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Review

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Zebrafish Vascular Development: General and Tissue-Specific Regulation

Hiroyuki Nakajima, Ayano Chiba, Moe Fukumoto, Nanami Morooka, Naoki Mochizuki

Department of Cell Biology, National Cerebral and Cardiovascular Center Research Institute, Suita, Japan

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Correspondence to
Naoki Mochizuki
Department of Cell Biology, National Cerebral and Cardiovascular Center Research Institute, 6-1 Kishibe-Shimmachi, Suita 564-8565, Japan.
E-mail: mochizuki@ncvc.go.jp

ORCID iDs
Hiroyuki Nakajima
https://orcid.org/0000-0003-4139-2779
Ayano Chiba
https://orcid.org/0000-0003-2536-2449
Moe Fukumoto
https://orcid.org/0000-0001-8081-1353
Nanami Morooka
https://orcid.org/0000-0002-7223-8398
Naoki Mochizuki
https://orcid.org/0000-0002-3938-9602

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ABSTRACT

Circulation is required for the delivery of oxygen and nutrition to tissues and organs, as well as waste collection. Therefore, the heart and vessels develop first during embryogenesis. The circulatory system consists of the heart, blood vessels, and blood cells, which originate from the mesoderm. The gene expression pattern required for blood vessel development is predetermined by the hierarchical and sequential regulation of genes for the differentiation of mesodermal cells. Herein, we review how blood vessels form distinctly in different tissues or organs of zebrafish and how vessel formation is universally or tissue-specifically regulated by signal transduction pathways and blood flow. In addition, the unsolved issues of mutual contacts and interplay of circulatory organs during embryogenesis are discussed.

Keywords: Endothelial cell; Signal transduction; Zebrafish; 3-D imaging

INTRODUCTION

Blood vessels form a network to deliver blood cells and plasma to peripheral organs and tissues, through arteries and arterioles, and function as drainage system from peripheral organs and tissues, through capillaries, venules, and veins.[1,2] The initial blood vessel network is formed by endothelial cells (ECs). Subsequently, mural cells, including pericytes and smooth muscle cells, emerge close to the EC-derived network to support blood vessels.[3] Furthermore, the adventitia surrounds the smooth muscle cell layer in arteries.

EC precursor cells, or angioblasts, first assemble and differentiate into ECs to form a primary vascular plexus through a process called vasculogenesis.[4] Those primitive ECs then give rise to either arterial ECs or venous ECs. Two types of EC precursors appear in the trunk and in the tail in zebrafish (**Fig. 1**, unpublished data). These 2 types of ECs emerge distinctly from the lateral plate mesoderm and the tail bud.[5,6] There is another type of segregation: artery and vein. The primitive vascular cord is divided into the dorsal aorta (DA) primordium and cardinal vein primordium in the trunk vessel of zebrafish.[5,7] In addition, the other type of arterial EC differentiation from venous ECs is reported in the tail fin of zebrafish.[8] The differentiation of angioblasts into ECs and that of primitive ECs into veins/arteries are predetermined by the hierarchy of gene expression. The gene-encoded molecules determine the fate and character of ECs.

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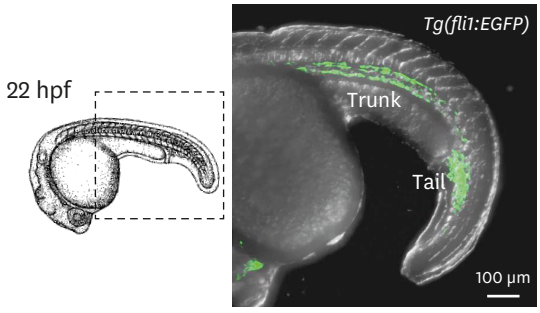

Fig. 1. Two types of emergence of endothelial precursors in the trunk and tail of the zebrafish embryo. A *Tg(fli1:EGFP)* embryo is imaged at 22 hpf. The endothelial cell precursors in the trunk and those in the tail appear simultaneously, but separately. The boxed region of the scheme of the embryo is imaged.
hpf, hours post-fertilization.

Zebrafish are widely used to clarify vascular development because of their translucency, the early growth of the circulatory system, their small size, and their extra-maternal growth. Importantly, many studies have shown that the morphological and molecular mechanisms involved in vascular development are conserved between zebrafish and humans.[9,10] These advantages enable developmental biologists to study circulatory system development using several kinds of live-imaging microscopes, including confocal, spinning disk, light-sheet, and super-resolution microscopes. In addition, multiple useful transgenic zebrafish lines for the fluorescent imaging of EC movement and signaling have been developed. These imaging techniques enable simultaneous investigations of the morphological changes and physiological functions of ECs using living embryos; these changes are triggered by EC-specific ligand/receptor-mediated intracellular signaling cascades. Thus, we review these EC-specific signaling and signal-dependent responses shown in zebrafish.

Recently, EC heterogeneity and organ/tissue-specific ECs have become a topic of interest in vascular biology and regenerative medicine.[11] For example, structural and cell biological differences are present among sinusoidal ECs, fenestrated ECs, and high ECs, which are found in the liver, endocrine organs, and lymph nodes, respectively.[12] Venous ECs and arterial ECs have specific characteristics in veins and arteries. During embryogenesis, tissue/organ-specific cell-cell interactions between parenchymal cells and ECs are regulated by angiocrine and paracrine mechanisms.[13] Moreover, the blood flow in lumenized blood vessels also affects the heterogeneity of ECs, indicating the importance of the environment of the surrounding blood vessels in addition to cell-autonomous regulation of ECs.

Beyond organ-specific blood vessel pathways, blood vessel formation follows a common pattern (i.e., angiogenesis). ECs sprout from pre-existing vessels; form branches by proliferation, migration, and intercellular adhesions; and thereby form new lumenized vessels.[14] Previous studies have tried to identify the molecular mechanisms underlying angiogenesis and maintenance/maturation in various tissues/organs.[15-18] However, some issues have yet to be fully clarified, including the origin of ECs, vascular niches, and tissue-specific mechanisms of angiogenesis.[19] We summarize the current understanding of vascular development and highlight the unanswered issues of vascular development in zebrafish.

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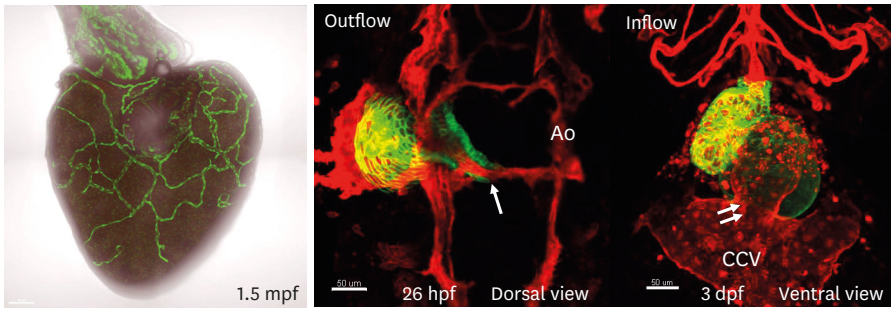
GENERAL CIRCULATORY SYSTEM

Circulation requires the heart, vessels, and blood as a pump, conduit, and carrier/content, respectively. The cardiomyocytes of the heart, ECs and mural cells of blood vessels, and blood cells are derived from mesodermal cells. The cooperative development of these 3 cell types efficiently and promptly starts the circulation to deliver oxygen and nourish parenchymal cell growth in the peripheral tissues and organs. At the very early stage of embryogenesis before the start of circulation, oxygen delivery depends upon diffusion.

The cooperation of blood cells and ECs is shown in hemogenic ECs, which are the origin of both hematopoietic stem and progenitor cells (HSPCs) and ECs.[20] Another aspect of the cooperation between these 2 cell types is that vascular ECs form a vascular niche for HSPCs in the caudal hematopoietic tissue (CHT) and the kidney marrow in zebrafish.[21] The molecular mechanism underlying the interaction of HSPCs and ECs has become a topic of interest in stem cell biology.

The coordinated growth of the myocardium and coronary vessels of the heart that supply oxygen to cardiomyocytes has been explored (**Fig. 2**, left). Endocardial ECs sprout and grow on the surface of the heart according to chemokine secretion from the myocardium.[22] Endocardial ECs also become the source of blood cells as the hemogenic endothelium in the heart. Following coronary vessel development, lymphatic vessels grow along pre-existing coronary vessels on the surface of the heart.[23] Of note, *cxcr4a* mutants, which lack coronary arteries, fail to exhibit lymphatic vessel growth. Therefore, the development of the myocardium, coronary vessels, and lymphatic vessels is regulated in a coordinated manner in the developing heart.

To pump out blood from the heart, the heart and great vessels must be connected (**Fig. 2**). However, it remains unclear how ECs connect to the endocardial cells of the heart (**Fig. 2**, right). The endocardial ECs of the outflow of the heart are likely to be connected to the ECs of the aortic arch, while those of the inflow tract are connected to the common cardinal vein (CCV). Although the migration and lumenization of ECs in the CCV have been investigated, as a process referred to as "lumen ensheathment,"[24] it remains elusive how endocardial ECs are connected to CCV ECs. These 2 connections allow the heart to pump out blood and establish the closed circulation in zebrafish.



Tg(fli1:EGFP) *Tg(fli1:myr-mC);(myl7:GFP-CAAX)*

Fig. 2. Vessels of the heart. The surface coronary vessels of a zebrafish heart at 1.5 mpf is visualized by fluorescence of *Tg(fli1:EGFP)* (left). The outflow tract (single arrow) and inflow tract (double arrows) of endocardial endothelial cells (red) are imaged with myocytes (green) using *Tg(fli1:myristoylated-mCherry);(myl7:GFP-tagged with CAAX motif of small GTPase Ras)* (right).
Ao, aortic root; CCV, common cardinal vein; mpf, months post-fertilization; hpf, hours post-fertilization; dpf, days post-fertilization.

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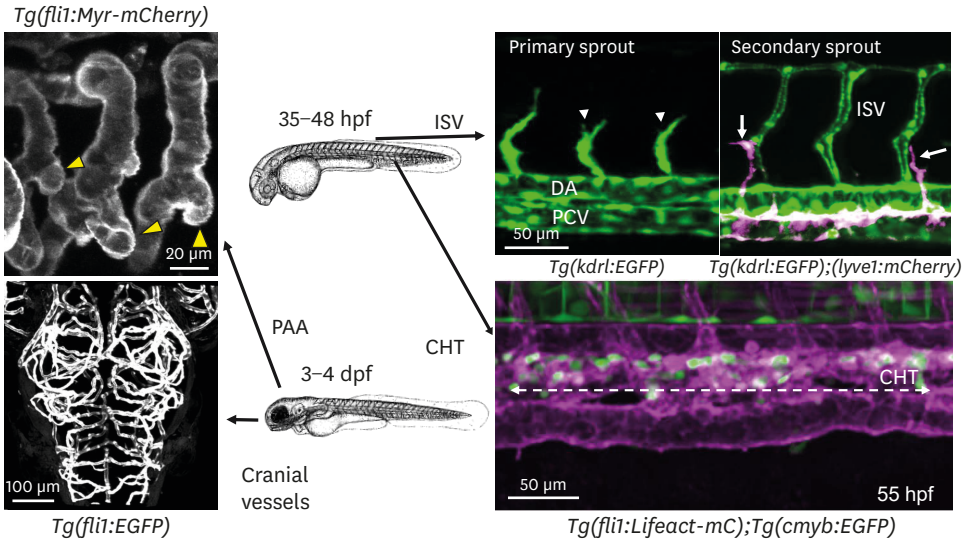

Fig. 3. Vessels in the brain, PAA, trunk, and CHT. The vessel images were obtained from the transgenic fish embryos indicated at the top or bottom of the panels.
Yellow arrowheads point to vessel protrusion. White arrowheads and white arrows indicate the primary sprouts from the DA and secondary sprouts from the PCV, respectively.
PAA, pharyngeal aortic arch; DA, dorsal aorta; PCV, posterior cardinal vein; ISV, inter-somitic vessels; CHT, caudal hematopoietic tissue; dpf, days post-fertilization; hpf, hours post-fertilization.

BLOOD VESSELS IN THE TRUNK, TAIL, AND BRAIN OF ZEBRAFISH

Previous studies mainly focused on the trunk blood vessels, such as the DA and posterior cardinal vein (PCV) (**Fig. 3**, top right). Angiogenic sprouting from the DA and PCV is categorized as primary sprouting and secondary sprouting.[10] The primary sprouts initially form inter-somitic vessels (ISVs) and the vessels that reach the most dorsal part form dorsal lateral anastomosis vessels (DLAVs). The secondary sprouts connecting to the preformed ISVs become venous ISVs (vISVs), whereas unconnected ISVs become arterial ISVs (aISVs). Thus, blood flow follows the path of DA-aISV-DLAV-vISV-PCV. The primary sprouts are mainly regulated by vascular endothelial growth factor (VEGF)-A and VEGF receptor (VEGFR) 2, while the secondary sprouts are promoted by VEGF-C and VEGFR3.[25-27]

The vessels in the posterior part (i.e., the caudal vessels) have also been characterized. The caudal vein plexus (CVP) changes its shape and differentiates into the caudal vein. ECs of the CVP proliferate in response to bone morphogenetic protein (Bmp).[28] We have demonstrated that Bmp-induced β-catenin-dependent signaling is activated in the ECs that preferentially differentiate into the caudal vein.[29] During somitogenesis, both arteries and veins need to extend posteriorly to oxygenate the posterior trunk and tail. We have previously shown that new ECs emerge in the tail during somitogenesis in addition to the proliferation of the pre-existing ECs that are located anteriorly.[30] Of particular note, the extension of the DA towards the tail depends upon the proliferation of the pre-existing ECs of the DA (unpublished data), whereas the origin of new venous ECs that contribute to the extension of trunk vein remains unclear. Interestingly, the CHT develops in parallel with venous development in the tail (**Fig. 3**, bottom right). Venous, but not arterial, ECs of the most dorsal CVP function as a vascular niche for HPSCs.[31] HPSCs that originate from the hemogenic ECs of the DA move to the CHT and finally migrate into the kidney marrow, which mirrors the function of the bone marrow in

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mammals, and remain there to produce various kinds of blood cells in juveniles.[21] Therefore, the transient vascular niche for HPSCs in the CHT consists of characteristic ECs that appear only for this period. The difference between these dorsal venous ECs and the ventral venous ECs of the CVP, which are β-catenin signaling-positive cells, is unclear. Therefore, the origin and function of these vascular niche ECs should be characterized in the future.

The blood vessels in the brain form a network to oxidize and nourish neural tissues. In the brain, a paired lateral DA is established and connected to the posterior DA.[32] During this period, the first primitive veins, the primordial hindbrain channels (PHBCs), develop. Following PHBC formation, the basilar artery and central arteries assemble in the hindbrain (**Fig. 3**, bottom left).[15,18] The blood-brain barrier (BBB) is a specific anatomical and functional structure between ECs and neurons that restricts the infiltration of inflammatory cells into the nervous system. In contrast, it efficiently exchanges molecules that are required for active metabolism in neurons and glial cells, including astrocytes, oligodendrocytes, and microglia. Astrocytes participate in forming the BBB, whereas other cells—including pericytes and capillary ECs—need to assemble together with astrocyte feet to form the BBB. We have previously shown that pericytes in the brain emerge after the establishment of the network of ECs.[33] The feet of pericytes seem to seal the junctions of ECs. However, we have not simultaneously monitored the assembly of ECs, pericytes, and astrocytes in the brain. Thus, more advanced *in vivo* imaging will help understand how the BBB functions to exchange molecules in the neural tissues.

In addition to exchanging molecules in the brain, the glymphatic system in the mouse has become an attractive focus of research into the clearance of wastes from the brain.[34] In zebrafish, waste from the central nervous system (CNS) can be removed through 2 pathways, the meningeal lymphatic vessels and the glymphatic system, which drains glial water and functions like the lymphatic system.[35] Upon injury of cerebrovascular system, lymphatic vessel invasion precedes the regeneration of blood vessels.[36] During the investigation of meningeal lymphatic vessels, mural lymphatic ECs (muLECs), also termed brain lymphatic ECs, were identified as a novel lymphatic cell type.[35,37] The muLECs express lymphatic markers, but do not form vessels. Therefore, the function of mural lymphatic vessels should be clarified in the clearance of waste products from the CNS.[38]

The aortic and pharyngeal arch artery (PAA) of zebrafish is unique, because zebrafish lack pulmonary circulation, which is required for mammals. The ECs of the PAA were shown to come from Nkx2.5 and Tcf21 double-positive mesodermal cells.[39,40] However, depletion of these progenitors of the pharyngeal arch does not result in deformation of the PAA, suggesting a compensatory function of ECs from other vasculature. The shape of the PAA is different from that found in sprouting angiogenesis, which is commonly regulated by VEGF-A. The branching or sprouting of pre-existing vessels is not observed during PAA formation, whereas protrusion of ECs is found (**Fig. 3**, top left, unpublished data). Edn1 and hand2 might be involved in the outgrowth of ECs to form a bulging protrusion of pre-existing vessels.[41]

ORGAN-SPECIFIC ECs: BLOOD VESSELS IN THE EYE, FIN, AND INTERNAL ORGANS OF ZEBRAFISH

The development of the eye requires 2 vascular systems: the intraocular hyaloid vessels and the superficial choroidal vessels.[42] The former vessels are transient and become retinal

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vessels. On the surface of the zebrafish eye, venous-derived superficial vessels develop prior to the formation of an elaborate network of choroidal vessels. Live imaging has revealed that ECs sprouting from the venous primordial midbrain channel form arteries through Notch signaling and chemokine signaling.[43]

Amputation and crush injury models have been developed to study fin regeneration.[8,44] Injury triggers tissue vascularization to oxygenate tissues. Like other organs and tissues, revascularization of injured bones has been investigated. In the caudal fin amputation model, the tip cells are derived from venous ECs and move backward to form the plexus. Therefore, the arteries of regenerated fins are derived from vein-derived tip cells. There is a striking difference between amputated and crushed fins. In the bone crush injury model, the main arteries in the intra-ray tissues are damaged. At a later stage, mis-patterned blood vessels are found in the crushed ray, probably due to the prolonged bone repair. These fin regeneration models may provide crucial insights into the relationship between tissue repair and vascular regeneration.

Internal organs such as the intestine, liver, and pancreas must be vascularized during development. The gut needs to efficiently absorb nutrition, which is transferred to the liver through the portal vein. The sinusoidal ECs have specific structures for nutrition absorption.[12] Moreover, as an endocrine organ, the pancreas has endocrine organ-specific capillary characteristics. In zebrafish, blood vessels in these organs originate from the floor of the PCV. The initial plexus that covers the internal organs is referred to as the sub-intestinal plexus.[17] Sub-intestinal vessel growth is first regulated by Bmp and secondarily by VEGF. Interestingly, the blood vessels in the internal organs are derived from venous ECs, like the superficial vessels of the eye and arteries of the fin after amputation.

ECs are required for parenchymal cell development and, conversely, parenchymal cells produce paracrine factors to stimulate maturation of the blood vessel network.[12] For example, tissue hypoxia induces angiogenesis by producing angiogenic signaling (VEGF). In turn, the tissue-specific paracrine system, from ECs to parenchymal cells, is important for organ and tissue development or maintenance. Therefore, this system is called angiocrine.[11] In zebrafish, ECs control hepatocyte polarization during liver development.[45] There are many angiocrine cues from ECs toward parenchymal cells in several organs.[11] For this reason, the importance of organotypic ECs has been highlighted in recent research.

ANGIOGENIC CUES AND FLOW-MEDIATED SIGNALING

Several conserved signals are mediated by ligand-receptor or mechano-transduction in ECs (**Fig. 4**). Some are commonly used beyond the vascular beds, while others are tissue-specifically or EC type-specifically regulated. In this section, we summarize those angiogenic and flow-mediated signals in zebrafish.

1. VEGF

Zebrafish have angiogenic signaling that is conserved in mammals.[38,46] VEGF is the common angiogenic cue during vascular development. Zebrafish have 4 VEGFR-related genes: *flt1*(VEGFR1), *kdr* (VEGFR2), *flt4* (VEGFR3), and *kdrl* (the fourth VEGFR), although mammals have 3 genes.[38] There are 3 agonists for VEGFRs; VEGF-A, VEGF-B, and VEGF-C. VEGF-A and its receptor Kdr- or Kdrl-mediated signaling is crucial for blood vessel formation in most vascular beds,[25] while VEGF-C and its receptor Flt4-mediated signaling is active during

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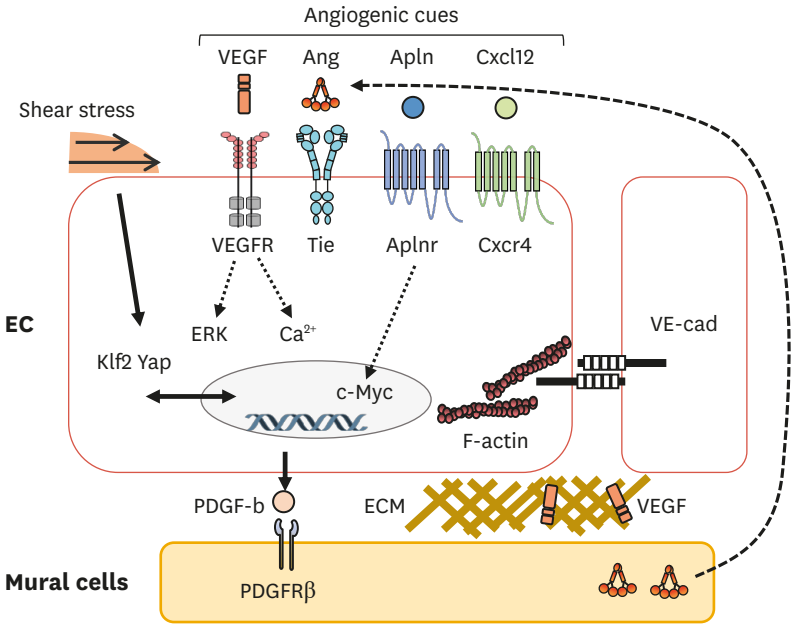

Fig. 4. The main signals that regulate vascular development and stabilization. These signals include tyrosine kinase-activating signaling (VEGF-VEGFR and Ang-Tie) and GPCR-mediated signaling (Apln-Aplnr and Cxcl12–Cxcr4). In addition to these receptor-mediated signaling pathways, mechanosignaling induced by blood flow (shear stress) also regulates the intracellular signaling of ECs, including Klf2 expression and nuclear translocation of Yap. PDGF-b released from ECs activates its PDGFR expressed on mural cells. Of note, Ang1 is released from mural cells to activate Tie2 expressed on ECs to regulate mutual interactions between ECs and mural cells. VE-cadherin is an endothelial-specific cadherin that is essential for adherens junction formation. Adherens junctions are strengthened by the actin cytoskeleton to maintain tissue integrity. The ECM can activate various signaling pathways directly to regulate vascular development.
VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; Ang, angiopoietin; Apln, apelin; Aplnr, apelin receptor; EC, endothelial cell; VE, vascular endothelial; ECM, extracellular matrix; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; GPCR, G protein-coupled receptor.

lymphangiogenesis.[27] The former is observed in primary sprouts from the DA, whereas the latter is found in secondary sprouts from the PCV. The expression of VEGFs and their receptors is spatially close to each other. In ISV sprouts, VEGF-A and VEGF-C are found in the notochord and DA.[32,47] VEGF-VEGFR signaling depends upon the tyrosine kinase activity of VEGFR. The downstream signaling of VEGFR has been extensively investigated to understand the physiological contribution of VEGF-dependent signals to angiogenesis. EC proliferation and migration are regulated by NFAT and ERK downstream of VEGFR2, while cell survival is controlled by PI3K-Akt-FoxO.[48]

2. Angiopoietin (Ang)

Another family of tyrosine kinase receptors, Tie1 and Tie2, are required for vascular remodeling, maturation, and lymphangiogenesis.[49] Ang1, Ang2a, and Ang2b are ligands for Tie1 and Tie2 in zebrafish. In mammals, Tie2 is thought to play a central role in EC biology, because cultured ECs expressing Tie2 respond to Ang1 and thereby exhibit distinct cell behaviors: EC migration or quiescence.[50,51] In contrast, *tie2* mutant zebrafish do not exhibit any abnormal phenotypes. The group of Stainier has extensively studied Tie2 in zebrafish and found that no mutants of *tie2* show any morphological deficiencies or growth defects.[52] Therefore, depletion of *tie1* is required to understand the roles of Ang-Tie signaling in zebrafish.

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3. Chemokines (Cxcl12–Cxcr4)

Chemokines contribute to cell migration in a tissue-specific manner. In mice, depletion of either *cxcl12* or its receptor *cxcr4* (a G protein-coupled receptor [GPCR]) results in impaired vascular development, especially in the gastrointestinal tract and kidney.[53,54] In zebrafish, Cxcl12b and Cxcr4a function to develop the initial network of brain vasculature, consisting of the PHBC and central artery.[18] Additionally, *cxcr4a* mutants exhibit impairment of coronary vessel growth.[22] Indeed, Cxcr4a is expressed on the ECs of coronary vessels. Chemokine-like factor superfamily MARVEL transmembrane 3 (CMTM3) is involved in the regulation of vascular endothelial cadherin (VE-cadherin)-dependent adherens junction. Silencing of CMTM3 using a morpholino in zebrafish impairs ISV formation.[55] In the fin, Cxcl12b functions as a blood vessel regulator during regeneration.[8] Cxcl12b is expressed in mesenchymal cells in the fin and promotes the growth of blood vessels.[56] Furthermore, lymphatic vessel growth along the aISVs is inhibited in either *cxcl12b* or *cxcr4a* mutants.[57]

4. Apelin (Apln)

Apln is a secretory peptide that acts on a GPCR, Apln receptor (Aplnr). Elabela (Ela) and Toddler, as well as Apln, activate Aplnr.[58] Using zebrafish, a gene knockout study elegantly uncovered the specific roles of Apln family members.[59] During the initial vasculogenesis, Ela guides angioblasts to the midline to form the axial vessel.[60] Then, Apln is required for angiogenic sprouting from the DA and subsequently for the leader cells to become tip cells, as evidenced by *alpn* or *aplnra/aplnrb* mutants. The essential roles of Aplnr-expressing ECs were confirmed by a cell transplantation assay. In addition to this study, the requirement of Apln-Aplnr signaling for lymphatic vessels has been demonstrated in *alpn* or *aplnr* morphants.[61] During fin regeneration, Apln is required for angiogenesis.[62]

5. Bmp

Both in mice and zebrafish, Bmp signaling controls venous identity via the Alk3 receptor and Smad1/Smad5.[63] Bmp-dependent venous differentiation and angiogenesis have been shown in the zebrafish caudal vein.[28,29] Alk1 or Alk2 depletion in mice suggests the pro- or -anti-angiogenic action of BMPs because of the combination of downstream transcription factors (Smads). Hereditary hemorrhagic telangiectasia is caused by mutations in various genes, including those encoding transforming growth factor-beta and its receptors. The loss of Alk1 has been investigated in zebrafish. While arterial EC proliferation is normal, the EC movement of lumenized vessels is altered in the brain.[64] During network formation in the brain, blood flow affects the maturation of vessels. Therefore, Alk1-dependent signaling for vessel formation functions together with blood flow-dependent signaling. Consistently, mutants of the Alk1 ligands, *bmp9* and *bmp10*, exhibit impaired vascular development.[65] Another study has demonstrated the importance of Bmp-mediated signaling in arteriovenous differentiation. Bmp endothelial precursor-derived regulator (BMPER) regulates the expression of an arterial marker (Ephrin-B2) and a venous marker (EphB4), as evidenced in *bmper* morphants.[66]

6. Ephrin/Eph

In addition to Bmp, arteriovenous fate is determined by the expression of Ephrin-B2 and EphB4. The repulsive action between Ephrin-B2 and EphB4 demarcates arteries and veins through bidirectional signaling. In zebrafish, the demarcation of the DA and PCV has been clearly revealed by high-resolution imaging.[7] Silencing of EphB4 using a morpholino in zebrafish resulted in defects of brain vessel formation,[67] and abnormal growth of caudal vessels was observed in *ephb4* morphants.[68]

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7. Extracellular matrix (ECM)

ECM molecules function as a scaffold of blood vessel growth. In zebrafish, Col1a2 and Col5a1 are expressed by fibroblasts along ECs of the ISVs. Gene silencing of *col5a1* resulted in hemorrhage in the trunk and irregular ISVs.[69] Upon binding to ECM, integrins activate intracellular signaling in ECs. Filopodia formation is consistently suppressed in *integrin b1b* mutants. The importance of integrin-dependent signaling has also been confirmed by the overexpressed dominant-negative form of integrin.[70] The adaptor molecule of integrin, Shc, has also been reported to be essential for ISV development.[71] Moreover, ECM binds to VEGF-A to guide angiogenesis. Synaptic proteins (b-neurexin and neuroligin) synergistically promote the CVP and subsequent sub-intestinal vessel growth with ECM-bound VEGF-A.[72] Epidermal growth factor-like domain 7 (Egfl7) is a secretory molecule highly expressed in ECs that binds to the ECM to regulate angiogenesis.[73] In zebrafish, Egfl7 is required for the establishment of the vascular lumen, likely by providing a permissive environment.[74] For dynamic remodeling of vessels during embryogenesis, the ECM must be reshaped to guide blood vessels. To this end, matrix metalloproteinase-2 regulates the deposition of the ECM. Lymphatic vessel development is consistently impaired in *mmp2* morphants.[75]

8. Blood flow

Blood flow regulates the development, remodeling, and stabilization of vascular networks in various tissues. ECs sense the flow as a force, although the identification of the mechanosensor is still undergoing. A mechanosensitive cationic channel, Piezo1, has been characterized as a sensor that directly responds to flow.[76] A mechanosensory complex at cell-cell junctions, consisting of VE-cadherin, PECAM-1, and VEGFR2, is thought to be a candidate mechanosensor.[77,78] Recently, plexin D1 has been characterized as a sensor for the mechanosensory complex.[79] Integrin-dependent signaling also functions as mechanosignaling.[80] The requirement of flow for vascular development has been reported by several groups using zebrafish. During the initial sprout of ISVs from the DA, flow-mediated pressure has been shown to be important for lumen formation.[81] In addition, blood flow facilitates artery-vein specification in the ISVs by regulating Notch signaling.[82] During this arterial and venous EC switch, the direction of flow determines the fate of ECs. In the DA, ventral extrusion of ECs is also negatively regulated by flow and cyclic stretching induced by heartbeats.[83] A transcription factor, Klf2, is downstream of the flow-dependent signaling cascade and regulates vascular stabilization of ECs through nitric oxide synthetase expression.[84] We have shown that a transcription cofactor, YAP, translocates into the nucleus in response to flow to stabilize the vessels in a tissue-specific manner in zebrafish.[85]

9. Models of diseases to understand pathophysiology

Zebrafish can be used for understanding cardiovascular diseases. Cranial hemorrhage is a common disease in developed countries, and hereditary cerebral cavernous malformation (CCM) has been molecularly and genetically investigated. The mutations found in CCM patients are Ccm1/Krit1, Ccm2/malcavernin and Ccm3/programmed cell death 10. These molecules assemble with heart of glass (Heg1), which is a single membrane-spanning molecule. In zebrafish, either *heg1* or *ccm2* morphants can form the normal patterned trunk vessels, but lack a lumen.[86] Krit1 is a binding partner of a small GTPase, Rap1, which is known to stabilize inter-EC-cell junctions. Consistent with Rap1-dependent cell-cell adhesion, *rap1* morphants exhibit abnormal hemorrhage in the brain.[87] CCM deficiency alters endocardial and endothelial gene expression (*klf2* and *klf4*).[88] Of note, blood flow inhibits vascular defects in the CCM zebrafish model, suggesting that blood flow-dependent signaling affects CCM-dependent signaling in zebrafish.[89]

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Importantly, most human genes related to cardiovascular diseases have zebrafish orthologs.[90] Therefore, as described above, analyses of disease-related genes using zebrafish have led to insights into the molecular mechanisms of cardiovascular diseases. Because sequence conservation between zebrafish and humans is relatively high, many drugs that target human cardiovascular diseases have similar pharmacological impacts on zebrafish.[90] In fact, several small molecules identified using zebrafish are now in the clinical trial phase.[91,92] Zebrafish are becoming a useful model in the field of cardiovascular diseases.

CONCLUSION

The usefulness of zebrafish when studying embryogenesis has been widely accepted in vascular biology. Herein, we summarize many reports demonstrating the essential roles of molecular pathways involved in general or tissue-specific vascular development. We emphasize again the important point that using zebrafish embryos enables us to visualize morphological alterations and cell signaling simultaneously. Molecular probes can be easily introduced into the genome, enabling the establishment of useful transgenic zebrafish. In addition, recent advancement of gene manipulation using TALEN or CRISPR/Cas9 systems has allowed researchers to test requirements and compensatory mechanisms in a tissue- or cell-type-specific manner. Using this excellent model system, we can continue to investigate how creatures live from fertilization to the end of life under conditions of environmental changes.

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