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## Arterial/Venous Segregation by Selective Cell Sprouting: An Alternative Mode of Blood Vessel Formation\*

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### Abstract

Blood vessels form de novo (vasculogenesis) or upon sprouting of capillaries from pre-existing vessels (angiogenesis). Using high resolution imaging of zebrafish vascular development we discovered a third mode of blood vessel formation whereby the first embryonic artery and vein, two unconnected blood vessels, arise from a common precursor vessel. The first embryonic vein formed by selective sprouting of progenitor cells from the precursor vessel, followed by vessel segregation. These processes were regulated by the ligand EphrinB2 and its receptor EphB4, which are expressed in arterial-fated and venous-fated progenitors, respectively, and interact to orient the direction of progenitor migration. Thus, directional control of progenitor migration drives arterial/venous segregation and generation of separate parallel vessels from a single precursor vessel, a process essential for vascular development.

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During early stages of vertebrate embryogenesis, coordinated sorting and segregation of arterial/venous-fated angioblasts into distinct networks of arteries and veins is essential to establish a functional vasculature. Recent studies in mouse and zebrafish have elucidated key roles for a number of signaling pathways and transcriptional regulators in arterial/venous specification (1, 2). However, we still lack a basic mechanistic understanding of how

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Materials and Methods

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mixed populations of specified arterial/venous cells coordinate their behavior to segregate and form distinct vessels.

During zebrafish vascular development, angioblasts migrate from the lateral plate mesoderm (LPM) to the midline (3, 4) and eventually give rise to the first embryonic artery (dorsal aorta; DA) and vein (caudal vein; CV). Notochord-derived Sonic Hedgehog, which induces the expression of vascular endothelial growth factor a (*vegfa*) in the ventral somites, is essential for angioblast differentiation (5). *Vegfa*-induced activation of Notch signaling (5, 6), as well as other factors (2, 7-9), subsequently promotes arterial specification in a subset of angioblasts (4), prior to arterial/venous segregation. To investigate mechanisms of arterial/venous angioblast sorting and segregation, we analyzed vascular development with high temporal resolution in *Tg(kdr1:GFP)<sup>sd43</sup>* (4) embryos. GFP-positive angioblasts coalesced at the midline to form a single vascular cord by the 21-somite stage (19.5 hours post-fertilization; hpf; Fig. 1A, B; fig. S1). Subsequent cord remodeling led to the formation of a lumenized DA primordium by 22hpf. From 21-23hpf, a subpopulation of angioblasts sprouted ventrally from the DA primordium and connected (anastomosed) with adjacent cells to form the CV primordium by 24hpf (Fig. 1A-C; figs. S1, S2, S4; movies S1, S2). Hence, the first artery and vein share a common vessel primordium. Dorsal intersegmental vessel (ISV) sprouting occurred at regular intervals along the DA and was initiated later than ventral sprouting (23hpf; Fig. 1B), suggesting that distinct mechanisms govern dorsal versus ventral sprouting behaviors. Thus, the DA forms by classical vasculogenesis whereas formation of the CV involves an alternative mechanism whereby selective sprouting of venous-fated angioblasts, subsequent sprout termination and cell-cell segregation allows distinct arterial and venous vessels to form. Hence, unlike angiogenesis in which new capillaries form a continuous network with the original vessel, two unconnected blood vessels can derive from a common group of cells.

Differences between DA and CV tube formation were further highlighted by contrasting modes of lumen formation. DA lumen formation involved hollowing of the vascular cord (10), whereas a functional CV lumen was generated differently (Fig. 1A, D, E; figs. S1, S2, S5, S6; movies S3, S4). Utilizing *Tg(gata1:dsRed)<sup>sd2</sup>* embryos that express dsRed in erythrocytes (11) and selective plane illumination microscopy (12), we found that initial hollowing of the CV primordium was followed by rapid luminal expansion upon the invasion of erythrocytes positioned ventral to the DA (fig. S6; movies S3, S4). Shortly thereafter, the CV lumen was cleared upon the flow-dependent displacement of erythrocytes, a process that failed to occur in *cardiac troponin t2 (tnnt2)* morpholino oligonucleotide (MO)-injected embryos, which lack circulation (13, Fig. 1E).

We hypothesized that arterial/venous segregation and generation of two separate vessels might be achieved by selective incorporation of venous-fated angioblasts into ventral sprouts whilst limiting the ventral migratory behavior of arterial-fated angioblasts. *Vegfa* was recently shown to differentially regulate angioblast sprouting behavior by promoting the expression of the Notch ligand *delta-like 4 (dll4)* (14-16). Expression of Dll4 in ISV tip cells activates Notch signaling in adjacent cells, thereby limiting their dorsal sprouting behavior. Injection of embryos with *vegfa* MO revealed that *Vegfa* signaling also limits the ventral migratory behavior of angioblasts (Fig. 2; table S1; fig. S7; movies S5, S6). In *vegfa* MO-injected embryos, angioblasts initially coalesced to form a vascular cord but then migrated ventrally excessively, failed to segregate and ultimately generated a single vascular tube encircling *Tg(gata1:dsRed)<sup>sd2</sup>*-positive erythrocytes. Hence, *Vegfa*-mediated arterial specification is required for the control and termination of angioblast ventral migration and maintenance of an intact DA. A similar phenotype was observed upon injection of MO to *plcγ1*, which encodes a key downstream component of *Vegfa* signaling (6), or upon inhibition of *Vegfa* receptor activation using SU5416 (Fig. 2; table S1; fig. S7; movies S5,

S7). ISV sprouting was also disrupted in *Vegfa* deficient embryos, consistent with opposing roles for *Vegfa* signaling in the induction of dorsal sprouting (6) and restraint of ventral sprouting. *Vegfa* influences dorsal sprouting behavior by indirectly activating Notch signaling (14-16). Accordingly, DAPT-mediated inhibition of Notch signaling promoted excessive ventral sprouting (Fig. 2B). Moreover, excessive ventral migration was also observed upon injection of MO to *gridlock/hey2*, which encodes a key transcriptional effector of Notch signaling (7). Thus, termination of ventral sprouting is regulated by genes downstream of *Vegfa* and Notch signaling.

In contrast, vein morphogenesis was strikingly disrupted by the broad range phosphoinositide 3-kinase (PI3K) inhibitor, LY294002 (Fig. 2B). Although LY294002 treatment did not affect total angioblast numbers, suggesting no proliferation defects (table S2), ventral sprouting was disrupted and significantly more cells were retained in the DA (Fig. 3A-C), consistent with a role for PI3K in angioblast ventral sprouting (note S1). Cell migration during angiogenic sprouting selectively requires signaling via the p110 $\alpha$  isoform of PI3K downstream of Vegf receptor activation (17). Consequently, we tested the hypothesis that a similar p110 $\alpha$ -dependent mechanism may control ventral sprouting and CV formation. We interrogated the group-I PI3K family (p110 $\alpha$ , p110 $\beta$ , p110 $\delta$  and p110 $\gamma$ ) with isoform-selective inhibitors (18, 19, table S3) to avoid the early developmental defects and compensatory interactions associated with genetically silencing individual isoforms (18, 20, 21). Consistent with a role for p110 $\alpha$ , exposure of embryos to inhibitors at concentrations that inhibit p110 $\alpha$  blocked vein morphogenesis (fig. S12; note S2). Application of LY294002 or the isoform selective inhibitor, AS605240, at concentrations that blocked ventral sprouting also disrupted dorsal sprouting (Fig. 3A-C), indicating a general requirement for PI3K in arterial/venous sprouting.

A common role for p110 $\alpha$  in dorsal/ventral angioblast sprouting indicated that similar signaling pathways regulate these processes. *Vegfa* promotes dorsal ISV sprouting upon activation of its receptor, Flk1, and downstream p110 $\alpha$ -mediated signaling (17). Restricted venous expression of the related *Vegfc* receptor gene, *flt4* (fig. S8), suggested a role for p110 $\alpha$  in vein morphogenesis downstream of Flt4-mediated signaling.

Although angioblast ventral sprouting and migration was significantly delayed in *vegfc* or *flt4* MO-injected embryos (fig. S13; table S4), possible functional redundancy with other Vegf signaling components, or compensation by alternate mechanisms, enabled vein formation at later time points. However, a role for Flt4 signaling in angioblast ventral sprouting was confirmed upon transplantation of cells from *Tg(kdrl:GFP)<sup>s843</sup>* donors into non-transgenic wild-type hosts. Significantly fewer *flt4* MO-injected donor-derived cells contributed to the CV than controls (Fig. 3D, E; table S5). Furthermore, the excessive contribution of *plcy1* MO-injected donor-derived cells to the CV was blocked upon *flt4* MO-co-injection. Hence, *Vegfa*/Flk1 and *Vegfc*/Flt4 signaling regulate multidirectional arterial/venous sprouting. However, *vegfc* is not expressed ventral to the DA but in the DA itself (fig. S14), indicating that other mechanisms must orient the direction of venous angioblast migration ventrally.

The EphB4 receptor tyrosine kinase and its plasma-membrane-spanning ligand, EphrinB2 (EfnB2), demarcate venous and arterial domains, respectively (22). Studies of EphB4- and EfnB2-null mice have revealed roles for forward and reverse EphB4-EfnB2 signaling in vascular morphogenesis (22-24); however, the exact function and mode of action of bidirectional EphB4-EfnB2 signaling remain unknown (1). Observations that zebrafish *Efnb2a* expression is restricted to the dorsal-most cells of the vascular cord (4, fig. S15) and is tightly regulated by *Vegfa* (figs. S11, S16), as well as Notch signaling (5-7) led us to hypothesize a role for Ephb4a/Efnb2a in the directional control of angioblast migration.

Consistent with this hypothesis, the generation of distinct arterial and venous vessels was disrupted, and the PI3K-dependent ventral migration of angioblasts deregulated, in *efnb2a* or *ephb4a* MO-injected embryos (Fig. 4A, B; fig. S17; table S6). In contrast to *vegfa* MO-injected embryos, *efnb2a* and *ephb4a* MO-injected embryos still formed ISVs (Figs. 2A, 4A, B), indicating that angioblasts migrate both dorsally and ventrally but fail to segregate. To elucidate the distinct functions of Efnb2a and Ephb4a in arterial/venous segregation, we transplanted cells from *Tg(kdrl:GFP)<sup>8843</sup>* donors into non-transgenic wild-type hosts. *efnb2a* MO-injected donor-derived cells contributed to the CV at over twice the frequency of controls (Fig. 4C; table S6; figs. S18, S19). In contrast, significantly fewer *ephb4a* MO-injected donor-derived cells contributed to the CV compared to controls. Thus, Efnb2a limits the ventral migration of arterial angioblasts, whereas Ephb4a promotes the ventral migration of venous angioblasts, suggesting a role for bidirectional Ephb4a-Efnb2a-mediated repulsion between arterial and venous cells in the directional control of angioblast sprouting (fig. S19). Furthermore, donor cells over-expressing *efnb2a* or *ephb4a* preferentially contributed to ISVs, consistent with increased repulsion of arterial angioblasts away from *ephb4a*-expressing venous cells in response to elevated Efnb2a, or a potential forced Ephb4a-Efnb2a cis-interaction in response to elevated Ephb4a (Fig. 4C; table S6; figs. S18, S19). Donor cells over-expressing reverse signaling deficient Efnb2a (25,  $\Delta$ C-Efnb2a) were not retained in the DA (Fig. 4C; table S6; figs. S18, S19), confirming a role for reverse Ephb4a-Efnb2a signaling in the regulation of arterial/venous segregation. Hence, Vegfa-induced *efnb2a* expression and bidirectional Ephb4a-Efnb2a signaling selectively exclude arterial angioblasts from venous sprouts whilst promoting the ventral migration of venous angioblasts. These data support the concept that Notch, EfnB2 and EphB4 can regulate the size of developing arteries and veins, and that endothelial cells from the DA may contribute to the formation of the CV in mouse (26). In summary, repulsive Ephb4a-Efnb2a signaling regulates the directional control of angioblast sprouting behavior.

Previous work has focused on identifying signals that modulate specification of arterial/venous angioblasts but how these signals ultimately affect cell behavior to sort and segregate angioblasts into distinct vessels was unknown. Our data provide a cellular framework for arterial/venous tube morphogenesis whereby coordinated regulation of dorsal and ventral angioblast sprouting behaviors by Vegf, Notch and Ephb4a/Efnb2a signaling efficiently fashions the embryonic vasculature (fig. S20; note S3), and sheds light on how multi-directional arterial and venous sprouting can be achieved whilst maintaining the integrity of a precursor vessel. Most importantly, we have uncovered an alternative mode of vascular development, which allows the segregation of cells into discrete arterial and venous vessels from one common precursor vessel.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

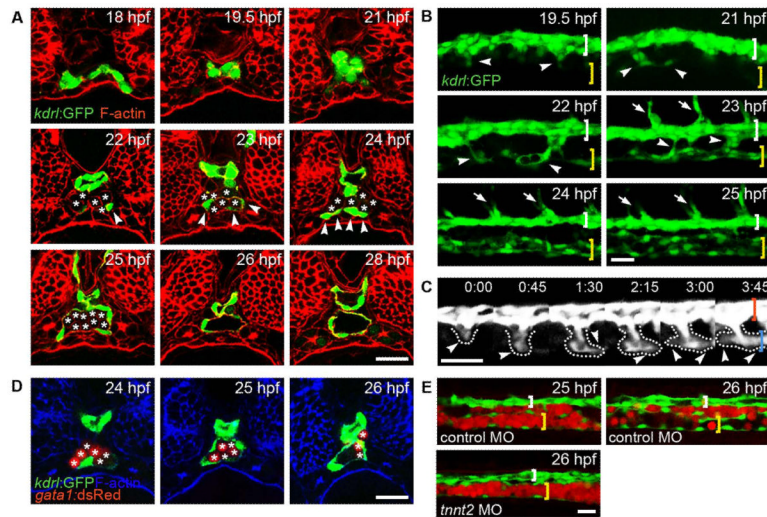
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## References and Notes

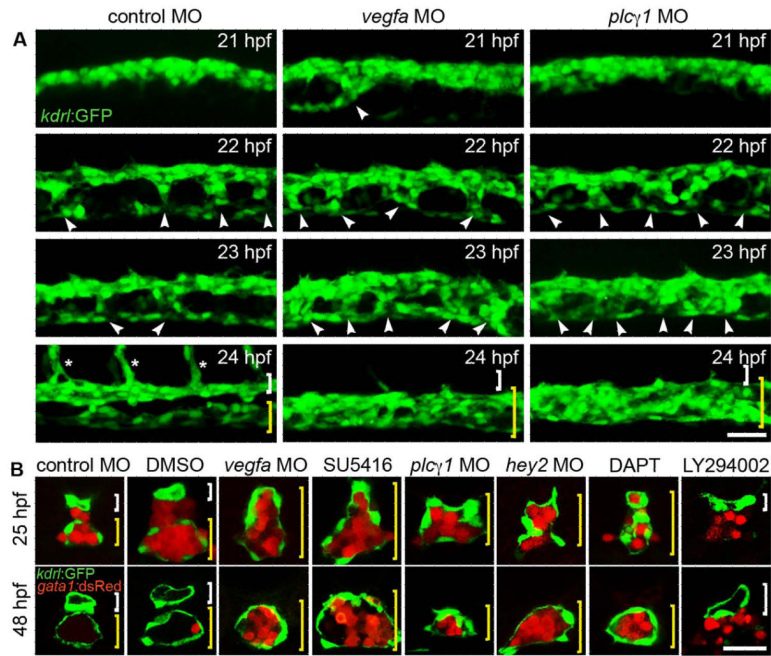
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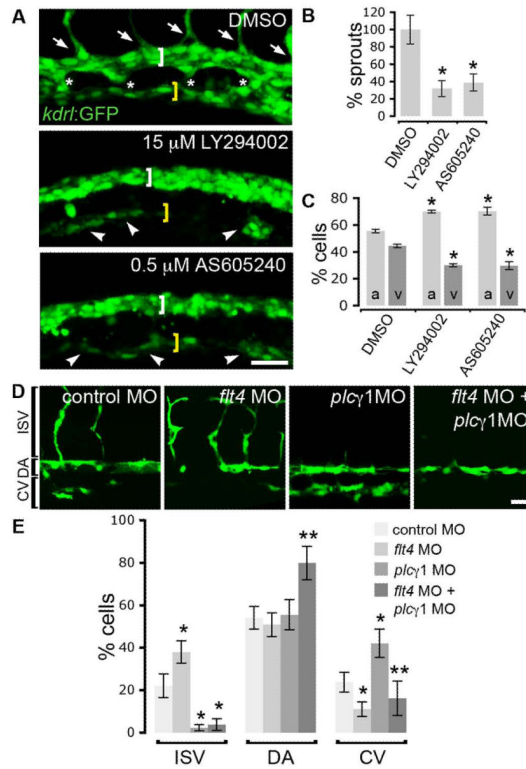
**Fig. 1. The CV forms by selective angioblast sprouting**

(A-E) Mid-trunk transverse sections (A, D), lateral views (B, E) or lateral time-lapse (C; movie S1) of *Tg(kdrl:GFP)<sup>843</sup>* (A-C) or *Tg(kdrl:GFP)<sup>843</sup>;Tg(gata1:dsRed)<sup>sd2</sup>* (D, E) embryos. Angioblasts coalesce and remodel to form the DA by 22hpf (white/red brackets; A-C). Between 21-24hpf, venous angioblasts sprout ventrally from the DA (arrowheads and dotted lines; A-C) and contribute to the CV primordium (yellow/blue brackets; A-C). ISVs sprout dorsally from 23hpf (arrows; B). By 25hpf, venous angioblasts surround *Tg(gata1:dsRed)<sup>sd2</sup>*-positive erythrocytes (asterisks, A, D; red cells, D, E). Erythrocyte displacement typically clears the CV lumen by 26hpf (A, D, E) but not in *tnnt2* MO-injected embryos (E). Scale bars, 35  $\mu$ m.



**Fig. 2. Vegf signaling limits ventral sprouting**

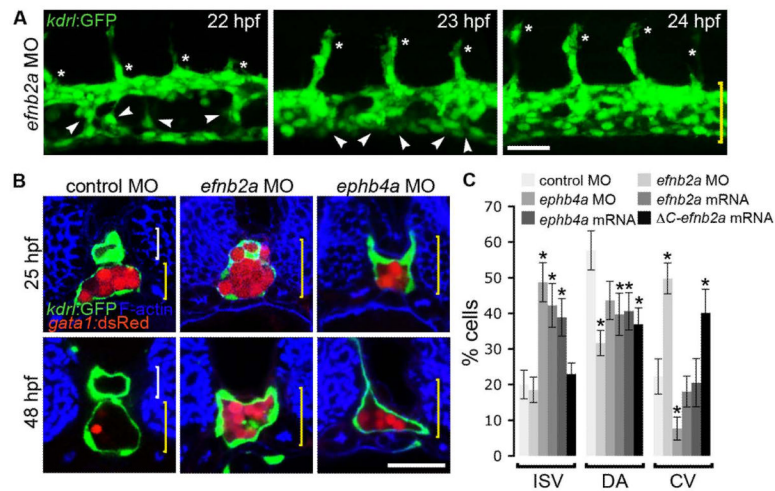
(A, B) Lateral views of *Tg(kdrl:GFP)<sup>s843</sup>* embryos (A) or transverse sections of *Tg(kdrl:GFP)<sup>s843</sup>;Tg(gata1:dsRed)<sup>sd2</sup>* embryos (B) injected with control, *vegfa*, *plc1* or *hey2* MO, or exposed to DMSO, SU5416, DAPT or LY294002. *vegfa* or *plc1* MO-injection blocks ISV sprouting (asterisks), promotes excessive ventral sprouting (arrowheads), leads to loss of the DA (white bracket) and generates a single venous tube (yellow bracket). SU5416, DAPT, or *hey2* MO-injection, also promote loss of the DA, whereas LY294002 blocks CV morphogenesis. Scale bars in, 35 μm.



**Fig. 3. Flt4/PI3K signaling mediates venous angioblast migration**

(A-C) *Tg(kdr:GFP)<sup>s843</sup>* embryos were exposed to DMSO, LY294002 or AS605240, processed for microscopy (23hpf; A) and then scored for (B) the number of ventral sprouts (as a percentage of DMSO treated embryos) or (C) the percentage of cells in the DA (white bracket; a in graph) or CV (yellow bracket; v in graph). PI3K-inhibition blocks ISV sprouting (arrows), venous sprouting (asterisks) and angioblast ventral migration (arrowheads). Lateral images (D) and quantification (E) of donor-derived *Tg(kdr:GFP)<sup>s843</sup>* cells in wild-type hosts at 48hpf. Fewer cells from *flt4* MO-injected donors contribute to the CV versus controls. Excessive ventral migration of angioblasts from *plcγ1* MO-injected donors is reduced upon co-injection with *flt4* MO. Scale bars, 35  $\mu$ m. Error bars represent mean  $\pm$  SEM. (\* $P$  < 0.05 versus control; \*\* $P$  < 0.05 versus *plcγ1* MO; Student's *t* test).





**Fig. 4. Ephb4a/Efnb2a-signaling determines the direction of angioblast migration** (A, B) Lateral projections of *Tg(kdr1:GFP)<sup>843</sup>* embryos (A) or transverse sections of *Tg(kdr1:GFP)<sup>843</sup>;Tg(gata1:dsRed)<sup>sd2</sup>* embryos (B) injected with control, *efnb2a*, or *ephb4a* MO (A, B). Reduced Efnb2a expression promotes excessive ventral sprouting (A; arrowheads), loss of the DA (B; white bracket) and formation of a single vessel (A, B; yellow bracket) without affecting initiation of ISV sprouting (A; asterisks). Similarly, posterior arterial/venous segregation is disrupted in *ephb4a* MO-injected embryos. (C) Quantification of donor-derived *Tg(kdr1:GFP)<sup>843</sup>* cells in wild-type hosts at 30hpf. More cells from *efnb2a* MO-injected or  $\Delta C$ -*efnb2a* over-expressing donors contribute to the CV versus controls, whereas less cells from *ephb4a* MO-injected donors contribute to the CV. Over-expression of *efnb2a* or *ephb4a* enhances the number of donor cells contributing to ISVs. Scale bars, 35  $\mu$ m. Error bars represent mean  $\pm$  SEM. (\* $P < 0.05$ ; Student's *t* test).