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To be or not to be: endothelial cell plasticity in development, repair, and disease

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Abstract

Endothelial cells display an extraordinary plasticity both during development and throughout adult life. During early development, endothelial cells assume arterial, venous, or lymphatic identity, while selected endothelial cells undergo additional fate changes to become hematopoietic progenitor, cardiac valve, and other cell types. Adult endothelial cells are some of the longest-lived cells in the body and their participation as stable components of the vascular wall is critical for the proper function of both the circulatory and lymphatic systems, yet these cells also display a remarkable capacity to undergo changes in their differentiated identity during injury, disease, and even normal physiological changes in the vasculature. Here, we discuss how endothelial cells become specified during development as arterial, venous, or lymphatic endothelial cells or convert into hematopoietic stem and progenitor cells or cardiac valve cells. We compare findings from in vitro and in vivo studies with a focus on the zebrafish as a valuable model for exploring the signaling pathways and environmental cues that drive these transitions. We also discuss how endothelial plasticity can aid in revascularization and repair of tissue after damage- but may have detrimental consequences under disease conditions. By better understanding endothelial plasticity and the mechanisms underlying endothelial fate transitions, we can begin to explore new therapeutic avenues.

Keywords

endothelial cell; vascular plasticity; transdifferentiation; zebrafish

INTRODUCTION

Cellular plasticity is the conversion of one cell type to another cell type through changes in gene expression, cell structure, and cell function. While cell fate changes occur throughout

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DISCLOSURES

None

development, cellular plasticity has also been observed in tissue regeneration and disease progression [1–3]. Endothelial cells (ECs) line the vascular system, including blood vessels, lymphatic vessels, and the heart, which are vital for the circulation of oxygen, nutrients, and immune cells throughout an organism. These cells undergo several fate transitions throughout development until becoming arterial, venous, or lymphatic ECs, hematopoietic stem and progenitor cells (HSPCs), or cardiac valve cells, for example (Figure 1a). In addition, ECs have dynamic responses to both local signals and hemodynamic forces. Thus, changes due to injury or disease can trigger EC plasticity leading to cell conversion. Here we discuss the role that EC plasticity plays in development, repair and revascularization, and disease, emphasizing the signaling pathways and hemodynamic forces that drive these responses. We compare both in vitro and in vivo studies, with a focus on zebrafish as an important model system for understanding vascular plasticity. Finally, we explore the broad range implications of EC dynamics and how it can lead to future therapies.

Zebrafish as a model for studying vascular development and plasticity

Zebrafish have emerged as a powerful model organism to study vascular development and plasticity. The ability to maintain large numbers of adult animals and generate large numbers of progeny facilitates genetic and experimental studies, while the ex utero development and optical clarity of zebrafish embryos and larvae allows for high-resolution imaging at all stages of development. Forward genetic screens using ENU mutagenesis, gene knockdown using morpholinos, and gene editing techniques such as CRISPR have uncovered the function of genes and signaling pathways vital for many different developmental processes. Gene expression is easily assessed in fixed samples using in situ hybridization and immunohistochemistry, while a myriad of different cell types, tissues, and organs can be visualized in living embryos using the numerous available transgenic fluorescent reporter lines. Using the zebrafish, scientists have been able to build upon their current understanding of vascular development established from other model systems, and uncover additional molecular mechanisms and cellular behaviors that drive vascular morphogenesis.

The vascular anatomy of zebrafish is highly conserved, as are the molecular mechanisms regulating vascular development [4–8]. As in other vertebrates, endothelial progenitors or angioblasts are specified in the mesoderm through the expression of ETS transcriptional regulator family members and migrate to form the axial vessels, the dorsal aorta (DA) and posterior cardinal vein (PCV). Initial formation of the major vessels during early development occurs via vasculogenesis, or the formation of vessels by the coalescence of these migratory primitive angioblasts. Subsequent vessel formation occurs via angiogenesis, or the sprouting and growth of new vessels from preexisting vessels. In the trunk, primary intersegmental vessels (ISVs) sprout from the DA and migrate dorsally along somite (future muscle blocks) boundaries to form an un-perfused network of arterially derived segments (Figure 1b) [9]. After this initial network is formed, secondary sprouts emerge from the PCV and either anastomose with the arterial ISVs to form venous ISVs or go on to form the lymphatic vasculature [9]. Since oxygenation of the early zebrafish embryo is dependent on diffusion and not blood circulation, vascular mutants that would normally be lethal early on in mammals can be assessed for several days in zebrafish [9,10]. This has helped identify mutations specifically affecting various aspects of vascular morphogenesis such as

endothelial specification, migration, proliferation, and lumenization (reviewed in [6]). Vascular specific transgenic lines have also been generated that label all or subsets of endothelial cells in living animals using fluorescent markers such as GFP (Figure 2a,b). Using these lines, as well as additional vascular labeling techniques such as microangiography and lymphangiography, the formation of blood and lymphatic vessels can be visualized in real-time through confocal microscopy and time-lapse imaging (Figure 2c–f). This has helped reveal the dynamic cellular behaviors that drive vessel formation during development. Additionally, transgenic lines can be used to enrich for different endothelial cell populations via florescence activated cell sorting (FACs), allowing for genome wide cell specific gene expression analysis.

Lineage tracing techniques such as Cre/lox and Flp/FRT for “permanent” labeling or Kaede and Dendra2 for photo-conversion labeling have been utilized in zebrafish and other model organisms to track endothelial cells and monitor their cell fate decisions (a few selected examples of which are shown in Table 1) [11]. Lineage tracing permits direct visualization of endothelial fate transitions such as the specification of arterial versus venous fate, the endothelial to hematopoietic transition (EHT), formation of the lymphatic system from venous endothelium, or endothelial to mesenchymal transition (EndoMT) during cardiac valve formation [12–17]. Lineage tracing tools have also facilitated tracking of individual endothelial cells during tissue repair and regeneration after injury [18]. Zebrafish have a robust capacity to regenerate injured tissues and the vasculature that perfuses these tissues, including the heart, brain, and caudal fin. This capacity makes zebrafish ideally suited for studying endothelial cell plasticity during the regeneration process, and recent studies using fish have already begun to reveal mechanisms utilized to rebuild vasculature in damaged tissue [18–21]. Zebrafish have also been used to model many human vascular diseases (a few selected examples of these models are shown in Table 2), and combined with lineage tracing and imaging techniques, provide a powerful tool for understanding the role of endothelial cell plasticity in disease progression.

VASCULAR PLASTICITY DURING DEVELOPMENT

Endothelial cell specification

Early endothelial cell fate is specified from the mesoderm through the expression of ETS transcriptional regulator family members. ETS variant 2 (Etv2) is a major driver of endothelial specific gene expression in mice and zebrafish and its loss leads to severe blood and vessel defects [22–25]. Ectopic expression of Etv2 *in vivo* is sufficient to drive precocious endothelial gene expression in *Xenopus* and zebrafish and even causes the transdifferentiation of fast skeletal muscle into functional endothelial cells [22,26]. Although Etv2 is a major regulator of angioblast specification, the transcription factor Npas4l is thought to act upstream. Npas4l was initially identified as the defective gene in *cloche,* a zebrafish mutant discovered over two decades ago that causes severe defects in both endothelial and hematopoietic cell lineages, suggesting these cell types derive from a similar origin [10]. The recent molecular cloning of Npas4l showed that its expression leads to the upregulation of the early endothelial and hematopoietic lineage markers Etv2 and Scl/Tal1, supporting the idea that it acts upstream as a master regulator of both lineages (Figure 3a)

[27]. Although there are Npas4l-like genes in other vertebrates including mammals [27], it is still not clear whether the critical function of Npas4l is conserved in other vertebrates.

Arterial and venous specification

Initial arterial-venous specification in vasculogenic vessels occurs early in embryonic vessel development, prior to the initiation of blood circulation. This suggests that this initial specification event is genetically programed and not driven by blood flow, although hemodynamic forces do play a major role in endothelial cell fate once flow initiates [28]. In mammalian and avian embryos, vascular plexi show distinct patterns of arterial and venous genes before remodeling of the capillary network into morphologically distinguishable arteries and veins. It was first shown in mice that the ligand EphrinB2 is localized to arterial cells while its receptor EphB4 is expressed in venous endothelial cells [29]. Additional studies in the chick embryo have shown that the Vascular endothelial growth factor (Vegf) co-receptors Neuropilin-1 (Nrp1) and Neuropilin-2 (Nrp2) are expressed in non-overlapping endothelial arterial and venous precursor cells respectively [30]. This signaling between arterial and venous compartments is thought to be vital for the separation of their cell fates but lies downstream of other signaling pathways that first distinguish arterial and venous precursors.

Studies in zebrafish have revealed an upstream signaling cascade in which Hedgehog signaling from the notochord activates Vegf signaling in the somites which in turn induces Notch signaling in the DA leading to an arterial fate (Figure 3a) [31,32]. Embryos deficient in any of these pathways lack EphrinB2 expression in blood vessels and contain defects in vascular morphology. Furthermore, loss of Notch signaling leads to an expansion of venous markers such as EphB4 and Flt4 into the arterial domain and overexpression activates arterial markers such as EphrinB2 into the venous vessels [31]. This suggests that in early development, the loss or gain of Notch is sufficient to drive arterial/venous cell fate changes. Similar findings have been found in mammalian vessel development, where loss of Notch signaling components causes severe vascular defects and impairment of arterial cell fate [33–35].

Venous identity was initially thought to be the default vessel state since lack of Notch signaling allows the expression of the definitive venous marker EphB4. However, a knockout mouse model of the nuclear orphan receptor COUP-TFII (Nr2f2) demonstrated that loss of this receptor leads to Notch expression within veins [36]. Work in zebrafish, however, suggests that COUP-TFII does not regulate Notch signaling but instead Notch signaling suppresses COUP-TFII in the DA [37]. Recent work using both zebrafish and mouse models to study early vascular development has found that Bone morphogenetic protein (BMP) signaling is required for venous specification. Loss of the BMP receptor Alk3 or its downstream effector SMADs leads to defects in vein formation and loss of EphB4 expression without affecting arterial identity [38]. Increased BMP signaling promotes the expression of the venous identity markers EphB4 and COUP-TFII. Thus, BMP and Notch signaling play antagonistic roles in driving arterial/venous specificity and modulation of these signaling pathways is sufficient to drive arterial-venous cell fate changes (Figure 3a).

Although acquisition of arterial or venous identity is established by genetically programed pathways during early development, changes to the vessel environment have been shown to influence and even switch endothelial cell fate as demonstrated when segments of the saphenous vein are grafted to injured arteries [39]. Studies using quail-chick chimeras have shown that grafted arteries or veins from quail are capable of integrating into both vessel types in chick hosts up until embryonic day seven. Although arterial vessel segments do not graft well into veins and vice-versa at later stages of development, isolated endothelial cells separated from their vessel wall components are still able to colonize both types of host vessels regardless of their vessel origin, suggesting that vessel wall composition can impact endothelial cell specificity [40]. Changes in oxygen tension have also been found to affect artery/vein identity. A decrease in oxygen due to hypoxic conditions was found to cause a decrease in arterial marker expression in mouse retinal arteries [41]. Together these findings suggest that endothelial cells are quite plastic and can change cell fate due to perturbations in their environment.

Besides vessel wall composition and oxygen tension, endothelial cells can respond and change fate due to hemodynamic forces. Perturbing blood flow patterns in early quail and chick embryos demonstrated that arteries could convert to veins and veins could convert to arteries upon a reduction or increase in blood flow respectively [42]. Additional studies have since shown that shear stress due to laminar flow activates Notch signaling which in turn can promote an arterial fate thus connecting differences in flow to genetic regulators of identity. This has been demonstrated in both human umbilical vein cells (HUVECs) and human aortic endothelial cells (HAECs) which show an upregulation in Notch and Notch-associated genes when exposed to fluid shear stress compared to cells under static conditions [43,44]. *In vivo* studies in zebrafish have indicated that patterning of intersegmental vessels (ISVs) in the trunk is dictated by differences in flow in which initiation of arterial flow in an ISV leads to a high probability that neighboring ISVs will connect to secondary sprouts to become venous (Figure 3b) [9]. This ensures that the number of arterial and venous ISVs is equal allowing for circulatory blood flow to be balanced. Recent work has shown that a global reduction in blood flow disrupts this balance leading to an increase in venous ISV formation [16]. In addition, the authors found that those ISVs receiving high levels of flow increase their Notch signaling preventing them from connecting to secondary sprouts. In contrast, those vessels with weaker flow had a higher tendency of becoming venous resulting in lower Notch signaling. This suggests that Notch also helps transduce mechanosensory cues in vivo to dictate vessel identity [16]. However, unlike in quail/chick chimeras, the change in vessel identity appeared to at least partially involve replacement of arterial endothelial cells with venous endothelial cells migrating from the adjacent PCV rather than direct endothelial cell transdifferentiation. It remains unclear to what extent the effects of flow on initial ISV pattering in the zebrafish trunk are due to cellular rearrangements versus changes in cellular arterial-venous identity. Blood flow has also been shown to drive vascular remodeling in mammals through endothelial migration, proliferation, and morphogenesis but again, whether this involves endothelial cell fate changes *in vivo* has yet to be explored [28,45].

The endothelial to hematopoietic transition

Shortly after the initial primitive embryonic arteries and veins form, a subset of endothelial cells within the aorta-gonad-mesonephros (AGM) region, in particular the ventral wall of the dorsal aorta, undergo an EHT to give rise to the HSPCs [1,46]. These stem cells are necessary for the formation of all the blood lineages of the adult animal. Lineage tracing experiments in mice were the first to indicate that HSPCs originate from the endothelium and not from the underlying mesenchyme [47]. Subsequently, live imaging in zebrafish embryos provided definitive evidence that HSPCs emerge via EHT from hemogenic endothelium in the ventral floor of the dorsal aorta (Figure 1c) [12,14,15]. Retinoic acid signaling has been shown to promote the specification of the hemogenic endothelium. Loss of the enzyme Raldh2, needed to make retinoic acid, leads to a loss of hemogenic endothelial cells and transplantation of AGM lacking Raldh2 results in failure to form blood cells capable of colonizing the peripheral blood of recipient mice [48,49]. The receptor tyrosine kinase c-Kit is expressed in hemogenic endothelial cells and appears to be required for hemogenic endothelial cell formation downstream from retinoic acid signaling. Re-expression of c-Kit in Raldh2 mutant embryos is sufficient to rescue hemogenic endothelial cell development but fails to rescue in the presence of the Notch inhibitor DAPT [50]. This suggests that retinoic acid signaling drives downstream expression of c-Kit, which may in turn activate Notch signaling to promote hemogenic endothelial specification [50].

In the absence of Notch signaling, embryos fail to form HSPCs [51,52]. While part of this loss can be explained by defective arterial differentiation of the dorsal aorta upstream from HSPC formation [31], some studies suggest that Notch signaling may play a more direct role in HSPC formation independent of its role in arterial cell fate specification. Transient over-expression of the notch intracellular domain *(NICD)* in wild-type zebrafish embryos is sufficient to cause an expansion of HSPCs and induce ectopic expression of HSPC markers within the aortic roof and vein [51]. Furthermore, loss of the Notch ligand Jagged1 but not Jagged2 in mouse embryos leads to a decrease in hemogenic endothelial cell specification without affecting arterial cell fate [53]. More recent work has shown that Notch ligand specificity may induce different levels of Notch activation leading to different cell fates. While activation by the ligand Delta4 drives high Notch activity causing endothelial cells to remain arterial, stimulation by the ligand Jagged1 leads to low Notch activation and a hematopoietic fate [54]. However, both ligands are homogenously expressed around the aortic endothelium and thus how Notch signaling is modulated to drive these different cell fates is still not well understood.

For EHT to be complete, specified hemogenic endothelial cells must exit the DA to become HSPCs. This process requires the transcription factor Runx1 with loss of Runx1 leading to a failure of hemogenic endothelial cells to undergo EHT and thus a reduction in HSPC formation [14,55]. Expression of *runx1* is regulated by Notch signaling and Runx1 in turn drives the expression of the HSPC marker *cmyb* [51]. However, *runx1* is only transiently expressed early in development, and only required for the initial specification of HSPCs, while Cmyb is necessary for both formation and ongoing maintenance of HSPCs [55–58]. Recent work has shed some light on the mechanism of Cmyb perdurance in the absence of Runx1. The transient expression of *runx1* leads to an increase in methylation of the *cmyb*

promoter via the DNA methyltransferase 3bb.1 (Dnmt3bb.1), promoting the continued expression of *cmyb* [59]. Overexpression of *dnmt3bb.1* in endothelial cells is also sufficient to drive ectopic *cmyb* expression and ectopic hematopoietic differentiation. These results demonstrate that epigenetic mechanisms also contribute to the transition of endothelial cells to the hematopoietic lineage. Whether the formation of additional HSPCs via EHT occurs later in development or in specific organs is not well explored, although recent work has suggested it does occur [60]. Future studies will be required to uncover the frequency and necessity of this form of endothelial cell plasticity throughout development or in adult life.

Lymphatic endothelial cell emergence

In addition to the blood vascular system, endothelial cells also line the lymphatic vasculature. The lymphatic system is an entirely separate and distinct vascular network from the blood vascular/circulatory system. It is required for tissue fluid homeostasis, for adsorption of lipids, and for immune cell trafficking, and plays an important role in cancer metastasis. The origins of lymphatic endothelial cells (LECs) have been debated for over a century, but recent live imaging in the larval zebrafish and lineage tracing in the mouse embryo confirmed that the earliest LECs emerge from primitive veins [17,61]. Prospero homeobox 1 (Prox1) was one of the first markers identified for LEC fate and has been shown to promote the expression of other lymphatic genes [62–64]. Loss of Prox1 in mice leads to a failure of lymphatic formation without affecting the blood vasculature suggesting its requirement is lymphatic-specific [65]. The mechanisms underlying emergence of LECs from venous endothelial cells are still being actively studied. Live imaging of the zebrafish trunk shows that precursor cells expressing low levels of Prox1 divide asymmetrically to produce daughter cells with distinct fates [66]. While one daughter becomes a LEC that upregulates Prox1 and migrates out of the PCV, the other daughter remains venous within the PCV and loses its Prox1 expression. Vegfr3/Vegfc signaling is required for this division and for secondary sprout formation, but not for initial LEC specification [66–68]. Current evidence suggests that a Prox1-Vegfr3 feedback loop forms in which Prox1 targets Vegfr3 expression which in turn upregulates Prox1 [66,68]. Prox1 is required not only for LEC specification but also to maintain lymphatic identity. Conditional knockout of *Prox1* in embryonic, postnatal, or adult mice leads to a reversion of LECs back to blood endothelial cells [69]. In addition, lymphatic vessels exposed to shear stress via blood flow are reprogramed to blood vessels [70]. Together, these studies indicate that a terminal lymphatic fate must be actively maintained and that LECs retain their plasticity even into adulthood.

Although they are largely excluded from the central nervous system, lymphatics have also recently been described within the brain meninges. Mice contain a network of lymphatic vessels within the dura mater of the meninges, important for the removal of excess fluids and macromolecules from this area [71,72]. Recent work has shown that zebrafish also have a complex intracranial lymphatic vessel network which develops from the facial lymphatic vascular plexus [73]. Since the facial lymphatic system originates from the common cardinal vein with other nearby veins contributing to its formation [74], this would suggest that the intracranial lymphatic system also has a venous origin. In addition to lymphatic vessels, zebrafish also contain a population of macrophage-like individual, isolated cells in the internal meninges (leptomeninges) that express lymphatic markers but that do not form

vessels and reside next to meningeal blood vessels [75–77]. These cells are also vein-derived, sprouting from the optic choroid vascular plexus, a primitive lymphovenous vascular plexus behind the eye. Although this cell population has been shown to also require Vegfc-Vegfd-Vegfr3 signaling for its development, the similarities and differences in the mechanisms leading to formation of these cells compared to vessel- forming LECs remain largely unexplored. Future studies should clarify the role of this endothelial derived meningeal cell population and their plasticity.

Endothelial-mesenchymal transition

A fourth major endothelial cell fate transition event occurs during cardiac valve development of the vertebrate heart. During this process, specialized endothelial cells that line the heart called endocardial cells undergo an EndoMT to form the interstitial cells of the cardiac valve leaflets (Figure 1d) [13,78–81]. This process is driven by many different signaling pathways including BMP, Transforming growth factor (TGF)β, and Notch, which are required for the specification, initiation, or cessation of EndoMT in the cardiac cushions, the sites of valve formation [82]. Inactivation of the ligand Bmp2 in the mouse myocardium or its receptor Bmp type 1A receptor (Bmpr1a) in the endocardium leads to a failure of EndoMT and thus a lack of mesenchymal cells in the atrioventricular (AV) cardiac cushion [83]. TGFβ signaling is also important for EndoMT initiation, with TGFβ2 deficient AV explants showing delays in this process [84]. In addition, embryos lacking TGFβ2 have enlarged cardiac cushions due to continual EndoMT suggesting that TGFβ2 is needed for EndoMT termination [84]. Notch signaling is also vital for EndoMT in both mice and zebrafish. A reduction in Notch signaling leads to a loss of mesenchymal cushion cells causing dysfunctional valves to form while constitutive activation leads to excessive EndoMT [85]. Although there are many points of crosstalk between these signaling pathways during heart valve development, all three pathways converge on regulating the expression of Snail family members, a group of transcription factors that are vital for the EndoMT process [82].

In addition to the Snail family members other transcription factors are also necessary for valve development, including Nuclear factor of activated T-cell 1 (Nfatc1). Loss of *Nfatc1* in mice and zebrafish causes valve morphogenesis defects in both organisms [13,86]. In mice, Nfatc1 is required in both the myocardium to reduce Vegf signaling allowing valve progenitor cells to undergo EndoMT and the endocardium to promote valve leaflet proliferation and elongation [86,87]. In zebrafish, global loss of *nfatc1* leads to a significant decrease in the mesenchymal interstitial cells that make up the valve due to a decrease in proliferation and recruitment of progenitor cells as well as a decrease in the EndoMT promoting factor *twist1b* [13]. More work needs to be done to better elucidate the cell specific role Nfatc1 plays in cardiac valve morphogenesis and EndoMT. Valve leaflets contain monolayers of endothelial cells surrounding the interstitial mesenchymal cells, indicating that while some endothelial cells undergo EndoMT others remain endothelial. However, the mechanisms regulating this choice are still not well understood. Since EndoMT is also required for cardiac regeneration after injury [88,89] and has been implicated in the progression of several diseases such as cerebral cavernous malformations (CCM), atherosclerosis, and cancer [1], understanding the molecular mechanisms that drive EndoMT and endothelial plasticity in general is of great therapeutic value.

PLASTICITY DURING VESSEL REPAIR AND REVASCULARIZATION

The plasticity of endothelial cells and their derivatives is not restricted to early developmental stages but also occurs in response to tissue injury due to trauma or disease. Reperfusion of damaged vessels is critical to ensure tissue survival, but vessel regrowth and reconnection depends on the tissue environment and the type of damage incurred. Amputation of the adult zebrafish caudal fin illustrates how arteriovenous fate transitions can occur after tissue damage. During tail regeneration, a vascular plexus forms that is predominately venous derived [90]. As the plexus remodels, new arteries and veins form with venous cells continuing to proliferate and contribute to the growing arteries [18,90]. Similar to arterial specification during development, this cell fate change from venous to arterial requires Notch signaling [90]. While arterial formation from venous cells does occur during later organogenesis stages in zebrafish and mice [91], the fin regeneration findings suggest that endothelial cells retain their plasticity even into adulthood. This has also been shown to be true in humans that receive vessel grafts. Saphenous vein grafts are commonly used for both venous and arterial vessel reconstruction. Recent work shows that vein grafts on the popliteal vein and artery due to an acute extremity injury are able to adapt to their environments [39]. The graft placed on the vein retained venous identity expressing markers such as EphB4 and COUP-TFII while the graft placed on the artery downregulated venous markers and started to express Ephrin-B2 and Delta-like ligand 4, markers of arterial identity [39]. This suggests that vessel identity is also plastic in human adult blood vessels. However, this plasticity may depend on the vessel the segment is grafted to or the type of injury that necessitated the need for a graft, since previous work shows vein to artery grafts losing venous identity but not gaining arterial marker expression [92]. It is also unclear to what extent the change in vessel identity results from cells moving from adjacent vessel segments into the graft, as opposed to a change in the arterial-venous identity of the grafted endothelial cells. Thus, more work needs to be done to understand what factors influence endothelial arteriovenous plasticity after injury.

The revascularization of tissue after injury is vital for its survival and proper organ function. This is especially true after cardiac injury, where lack of newly formed vessels can cause fibrotic scar formation [20,93,94]. In zebrafish, damage to the heart leads to the formation of new vessels from preexisting endothelium and studies have shown that the fast revascularization of vessels into the injured area seen after cryoinjury is due to vessel sprouting [20,95]. In mice, some studies have claimed that endocardial cells or resident cardiac fibroblasts contribute to newly forming coronary vessels after cardiac injury [96,97]. However, recent work has shown this not to be the case and similar to zebrafish, revascularization comes from preexisting endothelial cells [98,99]. Thus, unlike during the initial development of the coronary vessels from endothelial cells from the endocardium and sinus venosus, revascularization of the heart after injury is mostly from sprouting of preexisting nearby vessels [93,100].

In addition to resident tissue cells contributing to revascularization, some studies have suggested that circulating endothelial progenitor cells (EPCs) derived from the bone marrow contribute to newly forming adult organ vessels during normal physiological conditions and after myocardial ischemia, hind limb ischemia, skin wounding, or traumatic brain injury

[101–105]. In contrast, other studies have shown that the contribution of EPCs to new vessels is low and that circulating bone marrow derived cells are recruited to blood vessels in order to promote angiogenesis but are not actually incorporated into the vessel wall [106,107]. The range of EPC incorporation into newly forming adult vessels seems to vary greatly from study to study. This may be dependent on the type of tissue utilized in the study, or the type of injury/disease model used to stimulate neovascularization. However, even studies using a similar injury model for the carotid artery show differing results - one study has suggested that circulating EPCs contribute to its reendothelization [108], while other studies show that EPCs do not contribute to endothelial regeneration of the carotid artery and that endothelial cells migrate from the adjacent vessel segment [109,110]. While the question of whether EPCs actually get incorporated into newly forming vessels has been widely debated, it is generally agreed that bone marrow derived cells can provide therapeutic benefit in the context of ischemic tissues by promoting angiogenesis. A variety of clinical studies on peripheral artery disease and acute myocardial infarction have shown that bone marrow cell therapy can improve patient outcomes [111,112]. New technologies in lineage tracing and *in vivo* imaging may help us better elucidate how bone marrow derived cells, in particular EPCs, aid in the revascularization of tissue after ischemia.

In addition to blood endothelial revascularization after injury, lymphatic vessels also respond to tissue damage. In both mice and zebrafish, the cardiac lymphatic system is reactivated after injury in response to Vegfc signaling and expands into the wounded region [113–116]. In zebrafish, sprouting from pre-existing ventricular lymphatics does contribute to the newly forming lymphatic vessels, but the majority of new vessels seem to originate from the coalescence of LECs either from isolated LEC clusters normally found in the zebrafish heart or possibly from LECs detaching and migrating away from lymphatic vessels into the injured area [113]. Since isolated LEC clusters are only found transiently in mouse hearts until P23 it would be interesting to ascertain if lymphatic sprouting or LEC coalescence is utilized to form new lymphatic vessels following cardiac injury in mice. Furthermore, whether the endocardium or the cardiac blood endothelium can contribute to lymphatic revascularization after cardiac injury has not been explored. As noted above, the zebrafish meninges also contain a population of individual perivascular cells expressing lymphatic markers [75–77]. Although they do not contribute to lymphatic vessels under normal circumstances, one recent report suggests that these cells (variably called, fluorescent granular perithelial (FGP) cells, mural (mu)LECs, or brain (B)LECs) have the capacity to migrate into the brain parenchyma after brain injury to form lumenized lymphatic vessels that drain interstitial fluid and act as “tracks” for the growth of new blood vessels [117], suggesting they may be more plastic in the injury setting. This study was carried out in zebrafish larvae, however, and it remains to be seen whether these cells have the same plasticity after injury in the adult brain, which contains both FGPs/muLECs/BLECs and *bona fide* lymphatic vessels in the meninges [73]. In mice, photothrombosis induced stroke but not transient middle cerebral artery occlusion led to lymphatic vessel growth from the sagittal sinus into the alymphatic zone where the stroke occurred [118]. These findings suggest that lymphatic neovascularization can occur after mammalian brain injury but may depend on the severity of the injury incurred. In addition, whether these newly forming lymphatic vessels emerge from preexisting lymphatics in the meninges or derive from other

cell types within the brain still needs to be investigated, but better understanding how new lymphatics form after injury could greatly aid in treating several diseases such as stroke, traumatic brain injury, and neurodegenerative disorders.

Similar to its development, the cardiac valve also undergoes EndoMT after injury in zebrafish. Genetic ablation of the valve interstitial cells leads to the recruitment of endothelial cells that transdifferentiate into interstitial cells to build a new valve [88]. Furthermore, this regeneration process is promoted by TGFβ signaling. In sheep, tethering of the mitral valve leaflets to restrict valve closure led to an increase in endothelial cells undergoing EndoMT and an increase in leaflet area and thickness [119]. This suggests that changes in mechanical stress can induce endothelial cell plasticity to drive mitral valve growth, perhaps to help improve valve closure. EndoMT is also seen in the mitral valves of patients with ischemic mitral regurgitation which causes a reversal of blood flow [119]. Therefore, a compensatory mechanism seems to be initiated in these diseased hearts but it is not sufficient to prevent the backflow of blood. Better understanding how EndoMT is induced during injury or disease conditions could lead to new therapeutic approaches to further promote compensatory mechanisms.

ENDOTHELIAL PLASTICITY IN DISEASE

While endothelial plasticity can be beneficial when it comes to the repair and revascularization of ischemic tissue and/or damaged vessels, it can also be detrimental under pathological conditions, leading to severe outcomes. Arteriovenous malformations (AVMs) are aberrant shunts with high blood flow that bypass capillary beds and may cause hemorrhage or hypoxia in the surrounding tissue [120]. These malformations lack well defined arterial-venous identity and many animal studies have shown that loss or activation of genes that regulate arterial-venous specification, such as Notch, EphrinB2, and EphB4, can lead to the formation of AVMs [121,122]. AVMs can form throughout the body and occur in various human disorders such as capillary malformation-AVM (CM-AVM) and hereditary hemorrhagic telangiectasia (HHT). Recently, loss of function mutations in EPHB4 have been linked to a type of CM-AVM suggesting that loss of vessel identity can contribute to AVM formation [123]. HHT results from mutations in the BMP receptor ALK1/ACVRL1, the accessory type III receptor endoglin (ENG), or the downstream BMP transcription factor SMAD4, leading to downregulation of BMP signaling in endothelial cells [124]. Expression of EphrinB2, a marker of arterial identity, has been shown to be reduced in the arterial domain of AVMs in homozygous Alk1 mutant mice, although it appears unaffected in Eng mutants [125]. Smad4 has been recently implicated in the acquisition of venous identity in mice and fish [38], thus loss of any of these genes may affect the arterial or venous identity of endothelial cells leading to AVM formation. However, it remains unclear whether the formation of AVMs is the consequence of a failure in arterial-venous specification, or whether loss of arterial-venous identity is a secondary consequence of increased shear stress and enlargement of the malformed shunt vessels. Future studies using CM-AVM and HHT disease models in mice and fish will help us better ascertain whether loss of endothelial identity is the root cause of AVMs.

EndoMT is frequently seen in a variety of pathologies including tissue fibrosis, CCM, and atherosclerosis, to name a few [1]. In many fibrotic diseases there is an uncontrolled accumulation of fibrotic tissue due to activated fibroblasts or myofibroblasts that can lead to organ dysfunction [126]. While tissue resident fibroblasts are most likely the major cellular source contributing to fibrotic disorders, other cell types including endothelial cells may also contribute to disease progression by transdifferentiating into fibroblasts. EndoMT has been observed in patient samples and/or mouse models of systemic sclerosis, pulmonary fibrosis, cardiac fibrosis, and fibrotic kidney disease, making its role in disease progression an interesting avenue to explore [126–128]. In CCM, vascular lesions form from enlarged or irregular vessels within the central nervous system that can lead to hemorrhage [1,126]. This disease is caused by loss of function mutations in either *CCM1 (KRIT1), CCM2 (OSM),* or *CCM3 (PDCD10).* Mouse models for CCM1 have shown that endothelial cells lining the brain lesion had an increase in TGFβ signaling and mesenchymal marker expression compared to non-lesion controls suggesting that these cells may be undergoing EndoMT [129]. Similar results have since been shown in samples of human brain lesions from CCM patients [130,129]. This suggests that the same pathway that drives EndoMT during development could contribute to lesion formation in CCM mutants. In support of this idea, inhibiting TGFβ signaling in endothelial CCM1-deficient mice was sufficient to reduce the size and number of lesions and decrease vessel leakage [129]. Modeling CCM in other organisms such as zebrafish has revealed additional downstream signaling pathways [131] and further work in these model organisms can help us better understand if EndoMT contributes to disease progression as well as allow us to test a vast array of pharmacological therapies [132]. Future studies are needed to determine if TGFβ inhibition or other treatments would be effective in human patients.

Atherosclerosis is the build-up of plaque in artery walls leading to the obstruction of blood flow. Rupturing of plaques can have severe consequences, causing blood clots that lead to heart attack, stroke, or even death. While the pathology of atherosclerosis has been studied for quite some time, the role of EndoMT in its progression has only recently been considered [126]. Using ApoE-deficient mice on high fat diets which form atherosclerotic plaques, studies have shown that lineage-traced endothelial cells near the plaque undergo EndoMT by downregulating endothelial markers and upregulating mesenchymal markers [133,134]. Specifically, these lineage-traced endothelial cells become fibroblast-like and incorporate into the plaque [134], although whether they become *bona fide* fibroblasts remains unclear. Plaques within human patients have also been shown to contain cells that express both endothelial and mesenchymal markers suggesting that these cells may be undergoing a fate transition. Furthermore, the proportion of cells undergoing EndoMT seems to increase with an increase in disease severity indicating that EndoMT may help drive disease progression [133,134]. While this is just correlative, *in vitro* work has shown that endothelial derived fibroblasts express higher levels of matrix metalloproteinases and lower levels of collagen compared to normal fibroblasts, which may contribute to plaque rupture [134]. More work still needs to be done to fully elucidate the role EndoMT plays in atherosclerosis pathology.

Cellular plasticity is a major hallmark of cancer progression. While many tumor cells undergo some form of epithelial to mesenchymal transition, there are also many examples of

endothelial plasticity within cancer [3]. In Kaposi's sarcoma, highly vascularized tumors form from spindle cells which are thought to be endothelial and/or mesenchymal in origin [135,136]. These cells undergo reprogramming, forming aberrant phenotypes and expressing both blood and lymphatic cell markers [137,138]. Bone marrow-derived EPCs contribute to the vascularization of tumors in many cancers by secreting pro-angiogenic factors as well as incorporating into the newly forming vessels [139]. This has been shown to be the case for tumor angiogenesis in mouse models and within tissue samples of patient tumors [140,141]. In addition, cancer cells can take on endothelial-like characteristics forming vascular channels that carry blood and plasma from host blood vessels to the tumor [142]. Known as vascular mimicry, this phenomenon was first described in melanoma and has now been identified in other cancers such as sarcomas, carcinomas, and breast, prostate, and ovarian tumors [142,143]. EndoMT also occurs in cancer, with endothelial cells becoming fibroblast-like, apparently contributing to the cancer-associated fibroblast (CAF) population through cell conversion. Endothelial cells within tumors have been shown to express fibroblast markers such as fibroblast specific protein 1 and smooth muscle actin in mouse models of melanoma and pancreatic cancer, suggesting they are undergoing EndoMT [144], although again whether these cells fully lose their endothelial identity and assume a fibroblast identity still needs to be explored. CAFs are thought to facilitate tumor progression through depositing extracellular matrix and secreting paracrine factors that affect tumor cells as well as the surrounding microenvironment [145]. While human endothelial cells are capable of undergoing EndoMT *in vitro* when treated with different stimuli such as TGFβ, the prevalence and importance of EndoMT in human tumors needs to be further elucidated [146]. Since many types of cancer contain some form of endothelial plasticity which usually coincides with a more severe prognosis, better understanding the mechanisms that maintain or dysregulate endothelial identity could have great therapeutic value in preventing tumor metastasis.

CONCLUDING REMARKS

The ability of endothelial cells to undergo cell fate changes has been seen throughout development, during injury repair, and within different disease pathologies. This indicates that endothelial cells are not only plastic during embryonic stages but also into adulthood where they can respond to changes in local signals and hemodynamic forces. Zebrafish have emerged as a powerful model organism to study vascular plasticity due to their experimental accessibility and optical transparency that allows endothelial fate transitions to be lineage traced and observed in real time. Work in zebrafish has already furthered our knowledge of the molecular mechanisms that contribute to these changes in fate. As in mammals, endothelial cells in zebrafish undergo a number of major fate transitions during development, including specification of arteries and veins, formation of hematopoietic stem cells from the dorsal aorta, emergence of lymphatic endothelial cells from venous endothelial cells, and conversion of endocardium to cardiac mesenchyme to generate the heart valve. Endothelial plasticity also contributes to the repair and revascularization of tissue after injury. While there are various examples seen in zebrafish, chick, and mammals, the ability of the zebrafish to regenerate many organs after injury allows for the discovery of new examples of plasticity during repair. Many studies of endothelial plasticity in disease

have been done using human tissue samples or mouse models. However, with the development of many new zebrafish disease models such as HHT, CCM, atherosclerosis, and cancer (Table 2), zebrafish can become a useful tool in understanding just how prevalent and important endothelial plasticity is in disease progression. Altogether this review highlights the occurrences of endothelial plasticity during development, injury repair, and disease progression as well as the molecular mechanisms that drive these processes. Better understanding the causes and consequences of this plasticity could open up new approaches to regenerative therapies and disease treatments.

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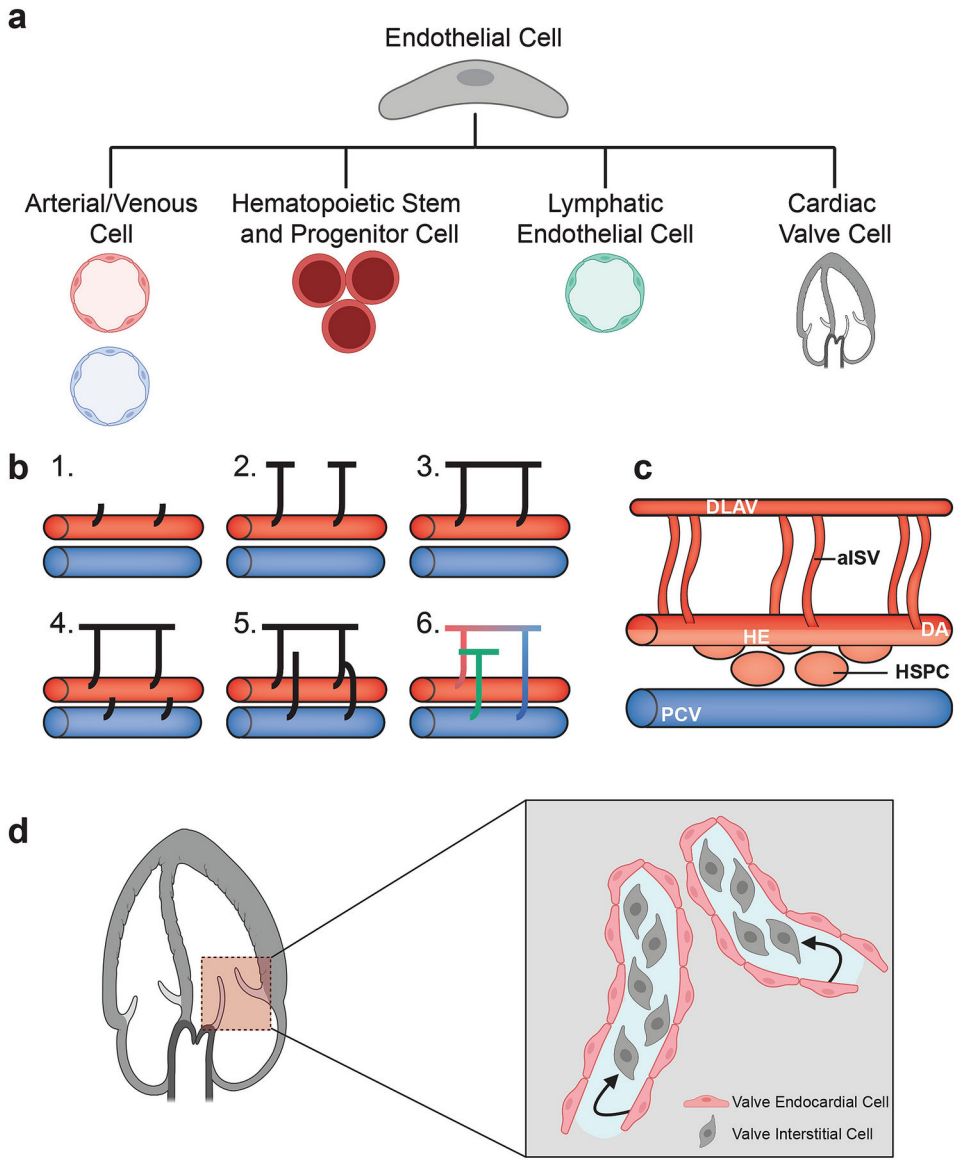

Fig 1:

Endothelial cell plasticity during development. (a) Endothelial cells undergo several fate transitions throughout development, forming arteries and veins, hematopoietic stem and progenitor cells, lymphatic vessels, and valve interstitial cells. Endothelial plasticity is also seen during tissue regeneration and under disease conditions. (b) Intersegmental blood vessels and lymphatic vessels emerge by sprouting angiogenesis. In zebrafish, primary sprouts emerge from the DA (1), sprouts grow dorsally and branch caudally and rostrally (2), branches interconnect to form the DLAV (3), secondary sprouts emerge from the PCV (4), some secondary sprouts connect to primary sprouts and others do not (5), primary sprouts that maintain their connection to the DA remain arterial ISVs (aISVs, red), while others that connect to secondary sprout segments from the PCV become venous ISVs (vISVs, blue). Secondary sprout segments that do not connect become lymphatic vessels (green). Adapted from Isogai et al. 2003 [9]. (c) Hematopoietic stem cells emerge through an endothelial to

hematopoietic transition by budding from the hemogenic endothelium (light red) in the ventral floor of the dorsal aorta. (d) Interstitial cells of the cardiac valve form via an endothelial to mesenchymal transition with endocardial cells converting into valve interstitial cells. aISV, arterial intersegmental vessel; DA, dorsal aorta; DLAV, dorsal longitudinal anastomatic vessel; HE, hemogenic endothelium; HSPC, hematopoietic stem and progenitor cell; PCV, posterior cardinal vein. Figures (a) and (d) were created using biorender.com.

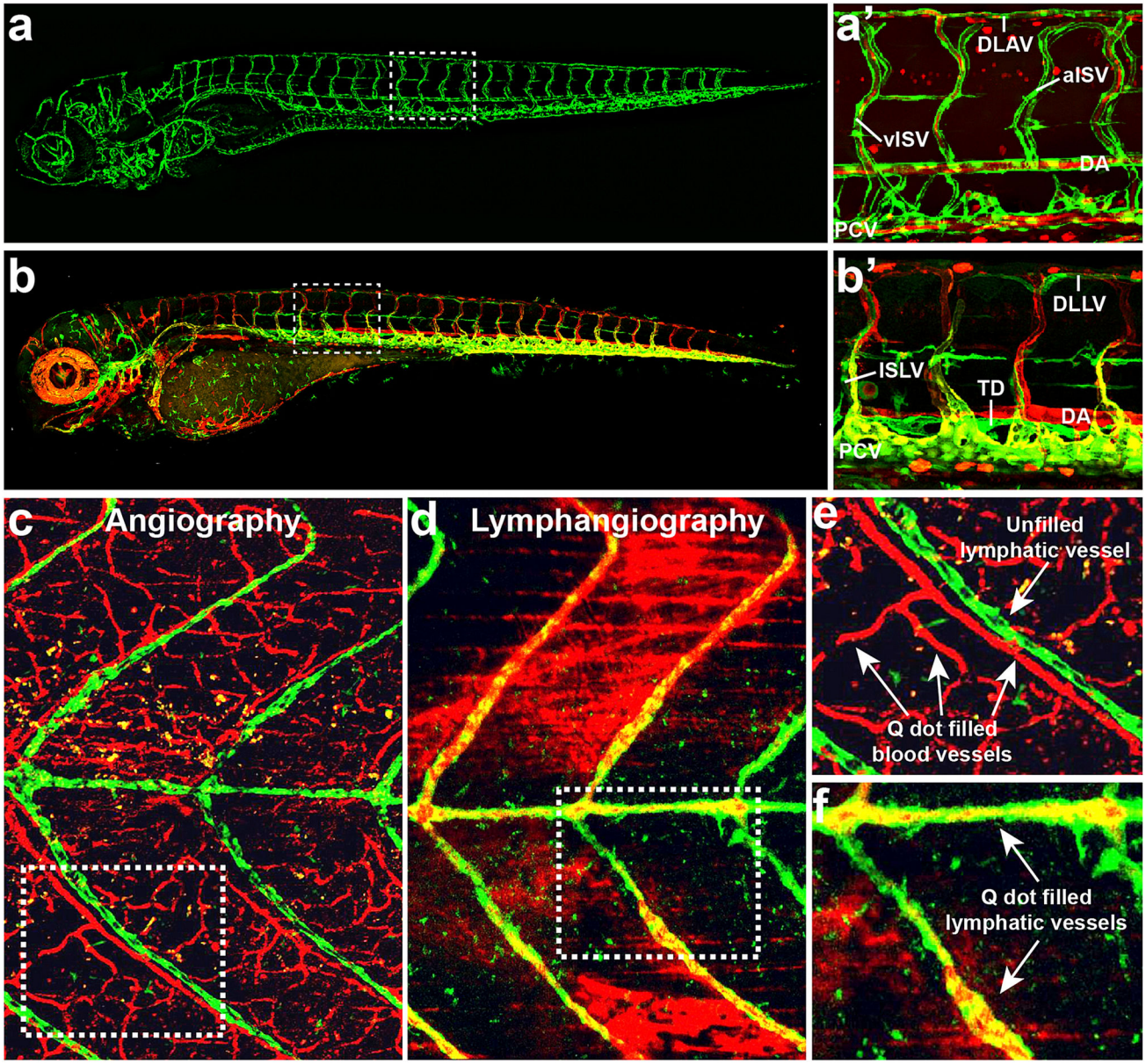

Fig 2:

Confocal images showing transgenic zebrafish with fluorescently "tagged" endothelial cells. (a) Fluorescent image of a (Tg(kdrl:eGFP), Tg(gata:dsRed)) transgenic zebrafish shows a 5 day post fertilization (dpf) larva whose blood vessels are labeled in green. (a') A close up of the trunk area (boxed in area from a) shows the major vessels within that region including the dorsal longitudinal anastomotic vessel (DLAV), dorsal aorta (DA), intersegmental arteries (aISV) and veins (vISV), and the posterior cardinal vein (PCV). Blood cells (red) can be seen within the vessels (green). (b) Fluorescent image of a (Tg(mrc1a:eGFP), Tg(kdrl:mcherry)) transgenic zebrafish depicts the arteries (red), veins (yellow), and lymphatic vessels (green) in a 5dpf larva. (b') A close up of the trunk area (boxed in area from b) shows the major blood vessels (DA, PCV) and lymphatic vessels within the region including the dorsal longitudinal lymphatic vessel (DLLV), intersegmental lymphatic vessel

(ISLV), and thoracic duct (TD). (c-f) Confocal images depicting angiography or lymphangiography of a 28dpf (Tg(mrc1a:eGFP) zebrafish whose lymphatics are labeled in green after either intracardiac injection of Qdot705 quantum dots to label blood vasculature (red) (c,e) or intramuscular injection of Qdot705 quantum dots into the tail to label the trunk lymphatic system (red) (d,f). Close up images of boxed in areas from c and d are shown in e and f, respectively. Panels (c-f) were reprinted with permission from Jung et al. 2017 [147].

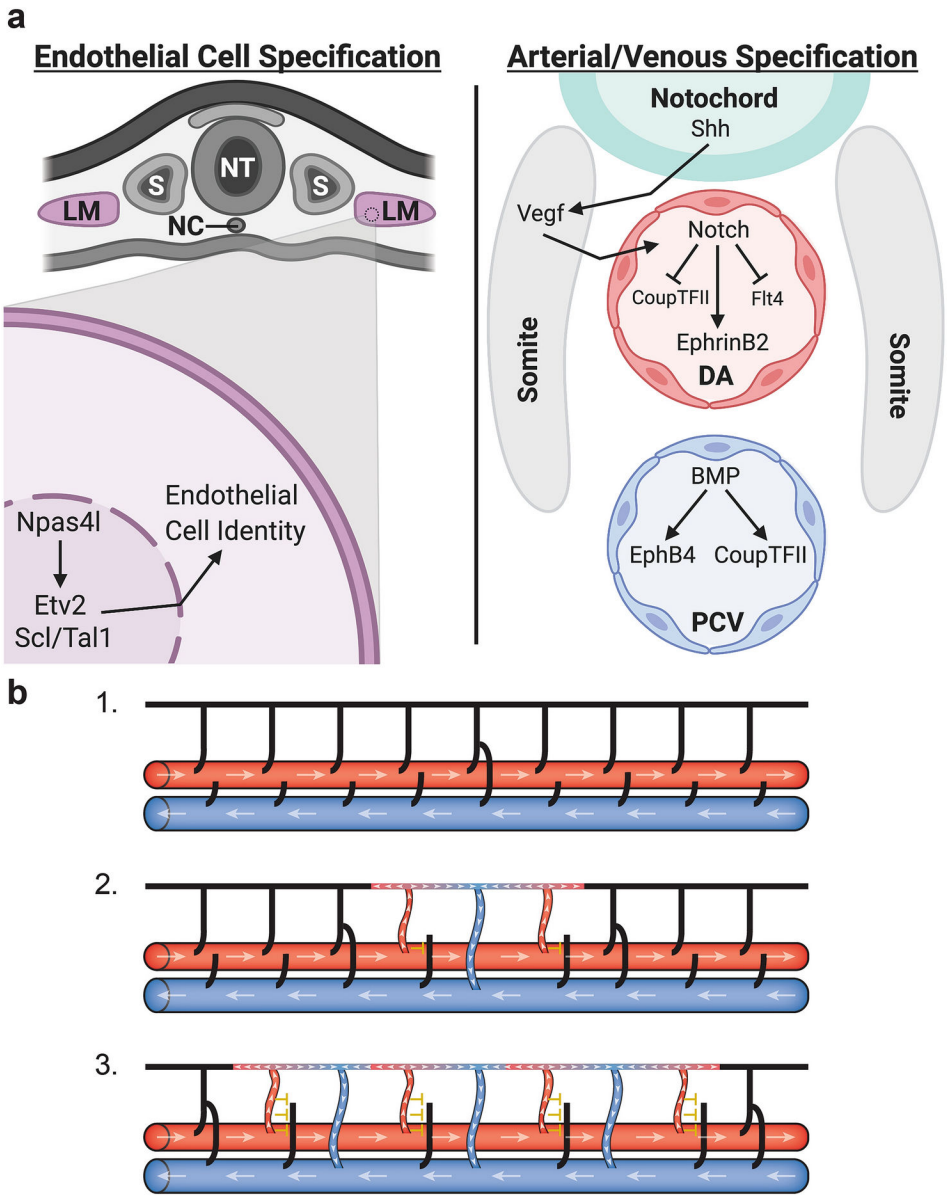

Fig 3:

Diagrams depicting the specification of arterial and venous identity through molecular mechanisms and hemodynamic forces. (a) Endothelial cells are first specified in the lateral mesoderm (LM); in zebrafish the master regulator Npas4l activates Etv2 and Scl/Tal1 to promote endothelial identity. Once specified, endothelial cells migrate out of the lateral mesoderm to form the major axial vessels - the dorsal aorta (DA) and the posterior cardinal vein (PCV). Sonic hedgehog (Shh) signaling from the notochord activates Vegf signaling in the somites which in turn activates Notch signaling in the DA leading to the expression of arterial genes and the inhibition of venous identity. In the PCV, BMP signaling promotes the expression of venous identity genes. (b) Hemodynamic forces help drive the formation of venous intersegmental vessels in the zebrafish trunk. Initially all intersegmental vessels lack flow (black) and are connected to the DA (large red horizontal vessel) (1). Once a secondary

sprout from the PCV (large blue horizontal vessel) connects to a primary sprout a circuit is created allowing blood flow (white arrowheads) to move up through adjacent primary sprouts (red) and return through the venous sprout (blue) (2). Blood flow through these primary sprouts prevents secondary sprouts from connecting (yellow inhibitory T) solidifying their arterial identity. Differences in flow then permit the next set of secondary sprouts to connect (3) leading to a largely alternating pattern of arteries and veins throughout the fish trunk and a close to 50:50 artery to vein ratio. Adapted from Isogai et al. 2003 [9]. DA, dorsal aorta; LM, lateral mesoderm, NC, notochord; NT, neural tube; PCV, posterior cardinal vein; S, somite. Figure (a) was created using biorender.com.

Table 1:

Selected Transgenic Zebrafish Lines for Endothelial Cell Lineage Tracing

Zebrafish Transgenic Line	Selected References
Endothelial cell driver	[148]
Tg(fli1ep:GAL4FF)$^{ubs2-4}$	[148]
TgBAC(cdh5: GAL4FF)^{mu101}	[149]
Tg(kdrl: Cre)^{s898}	[12]
Tg(flt4:Gal4FF)^{hu9236}	[150]
Hematopoietic stem cell driver	
TgBAC(gata2b:KalTA4)^{sd32}	[151]
Lymphatic cell driver and reporter	
Tg(prox1a^{BAC}:KalTA4–4xUAS-E1b:uncTagRFP)^{nim5}	[150]
Vascular specific photoconversion	
Tg(kdrl: Kaede)^{wz3}	[152]
Tg(flk1:Dendra2)	[153]
Gal4 driven photoconversion	
Tg(UAS:Kaede)^{rk8}	[154]
Cre driven permanent labeling	
Tg(βactin2:loxP-STOP-loxP-DsRed-express)^{sd5}	[12]
Tg(−3.5ubi:loxP-EGFP-loxP-mCherry)	[155]
Tg(ubi:Zebrabow)	[156]

Table 2:

Selected Zebrafish Disease Models to Study Endothelial Plasticity

Disease	Type of Model	Selected References
Arteriovenous Malformations (AVMs)/ Hereditary Hemorrhagic Telangiectasia (HHT)	Alk1 mutant	[157]
	Eng mutant	[158]
Cerebral Cavernous Malformations (CCM)	CCM1 (Krit1) mutant	[159]
	CCM2 (OSM) mutant	[159]
	CCM3 (PDCD10) morphant	[160]
Atherosclerosis	High Cholesterol Diet (HCD)	[161]
	Apoc2 mutant	[162]
	Ldlr mutant + HCD	[163]
Karposi's Sarcoma	Primary Effusion Lymphoma (PEL) Xenograft	[164]
Melanoma	p53 mutant with $BRAF^{V600E}$ expression in melanocytes	[165]
Pancreatic Cancer (exocrine)	Expression of $Kras^{G12V}$ in pancreatic progenitor cells	[166]
Pancreatic Cancer (endocrine)	Expression of MYCN in β cells	[167]