf embryos in 4% PFA and 0.5% Triton X100 in PBS. The embryos were washed four times over 2 hours in 1% goat serum, 0.5% Triton in phosphate-buffered saline (PBS). Embryos were then washed three times in NTMT [100 mM Tris (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20], before staining in NBT/BCIP (Boehringer Mannheim). Blood was stained with biotinylated Isolectin B4 (Sigma), and detected with the Vectastain kit (Vector Laboratories). For laser labeling studies, one-cell embryos were injected with 5% DNMB caged fluorescein (Molecular Probes) in 200 mM KCl, and allowed to develop in the dark as described (Serbedzija, 1998). Between the 6- and 12-somite stages of development, cells in the LPM were activated by an nitrogen laser tuned to 352 nm (Laser Sciences, Franklin MA, and Photonics, Arlington Heights, IL) attached to a Zeiss Axioplan fluorescent microscope. Embryos were then allowed to develop to 40 hpf in the dark, and fixed in 4% paraformaldehyde (PFA). Embryos were stained with a 1/500 dilution of anti-fluorescein-peroxidase antibody (Boehringer Mannheim), developed using diaminobenzidine. In addition to the specific labeling, periderm is also frequently labeled at the site of activation. Embryos were mounted and sectioned at 5 µM in JB-4 plastic medium (Polysciences).

Mosaic transgenics
A construct encoding the mouse tie-2 promotor and enhancer (Schlaeger, 1997) was digested with *Sal*I and injected into single-cell stage wild-type and *obd* mutant embryos at a concentration of 200 ng/µl. Embryos were examined between 24 and 46 hours post fertilization (hpf). For sectioning, embryos were fixed in 4% PFA/PBT, permeabilized with cold methanol/acetone, and stained with a 1/2000 dilution of JL-8 antibody (Clontech). Staining was visualized with a goat anti-mouse Alexa Fluor 568 antibody (Molecular Probes).

Genetic screening and embryo staining
The screen for recessive ENU-induced mutants in TopLong fin strain zebrafish has already been described (Chen et al., 2001). To identify vascular patterning mutants, endogenous AP activity was assayed as before. To assay motoneuron pattern, a 1/300 dilution of znp-1 supernatant (Developmental Studies Hybridoma Bank) (Trevarrow et al., 1990) was applied to embryos after fixation in 4% PFA and permeabilization in methanol acetone. Staining was visualized by diaminobenzidine (Vectastain). *fli1*, *tie1*, *delta D*, *fgf8* or *vegf* expression (Brown et al., 2000; Haddon et al., 1998; Liang et al., 1998; Lyons, 1998; Reifers et al., 1998) was visualized by in situ hybridization with digoxigenin labeled probe as described (Jowett and Lettice, 1994). Signal was detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody, and reaction with NBT/BCIP (Boehringer Mannheim).

Microangiography
Microangiography was performed as described (Weinstein et al., 1995) with the exception that 0.2 µM yellow-green polystyrene Fluospheres (Molecular Probes) in 1% bovine serum albumin (BSA) were sonicated for 2 minutes at full power before injection into the sinus venosus.

Transplantation
Dechorionated one-cell donor embryos were injected with tetramethylrhodamine biotin dextran (Molecular Probes). Cells from the animal pole were transplanted to the margin of mutant recipient embryos at the sphere stage of development, as this gave high numbers of transplanted cells in the somite.

Morpholino treatment
Dechorionated one-cell wild-type and mutant embryos were injected with VEGF morpholino antisense oligonucleotideA-1 (5′GTATCAAATAAACAACCAAGTTCAT-3′) described previously (Nasevicius et al., 2000). Stock concentrations from 150 µM to 1 mM were injected.

RESULTS

Three cell fates in the zebrafish angiogenic intersegmental sprout

Our focus here is particularly on the intersegmental vessels (ISVs) that run between each pair of somites from the dorsal aorta to the DLAV. The ISVs are in physical proximity to the somite-notochord interface ventrally, and the somite-neural tube interface more dorsally, as shown in Fig. 1. There are two parallel DLAVs on the dorsal side of the embryo into which the left or right ISVs connect.

The ISVs are first revealed by *flk1* labeling at approximately 23 hpf (Fouquet et al., 1997), and are partially patent at 1.2 to 1.5 dpf (Isogai et al., 2001). They show robust circulation by 2 dpf. Although the location of the vessels remains stable, the direction of flow is not predictable for any given ISV at the onset of circulation. Eventually half of the ISVs will carry blood from the aorta to the DLAVs, and half from the DLAV to the posterior cardinal vein. The processes that mediate the remodeling of the vessels into arterial or venous, and the re-routing of the circulatory pattern are completely unknown.

ISVs are arranged in an extremely regular array. In the medial-lateral plane, the ventral portion of the ISVs runs between the notochord and somite, and the dorsal portion between the neural tube and somite boundaries (Fig. 1A). The launching site of the ISV is in a small triangle of space bounded by two somites (Fig. 1C). Ventrally, the ISVs follow the chevron shape of the somite. More dorsally, when adjacent to the neural tube, the ISVs no longer run between somite borders, and rather adopt a straight course to the DLAV (Fig. 1B). In the vicinity of the notochord we do not observe cells or their processes outside of boundaries defined by the somite borders. The initiation of the angiogenic sprout, the processes of the cell, and at least the ventral part of the migratory path all appear to be constrained by somite borders.

In order to define the cellular architecture of the ISV, we labeled the angioblastic lineage with GFP under control of a *tie-2* promotor, previously shown to be expressed in zebrafish endothelial cells (Motoike et al., 2000). We find that transient expression of the construct labels cells in a highly mosaic fashion, allowing analysis of individual cells. By examining mosaic embryos repetitively over 20 hours, we tracked the migration of individual angioblasts (Fig. 2). Beginning at 25 hpf, scattered GFP-expressing angioblasts are evident in the axial vessels and began to send projections dorsally into the somitic region. We followed the migration and fates of these cells as they assume positions in the ISVs and DLAV.

There are three different stereotypical endothelial cell fates in the sprout. The first type of cell leaves the aorta and migrates between the somites until reaching the DLAV, at which point it spreads anteroposteriorly into the nascent DLAV, but leaves a tail between the somites. Hence, the cell resembles a T-junction (Fig. 2A). The next aortic migrant cell follows the same track, and stretches along nearly the entire length of the ISV from aorta to DLAV. The nucleus of this cell is positioned midway along the ISV. This appears to be a connector cell (Fig. 2B). The third component of the sprout
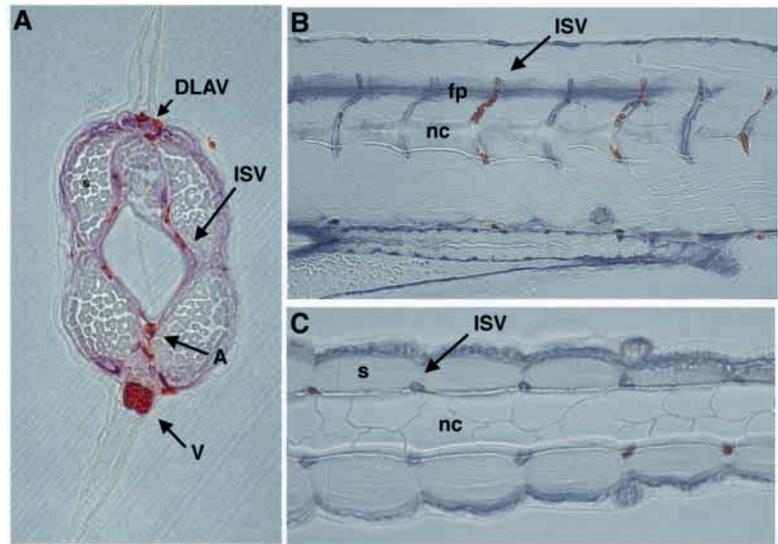

Fig. 1. ISV relationships to somite, notochord and neural tube. (A) Cross section of posterior trunk shows close apposition of a pair of ISVs with somite, notochord and neural tube. (B) Sagittal section shows extremely regular pattern of ISVs closely associated with the somite boundary in the ventral trunk. Anterior is towards the left, and dorsal is upwards. (C) In this transverse section, pairs of ISVs are located at the somite boundaries that surround the notochord. Anterior is towards the left. Vessels are labeled by reaction of endogenous alkaline phosphatase. Blood is labeled with Isolectin B4. DLAV, dorsal longitudinal anastomotic vessel; ISV, intersegmental vessel; A, dorsal aorta; V, posterior cardinal vein; fp, floor plate; nc, notochord; s, somite.

is that most proximal to the aorta, which comprises of the sprout from a cell in the aorta, forming the mirror image of the T in the DLAV (Fig. 2C). We find these cell types when labeling angioblasts by uncaging of fluorescent dye in angioblasts. In cross-section, the circumference of the ISV appears to be constituted by one, and sometimes two, connector cells (Fig. 2D). In this fashion, the entire ISV and DLAV is estimated to consist of three (or at most 4) endothelial cells per somite.

Angioblasts for all components of the angiogenic sprout arise in the lateral posterior mesoderm

We wished to identify the origin, migratory path and cellular characteristics of the angioblasts that form the ISVs. Previously we have shown that precursors for the dorsal aorta and posterior cardinal vein can be labeled by uncaging a caged fluorescein marker in cells of the LPM using a laser (Zhong et al., 2001). To identify ISV precursors, we focused the laser to label five to ten cells of the LPM (Fig. 3A). We activated the LPM at different somite levels from anterior to posterior, mediolaterally, and in embryonic stages from seven somites up to 12 somites. We observed that only a small proportion of labeled cells were angioblasts, and most LPM cells contributed to other tissues, notably pronephric duct and blood.

We find that the LPM contains precursors for all vessels of the trunk: aorta, posterior cardinal vein, DLAVs, SIVs and ISVs. All three cell subtypes of the ISVs are labeled (Fig. 3B-D). No definable region of the LPM preferentially provides progeny to any type of vessel.
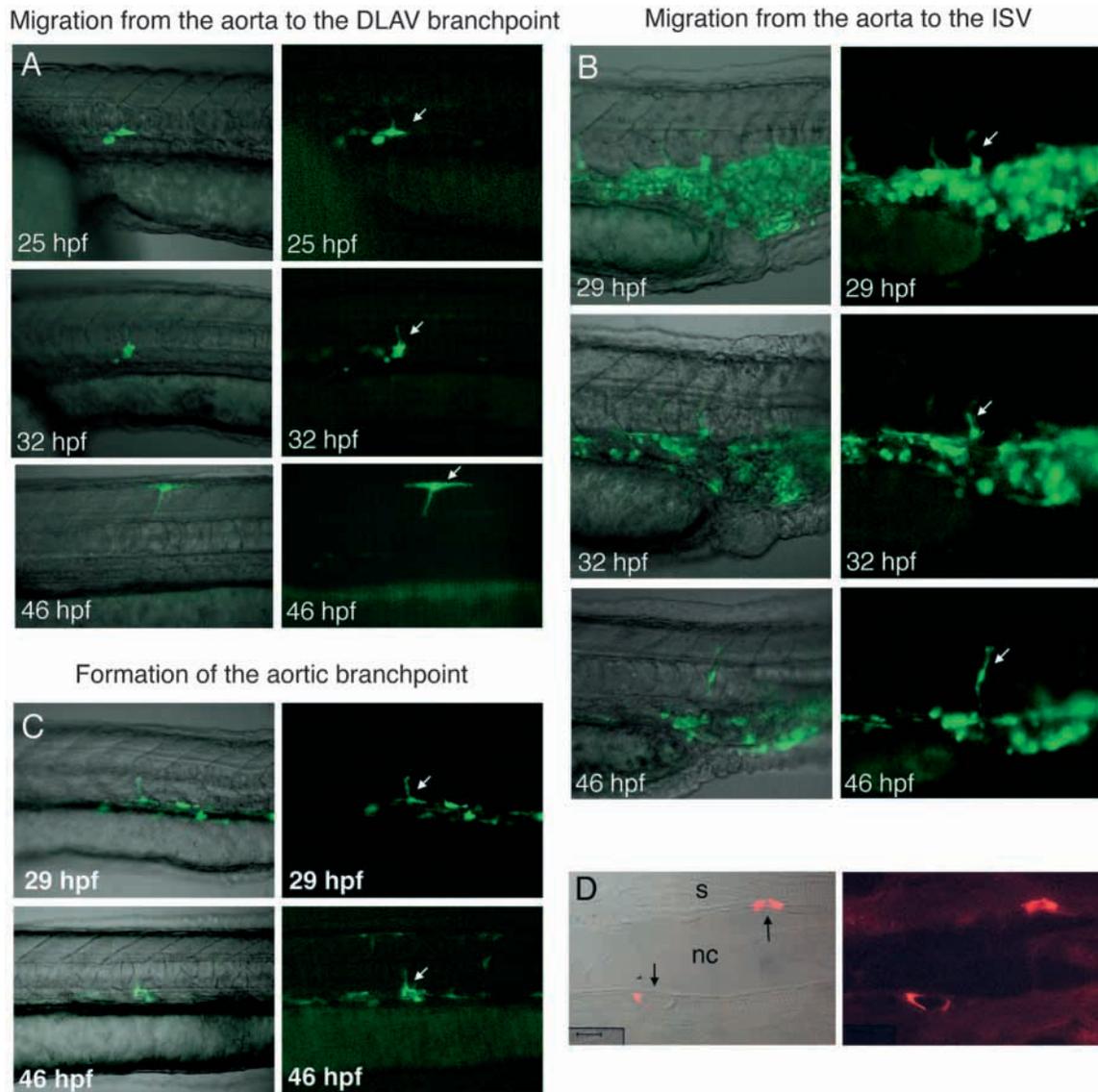
Migration from the aorta to the DLAV branchpoint

Migration from the aorta to the ISV

Formation of the aortic branchpoint

Fig. 2. Time-lapse of angioblasts migrating from the aorta to assume the three stereotypical cell fates in the ISV: single cells expressing GFP under the *tie2* promotor were photographed over a 21 hour period. Examples are shown of cells assuming the three positions. (A) T-shaped cell based in the DLAV projecting into the ISV. Arrows show the cell turning its migration; (B) ISV connector cell (arrow); and (C) aorta and ISV T-shaped cell (arrow). (D) Transverse sections of these *tie2* GFP labeled embryos show that one to two cells (arrows) surround each ISV. In A-C, the right panel shows the fluorescent image and the left panel shows the fluorescent image superimposed on the phase contrast one. Anterior is towards the left, and dorsal is upwards. Scale bar: 10 μm.

There is clearly an anteroposterior pattern in the LPM that is reflected in the ultimate vascular destination. Progeny assume positions in the vessels at approximately the same anteroposterior level as the LPM precursors that were activated, and they contribute to the vasculature between only one to two pairs of somites. The same three types of progeny are revealed regardless of the anteroposterior level, whether activated adjacent to the first or to the last-formed somites of a ten somite embryo. However, as the embryo develops to ten somites and beyond, angioblasts are more commonly revealed after labeling in the posterior regions of the LPM, suggesting that there is an anteroposterior gradient in the initiation of medial migration of angioblasts, such that the angioblasts adjacent to the more mature anterior somites migrate slightly

earlier. This anterior to posterior maturation program is also suggested *tie1* expression at the 12- to 14-somite stages (data not shown).

There does not appear to be a left-right preference in the tracking of LPM angioblasts. DLAVs and ISVs are labeled ipsilateral and contralateral to the LPM activation side. Labeled cells in the DLAV are also observed on both left and right sides (Fig. 3D). As these cells transit through the midline dorsal aorta before sprouting to form the derivatives, left-right laterality may be lost at the midline. Similar contralateral labeling of ISVs has also been observed in quail-chick transplantation studies (Ambler et al., 2001), although it has not been seen in other studies (Wilting, 1995; Pardanaud et al., 1996). By contrast, labeling of pronephric duct is exclusively
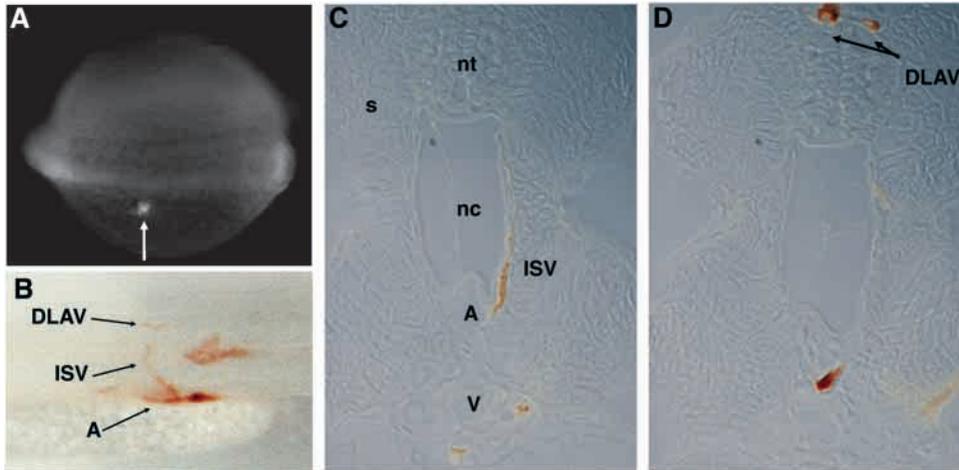
Fig. 3. Lineage tracing of trunk shows ISVs derive from the LPM, via the axial vessels. (A) Fluorescein dye is uncaged by the laser in cells in the LPM of a nine somite embryo adjacent to somite nine. (B) Laser activation of cells in the LPM leads to labeling of aortic, connector and T-cells. (C) Cross section of a labeled ISV. (D) Cross section of ipsilateral and contralateral labeled DLAV cells. In A,B, anterior is towards the left. Dorsal is upwards. DLAV, dorsal longitudinal anastomotic vessel; ISV, intersegmental vessel; A, dorsal aorta; V, posterior cardinal vein; nt, neural tube; nc, notochord; s, somite.

limited to the side of the LPM labeling (Serluca and Fishman, 2001) (this study).

In chick, the roof of the aorta and the ISVs have been shown to be mosaic, being derived from splanchnopleural mesoderm and somite when evaluated by quail-chick transplantation (Pardanaud et al., 1996). We labeled lateral, central and medial somite with the laser at the 11 somite stage of development. Although the somites were strongly labeled, we did not observe any endothelial cells deriving from labeled somite (*n*=0/63, data not shown). Thus the development of the ISV vasculature in zebrafish may be different from that of the chick, and appears to be exclusively derived from the LPM.

The sub-intestinal venous vessels (SIVs) we find to be outgrowths of the posterior cardinal vein. The SIVs develop in the ventral trunk of the embryo underneath the somites and grow over the yolk. They are spatially restricted to a segment of anterior trunk. By using the laser, LPM activation at the six somite level, which labeled the vein, also resulted in labeling of projections spreading over the yolk where they will form the SIVs (*n*=8/8).

The pattern of angiogenic vessels is abnormal in *out of bounds*

We performed a genetic screen to identify mutants with defects in vessel patterning (Chen et al., 2001). From 750 mutagenized genomes, we isolated one vessel patterning mutation, termed *out of bounds* (*obd*). This is a recessive and fully penetrant mutation with two non-complementing alleles (fs31-l and fv109-k). The mutation is embryonic lethal in more than 80% of homozygotes. However, some homozygous embryos do survive to adulthood and are fertile.

obd mutant embryos appear normal until midway through somitogenesis. At this time, around the 17-18 somite stage, the aorta of

obd mutant embryos launches precocious sprouts, as observed by staining with *fli1* (Fig. 4A). Sprouts in wild-type embryos do not appear until the 24S somite stage (Fouquet et al., 1997) and thus *obd* ISVs develop more than 3 hours in advance of the normal schedule. These precocious sprouts originate from
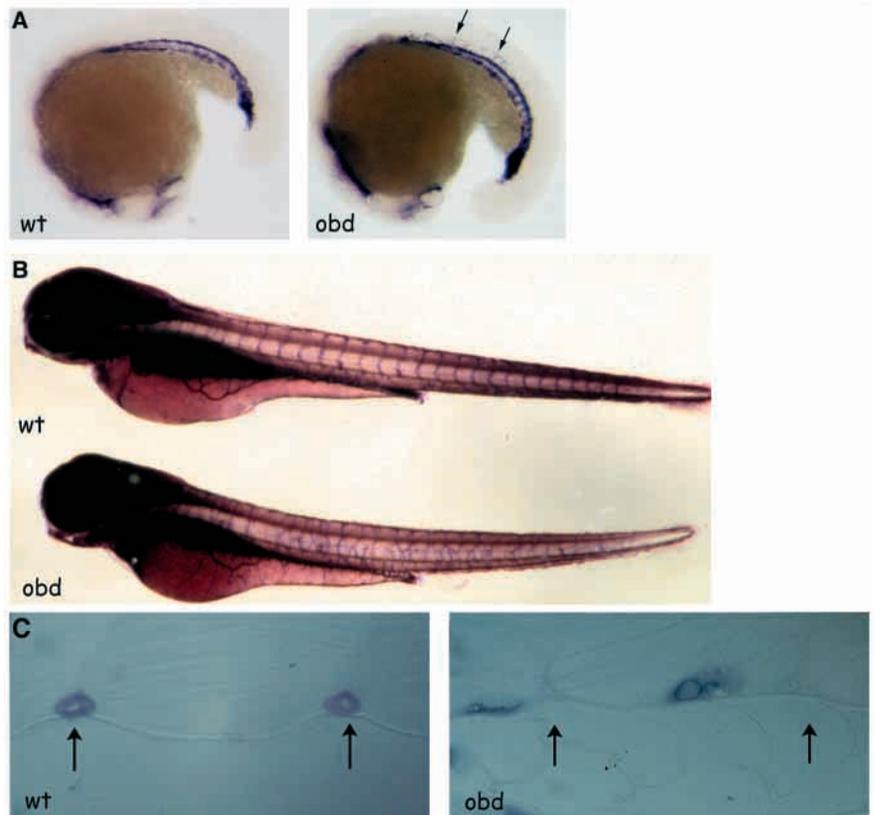

Fig. 4. *out of bounds* mutant angioblasts migrate precociously, sprout from anomalous positions and generate abnormal patterns. (A) In situ hybridization of *fli1* on 18 somite *obd* embryos demonstrates ectopic angioblast migration into the somite region in mutant embryos compared with wild type. Arrows denote ectopic cells. (B) The dorsal aorta and posterior cardinal vein are normally patterned as revealed by alkaline phosphatase staining in *obd*, while angiogenic vessels of the SIVs and ISVs are completely disorganized in the trunk region. (C) Transverse sections of alkaline phosphatase stained embryos demonstrate that ISVs are positioned at the intersegmental boundaries (arrows) in wild type, and are positioned randomly within the somites of *obd* mutants.
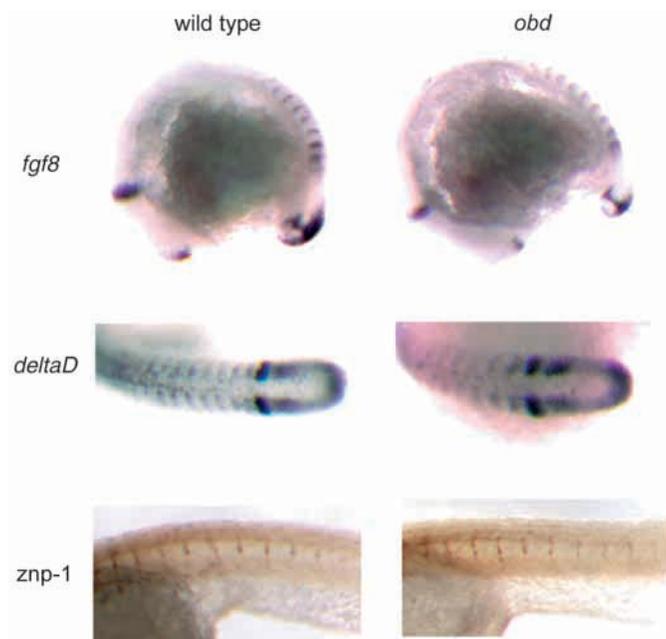
Fig. 5. Anteroposterior patterning of the somite and primary motoneuron patterning are unaffected in *obd* mutants. In situ hybridization of wild-type zebrafish with probes for *fgf8* or *deltaD* demonstrates a segmental expression pattern in the anterior somite. The expression pattern in *obd* homozygous mutant animals is identical to that in wild type. Antibody staining with znp-1 marks primary motoneurons in zebrafish. The pattern is identical in *obd* and wild-type embryos.
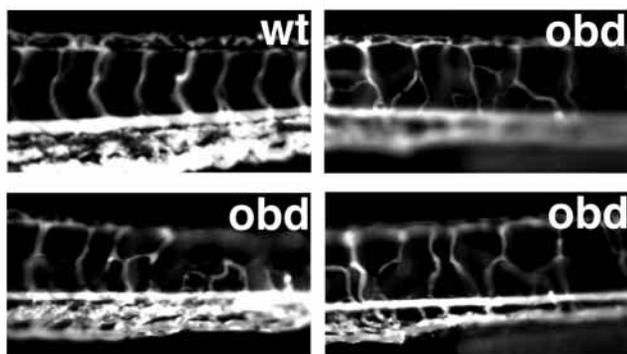

Fig. 6. *obd* ISVs are patent but tortuous. Angiograms of the trunk region of wild-type and three *obd* mutants demonstrate the abnormal and variable pattern of patent *obd* mutant ISVs. The spacing of ISVs in *obd* appears most affected ventrally where the vessels originate, as opposed to dorsally where they terminate.

abnormal positions along the aorta and traverse domains normally forbidden to sprouts in wild-type embryos. Interestingly, there is still approximately one vessel per somite in *obd*. Thus, neither their site of origin nor path of migration is restricted to the intersegmental space. By 24 hpf, the ISV sprouts of *obd* embryos appear chaotic compared with wild type, particularly in their ventralmost region. The dorsal portion of the ISVs appears far less affected, tending to run along a more-or-less straight path to the DLAV. *obd* ISVs are patent, and begin to carry blood flow at the same time as wild-type embryos. The pattern of vessels is highly disorganized,
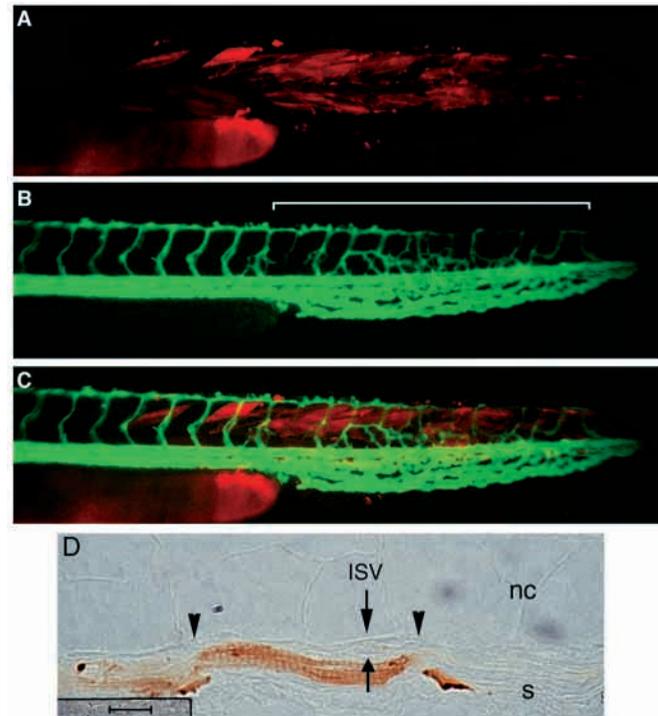

Fig. 7. *obd* cells in ventral somite perturb wild-type endothelium: *obd* acts cell non-autonomously. (A) Transplanted *obd* somite labeled with rhodamine-dextran in the tail of an unlabeled wild-type embryo at 52 hpf. (B) Angiogram of the same embryo showing normally patterned ISVs in regions of no transplant (or where dorsal *obd* somite was transplanted) and vascular disorganization in the tail region where *obd* ventral somite was transplanted (bracket). (C) Overlay of A,B. (D) ISVs composed of wild-type endothelial cells grow mid-somite (borders of the somites are indicated by arrowheads), in the vicinity of rhodamine dextran biotin-labeled *obd* somite. ISV, intersegmental vessel; nc, notochord; s, somite. Scale bar is 10 μm.

however (Fig. 4B): in transverse section, the ISVs may be found in any position along the AP axis of the somite (Fig. 4C).

It is of interest that somite patterning in general is not perturbed in *obd* mutant embryos. While *obd* ISVs are not restrained along the AP axis to be between somites, they are restrained in the mediolateral plane (Fig. 4C). The patterning of somites is normal as revealed by *deltaD* or *fgf8* expression, and primary motoneuron axons track as in wild-type embryos, as revealed by staining with the znp-1 antibody (Fig. 5).

By 48 hpf, blood flow through the ISVs has begun. At this time, circulation through the trunk of *obd* embryos is clearly aberrant. Not only are the ISVs tortuous, but instead of extending to the most dorsal side of the embryo, some loop back to the aorta or cross somites to connect to adjacent ISVs (Fig. 6).

The patterning defect in *obd* appears to be cell non-autonomous

The *obd* phenotype suggests that the *obd* gene is particularly important in defining the timing and site of ISV sprout launching, as well as in controlling its path. Thus, naturally we wanted to distinguish whether *obd* acts autonomously in an endothelial cell, or in a cell non-autonomous manner. We
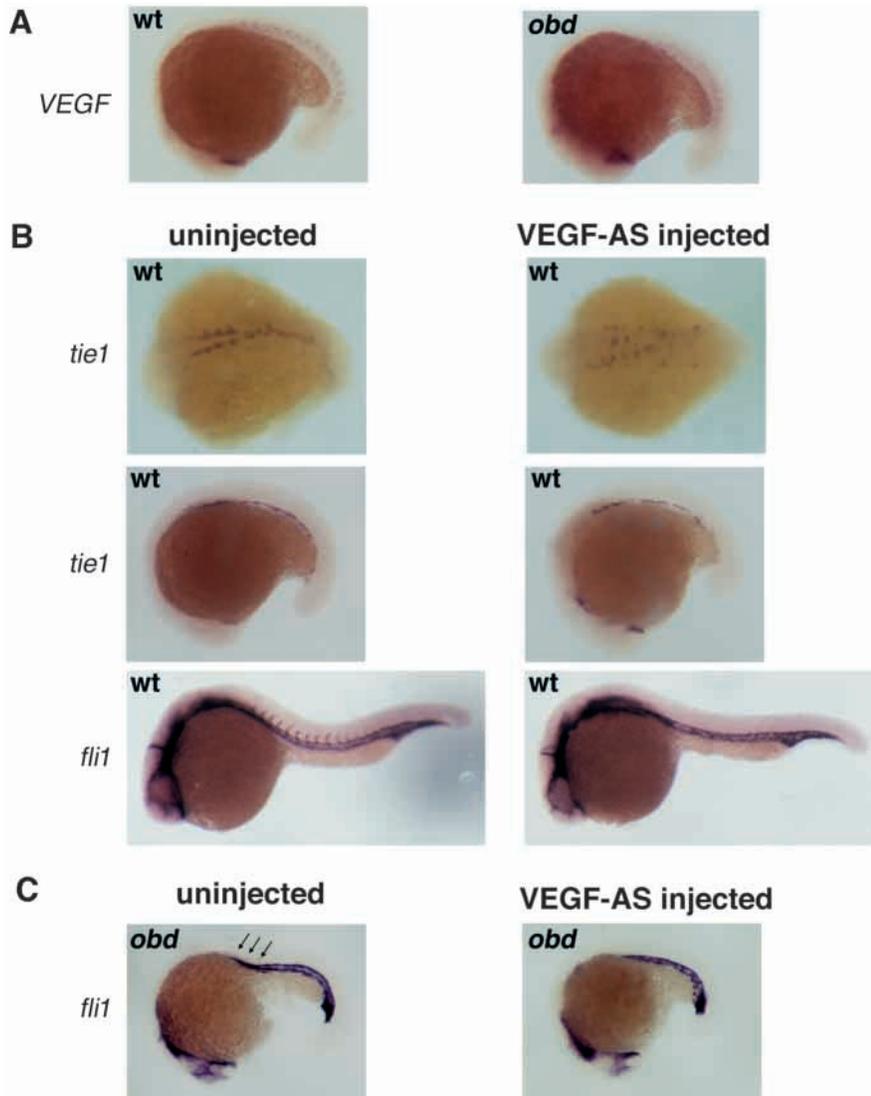
Fig. 8. VEGF antisense delays precocious migration of *obd* angioblasts. (A) VEGF is normally expressed segmentally, beginning at the 17 somite stage, in both wild-type and *obd* embryos. (B) In a wild-type untreated embryo at 14 somites, angioblasts have migrated from the LPM to the midline while in a 1 mM VEGF-antisense treated embryo, *tie1*-positive cells are still localized laterally (top row). The dorsal aorta and vein are formed in both treated and untreated embryos at 19 somites, although the vessels in the injected embryo are discontinuous (middle row). At 24 hours, angiogenic sprouts are evident in an untreated embryo but are not seen in the treated embryos (bottom row). (C) The ectopic sprouts evident in a 19 somite *obd* embryo (arrows), are completely inhibited in the VEGF-AS-treated *obd* mutant embryo.

examined this issue by reciprocal transplantation between wild-type and *obd* homozygous mutant embryos. We find that *obd* cells transplanted in the ventral somite can induce wild-type endothelial cells to acquire an *obd*-like pattern in the ISV region (Fig. 7). *obd* cells transplanted into dorsal somite, notochord, neural tube or hypochord do not perturb the ISV pattern of wild-type endothelial cells (data not shown). Wild-type somite in *obd* mutants appears to rescue some of the patterning defects. However, because a small proportion of ISVs in *obd* mutants can have a normal pattern, it is difficult to assess the degree of rescue. It is rare to transplant cells in a

manner to incorporate into ISVs themselves, but where we have observed *obd* ISVs in wild-type embryos, their pattern is normal (data not shown).

Abnormal, rather than precocious migration causes the *obd* phenotype

obd ISVs sprout precociously, so it was possible that premature sprouting meant that vessels lacked time-dependent guidance signals, and therefore adopted an aberrant sprouting pattern. To address this issue, we explored means to reversibly delay ISV sprouting in *obd* embryos. We found that we could do so by inhibition of VEGF.

VEGF is normally expressed segmentally in the ventral-medial somite, adjacent to the notochord. The expression of VEGF in *obd* is normal (Fig. 8A). We find that injection of VEGF antisense morpholino into wild-type embryos does not diminish the apparent number of angioblasts, as assayed by *tie1* in situ hybridization. It does, however, as noted previously (Nasevicius et al., 2000), completely inhibit ISV formation until 48 hpf. We find that there are several explanations for this effect. First, angioblast migration from the LPM to the midline is impaired (Fig. 8B). Although delayed, cells do eventually migrate to the position of artery and vein by the 19-somite stage. This dose completely prevents the formation of ISV sprouts in wild-type embryos. Additionally, precocious migration of angioblasts in *obd* is completely inhibited (Fig. 8C). Interestingly, *obd* sprouting is more sensitive to VEGF than is wild type and the suppression of precocious sprouting is evident at very low doses (as low as 150 µM).

We were interested to observe the vascular pattern after recovery to see if there were time-dependent signals for vascular patterning. By 72 hpf, about 20% of VEGF morpholino-treated embryos recover circulation. In wild-type embryos with recovered circulation at 3 dpf, the vessel pattern is completely normal. In VEGF morpholino-treated *obd* embryos, the recovered vessel pattern is indistinguishable from untreated *obd* embryos, even though precocious sprouting was completely inhibited during the usual time window (data not shown). Thus, although the precocity of migration is a hallmark of *obd* embryos, it is not essential to the patterning phenotype. Signals that guide vessel patterning of the ISVs in the trunk appear to be present for a longer time window than the period in which sprouting usually occurs.
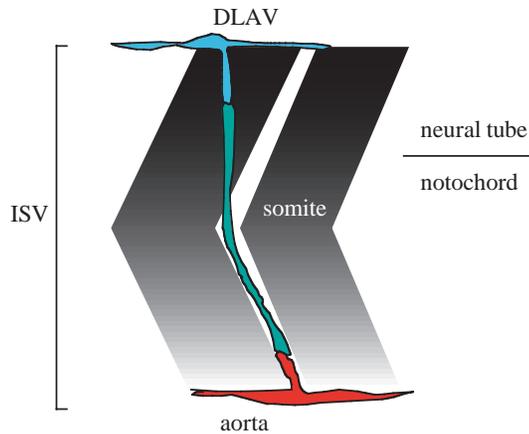
Fig. 9. Model of the construction of a zebrafish ISV. An intersegmental vessel (ISV) is composed of three types of endothelial cells, distinguished by their morphologies. The dorsal connection to the DLAV is a T-shaped cell (blue); the ventral connection to the aorta is an inverted 'T' (red). The connecting cell (green) courses between the somites ventral to the notochord-neural tube interface, and appears not to follow the somite boundary dorsally, where it runs directly to the DLAV..

DISCUSSION

We have focused here upon defining the in vivo rules that pattern vessels formed by angiogenesis, specifically those that sprout from the aorta in the trunk. We have defined the site of origin of angiogenic precursor cells, their migratory paths and cell fates, as well as the migration constraints that yield the beautiful array of repetitive sprouts, one per segment, running between adjacent somites. We find by lineage tracking that angiogenic precursors arise in the lateral posterior mesoderm, at an axial level that corresponds to the eventual sprout, and migrate medially to the aorta, from whence they sprout dorsally to connect the aorta with the DLAV. Three cell types constitute the sprout (Fig. 9). Two resemble T-joints, one pointing dorsally off the aorta and one pointing ventrally off the DLAV. The third constitutes a connecting tube in between. Thus, the angiogenic sprout has an organotypic form that is the consequence of the intercalation of cells of particular shapes.

The global pattern of angiogenesis in the trunk requires that the launching of each sprout and its dorsal growth are precisely oriented and constrained to specific pathways. These guidance cues are disrupted by the ENU-induced mutation *obd*. In *obd*, the sprouts originate and traverse the ventral somites in an irregular fashion, outside of normal boundaries. Thus, vascular patterning appears to use systems similar to those for pathway generation in the nervous system (Tessier-Lavigne and Goodman, 1996), with guidepost regions that dictate the site of origin of sprouts and repulsive signals to restrain growth within particular pathways.

The lineage and migration of angioblasts

The lateral posterior mesoderm of the zebrafish provides angioblasts for all major vessels of the trunk, including the aorta and vein, which form by vasculogenesis, and the ISVs, SIVs and DLAVs, which form by angiogenesis. In the chick, transplantation experiments suggest that cells from the somite

also provide angioblasts, subsequently populating the roof of the aorta and the ISVs (Wilting, 1995; Pardanaud et al., 1996; Ambler et al., 2001). DiI-labeled sclerotome cells in zebrafish also contribute to both aorta and ISVs (Morin-Kensicki and Eisen, 1997). While not ruling out the possibility of a mosaic origin of trunk vessels in the zebrafish, in our experiments it is clear that the majority of the trunk vasculature is derived from cells of the LPM, via the axial vessels. It is certainly possible that there is a species difference in source of angioblasts. Among these various methods, the advantage of laser uncaging is its ability to label only a few cells with great precision.

There is a strong correlation between the anteroposterior position of a precursor in the zebrafish LPM, and that of the ISV site. This also is different from the long distances migrated by angioblasts in the chick after they are transplanted (Pardanaud et al., 1996). It is possible that the physical manipulations of transplantation may disturb some normal constraints, or it may be that somitically derived angioblasts in the chick are more invasive than LPM-derived angioblasts.

The generation of the ISV is by carefully choreographed cell migration. In our model (Fig. 9), the first cell to leave the aorta migrates dorsally between the somites, and at the DLAV assumes a T shape, with the junction becoming part of the DLAV. The next cell migrates between the somites until reaching the stem of the T, when it stops and seems to form a tube. The direct connection to the aorta is formed last, by extension of an inverted T stem, a mirror image of the cell at the DLAV. Using a mosaic transgenic marker, single labeled cells assume positions in the DLAV, ISVs or aorta without their neighboring cells being labeled. This strongly suggests that these sprouting vessels of the trunk are formed by migration of cells from the aorta with minimal or no proliferation. Hence, despite the fact that the ISV is generated as a sprout, it seems more appropriate to describe this process as vasculogenesis type II, meaning that there is migration of angioblasts followed by tubular formation, rather than using the traditional definition of angiogenesis, which includes cellular proliferation as well as migration (Risau, 1997). A similar vasculogenic type II origin of ISVs has been recently been suggested in a chick chimera model (Ambler et al., 2001). This may be an important distinction, in that growth factors, such as VEGF, enhance cell division as well as directed migration, while others, such as angiopoietin 1, affect migration without inducing cell division (Koblizek et al., 1998).

It is notable as well that regions of the ISV are reproducibly configured by endothelial cells with distinctive shapes. These are effectively different cell fates. How do they arise? It seems reasonable to speculate that during the ventral-to-dorsal migration, angioblasts encounter distinct signals, or different levels of a signal arrayed in a morphogenetic gradient that dictates the fate of the individual cell and the shape of the vessel. Perhaps also the establishment of contact with other angioblasts already in position in the nascent ISV would inhibit migration of further angioblasts. The position of nuclei in the ISV also appears stereotypical and may reflect guidance constraints.

obd is part of a patterning system for ventral ISV

What signals could direct ISV patterning? Hypoxia is detected via oxygen-sensitive elements in the HIF1α gene, and induces expression of VEGF and subsequent angiogenesis (Shweiki et

al., 1992). However, hypoxia cannot account for the exquisite patterning of ISVs. The zebrafish embryo does not depend upon circulation for oxygen delivery, and mutants without blood flow have a trunk vessel pattern that is indistinguishable from wild-type (F. Senluca and M. C. F., unpublished).

VEGF is, however, likely to be crucial to vasculogenesis, in supporting the migration of angioblasts from the LPM to form the dorsal aorta (Cleaver and Krieg, 1998). VEGF is also segmentally expressed in the trunk during somitogenesis and is clearly important to sprouting. Mice hemizygous for VEGF have few or no ISVs despite having an aorta (Carmeliet et al., 1996; Haigh et al., 2000). In zebrafish, morpholino antisense blocks ISV formation (Nasevicius et al., 2000). Nonetheless, there is little evidence for a role for VEGF in patterning (Flamme et al., 1995), and VEGF is expressed normally in *obd*. Additionally, we have shown that inhibition of VEGF translation during vessel sprouting does not affect pattern formation.

Angiogenesis, including of the intersomitic vessels, is abnormal in mice mutant for *ephrinB2, EphB2/EphB3* or Eph B4 (Adams et al., 1999; Wang et al., 1998; Gerety et al., 1999). Overexpression of dominant negative EphB4 in frog also results in an ISV patterning defect (Helbling et al., 2000). The ISV phenotype does not affect the localization of sprouting, and has been attributed to perturbation of remodeling rather than of initial patterning.

One gene that is crucial to the patterning per se is *obd*. We have examined some candidate genes for linkage to *obd* (*ephrinA2*, *ephrinA5a*, *ephrinA5b*, *ephrinB2a*, *Eph zek1*, *Eph zek2*, *Eph zek3*, *Eph rtk4*, *Eph rtk5*, *Eph rtk6/EphA2/eck*, *Eph rtk7*, *Eph rtk8*, *tie1*, *tie2*, *angiopoietin 1*, *angiopoietin 2*, *vegf*, *flk1* and *flt4*; S. C., J. D. Mably, J. Chan and M. C. F., unpublished), but none of these genes is linked. Of course, this does not rule out the possibility that *obd* is an unmapped member of the ephrin family. Mutation of *obd* does not appear to affect generation of the normal aorta or posterior cardinal veins. Nor does it interfere with its ability to generate a sprout, to grow from aorta to DLAV or to become a functional vessel. Rather, it specifically perturbs the site of origin of the sprout along the aorta and the subsequent direction of growth. Normally the sprout is constrained to a region between the somites. Sprouts in *obd* grow in the same plane as in wild type, abutting either notochord or neural tube, but cross somite borders. This is not due to the accompanying precocity of sprouting, as sprouting can be delayed by VEGF antisense morpholinos while the pattern of vessels remains aberrant. Taken together, the precocity of sprouting, the abnormal launching site and the aberrant path of angiogenesis, all indicate that *obd* is part of a normal inhibitory system that guides vessel patterning along its ventral course. It is not clear what changes occur more dorsally, where ISVs normally no longer obey somite boundaries and where *obd* appears to be less crucial, The position of change in ISV pathway correlates with the notochord-neural tube interface, so it is conceivable that these tissues generate different guidance signals, either directly or via the somite. It is interesting that we have also discovered a mutation, fv055b, that causes increased dorsal branching of the ISV, but does not affect ventral pattern (S. C., D. M. G. and M. C. F., unpublished).

obd is also a key temporal regulator of migration. Normally, it appears to counter a VEGF-induced tendency to sprout at early stages. VEGF is expressed 3.5 hours before sprouting normally begins, and the precocious sprouting in *obd* mutant embryos can be suppressed by VEGF morpholinos. This balance of angiogenic promoting and inhibiting molecules is also important in determining tumor angiogenesis (Holash et al., 1999). It will be of interest to determine whether sprouting of vessels in other locales, both normal and pathological, generate vessels by accumulation of cells with distinctive fates, and how local guidance cues determine the site of sprouting.

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