Angiogenic network formation in the developing vertebrate trunk

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Summary

We have used time-lapse multiphoton microscopy of living *Tg(fli1:EGFP)^{y1}* zebrafish embryos to examine how a patterned, functional network of angiogenic blood vessels is generated in the early vertebrate trunk. Angiogenic vascular sprouts emerge from the longitudinal trunk axial vessels (the dorsal aorta and posterior cardinal vein) in two spatially and temporally distinct steps. Dorsal aorta-derived sprouts form an initial primary network of vascular segments, followed by emergence of vein-derived secondary vascular sprouts that interact and interconnect dynamically with the primary network to initiate vascular flow. Using transgenic *silent heart* mutant embryos, we show that the gross anatomical patterning of this network of vessels does not require blood circulation. However, our results suggest that circulatory flow dynamics play an important role in helping to determine the pattern of interconnections between the primary network and secondary sprouts, and thus the final arterial or venous identity of the vessels in the functional network. We discuss a model to explain our results combining genetic programming of overall vascular architecture with hemodynamic determination of circulatory flow patterns.

Key words: Zebrafish, Transgenics, Intersegmental vessels, Vascular development

Introduction

Studies in developing vertebrates have uncovered many genes crucial for embryonic endothelial specification and for blood vessel differentiation and growth (reviewed by Roman and Weinstein, 2000), but we still know little about what guides the patterning of developing blood vessels and determines the anatomical architecture of the vascular system. Many basic questions remain unanswered, including what cues guide vessel positioning relative to other tissues and organs, how vessel interconnection or 'wiring' is determined, and how arterial and venous vessels form parallel and often juxtaposed yet at the same time distinct and separate networks. The genetic and experimental accessibility and optical clarity of the developing zebrafish makes this a useful model system in which to examine the mechanisms of blood vessel formation and patterning during development (reviewed by Vogel and Weinstein, 2000). The intrinsic advantages of the fish are further augmented by novel experimental methods for visualizing blood vessels such as confocal microangiography (Weinstein et al., 1995), and by the elucidation of a complete atlas of the developing vascular system of the zebrafish (Isogai et al., 2001). Transgenic zebrafish expressing green fluorescent protein (GFP) throughout the vasculature are a particularly powerful new tool for dissecting the dynamics of vessel formation. Germline transgenic lines have been generated expressing GFP in vascular endothelium under the control of either the murine Tie2 or zebrafish *fli1* promoters (Lawson and Weinstein, 2002; Motoike et al., 2000), permitting in vivo time-lapse imaging of vascular endothelial cells and their angioblast precursors. The robust expression of EGFP in vascular endothelium of the *fli1* promoter-driven lines [*Tg(fli1:EGFP)^{y1}*] makes it possible to perform sensitive long-term imaging of blood vessels in normally developing, living embryos (Lawson and Weinstein, 2002).

The blood vessels of the developing trunk are ideal for studying the cues and mechanisms guiding vascular patterning during development. The vascular anatomy of the developing trunk is both reproducible in gross anatomy from animal to animal, and characteristically conserved in its basic plan with some species-specific variations (Fig. 1). All vertebrates possess longitudinal axial vessels (dorsal aorta and posterior cardinal vein) that form by vasculogenesis, or the co-migration and coalescence of angioblast progenitor cells originating in the trunk lateral mesoderm to form vessels de novo (Risau and Flamme, 1995). There is also a conserved network of secondary vessels including dorsoventrally aligned intersegmental vessels at the vertical myotomal boundaries between somites, and longitudinal parachordal vessels to either side of the notochord. These secondary vessels are believed to form via angiogenesis, or the sprouting and growth of new vessels from preexisting vessels, although their formation has not been examined in detail. Secondary angiogenic trunk vessels form in metameric units along the trunk, making them ideal for efficient descriptive survey of developmental mechanisms and well-controlled experimental analysis. In this study we use multiphoton time-lapse imaging to examine the

formation of the trunk angiogenic vascular network in living *Tg(fli1:EGFP)^{y1}* transgenic zebrafish embryos. We find that these vessels form by a novel two-step process. Based on our observations, we propose a model for how genetically programmed assembly of vessel tracts is combined with flow dynamic regulation of vessel interconnections to assemble a network with both defined and conserved anatomy and optimized hemodynamic properties. We also discuss possible broader implications of our results for mechanisms of vascular network formation.

Materials and methods

Zebrafish

Zebrafish (*Danio rerio*) embryos were obtained from natural spawnings of laboratory lines. Embryos were raised and fish maintained as described (Kimmel et al., 1995; Westerfield, 1995). The *Tg(fli1:EGFP)^{y1}* transgenic zebrafish line used in this study has been described elsewhere (Lawson and Weinstein, 2002). *silent heart* (*sih*) mutants (Stainier et al., 1996) were crossed to *Tg(fli1:EGFP)^{y1}* transgenic zebrafish to generate *Tg(fli1:EGFP)^{y1}/+, sih/+* double heterozygotes. These were incrossed to obtain doubly homozygous embryos for analysis. Imaged embryos after 3 dpf were treated with 1-phenyl-2-thiourea (PTU) to inhibit pigment formation (Westerfield, 1995) or albino *Tg(fli1:EGFP)^{y1}/+; alb^{b4}/alb^{b4}* transgenic embryos (Lawson and Weinstein, 2002) were used, with similar results.

Microscopy

Transmission videomicroscopic imaging of zebrafish embryos and larvae was performed using a Zeiss Axioplan 2 compound microscope equipped with a Dage SIT-68 camera and an S-VHS recorder. Confocal microscopic imaging of *Tg(fli1:EGFP)^{y1}* zebrafish embryos and larvae was performed using a Radiance 2000 imaging system (BioRad). Standard confocal imaging of EGFP (used for some of the single, isolated images) was performed using the 480 nm laser emission supplied by a Krypton-Argon laser. Multiphoton imaging of EGFP (used for time-lapse sequences and most images in figures) was performed using 950 nm pulsed mode-locked laser emission from a tunable Ti-Sapphire laser (Tsunami laser, Spectra Physics). Time-lapse imaging was performed with the minimal necessary laser power, and development of imaged vessels was not significantly delayed compared with the vessels in adjacent unimaged regions of the trunk.

Embryos were held for time lapse analysis in an imaging chamber prepared from a modified 60 mm petri dish. The embryo medium was prepared with tricaine (0.016%) to inhibit movement of the embryo, and with PTU (0.002%) when non-albino mutant embryos after 3 dpf were imaged, to prevent pigment development. Embryos held in this way maintained heartbeat and robust circulation throughout the imaging period (up to 24 hours). Stacks of frame-averaged (5 frames) confocal optical slices were collected digitally, at 1.67 to 20 minute intervals (as noted) for time-lapse sequences. 2D or 3D reconstructions of image data were prepared using the Lasersharp (BioRad) or Metamorph (Universal Imaging) software packages. The images shown in this paper are single-view 2D reconstructions of collected image *z*-series stacks, reconstructed at a single angle of zero degrees. 3D reconstructions and raw image stacks of single images, and Quicktime timelapse movie sequences, are available for viewing online at http://dev.biologists.org/supplemental.

Quantitative analysis of intersegmental vessel arterial-venous (AV) identity

Arterial-venous identity was determined for each one of the intersegmental vessels in each of six different albino zebrafish larvae on days 2, 3, 4, 5, 6 and 7 post-fertilization. These data can be accessed in Tables S1-S6 at http://dev.biologists.org/supplemental (follow the 'numerical data' link). Assignment of artery or vein identity was made based on two criteria: first, direction of flow of blood cells transiting the segment; and second, whether the segment was visibly joined ventrally to the posterior cardinal vein or to the dorsal aorta. If blood cells could not be observed transiting through the segment and/or a link to the dorsal aorta or posterior cardinal vein could not be verified, vessel identity was recorded as 'undetermined' (no entry present). A final 'definitive' or 'composite' arterial or venous assignment was made if an intersegmental vessel maintained a solely venous or solely arterial identity on at least three of these days and was otherwise of undetermined identity, or if the intersegmental maintained its identity from day 4 onwards. When intersegmental vessels on both right and left sides were functioning, AV identity of each was determined and listed. When only one intersegmental was functioning, the particular side that vessel was present on could not be definitively determined using the microscopic assay employed, and the vessel position was listed as unknown. The definitive or composite vessel identities are listed for each fish in the raw data tables and are compiled together in Table S7 at http://dev.biologists.org/supplemental. Our assignment criteria take into account that intersegmental vessels initiate blood cell circulation asynchronously, even as late as day 4 or even 5, and that intersegmental vessels also occasionally temporarily stop carrying blood cell flow altogether, resuming circulation at a later point. Using the data on AV identities in these six fish, we quantified AV identity in nearest- or next-nearest neighboring intersegments to determine the correlation between vessel identities in spatially juxtaposed intersegments, and also performed a statistical analysis of the data set (see Tables S8 and S9 at http://dev.biologists.org/supplemental). The results of these calculations are presented graphically in Fig. 8.

Results

Vascular anatomy of the developing trunk

The 3 days post-fertilization (dpf) patent (functioning) vasculature of the zebrafish is shown in Fig. 1 (Isogai et al., 2001) (see Movie 1 at http://dev.biologists.org/supplemental/). The longitudinally aligned major axial vessels of the trunk, the dorsal aorta (DA) and posterior cardinal vein (PCV), come online at approximately 1 dpf (Isogai et al., 2001). The axial vessels are linked by dorsoventrally aligned intersegmental arteries (ISA) and intersegmental veins (ISV), nearly all of which are functioning by 3 dpf. Intersegmental arteries and intersegmental veins connect ventrally to either the dorsal aorta or posterior cardinal vein, respectively, and run dorsally between and adjacent to the notochord and neural tube and the somites. The intersegmental vessels on each side of the trunk are joined together just dorsal to the neural tube by two separate dorsal longitudinal anastomotic vessels (DLAV), which are only sparsely interconnected at 3 dpf. There are two intersegmental vessels at each vertical myotomal boundary (myoseptum), one on either side of the trunk. These two intersegmental vessels can be two arteries, two veins, or one artery and one vein, one on either side of the trunk (see below). In addition to these functioning vessels, the parachordal vessels (PAV, in yellow), which run longitudinally along the horizontal myoseptum, are also depicted in this diagram. At this stage they are not yet carrying circulation and in most cases do not even possess a lumen. The pattern of connection of the parachordal vessels is described further below. As noted in the introduction, the vessels shown in Fig. 1 are not unique to zebrafish but are basic features of the early trunk vasculature of vertebrates.

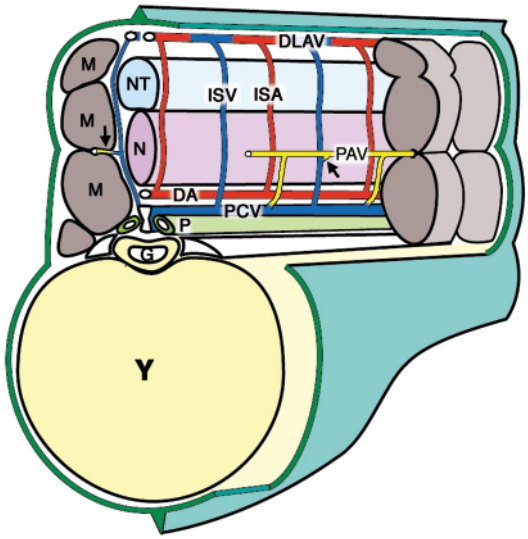

Fig. 1. Anatomy of the zebrafish trunk and its blood vessels at ~3 days post-fertilization. At this stage, there is active flow through the dorsal aorta, (DA), posterior cardinal vein (PCV) and most intersegmental arteries (ISA) and intersegmental veins (ISV). The ISA and ISV are linked together dorsally via paired dorsal longitudinal anastomotic vessels (DLAV). All of these vessels are shown relative to adjacent tissues and structures in the mid-trunk including the gut (G), myotomes (M), notochord (N), neural tube (NT), left pronephric duct (P) and yolk mass (Y). In addition to the functioning vessels noted above, parachordal vessels (PAV) run longitudinally to either side of the notochord, along the horizontal myoseptum. The parachordal vessels are linked to the posterior cardinal vein and intersegmental veins (arrows), but generally not to intersegmental arteries. At 3 dpf, the parachordal vessels do not yet carry flow. Anterior is towards the left and above the plane of the page, and dorsal is upwards.

Primary angiogenic sprouts emerge from the dorsal aorta

As described previously (Fouquet et al., 1997), the dorsal aorta condenses as a distinct cord of angioblast cells at the trunk midline beginning at approximately the 15 somite stage (16.5 hours), developing an open (patent) lumen by about 28 somites (23 hpf), with circulation initiating shortly thereafter. In contrast to the axial vessels, the later-forming intersegmental arteries, intersegmental veins and parachordal vessels are believed to form by angiogenesis (Childs et al., 2002), or the sprouting and growth of new vessels from preexisting vessels. To determine the mechanism by which these blood vessels form, we used multiphoton laser-scanning microscopy (Denk and Svoboda, 1997) of living *Tg(fli1:EGFP)*^{y1} zebrafish embryos. Our findings are summarized schematically in Fig. 8, and detailed in the remainder of this paper.

Beginning at ~20 hpf, pairs of endothelial sprouts emerge bilaterally from the dorsal aorta adjacent to the vertical boundaries between myotomes (Fig. 2A,B) (Movies 2, 3 at http://dev.biologists.org/supplemental/). These sprouts emerge solely from the DA – no sprouts emerge from the posterior cardinal vein at this stage. We designate these first sprouts 'primary' to differentiate them from later-appearing 'secondary' sprouts. The terms 'arterial' and 'venous' are not used because the eventual functional identity of primary vessels can be either (see below). Primary sprouts grow dorsally between the somites and notochord and then between the somites and neural tube, tracking along vertical myotomal boundaries (Fig. 2C-E). They grow in a saltatory fashion with numerous active, filopodia rapidly extending and retracting in all directions around the elongating vessels, particularly near the dorsalmost leading extension (Fig. 2C and Movies 3-5). Processes frequently extend up to tens of μm and then retract in successive frames of time-lapse sequences collected at 5 or even 1-3 minute intervals. As growing intersegmental vessels approach the dorsolateral roof of the neural tube (at approximately 28 hpf) they divide into two major branches that turn caudally and rostrally (Fig. 2E,F; Movies 4, 5 at http://dev.biologists.org/supplemental/), elongate, and then fuse together with branches from adjacent segments to form the bilateral dorsal longitudinal anastomotic vessels (Fig. 2G). By 1.5 dpf, two completed lattices of endothelial vessels are present on each side of the trunk (Fig. 2H), composed entirely of primary intersegmental vessel segments that emerged from the dorsal aorta. Our observations as well as previous work suggest that each of the primary segments is composed of three linked endothelial cells (S.I. and B.M.W., unpublished) (Childs et al., 2002). In the completed primary network, endothelial cell bodies are located approximately: (1) at the DLAV-primary segment junction, (2) at the level of the parachordal vessels and (3) at the dorsal aorta-primary segment junction. We have also observed that the formation of the primary network described here occurs with little or no cell division, with cells migrating to their positions from the dorsal aorta. There is undoubtedly additional cell division at later stages of development, but we have not examined these later stages in detail to observe when and how this occurs. Dorsally, the two parallel dorsal longitudinal anastomotic vessels are only sparsely linked by filopodial connections and are essentially distinct and separate vessels at this stage (Fig. 2I). Throughout this entire period, and often even after completion of the primary intersegmental vessel lattice, few of these primary intersegmental vessels have actually formed open lumens. Most remain as cords or strands of endothelial cells. Where lumens are present, they are usually found first in the ventralmost regions of the vessels, proximal to the dorsal aorta (Fig. 2F,G).

Secondary angiogenic sprouts emerge from the posterior cardinal vein

As formation of the primary, aorta-derived vascular network is completed a new, secondary set of vascular sprouts begins to emerge, beginning at ~1.5 dpf (Fig. 3). Like primary sprouts, these new secondary sprouts arise bilaterally in every myotomal segment and their behavior is highly dynamic (Movies 6, 7 at http://dev.biologists.org/supplemental/). Unlike primary intersegmental sprouts, however, secondary sprouts emerge exclusively from the posterior cardinal vein, and not the dorsal aorta. They emerge less synchronously than the primary intersegmental sprouts, with new secondary sprouts appearing as late as 2.5 dpf. Secondary sprouts grow dorsally, often towards and/or alongside the nearest primary vessel. Approximately half of the secondary sprouts eventually make a connection to the adjacent primary vessel segment, linking the posterior cardinal vein to the primary vascular network (Fig. 3A,B; Movie 6 at http://dev.biologists.org/supplemental/). Once this connection to the posterior cardinal vein is made and the

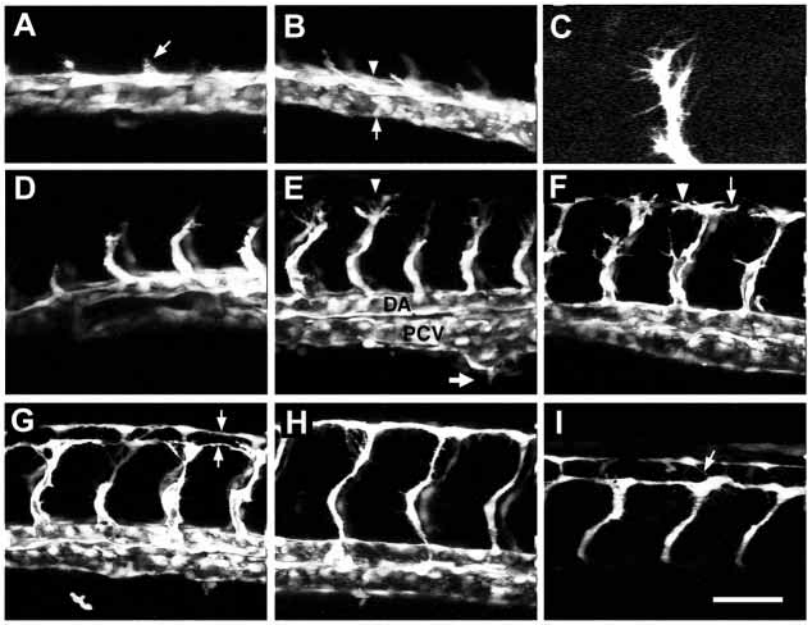

Fig. 2. Formation of the primary angiogenic network. Primary sprouts emerge bilaterally from the dorsal aorta at each vertical myoseptal boundary, then elongate dorsally, ramify and interconnect along the dorsolateral roof of the neural tube to form paired dorsal longitudinal anastomotic vessels. Images shown are lateral (A-F,H) or dorsolateral (G,I) views of the trunk vasculature of different *TG(fli-egfp)^{y1}* embryos at ~0.8–1.5 dpf. Images were collected by standard confocal microscopy. (A) Primary sprouts (arrow) just beginning to emerge from the dorsal aorta. (B) Paired primary sprouts appear bilaterally at or adjacent to each vertical myoseptum. The dorsal aorta (arrowhead) and posterior cardinal vein (arrow) are noted. (C) Close-up high-contrast image of filopodia extend from a growing primary sprout (see Movie 4 at http://dev.biologists.org/supplemental/). (D) Paired primary sprouts extending in the anterior trunk. (E) Primary sprouts in the posterior trunk. The dorsal aorta (DA) and posterior cardinal vein (PCV) are labeled, and end of the yolk extension is noted with an arrow. Increased numbers of filopodia are observed as sprouts approach the dorsolateral surface of the neural tube (arrowhead). (F) Primary sprouts split into rostral (arrowhead) and caudal (arrow) branches at the level of the dorsolateral surface of the neural tube. (G) Rostral and caudal branches from adjacent primary sprouts interconnect to form the paired dorsal longitudinal anastomotic vessels (arrows). (H) Completed primary network. Venous sprouts are still absent. (I) The two dorsal longitudinal anastomotic vessels are separate and only sparsely linked by filododial connections (arrow). The dorsal aorta and posterior cardinal vein are more ventral and are not imaged in this confocal stack. Anterior is towards the left. Scale bar: 50 μm in A,B,D-I; 25 μm in C. 3D reconstructions of these images are available at http://dir.nichd.nih.gov/lmg/uvo/ISV3_D.html

secondary vessel segment starts to carry robust venous (cardinal vein-directed) blood flow, the adjacent ventralmost regions of the primary vessel will regress and disappear (Fig. 3C,D; Movie 9 at http://dev.biologists.org/supplemental/) and the vessel assumes its final identity as an intersegmental vein, despite the fact that nearly all of the endothelial wall of this vessel was derived from the dorsal aorta.

The remaining secondary sprouts elongate dorsally but do not connect to adjacent primary vessel segments (Fig. 3E; Movie 7 at http://dev.biologists.org/supplemental/). A few of these 'non-connecting' sprouts simply regress and disappear (data not shown), but most instead contribute to formation of and serve as ventral venous roots for a separate set of vessels, the parachordal vessels (Figs 1, 4; Movie 8 at http://dev.biologists.org/supplemental/) (see Isogai et al., 2001). Parachordal vessels form along the horizontal myosepta to either side of the notochord (Fig. 1). They form by angiogenic growth from secondary sprouts from the posterior cardinal vein and from additional sprouts that emerge from (future) intersegmental veins at the level of the horizontal myoseptum (Fig. 4). Parachordal sprouts only rarely emerge from intersegmental arteries, and intersegmental arteries for the most part do not initially connect to the parachordal system.

Circulatory flow does not contribute to primary network formation

As we have noted, the basic anatomical pattern of trunk vessels in vertebrates is both reproducible and well conserved (Isogai et al., 2001). Our results show that these vessels form essentially 'as is' in the zebrafish, without an intermediate, more complex vascular plexus that is later pruned and remodeled. This suggests that the anatomical pattern of the intersegmental and parachordal vessels is not only reproducible but also tightly regulated by spatially and temporally defined genetic cues. We hypothesized that the formation and patterning of the primary intersegmental vessel network is not influenced by flow dynamics and would proceed normally in the absence of circulation. To determine if this is the case, we crossed *Tg(fli1:EGFP)^{y1}* fish to heterozygous carriers of the *silent heart* (*sih*) mutation. The hearts of *sih* homozygous animals fail to beat due to a defect in expression of an important cardiac myofibrillar component, cardiac troponin T (Sehnert et al., 2002). Despite the lack of circulation in zebrafish embryos homozygous for *sih*, mutants are normal in other respects and continue to grow and develop for several more days (Stainier et al., 1996). By using the fli1-egfp transgene in *sih* mutant embryos, it is possible to assess the direct effects of lack of blood flow on formation of the vasculature.

We find that the primary vessel network forms normally in *TG(fli1:egfp)^{y1}* embryos mutant for *sih* which lack blood circulation (Fig. 5A). Primary sprouts emerge from the DA, elongate and branch to form two complete lattices, including two continuous DLAV. The timing and dynamics of primary vessel lattice formation are similar in *sih* mutant animals and their phenotypically wild-type siblings. Secondary intersegmental vessel sprouts appear at the proper time in mutant animals (Fig. 5B), and, as in wild-type animals, many sprouts contribute to the parachordal system (Fig. 5C). The connection of secondary sprouts to primary segments cannot be definitively assayed in the absence of blood flow, but it is not obviously evident in *sih* mutants. The formation of additional, supernumerary vessels in the trunks of *sih* mutants is not observed even at 3 dpf, although enlargement of dorsal regions of the intersegmentals and the dorsal longitudinal anastomotic vessels is observed (Fig. 5D).

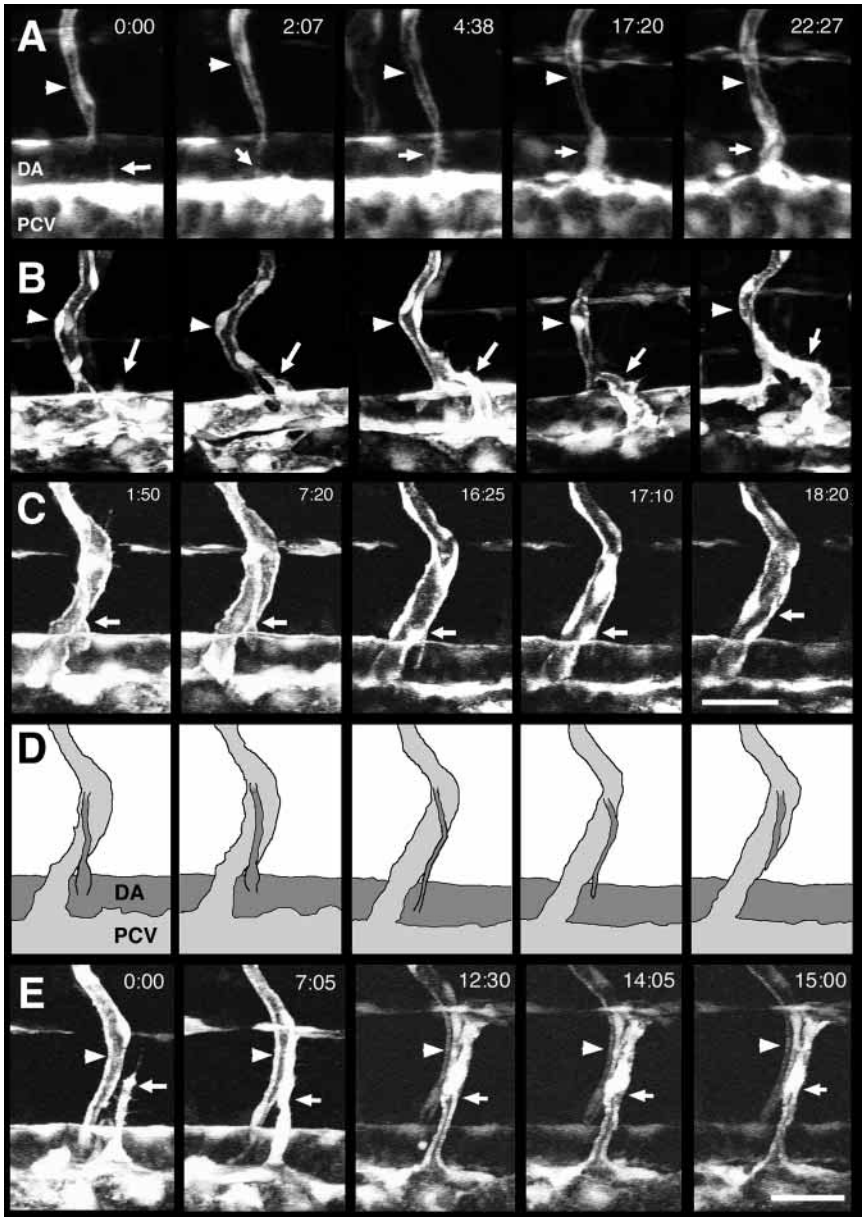

Fig. 3. Emergence and fate of secondary angiogenic sprouts. Images shown are lateral views of vessels at single intersomitic positions in the mid-trunk of *TG(fli-egfp)*^{y1} zebrafish embryos. Sequential image stacks were collected by multiphoton confocal microscopy. Time-lapse movies of the sequences in A, C, E are available as Movies 6, 7 and 9 at http://dev.biologists.org/supplemental/ The images shown in these panels are from selected frames of these movies, labeled with the time in hours:minutes from the first frame (arbitrarily designated time zero). (A) A secondary sprout (arrow) emerges from the posterior cardinal vein immediately adjacent to a primary segment (arrowhead). After an extended time it fuses with the primary vessel, lumenizes and begins to carry blood. Images are from ~1.5-2.5 dpf. The dorsal aorta (DA) and posterior cardinal vein (PCV) are noted. (B) A secondary sprout (arrow) emerges from the posterior cardinal vein slightly away from the vertical myoseptum. It elongates towards and reaches the proximal part of the primary segment (arrowhead), forming a patent connection and lumenizing. Images are from ~1.5-2.0 dpf. (C) A nonfunctional vestigial ventral segment connecting a primary segment to the DA (arrows) thins and then regresses completely adjacent to a large robustly patent secondary connection to the posterior cardinal vein. This vessel is thus an intersegmental vein. Images are from ~1.7-2.5 dpf. (D) Explanatory diagram showing the vessels in C. (E) A secondary sprout elongates next to a primary segment, partially lumenizing but failing to connect. Images are from ~1.6-2.2 dpf. Anterior is towards the right in A and B, and to the left in C-E. Primary (arrowhead) and secondary (arrowhead) intersegmental vascular segments are noted. Scale bar: 25 μm. 3D reconstructions of these images are available at http://dir.nichd.nih.gov/lmg/uvo/ISV3_D.html

Although primary intersegmental blood vessel pattern does not appear to require circulatory flow, experimental or genetic manipulation of dorsal aorta formation or somite identity can dramatically affect trunk vessel patterning. For example, cyclopamine treated zebrafish embryos or those injected with Vegf morpholino fail to form a dorsal aorta or lack proper dorsal aorta arterial identity, respectively (Lawson et al., 2002). The artery-derived primary network fails to form in embryos injected with a morpholino directed against Vegf (Fig. 5E,F), or in mutants we have recently identified that disrupt signaling downstream from Vegf (Lawson et al., 2003) (see Discussion).

Arterial-venous identity of the intersegmental vessels and interconnection of the network

The primary vascular network forms in essentially the same manner in every myotomal segment, but the eventual functional fate of these vessels varies. Depending on whether or not a functional connection is made to a secondary sprout, approximately half of the primary segments eventually become part of intersegmental veins, while the remainder become intersegmental arteries. As previously noted (Isogai et al., 2001), the anteroposterior sequence of intersegmental arteries and intersegmental veins in the zebrafish trunk does not appear regularly ordered (e.g. artery, vein, artery, vein, artery, vein, etc.). Furthermore, with the exception of the first five pairs of vessels the arterial or venous identity of intersegmental vessels along the trunk differs in every individual animal (Isogai et al., 2001). To examine whether the arrangement of intersegmental arteries and intersegmental veins is in fact random, we performed a statistical analysis of the pattern of intersegmental arteries and veins in the trunks of six different embryos/larvae. A detailed description of this analysis and the resulting data are provided in the Materials and methods section and in the web supplement at http://dir.nichd.nih.gov/lmg/uvo/ISVdata.html.

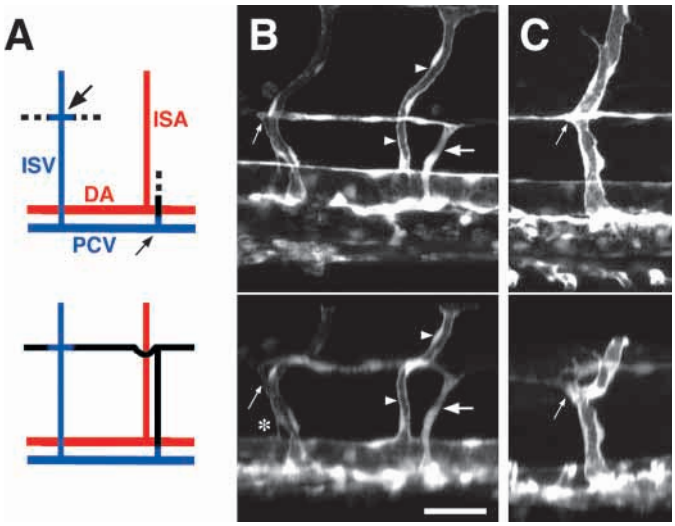

Fig. 4. Formation of the parachordal vessels. Multiphoton confocal images (B,C) show lateral (top) and ventrolateral (bottom, image tilted upwards –40 to –50 degrees relative to the top images) views of vessels on one side of the mid-trunk of *fli-egfp* transgenic zebrafish embryos at ~1.8- 2.2 dpf. (A) Parachordal vessels form from sprouts derived from both the posterior cardinal vein (small arrow) and from future intersegmental veins (large arrow). (B) Parachordal segment connected to an intersegmental vein at one end (small arrow), and connected to the posterior cardinal vein at the other end via a ventral root (large arrow) adjacent to an intersegmental artery (arrowheads). A vestigial connection to the dorsal aorta persists on the intersegmental vein (asterisk). (C) Intersegmental vein connected to the adjacent parachordal vessel (arrow). Anterior is towards the left in all panels. Scale bar: 25 μm. 3D reconstructions of these images are available at http://dir.nichd.nih.gov/lmg/uvo/ISV3_D.html

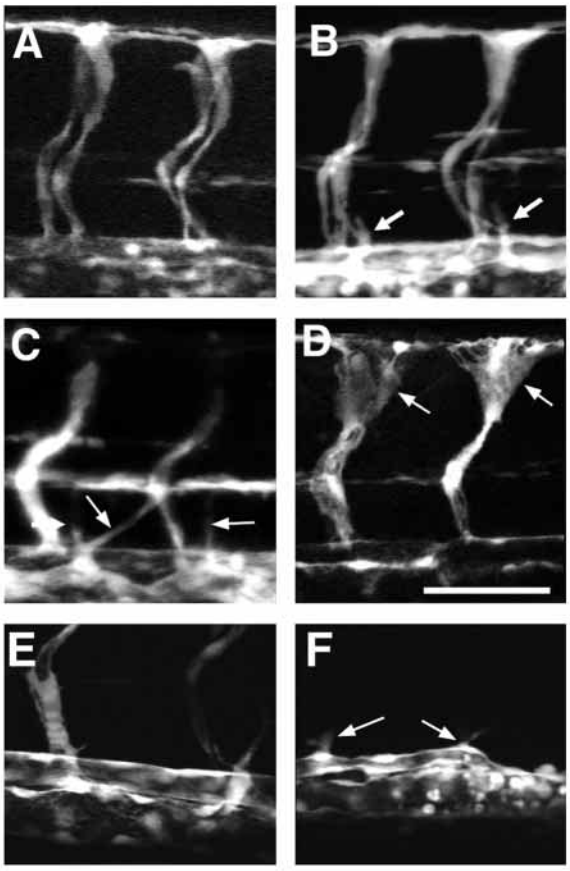

Fig. 5. Primary network formation proceeds normally in embryos that have no blood circulation. (A-D) Lateral views of the mid-trunk of *Tg(fli1:EGFP)^{y1}/Tg(fli1:EGFP)^{y1}*, *sih/sih* or *Tg(fli1:EGFP)^{y1}/Tg(fli1:EGFP)^{y1}* (E,F) embryos. (A) The primary vascular lattice appears normal at 1.5 dpf. (B) Secondary sprouts (arrows) are apparent by 2 dpf. Their appearance is also normal. (C) Secondary sprouts (arrows) contribute to the parachordal system by 2.5 dpf, as they do in wild-type embryos. (D) By ~3.5 dpf primary segments display dorsal enlargement and ramification (arrows), but lack obvious vessel lumenization. (E) Control morpholino and (F) anti-Vegf morpholino injected transgenic at 1.5 dpf. Anti-Vegf morpholino-injected animals do not form the primary angiogenic network, although initial sprouting is sometimes observed (arrows in F). Images were collected by multiphoton confocal microscopy. Anterior is towards the left in all panels. Scale bar: 50 μm. 3-D reconstructions of these images are available at http://dir.nichd.nih.gov/lmg/uvo/ISV3_D.html

This analysis revealed that while the pattern is not regular, it is also not random. There is a highly significant bias toward preserving hemodynamic balance between adjacent intersegmental arteries and intersegmental veins (see web supplement at http://dir.nichd.nih.gov/lmg/uvo/ISVdata.html, bottom of the web page). In other words, veins tend to be surrounded by arteries while arteries tend to be surrounded by veins.

To examine how early patterns of secondary sprout emergence and interconnection relate to the later AV identity of trunk intersegmental vessels, we imaged all of the trunk vessels on the left side of two different embryos (a total of 26 intersegments) at approximately 1.8-2.2 dpf, then scored the final AV identity of the same intersegmental vessels at 7 dpf. The results are diagrammed in Fig. 6, and the corresponding images are available at http://dir.nichd.nih.gov/lmg/uvo/ISVhome.html. When a secondary sprout forms a root for the parachordal system, the adjacent primary segment almost always becomes an intersegmental artery. At ~2.2 dpf a parachordal root was found adjacent to 10/13 future intersegmental arteries but only 1/13 future intersegmental veins. The presence of parachordal sprouts emanating from an intersegmental vessel at the level of the horizontal myoseptum is also strongly correlated with a venous fate for that vessel. 12/13 future intersegmental veins were connected to the parachordal system by 2.2 dpf, whereas only 1/13 future intersegmental arteries were connected at the same time point. The one exceptional intersegmental artery (noted with an asterisk in Fig. 6) possessed only a thin connection to the parachordal system at 2.2 dpf, and did have an adjacent parachordal root like other future intersegmental arteries. The results of this survey suggested that there is a more or less binary fate choice for secondary sprouts between serving as parachordal roots or connecting to the primary network, and that this is predictive of future intersegmental identity. Does this choice depend on flow dynamics?

One way for flow dynamics to determine intersegmental AV might be through transient intermediates in which developing intersegmental vessels possess patent connections to both the dorsal aorta and posterior cardinal vein, permitting preferred flow patterns to make the ‘choice’ (Fig. 7A). We examined

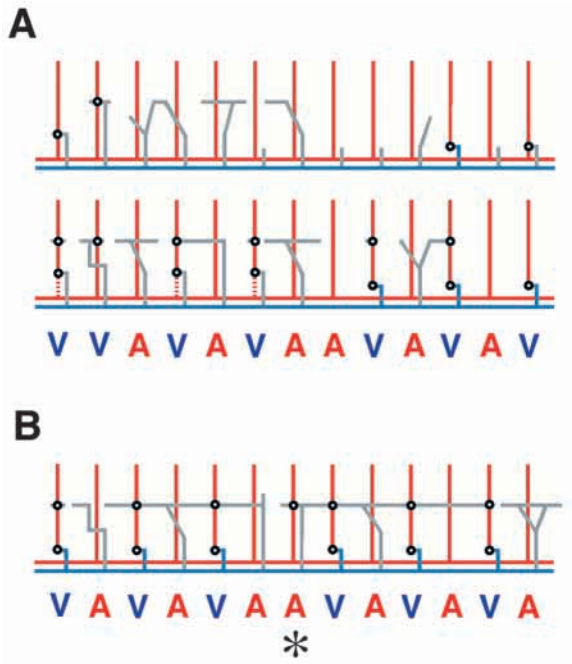

Fig. 6. Secondary sprouts contribute to either intersegmental veins or parachordal vessels. Blood vessels on the left side of the trunk were imaged in two separate *Tg(fli1:EGFP)^{y1}* animals at ~1.8 and 2.2 dpf (A) or 1.9 dpf (B) and the final AV identity of each intersegmental vessel was determined at 7 dpf. The data for trunk vascular wiring are presented schematically. Horizontal lines show the dorsal aorta (red), posterior cardinal vein (blue) and parachordal segments (gray). Vertical lines show primary segments (red), secondary segments connecting to form intersegmental veins (blue), and secondary segments forming ventral parachordal roots or whose fate has not yet been determined (gray). Connections to intersegmental vessels are noted with black rings; vessels depicted as crossing one another without a black ring are adjacent, but not connected. Asterisk notes the lone ISA that did form a connection to the parachordal system. See text for additional details.

~800 trunk intersegments in a large number of embryos between 2.0 and 2.5 dpf, when flow can be observed in most intersegments, and found that most only had a single obvious patent ventral connection. However, 25 (~3%) of the intersegments had patent connections to both the dorsal aorta and posterior cardinal vein, and blood cells could be seen transiting from the dorsal aorta into the base of the intersegmental vessel and then directly back to the posterior cardinal vein (Fig. 7; Movies 10, 11 at http://dev.biologists.org/supplemental/). In almost every case, there was no net blood flow through dorsal regions of these 25 intersegmental vessels, although they were generally patent and blood cells could be observed oscillating back and forth in response to pulsatile blood flow. The same 25 intersegments were re-examined 2 days later (at 4 dpf) to determine if and how these dual connections had resolved. In one case, the dual connection had still not resolved, and in three cases the vessel was not functioning at all. Of the other 21 intersegmentals, 11 had resolved in favor of an intersegmental artery, while 10 had resolved in favor of an intersegmental vein. This balanced outcome was in keeping with the observed balance between arterial and venous inputs from vessels surrounding these 'dual segments' (data not shown) and lack of flow through dorsal regions of these vessels, and suggests that the formation or persistence of joint connections might reflect an initial lack of a clear flow dynamic choice (see Discussion).

Discussion

We provide the first comprehensive look at how a defined, patterned network of angiogenic blood vessels takes shape and begins to function in a living vertebrate embryo. Our images of embryonic trunk vessel formation reveal a novel two-step mechanism, shown schematically in Fig. 8A. A complete artery-derived primary vascular network forms first, followed by emergence and growth of a set of secondary, vein-derived sprouts. These secondary sprouts interact dynamically with the primary network to determine both the final pattern of interconnections and final AV identity of the intersegmental vessels as well as the initial arrangement of connections to the parachordal vessel system. This two-step mechanism with initial assembly of artery-derived vascular components is interesting in light of recent work suggesting that the key proangiogenic signaling molecule *vascular endothelial growth factor* (*vegf*), in addition to its well-documented roles as an endothelial mitogen, promigratory factor and vascular permeability factor (Ferrara and Gerber, 2001), is also crucial for proper arterial differentiation and preferentially promotes the formation of arterial blood vessels (Lawson et al., 2002; Mukouyama et al., 2002; Stalmans et al., 2002; Visconti et al., 2002). In murine skin, initial artery formation adjacent to sensory nerves is driven by Vegf expression by these nerves (Mukouyama et al., 2002). The dorsal aorta-derived primary angiogenic sprouts we have described grow dorsally along the medial aspects of the somites, the major location of *vegf* expression in the developing trunk (Lawson et al., 2002; Liang et al., 1998). Cyclopamine treatment or anti-*vegf* morpholino injection, both of which reduce or eliminate somitic expression of *vegf*, result in failure to form the primary angiogenic sprouts, although the axial vessels still form (N.D.L., unpublished) (Nasevicius et al., 2000)). Zebrafish mutants affecting the Vegf receptor *flk1* are also defective for primary network formation (Habeck et al., 2002). We have recently identified an additional mutant with defects in arterial differentiation that does not form primary sprouts but does generate secondary sprouts (Lawson et al., 2003). Molecular cloning of the mutation revealed that it is a defect in a zebrafish *phospholipase C gamma* (*plcg*) (*plcg1* – Zebrafish Information Network) gene. Plcg genes are important effectors of signaling by receptor tyrosine kinases such as the *vegf* receptors, and further analysis showed that zebrafish *plcg* functions as a major downstream component of the *vegf* signaling pathway. Based on these and other results we have recently proposed that Vegf-driven vessel formation occurs via a two step-process with emergence of arterial components first followed by assembly of venous components [see Weinstein and Lawson (Weinstein and Lawson, 2002) for further discussion of the proposed two-step model].

Our studies show that circulatory flow appears to play a minimal role in the gross anatomical patterning of trunk blood vessels (i.e. the positioning of vessel tracts relative to other tissues and organs), and in the formation of the primary vessel network in particular. We used *sih* mutants to examine to what

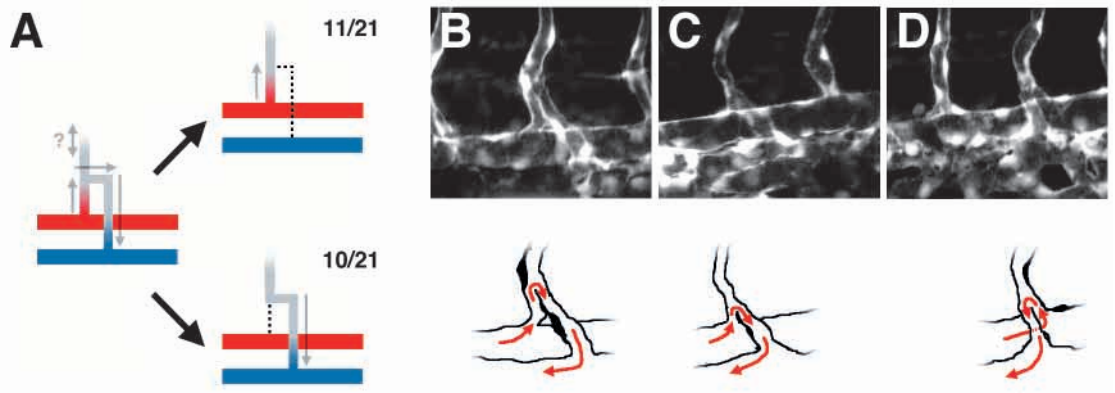

Fig. 7. Circulation through segments with functional connections to both the dorsal aorta and the posterior cardinal vein. (A) The ventral half of a primary segment connected to both the dorsal aorta (red) and the posterior cardinal vein (blue). Blood flow through the vessels is noted (gray arrows). Numbers shown are fraction of 21 vessels examined that initially (at 2 dpf) had functioning dual connections that later resolved in favor of either an intersegmental artery (top, 11/21) or an intersegmental vein (bottom, 10/21) between 2 and 4 dpf (see text for details). (B-D) Representative examples of 'dual connection' segments in which blood is flowing from dorsal aorta to posterior cardinal vein. Images shown (top) are lateral views of the trunks and tails of *fli-egfp* transgenic zebrafish embryos at approximately 2-2.2 dpf. (C,D) The vessels on opposite sides of the tail of at the same anterior-posterior position. Images were collected by multiphoton microscopy. Accompanying illustrative diagrams (bottom) show blood flow patterns through the dual-connected intersegments (flow direction is noted with red arrows). 3D reconstructions of these images are available at http://dir.nichd.nih.gov/lmg/uvo/ISV3_D.html Movies showing blood flow through these vessels are available at http://dev.biologists.org/supplemental/.

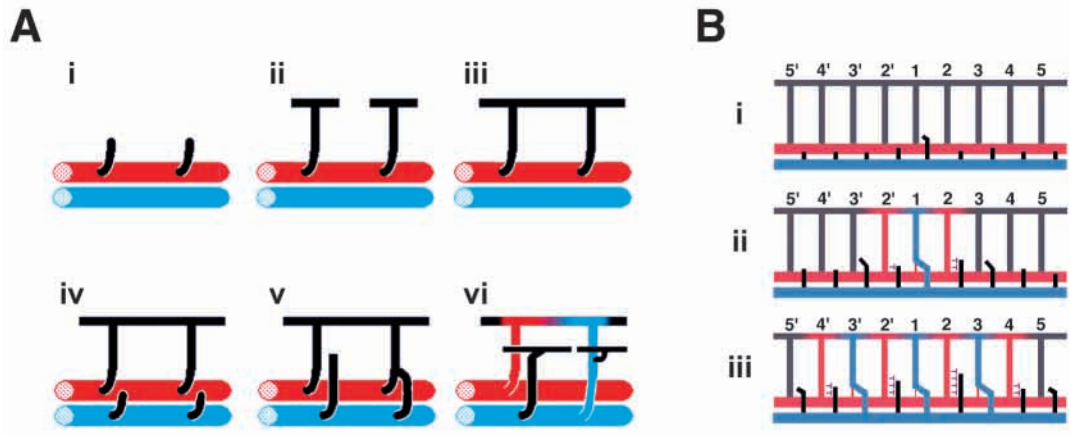

Fig. 8. (A) Steps leading to assembly of the trunk angiogenic vascular network and (B) a proposed model for determination of secondary sprout fate and intersegmental vessel AV identity. For clarity, both diagrams show the vessels on only one side of the trunk. (A, part i) Primary sprouts emerge bilaterally exclusively from the dorsal aorta (red). (A, part ii) Primary sprouts grow dorsally, branching cranially and caudally at the level of the dorsolateral roof of the neural tube. (A, part iii) Branches interconnect on either side of the trunk to form two dorsal longitudinal anastomitic vessels (DLAV). (A, part iv) Secondary sprouts begin to emerge, exclusively from the posterior cardinal vein (blue). (A, part v) Some secondary sprouts connect to the base of primary segments, while others do not. (A, part vi) Primary segments with patent connections to secondary segments become intersegmental veins (blue), while primary segments that remain connected only to the dorsal aorta become intersegmental arteries (red). Most of the secondary sprouts that do not connect to primary segments serve instead as ventral roots for the parachordal vessels. Intersegmental veins form additional connections to the parachordal vessels at the level of the horizontal myoseptum. (B) How flow dynamics might help to guide the patterning of vessel connections (see Discussion for details). Primary segments without blood flow are shown in gray, while those carrying arterial or venous blood flow are shown in red and blue, respectively. Unconnected (growing) secondary sprouts are shown in black. Flow through a primary segment inhibits connection to the segment by an adjacent secondary sprout (inhibitory cues are shown as sideways 'T' symbols).

extent flow-based cues guide trunk vessel formation and patterning. *sih* mutants lack a heartbeat and have no blood circulation, although they appear normal in most other respects (Stainier et al., 1996). Gross anatomical patterning of the early trunk vessels is relatively unaltered in *sih* mutants. Primary sprouts emerge, elongate and form bilateral lattices of vessels in mutants with morphology and kinetics similar to wild-type embryos. Secondary sprouts also emerge and contribute to parachordal vessel formation as in wild-type embryos. Previously published reports have also indicated that subjecting developing zebrafish embryos to hypoxic and hyperoxic conditions and disrupting hemoglobin transport does not appreciably alter early trunk vascular patterning (Pelster and Burggren, 1996). These results support the view we have previously put forward (Weinstein, 1999) that 'hard-wired' genetic cues play a preeminent role in the defining the overall anatomical architecture of early, major blood vessels in the trunk, and most likely other locales as well. The nature of the cues that determine the pattern of the primary angiogenic vessels of the trunk remains to be determined.

Although flow dynamics do not appear to strongly influence the gross anatomical structure of the trunk angiogenic network, they may play a crucial role in determining and/or refining the pattern of connections between vessels that allows this network to function properly. Trunk intersegmental vessel AV identity is not fixed until after secondary sprout emergence and connection. Primary segments that acquire robust connections to secondary segments become intersegmental veins, whereas

those that do not become intersegmental arteries. Although there is not a regularly alternating or reproducible distribution of intersegmental arteries and veins along the trunk, there is a strong bias toward maintaining a local balance between arterial feed and venous return in the intersegmental vessel system. The simplest explanation for how this bias could be generated is that flow dynamics determine this choice once patent connections begin to be made between primary and secondary segments and circulation begins. Based on our observations we suggest a model for determination of secondary sprout fate and intersegmental vessel AV identity based on four 'rules'. First, formation of the primary network and emergence of secondary sprouts is genetically programmed and fixed, as we have noted above. Second, secondary sprout connection to primary segments occurs stochastically. Third, a crucial caveat to the second rule is that blood flow through a primary vessel segment strongly inhibits the adjacent secondary segment from connecting to it. We have previously noted for many different developing vessels that the initiation of blood flow through a developing angiogenic vessel correlates with a dramatic reduction in its dynamic activity [see Movie 5 by Lawson and Weinstein (Lawson and Weinstein, 2002)], and we have observed the same phenomenon in the primary vascular network (this work and S.I., unpublished). Fourth, a patent vessel segment with little or no blood flow will eventually undergo regression. This has also been previously noted by other investigators in other systems and is likely to be a general feature of developing blood vessels.

Fig. 8B shows how we propose these four rules act together to generate a hemodynamically balanced intersegmental vessel network. The primary angiogenic network forms in a defined, programmed pattern, as noted above, but there is no circulation through this initial network as it has no venous return route. As it emerges, the first secondary sprout to form a patent connection to a primary segment (segment 1 in Fig. 8B) provides a venous return route for the adjacent primary segments 2 and 2′, permitting robust blood to begin flowing through all three vessels (Fig. 8B, part ii). Blood flow through 2 and 2′ prevents secondary sprouts from connecting to these segments, 'fixing' their identity as arteries. However, little or no blood flows through the more distant segments 3 and 3′ as a result of venous flow beginning in segment 1 (S.I., unpublished) so secondary sprouts are able to connect to these segments. Once this connection is made, venous blood flow begins through 3 and 3′ as a result of their proximity to 2 and 2′ (Fig. 8B, part iii), which in turn initiates arterial blood flow in segments 4 and 4′. Flow through 4 and 4′ prevents secondary sprout connection to these vessels and fixes their identity as arterial, as for 2 and 2′. Robust venous flow through segments 1, 3, and 3′ reduces blood flow through the (primary) connections these vessels still retain to the dorsal aorta, and with time these connections regress and disappear. In order to generate a strictly alternating pattern of intersegmental arteries and veins throughout the entire trunk, secondary sprout connection would have to initiate at only a single primary segment in the trunk and propagate outward from this point in a temporal wave. This violates the first rule and is contrary to our observations of actual patterns of secondary sprout emergence (data not shown). But, as we have noted, the pattern of intersegmental vessels in the zebrafish trunk is neither regularly alternating nor reproducible from animal to animal, but is biased toward balanced flow as would be the case.

This model can also account for the existence and persistence of 'dual-connection' segments (Fig. 7). As secondary vessel connection is stochastic, and does initiate at multiple points throughout the trunk, primary segments will occasionally find themselves surrounded by a relative balance between arterial and venous hemodynamic forces even after a patent connection to a secondary sprout is established. With a dual connection ventrally and no flow dorsally, blood will as a matter of course flow directly from dorsal aorta to posterior cardinal vein as shown in Fig. 7A. If both connections possess robust blood flow, neither one will regress and the dual connection will persist. This state of affairs will continue until shifts in surrounding hemodynamic forces result in initiation of robust arterial or venous flow through dorsal portions of the vessel. This will reduce or eliminate flow through one of the two ventral connections, leading to its regression and to the assumption of a definitive venous or arterial intersegmental identity. Observation of dual-connected segments supports this interpretation. Almost all of these segments lack dorsal blood flow at 2 dpf. Examination of the intersegmental vessels surrounding dual connected segments reveals that in almost every case there is a relative balance of arterial and venous blood flow (data not shown) and that these segments resolve in approximately equal numbers to form intersegmental arteries and intersegmental veins, although this resolution can take an extended period of time..

The two-step model that we have proposed has many appealing features. It allows for effective interplay between genetically programmed patterning cues and flow dynamics, ensuring that a vascular network will be both properly positioned within the context of the embryo as a whole and wired together for optimal hemodynamic function. It also provides for a remarkably self-assembling and self-correcting system that ensures venous drainage is provided for arterial blood vessels. In addition, it is potentially adaptable to many different vascular beds, as it relies upon simple and widely applicable properties of developing vessels. There is in fact ample evidence in the scientific literature to suggest that other vessels beside the initial trunk network might form by a similar two-step process during development [see Weinstein and Lawson (Weinstein and Lawson, 2002) for discussion of additional evidence for sequential assembly of arterial and venous vascular components]. With the experimental tools available in the zebrafish, further analysis of trunk vessel formation should permit the testing of the validity of this model and eventually elucidate the nature of the genetic and hemodynamic cues that direct vascular network assembly.

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