

Chemokine signaling guides regional patterning of the first embryonic artery

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The aorta traverses the body, yet little is known about how it is patterned in different anatomical locations. Here, we show that the aorta develops from genetically distinct endothelial cells originating from diverse locations within the embryo. Furthermore, chemokine (C-X-C motif) receptor 4a (*cxcr4a*) is restricted to endothelial cells derived from anterior mesoderm, and is required specifically for formation of the lateral aortae. *Cxcl12b*, a *cxcr4a* ligand, is expressed in endoderm underlying the lateral aortae, and loss of *cxcl12b* phenocopies *cxcr4a* deficiency. These studies reveal unexpected endothelial diversity within the aorta that is necessary to facilitate its regional patterning by local cues.

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The aorta is the first artery to develop in vertebrate embryos and is thought to form largely through vasculogenesis, or the de novo formation of blood vessels (Coffin and Poole 1988; Cleaver and Krieg 1999). In zebrafish embryos, the dorsal aorta (DA) forms as a single vessel in the trunk region of the embryo (Lawson and Weinstein 2002) while in more anterior regions the lateral dorsal aortae (LDA) form as paired vessels on either side of the embryo (Isogai et al. 2001). Recent results have shown that midline structures within the trunk, such as the notochord, are necessary for DA patterning during this process (Fouquet et al. 1997; Sumoy et al. 1997; Cleaver and Krieg 1998). In some cases, the midline serves as a source of angiogenic signals such as vascular endothelial growth factor (Vegf) that promote the migration of

arterial endothelial cell progenitors to the midline, where they form the DA (Cleaver and Krieg 1998). Alternatively, the notochord secretes morphogens such as Sonic hedgehog (Shh) that instruct surrounding tissues to express Vegf, which in turn promotes patterning of trunk arteries (Lawson et al. 2002, 2003). This process is facilitated, in part, by the early differentiation of arterial and venous endothelial cell fates that allow distinct cell populations to respond differentially and appropriately to diverse morphogenetic signals (Lawson et al. 2002; Covassin et al. 2006). While zebrafish embryos deficient for Shh or Vegf or lacking the notochord itself display defective DA formation, the anterior vasculature, including the LDA, appears unaffected in these cases (see Supplemental Fig. 1; Stainier et al. 1996; Fouquet et al. 1997; Sumoy et al. 1997; Nasevicius et al. 2000), suggesting the existence of distinct mechanisms that may contribute to the patterning of the aorta in other anatomical locations.

In addition to Vegf, a number of other ligands and their receptors play important roles during the development of the vascular system. Among these are chemokines, which are small (8- to 14-kDa) vertebrate-specific proteins that can be categorized into four subgroups according to the presence and position of conserved cysteine residues (C, CC, CXC, and CX3C) (Raz and Mahabaleshwar 2009). These secreted proteins were initially identified as important mediators of the immune response (Luster 1998). More recently, chemokines of the CXC class have been implicated in a wide variety of developmental processes, such as primordial germ cell migration, axon growth, lateral line formation, and endoderm development (Raz and Mahabaleshwar 2009). In these settings, chemokines regulate the migratory behavior of individual cells or groups of cells. Of interest, mice deficient for either chemokine (C-X-C motif) receptor 4a (*cxcr4*) or its ligand, *cxcl12/sdf1*, show specific defects in the gut vasculature (Nagasawa et al. 1996; Tachibana et al. 1998), suggesting a role of this ligand/receptor pair in vascular patterning.

In this study, we demonstrate that the developing aorta is comprised of several genetically distinct arterial endothelial cell populations that arise from different anatomical locations. In particular, we find that the zebrafish *cxcr4* ortholog *cxcr4a* is specifically expressed in arterial endothelial cells derived from anterior but not posterior lateral mesoderm. Embryos deficient for *cxcr4a* or its ligand, *cxcl12b*, display defects in anterior LDA formation, while DA formation in the trunk is not affected. Interestingly, *cxcl12b* is expressed in the endoderm immediately adjacent to the developing LDA, and embryos lacking endoderm fail to form an LDA. Together, our observations reveal new levels of differentiation within the developing vascular system and implicate this diversity in governing the appropriate response to local extrinsic guidance cues during blood vessel patterning.

Results and Discussion

To better understand the mechanisms that contribute to the formation of the anterior aorta, we performed two-photon time-lapse analysis of *Tg(kdrl:egfp)^{ja116}* embryos in which endothelial cells are labeled with enhanced green fluorescent protein (*egfp*) (Choi et al. 2007). These movies revealed that formation of the LDA occurs in

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a bidirectional manner: Starting at 14 h post-fertilization (hpf), we observed endothelial cells from the anterior lateral plate mesoderm migrating posteriorly (Fig. 1A, anterior LDA, cells labeled in red), while at the same time, endothelial cells from posterior regions of the embryo started to migrate anteriorly (Fig. 1A, posterior LDA, cells labeled in green). These cells continued to migrate toward each other, finally fusing to form a continuous vessel by 20.5 hpf (Fig. 1B-F; see also Supplemental Movies 1, 2). At ~17 hpf, venous endothelial cells also started to sprout parallel to the LDA in a similar bidirectional manner, completing the formation of the major head vein, called the primordial hindbrain channel (PHBC), by 24 hpf (Fig. 1C-F, arrowheads, PHBC labeled in blue). While the LDAs formed via angiogenic sprouting, the single axial DA in the embryonic trunk formed via the medial migration of endothelial cells from the adjacent lateral plate mesoderm, connecting to the LDAs to form a Y-shaped structure (Supplemental Figure S2A-F; Supplemental Movies 3, 4). Thus, based on these observations, we can delineate at least three different endothelial cell populations that contribute to the primitive aorta: (1) cells from the anterior lateral mesoderm that contribute to the anterior LDA, (2) cells from the posterior lateral mesoderm that contribute to the posterior LDA, and (3) posterior lateral mesoderm endothelial cells that migrate medially to form the DA (see also Figs. 1F, 2I).

We reasoned that these different migratory behaviors might correspond to genetic differences within aortic endothelial cells. To determine if this was the case, we searched an online expression pattern database available through the Zebrafish Information Network (<http://www.zfin.org>) for genes expressed specifically in arterial endothelial cells. We subsequently performed whole-mount in situ hybridization with candidate genes at different embryonic stages to more closely monitor their expression pattern within the developing aorta. We found several genes that were expressed exclusively in individual domains delineated by our time-lapse studies. We observed that *cxcr4a* displays strikingly specific expression in arterial endothelial cells within the anterior, but not posterior, lateral mesoderm starting as early as the

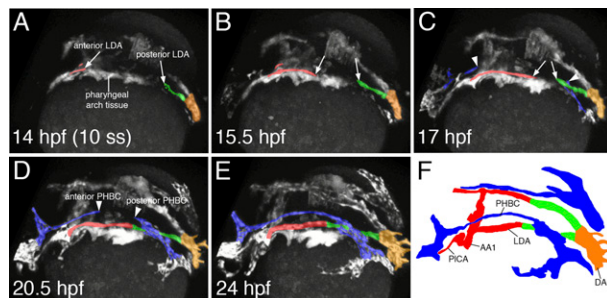


Figure 1. Two-photon time lapse of LDA formation in live zebrafish embryos. (A-F) Dorsolateral view of LDA formation. Anterior LDA is pseudocolored in red, posterior LDA is shown in green, axial DA is indicated by orange, and the PHBC is shown in blue. Arrows mark migrating cells of the LDA, which forms in a bidirectional manner. Arrowheads indicate similarly migrating PHBC cells. (F) Labeled camera lucida image indicating position of vessels in E. (DA) Dorsal aorta; (LDA) lateral dorsal aorta; (PHBC) primordial hindbrain channel; (PICA) primitive internal carotid artery; (AA1) aortic arch 1.

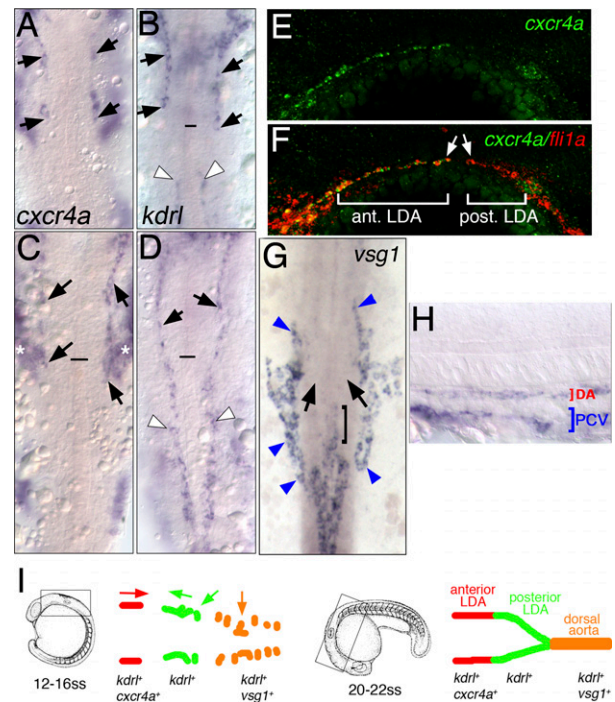


Figure 2. LDA progenitors are genetically distinct. (A-H) In situ hybridization with indicated markers. (A-D, G) Dorsal view, anterior is up. (E, F, H) Lateral view, anterior to the left, dorsal is up. (A-D) Expression of *cxcr4a* (A, C) and *kdrl* (B, D) at the 18-somite stage and the 22-somite stage, respectively. Anterior LDA (black arrows) and posterior LDA (white arrowheads) are indicated. Black line marks the anterior end of the notochord. Asterisks in C mark expression of *cxcr4a* in pharyngeal arch tissue. (E, F) Confocal images of 22-somite-stage embryos following double-fluorescent in situ hybridization. Endothelial cells are labeled by expression of *fli1a* in red and *cxcr4a* in green. (G, H) Expression of *vsg1* at the 22-somite stage. (G) Dorsal view. (H) Lateral view. (G) Black arrows mark posterior LDA, which does not express *vsg1*. Bracket marks anterior end of DA. Blue arrowheads mark venous cells. (I) Schematic drawing of DA domains delineated by different migratory behaviors and gene expression profiles at the 18-somite stage and the 22-somite stage.

12-somite stage (15 hpf) (Supplemental Fig. S3). In contrast, the duplicate *cxcr4b* gene does not appear to be expressed in endothelial cells (ZDB-PUB-010810-1, <http://www.zfin.org>). During the convergent migration of the anterior and posterior LDA branches (17–18 hpf), *cxcr4a* expression can be observed exclusively in the anterior endothelial cell population (Fig. 2A; Supplemental Fig. S3), while the zebrafish *vegf receptor-2* ortholog *kdrl* is expressed in both anterior and posterior branches (Fig. 2B). We did not detect *cxcr4a* expression in venous endothelial cells at this stage. Expression of *cxcr4a* is similarly restricted to the anterior domain of the LDA at the 22-somite stage (Fig. 2C; Supplemental Fig. S3), at which time these blood vessels have fused and are labeled throughout by *kdrl* (Fig. 2D). By 24 hpf, *cxcr4a* expression is apparent throughout the LDA and in the axial DA within the trunk (Supplemental Fig. S3). More detailed analysis using two-color fluorescence in situ hybridization demonstrated coexpression of *cxcr4a* and the pan-endothelial marker *fli1a* in the anterior, but not posterior, domains of the LDA (Fig. 2E, F). In contrast, we observed expression of *vsg1*, a zebrafish ortholog of *plasmalemma vesicle-associated protein (plvap)*, in the

DA, but not in the branches of the LDA at the 18-somite stage and the 22-somite stage (Fig. 2G,H); *vsg1* expression was also observed in venous endothelial cells at this stage (Fig. 2G,H). Together with our time-lapse analyses, these results demonstrate that the aorta is comprised of genetically distinct arterial endothelial cells that arise from different anatomical origins (Fig. 2I).

Chemokine signaling is important for directing cell migration in a variety of tissue types (Raz and Mahabaleshwar 2009). Therefore, we reasoned that *cxcr4a* might play a crucial role in guiding the migration of arterial endothelial cells during formation of the LDA. To determine if this was the case, we used zinc finger nucleases (ZFNs) to introduce a targeted deletion within exon 2 of *cxcr4a* (Fig. 3A). We constructed individual ZFN monomers by modular assembly (see the Supplemental Material) and introduced them together into zebrafish embryos via mRNA coinjection at the one-cell stage. We subsequently identified mosaic founders by assaying for loss of a DdeI restriction site within the target sequence (Fig. 3A,B) in progeny embryos. F2 families of heterozygous carriers derived from initial founders were used to generate *cxcr4a* mutant embryos. The *um20* allele used for subsequent analysis causes an in-frame 5-amino-acid deletion within the second transmembrane domain of the Cxcr4a receptor that eliminates its membrane localization (Fig. 3B; Supplemental Fig. S4). Compared with their wild-type siblings, *cxcr4a^{um20}* mutants displayed mild cardiac edema associated with a delay in the onset of circulation between 24 and 32 hpf (Fig. 3C,F; data not shown), while their general morphology was otherwise indistinguishable (Fig. 3C,F; Supplemental Fig. S5). By day 2 of development, we observed that approximately half of *cxcr4a^{um20}* mutant embryos failed to exhibit active circulation (Supplemental Table 1). We further observed endoderm defects, which have been reported in zebrafish embryos injected with a morpholino targeting the endogenous *cxcr4a* start codon (Mizoguchi et al. 2008; Nair and Schilling 2008). In particular, we noted precocious endoderm migration during gastrulation and ectopic clusters of *insulin*-positive pancreatic islet cells at day 2 of development (Supplemental Fig. S6). As with the observed circulatory defects, abnormal islet cell localization was partially penetrant (Supplemental Table 1). Importantly, the partially penetrant circulatory and pancreas phenotypes could be observed independently in some *cxcr4a^{um20}* mutant embryos (Supplemental Table 1), suggesting an autonomous role for *cxcr4a* in each tissue type.

To determine the primary vascular defects caused by loss of *cxcr4a* function, we observed blood vessel morphology in wild-type and *cxcr4a*-deficient embryos bearing the *Tg(kdrl:egfp)^{Ja116}* transgene. At 32 hpf, wild-type siblings displayed completely formed LDA and PHBC, as well as a normal vascular network in the trunk (Fig. 3D,E). In contrast, we observed incomplete formation of the LDA in *cxcr4a^{um20}* mutant embryos (Fig. 3G) or embryos injected with a *cxcr4a* morpholino (Supplemental Fig. S7; Supplemental Tables 2, 3). Formation of the PHBC and the major trunk vessels was normal in *cxcr4a*-deficient embryos at this developmental stage (Fig. 3G,H; Supplemental Fig. S7). As mentioned above, *cxcr4a* deficiency causes endoderm defects that may affect LDA development. However, transplantation of wild-type endoderm into *cxcr4a^{um20}* mutant embryos failed to rescue LDA formation (Supplemental Fig. S8; Supplemental

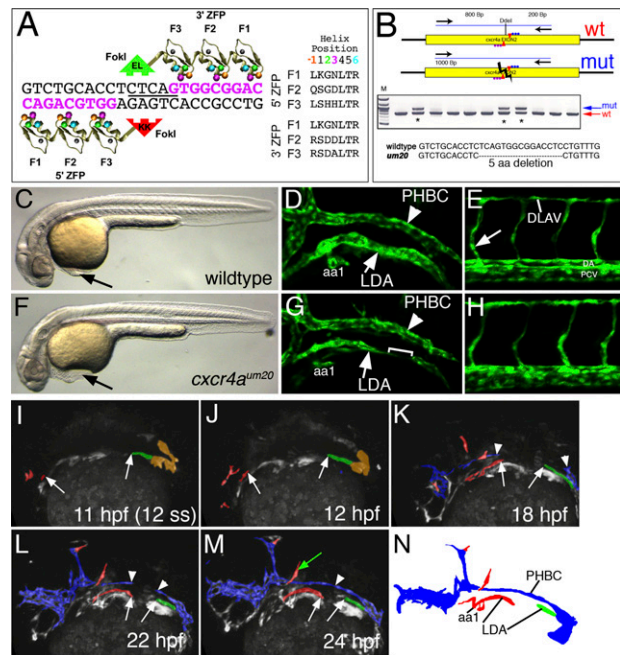


Figure 3. LDA formation defects in *cxcr4a*-deficient embryos. (A) Schematic depicting ZFN monomers designed against *cxcr4a* exon 2 target site. Nucleotides in pink represent the recognition elements. The DdeI site within the spacer region is underlined. Recognition helices for each finger are indicated. (B) PCR genotyping analysis of embryos derived from putative founder fish. Failure to digest with DdeI is indicative of a possible target site deletion. An asterisk marks genotypes that bear mutagenic lesions. (C,F) Transmitted light images of wild-type (C) and *cxcr4a* mutant embryos (F) at the 32-hpf stage. Lateral views, dorsal is up, anterior is to the left. (D,E,G,H) Confocal images showing anterior regions (D,G) or posterior regions (E,H) of wild-type (D,E) or *cxcr4a* mutant (G,H) embryos. (LDA) Lateral dorsal aorta; (PHBC) primordial hindbrain channel; (aa1) aortic arch1. Bracket in G indicates a gap within LDA. (DLAV) Dorsal longitudinal anastomotic vessel; (DA) dorsal aorta; (PCV) posterior cardinal vein. The arrow in E indicates the intersegmental blood vessel sprout. (I–M) Stills of two-photon time-lapse (see Supplemental Movie 5) showing LDA formation in *cxcr4a^{um20}* mutant embryos. Lateral views, anterior is to the left. Time points are indicated. Red labels arterial LDA cells, green indicates posterior LDA cells, and blue labels venous endothelial cells. Arrows mark anterior and posterior LDA, while arrowheads mark the forming PHBC. The green arrow in M marks ectopically located endothelial cells. (N) Camera lucida drawing of embryo in M.

Table 5), indicating that the observed vascular defects were not a result of abnormal endoderm patterning.

To further investigate defects in LDA formation, we performed two-photon time-lapse analysis of *Tg(kdrl:egfp)^{Ja116};cxcr4a^{um20}* mutant embryos. Between 14 and 24 hpf, we observed that initial sprouts of the anterior and posterior origins of the LDA formed normally, but failed to connect as in wild-type embryos (Fig. 3I–N; Supplemental Movie 5). Time-lapse movies of *cxcr4a* mutant embryos up to 36 hpf revealed failure to connect the branches of the LDA, suggesting that this defect was not due to developmental delay (Supplemental Movie 6). We noted similar LDA defects in time-lapse movies of *cxcr4a* morpholino-injected embryos (Supplemental Movie 7). In all cases, formation of the PHBC and trunk blood vessels was unaffected by the loss of *cxcr4a* function. Interestingly, we observed ectopic migration of arterial endothelial

cells and inappropriate arteriovenous connections in embryos lacking *cxcr4a* function (Supplemental Movie 5; Fig. 3M, green arrow; Supplemental Movie 6, respectively). This cell behavior was not found in wild-type embryos. Taken together, our observations indicate that *cxcr4a* is specifically required to guide a subset of arterial endothelial cells during formation of the anterior LDA.

The zebrafish genome encodes two duplicate *Cxcr4* ligands, referred to as *cxcl12a* and *cxcl12b*. While *cxcl12a* acts primarily through *cxcr4b* to promote sensory axon pathfinding and germ cell migration (Doitsidou et al. 2002; Miyasaka et al. 2007), *cxcl12b* and *cxcr4a* are required for endoderm formation (Mizoguchi et al. 2008; Nair and Schilling 2008). Given the importance of *cxcr4a* for LDA formation, we asked if *cxcl12b* might be involved in LDA patterning. In order to address this question, we first analyzed the expression pattern of *cxcl12b* during LDA formation. Whole-mount in situ hybridization revealed that *cxcl12b* is expressed in tissue underlying the arterial endothelial cells that will comprise the LDA before and after their migration (Fig. 4A,C–E; Supplemental Fig. S9). This expression was severely reduced in embryos injected with a morpholino targeting *sox32* that lack the endoderm (Fig. 4B; Chung and Stainier 2008), but was normal in *cxcr4a^{um20}* mutant embryos (data not shown). We next addressed the effect of loss of either *cxcl12b* or the endoderm itself on vascular func-

tion and LDA formation. In comparison with control morpholino-injected embryos (Fig. 4F–H), embryos injected with a morpholino targeting *cxcl12b* displayed complete loss of circulation, or slow circulation associated with pericardial edema (Fig. 4F,I, arrow; Supplemental Table 4), although heart morphology appeared grossly normal (data not shown). Similar to *cxcr4a^{um20}* mutant embryos, *cxcl12b*-deficient embryos also displayed defects in LDA formation (Fig. 4J; Supplemental Movie 8; Supplemental Fig. S7; Supplemental Tables 2, 3). In contrast, embryos injected with a morpholino targeting the closely related *cxcl12a* paralog displayed normal circulation and LDA formation (Supplemental Tables 2, 3; data not shown). The effect on LDA formation was even more pronounced in embryos injected with *sox32* morpholino (Fig. 4F,K,L; Supplemental Fig. S7). Here, both anterior and posterior branches of the LDA failed to migrate (Fig. 4L), and injected embryos never established circulation (Fig. 4F; Supplemental Movie 9; Supplemental Fig. S7). These observations indicate that endoderm-derived *cxcl12b* serves as an important guidance cue to direct migration of *cxcr4a*-positive arterial endothelial cells during formation of the LDA.

Proper assembly of a mature circulatory system is essential for cardiovascular function during embryonic development and postnatal life. An important step in this process is the appropriate response of endothelial cells to extrinsic guidance and growth factor cues in the developing embryo. In our present study, we show that aorta development entails coordinate migration of distinct populations of arterial endothelial progenitors. We provide further evidence that this process is facilitated by an unexpected molecular diversity within these cells. In this case, restricted *cxcr4a* expression directs a subset of arterial endothelial cells from the anterior lateral mesoderm to follow an endoderm-derived chemokine that guides formation of the LDA. Although defects in LDA formation have not been described in mouse embryos deficient for either *cxcr4* or *cxcl12b*, defects in other endoderm-associated blood vessels, such as those in the gut, are evident (Nagasawa et al. 1996; Tachibana et al. 1998), suggesting a possible conserved role for this pathway. Furthermore, mouse embryos with deficient endodermal differentiation display specific defects in anterior DA patterning (Sakamoto et al. 2007), while posterior aorta development is not affected, suggesting that genetic differences between anterior and posterior regions of the LDA might also exist in mice.

Interestingly, *cxcr4a* and *cxcl12b* are dispensable for trunk blood vessel formation in zebrafish, which instead is highly dependent on *Vegf* signaling (Lawson et al. 2003). In this setting, different *Vegf* receptors are restricted to distinct endothelial cell populations and are specifically required for trunk artery patterning (Covassin et al. 2006; Siekmann and Lawson 2007), analogous to the role for *cxcr4a* in patterning the anterior vasculature. Similarly, PHBC development proceeds normally in the absence of endoderm or *cxcr4a* and instead requires *Vegfc* signaling through *Flt4*, which is largely restricted to venous endothelial cells (Covassin et al. 2006). Thus, endothelial cell differentiation appears to play an essential role in vascular morphogenesis by establishing the appropriate complement of signaling molecules that enable some, but not all, cells to respond to a particular extrinsic factor. This differentiation process enables the

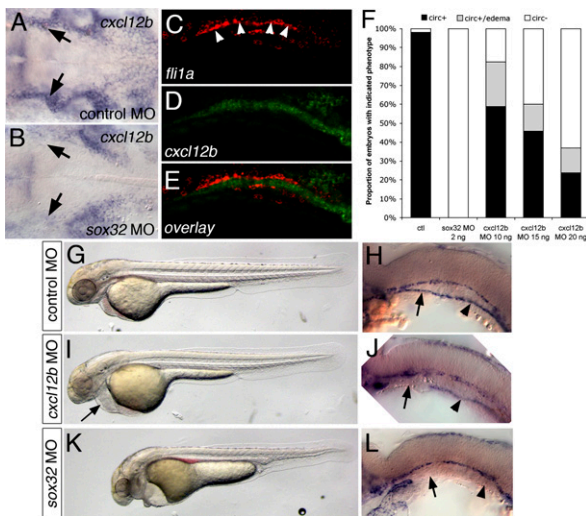


Figure 4. Endoderm-derived *cxcl12b* is required for LDA formation. (A,B) In situ hybridization at 24 hpf showing *cxcl12b* expression in tissue underlying the LDA (arrows). (B) Loss of endoderm in *sox32* morpholino-injected embryos leads to severe reduction in *cxcl12b* expression. Dorsal view, anterior is to the left. (C–E) Confocal micrographs of 22-hpf embryo subjected to double-fluorescent whole-mount in situ hybridization, lateral view, anterior is to the left, dorsal is up. (C) *fli1a* expression, marking the LDA. (D) *cxcl12b* expression. (E) Overlay; *fli1a* is red, *cxcl12b* is green. (F) Influence of loss of endoderm or *cxcl12b* on circulation. (ctl) Control morpholino-injected; (circ⁺) embryos with circulation; (circ⁻) embryos without circulation. (G–L) Vascular defects in embryos lacking endoderm or *cxcl12b*. (G,I,K) Bright-field views at 32 hpf, anterior is to the left, dorsal is up. (H,J,L) In situ hybridization for *kdrl* at 24 hpf. Black arrows indicate anterior LDA, arrowheads indicate posterior LDA. (G,H) Embryos injected with control morpholino. (I,L) Embryos injected with 15 ng of *cxcl12b* MO. (K,L) Embryos injected with 2 ng of *sox32* morpholino. Arrows mark the anterior LDA, arrowheads mark the posterior LDA.

coordinated migration, coalescence, and sprouting of endothelial cells throughout the diverse extracellular milieu in distinct anatomical locations within the embryo. Ultimately, the intimate connection between these two processes—differentiation and morphogenesis—enables the formation of an appropriately patterned and patent vascular system that can bear circulatory flow.

Materials and methods

Zebrafish strains

Zebrafish were maintained as described previously (Westerfield 1993). The establishment and characterization of the *Tg(kdr:egfp)^{la116}* transgenic line have been described elsewhere (Choi et al. 2007). The *ntl^{b160}* mutant zebrafish (Halpern et al. 1993) were obtained from Erez Raz.

Generation of *cxcr4a^{um20}* mutant zebrafish

ZFNs specific for exon2 of *cxcr4a* were designed as described in the Supplemental Material. Generation and identification of mutants were essentially carried out as described previously (Meng et al. 2008). Primers for genotyping were *cxcr4a* FWD1, 5'-CCAACTTTGAGGTCCCGTGTG ATG-3'; and *cxcr4a* REV1, 5'-CTGTGGACACGGATGACATTCCTG-3'. PCR products were digested using DdeI. We identified several founder fish that carried different genomic lesions. From one of these, we established the *cxcr4a^{um20}* line, containing a 5-amino-acid in-frame deletion from position 87 to 91 in the *cxcr4a* ORF.

Time-lapse, two-photon, and confocal microscopy

Confocal microscopy of wild-type and *cxcr4a^{um20}* embryos was carried out as described previously (Covassin et al. 2006). Time lapses were essentially carried out as described (Lawson and Weinstein 2002); however, we used a two-photon microscope to reduce photobleaching. Live embryos were mounted in low-melting-point agarose in tissue culture dishes at the 10-somite stage and imaged with Bio-Rad Radiance MP2100 two-photon microscope, consisting of a Spectra Physics laser system (MillenniaV, 5-W, 532-nm diode-pumped laser, which pumps a mode locked Tsunami Ti:sapphire laser). We used a BX50WI Olympus upright microscope with an Olympus XLUMPlanFl 20×, NA 0.95 water-dipping objective. Both the objective and the sample stage were temperature-controlled to 28°C. The two-photon excitation wavelength used in these experiments was 830 nm, and an eGFP/Red emission filter cube from Chroma technology was used giving an observation bandpass of 485–535 nm. To prevent movement of the embryo, medium was supplemented with 0.1% Tricaine. Pigmentation of the embryos was inhibited by adding Phenylthiourea to a final concentration of 0.003%. Confocal stacks and two-photon movies were assembled using Imaris Software (Bitplane). Movies were labeled using Flash Player (Macromedia).

Morpholinos and whole-mount in situ hybridization

Morpholinos for *cxcr4a* (MORPH 1667, 5'-AGACGATGTTCGTAAT AAGCCAT-3'), *cxcl12b* (MORPH 1736, 5'-CGCTACTACTTTGCTA TCCATGCC-3'), *cxcl12a* (MORPH 1176, 5'-ACTTTGAGATCCATG TTTGGCAGTG-3'), and *sox32* (MORPH 0008, 5'-CGGTCGAGAT ACATGCTGTTTTGCG-3') were initially obtained from Open Biosystems. Sequence-identical morpholinos were then reordered from GeneTools. All morpholinos were dissolved in H₂O. The morpholinos for *cxcr4a* and *sox32* have been published previously (Hollway et al. 2007; Chung and Stainier 2008). Morpholinos for *cxcl12a* and *cxcl12b* differ in one nucleotide from previously published morpholinos (Miyasaka et al. 2007). As a control, we injected standard control morpholinos (5'-CCTC TTACCTCAGTTACAATTTATA-3') into sibling embryos. Whole-mount in situ hybridization and imaging for *kdr* were carried out as described (Lawson et al. 2002). A plasmid with zebrafish *cxcr4a* was kindly provided by Beth Roman (University of Pittsburgh). The probe for *cxcl12b* was provided by Nobuhiko Miyasaka (RIKEN Brain Science Institute, Saitama) (Miyasaka et al. 2007). To generate the probe for *vsg1*, we obtained an EST

for *vsg1* from Open Biosystems (NAA06F04), digested it with SmaI, and used Sp6 to generate the riboprobe. Double-fluorescent in situ hybridization was carried out as described previously (Julich et al. 2005). The probe for *fli1* was described previously (Lawson et al. 2001).

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