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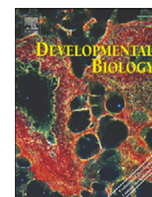

Review

Vascular morphogenesis in the zebrafish embryo

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ABSTRACT

During embryonic development, the vertebrate vasculature is undergoing vast growth and remodeling. Blood vessels can be formed by a wide spectrum of different morphogenetic mechanisms, such as budding, cord hollowing, cell hollowing, cell wrapping and intussusception. Here, we describe the vascular morphogenesis that occurs in the early zebrafish embryo. We discuss the diversity of morphogenetic mechanisms that contribute to vessel assembly, angiogenic sprouting and tube formation in different blood vessels and how some of these complex cell behaviors are regulated by molecular pathways.

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Introduction

Branched tubular organs, such as the insect tracheal system or the vertebrate cardiovascular system, kidney or lung, are found throughout the animal kingdom. Formation of such tubular networks from precursor cells or tissues involves a variety of morphogenetic processes, such as tube formation, elongation, branching and fusion. These processes are brought about by complex cellular behaviors, which include cell polarization, cell migration, cell rearrangements, cell shape changes and cell division. Although tubular organs are extremely diverse in anatomy and function, the cellular activities that govern tube formation and branching morphogenesis appear to be quite similar (Baer et al., 2009; Andrew and Ewald, 2010). In this review, we describe the current understanding of blood vessel formation in the early zebrafish embryo. We are placing special emphasis on the morphogenetic processes that contribute to vascular development and discuss the regulatory components that accompany these events.

In vertebrates, the cardiovascular system constitutes a highly ramified network of tubes that transports gas, nutrients, hormones and metabolites throughout the body. It also has important roles in the regulation of homeostasis and wound healing and is involved in the pathology of numerous diseases including cancer and inflammation (Carmeliet, 2003). The cardiovascular system emerges as one of the first organs during embryonic development and retains morphogenetic plasticity in adult life. Blood vessels are an integral component of all organs and are vital not only for their function but also for their formation during embryonic development (Nikolova and Lammert, 2003; Red-Horse et al., 2007; Sakaguchi et al., 2008). Blood vessels are highly diverse: they differ in size and are specialized depending on their function and the tissue or organ they are embedded in (Aird, 2007; Rocha and Adams, 2009). In general, they consist of an inner epithelium (endothelium) lining the lumen; depending on the type of vessel, this endothelium is surrounded by a basal lamina and by mural cells, such as pericytes and smooth muscle cells, which both support and regulate the function of the endothelium (Armulik et al., 2005).

Over the last decade, the molecular pathways controlling vascular development have attracted much attention, and a large number of key molecules has been identified that regulate different aspects of blood vessel morphogenesis. The basic frameworks of the vascular anatomy are conserved among vertebrates, which makes it possible to assign homologies between distinct blood vessels and to directly compare the formation of these vessels in different vertebrate species (Isogai et al., 2001; see Fig. 1). The zebrafish embryo has proven to be a useful model to study vascular morphogenesis in vivo. The vasculature can be easily visualized using a variety of labeling techniques, such as endothelial specific expression of fluorescent protein or by microangiography (Fig. 1). Its small size, experimental accessibility, optical clarity and rapid development allow to observe cellular activities, such as cell migration, cellular rearrangements and cell divisions, as they occur during blood vessel formation in the embryo. It is also possible to follow cardiovascular mutant phenotypes for several days because oxygenation of the early zebrafish embryo does

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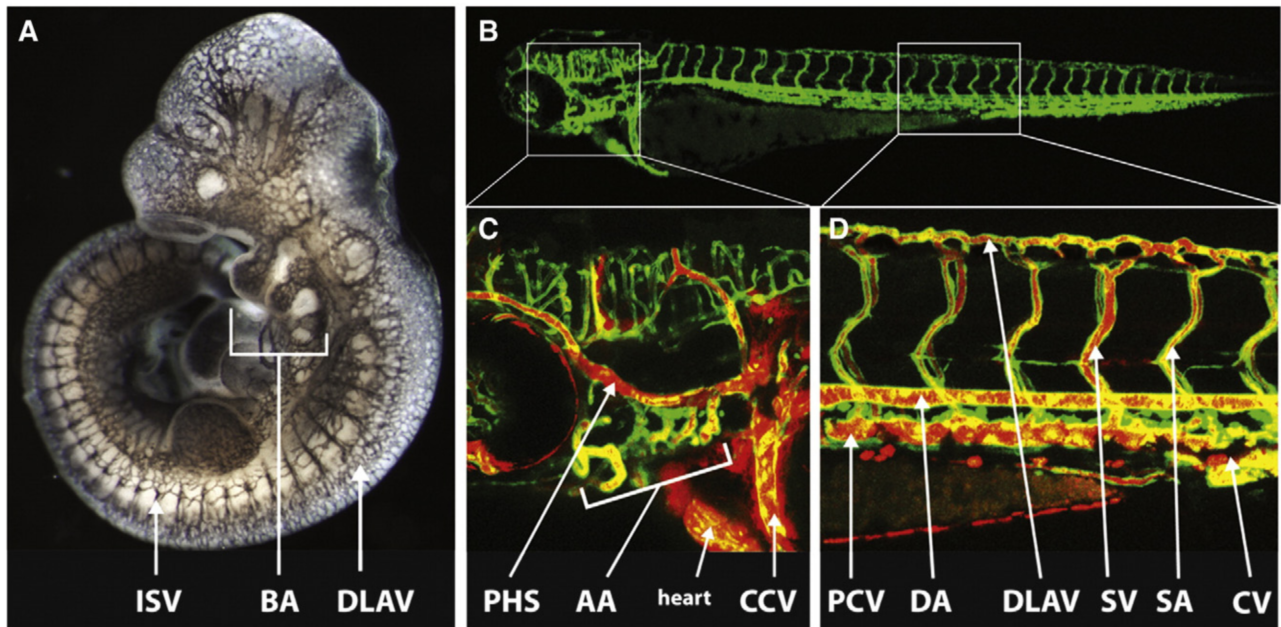

Fig. 1. The vascular system in mouse and fish embryos. (A) Visualization of the vascular system by immunohistochemical localization of PECAM-1 in a day 10 mouse embryo (photo courtesy of Ralf Adams, MPI, Münster, Germany). Owing to the opacity of the mouse embryo, only superficial blood vessels can be seen. BA: branchial arches (1st and 2nd); ISV: intersegmental vessel; DLAV: dorsal longitudinal anastomotic vessel. (B–D) The vascular system in a 3-day-old zebrafish embryo visualized by reporter gene analysis (*TG:flk1:EGFP* in green) and by microangiography using quantum dots (red in panels C and D). Some blood vessels are indicated according to Isogai et al. (2001). AA: aortic arches (1–6); CV: caudal vein; CCV: common cardinal vein; DA: dorsal aorta; PCV: posterior cardinal vein; PHS: primary head sinus; SA: segmental artery; SV segmental vein. At these stages, anatomical similarities between the two species are best observed in the branchial arches and in the ISV of the trunk. ISV and DLAV form quite similarly in both species (Isogai et al., 2003; Walls et al., 2008).

not rely on blood circulation. Furthermore, functional studies by forward and reverse genetics have shown that the molecular components that regulate vascular development are conserved between mammals and fish (Beis and Stainier, 2006; Lawson and Weinstein, 2002b; Thisse, 2002). Thus, the zebrafish embryo presents a unique system in which live imaging can be combined with functional studies to gain a more complete insight into how the molecular and morphogenetic mechanisms are integrated at the (sub)cellular level to shape the vascular tree.

Vasculogenesis

The formation of vertebrate blood vessels is commonly subdivided into two distinct morphogenetic processes, called vasculogenesis and angiogenesis. Vasculogenesis is defined by in situ aggregation of angioblasts into a blood vessel (Coffin and Poole, 1988; Poole and Coffin, 1989; Risau, 1995; Risau et al., 1988), while further sprouting of vessels from existing vessels occurs via a process called angiogenesis (Risau, 1995).

Origin and specification of endothelial cells

Angioblasts are precursors of endothelial cells not yet incorporated into blood vessels. They originate from the ventrolateral mesoderm (Kimmel et al., 1995; Stainier et al., 1995). Analyses of genes expressed in the hematopoietic and endothelial cell lineages have revealed a remarkable conservation between vertebrate species. In particular, transcription factors belonging to the ETS, GATA and LMO families have been shown to control specification of these lineages in mammals as well as fish (De Val et al., 2008; Detrich et al., 1995; Liu and Patient, 2008; Thompson et al., 1998; Zon et al., 1991). At the beginning of somitogenesis, transcription factors, such as *scl*/*tal1* and *lmo2*, which specify angioblasts and hematopoietic cells, are expressed in two domains along the body axis, the anterior and the posterior lateral mesoderm (Dooley et al., 2005; Liao et al., 1998; Patterson et al., 2007). During somitogenesis these cell populations acquire unique gene expression profiles. For example, *flk1*-positive/*scl*-positive precursor cells differentiate into *flk1*-positive/scl-negative and *flk1*-negative/*scl*-positive cells, which will give rise to endothelial and hematopoietic cells, respectively (Gering et al., 1998). There seems to be no transcriptional factor regulating exclusively the endothelial specification but a combination of multiple factors with overlapping expression patterns (reviewed by De Val and Black, 2009).

Formation of the dorsal aorta and the cardinal vein

The basic anatomy of the initial embryonic circulatory system is quite similar among vertebrates. In addition, the first embryonic vessels to appear, the dorsal aorta (DA) and the posterior cardinal vein (PCV), are formed by a distinct morphogenetic mechanism called vasculogenesis in all vertebrates (Isogai et al., 2001). In zebrafish, angioblasts are specified well before the first blood vessels are formed. Expression of molecular markers such as *fli1a* shows that angioblasts are located in two lateral stripes at 12–14 hpf. By 28–30 hpf, the DA and the PVC can be discerned and are fully lumenized (Roman et al., 2002). In vivo imaging, using a *Tg(fli1a:EGFP)* reporter fish line, has shown that angioblasts migrate as individual cells towards the embryonic midline where they coalesce (Lawson and Weinstein, 2002b). During recent years, a considerable amount of research has focused on how this migration process is regulated, how these cells form the axial vessels and how DA and PCV are specified. As indicated in Fig. 2, the PCV forms subsequently to the DA (Eriksson and Löfberg, 2000; Herbert et al., 2009; Jin et al., 2005), and this relationship appears to be conserved among vertebrates (Coffin and Poole, 1988; Hirakow and Hiruma, 1981; Meier, 1980).

Formation of the DA in zebrafish has been studied by transmission electron microscopy (TEM) (Eriksson and Löfberg, 2000; Meier, 1980) and more recently by analysis of transgenic zebrafish embryos (Herbert et al., 2009; Jin et al., 2005; Lawson and Weinstein, 2002b). During vasculogenesis, angioblasts are attracted towards the midline by guidance cues thought to emanate from the endoderm

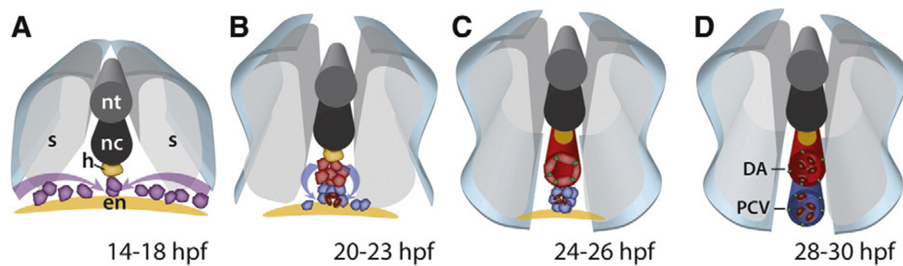

Fig. 2. Phases of vasculogenesis in the zebrafish embryo. Schematic cross sections of the trunk region at representative stages of development (according to Herbert et al., 2009 and Jin et al., 2005). (A) Medial migration. From 14 hpf onward, angioblasts (purple) that originate in the lateral plate mesoderm migrate over the endoderm towards the midline just below the hypochord, where they aggregate to form a vascular cord (B). (B) Arterio-venous segregation and ventral sprouting. At around 17 hpf, angioblasts start to express markers of arterio-venous differentiation, such as *ephrin-b2a* in arterial cells (marked in red). These cells are located in the dorsal portion of the vascular rod and will give rise to the DA, whereas *ephb4a* expressing cells are located more ventrally and will contribute to the PCV and CV. At 21 hpf, angioblasts located in the ventral part of the vascular cord start migrating ventrally and accumulate below the forming DA (B, C). (C) Lumen formation. The DA forms and lumenizes prior to the PCV and CV in the absence of blood cells (brown) by cord hollowing. Venous angioblasts aggregate and coalesce around the blood cells to ultimately form a tube. (D) Functional Vasculature. At 30 hpf, both vessels are fully formed and carry blood flow. Endothelial cell junctions are indicated in green.

(Jin et al., 2005; Fig. 2A). Once the angioblasts have reached the embryonic midline, they form aggregates and tube formation commences (Fig. 2B). TEM studies have shown that angioblasts initially form "aggregates of tightly packed cells" between hypochord and the underlying mesoderm (Eriksson and Löfberg, 2000). These aggregates are discontinuous along the anterior–posterior axis and the cells are spherical at the beginning of the process. At around the 17-somite stage (17.5 hpf), more flattened tube forming cells are found posterior to the 7th–9th somite in the fish embryo. From analysis of transgenic zebrafish, it has been suggested that endothelial cells migrate in two waves to the midline and it has been suggested that the first wave contributes to the DA while the cells of the second wave will form the primary vein (Jin et al., 2005). Alternatively, endothelial cells from both migratory waves may join in a single medial cord and segregate independently from this structure. The latter possibility has recently gained strong support from in vivo time-lapse analyses, which showed that the precursor cells of the caudal vein dissociate from the primordium of the DA by a process termed ventral sprouting (Herbert et al., 2009). Ventral sprouting is initiated around 20 h post-fertilization shortly before the emergence of dorsal sprouts, which will give rise to segmental arteries (SA) (see below) at a time when the DA is not yet lumenized. This finding together with the observation that the expression of the arterial marker *ephrinb2a* (*efnb2a*) is restricted to a subpopulation within the vascular cord suggested that the primary arteries and veins are derived from a common primordium that contains a mixed population of arterial as well as venous angioblasts (Herbert et al., 2009; Jin et al., 2005).

Specification of arterial versus venous fates has been shown to depend on the interaction of the VEGF and Notch signaling pathways (reviewed by Lawson and Weinstein, 2002a; Siekmann et al., 2008). Sonic hedgehog (SHH) signals from the notochord lead to an activation of *vegf-a* expression in ventral somites (Lawson et al., 2002). VEGF-A is sensed by the angioblasts via VEGF-receptor-2/KDR/FLK1 (KDR-Like/KDRL in zebrafish), which leads to the activation of Notch signaling and the transcriptional activation of other factors which results in arterial differentiation in a subset of angioblasts (Cermenati et al., 2008; Lawson et al., 2003; Pendeville et al., 2008; Zhong et al., 2001). In contrast to arterial development, specification of venous fates is independent of VEGF-A signaling (Covassin et al., 2006; Lawson et al., 2003). In agreement with these concepts, modification of VEGF or Notch signaling levels influences the segregation and ventral sprouting behavior. Angioblasts showed excessive ventral migration, when VEGF-A and Notch signals were blocked, whereas downregulation of FLT4 led to a reduction in ventral sprouting (Herbert et al., 2009).

Arterial and venous specification of angioblasts is reflected by particular gene expressions (Lawson and Weinstein, 2002a). Notably, two members of the Eph-ephrin subclass of the receptor tyrosine kinase family are differentially expressed in arteries (EphrinB2/*Efnb2*) and veins (*EphB4*) (Adams et al., 1999; Lawson et al., 2001). Genetic analyses in mouse have demonstrated an important role for bidirectional Ephb4-Efnb2 signaling for vascular morphogenesis (reviewed by Adams and Alitalo, 2007). In mouse and fish, EPHB4-Efnb2 signaling has been shown to be involved in sorting of neuronal cells, which leads to their segregation into adjacent hindbrain segments (Kemp et al., 2009; Mellitzer et al., 1999). To test whether these factors also play a role in the segregation of arterial and venous angioblasts in the primary vascular cord, Herbert and colleagues (2009) modified EPHB4a and EfnB2a levels and interfered with forward and reverse properties of EPHB4a-EfnB2a signaling. Either overexpression or knockdown of EPHB4a/Efnb2a function caused aberrant migration of transplanted angioblasts consistent with defects in arterio-venous segregation. Taken together, these findings show that repulsive EPH4a-EfnB2 signaling regulates arterio-venous segregation, thereby controlling the directionality of angioblast sprouting.

Anterior–posterior differences in artery formation

While the process of vasculogenesis has been best described in the dorsal aorta of the trunk, there is increasing evidence of regional differences in the way the primary vessels form and it has been proposed that distinct cues guide endothelial cells in different domains of the body (Coffin and Poole, 1991; Eriksson and Löfberg, 2000). In agreement with this view, several zebrafish mutants have been isolated that exhibit vascular defects in particular regions of the body (Jin et al., 2007).

Some experiments have shed light on the differences that regulate the formation of the DA of the trunk and the paired lateral dorsal aortae (LDA), which is located in anterior body regions. In an earlier study, the role of the endoderm for formation of the DA was examined in *casanova* (*sox32*) mutants that lack endoderm (Jin et al., 2005). In these embryos, medial migration of angioblasts was slowed but the DA formed normally, suggesting that endoderm is dispensable for DA formation. However, it has more recently been shown that the endoderm plays an essential role for LDA formation in the anterior region of the embryo (Siekmann et al., 2009). Strikingly, mutants for the chemokine receptor *cxcr4a*, which is expressed in the LDA, lack the LDA. CXCR4 is known to bind to CXCL12, which is specifically expressed in the anterior endoderm underlying the developing LDA. Furthermore, loss of CXCL12 function phenocopies the *cxcr4a* deficiency. These findings illustrate the molecular diversity in endothelial cells and the importance of local extrinsic cues for the formation and patterning of the primary aorta.

Transforming a cord into a tube: lumen formation in the primary blood vessels

The morphogenesis of biological tubes has been a longstanding interest in developmental biology and it has been shown that tubes can form in very different ways (Baer et al., 2009; Lubarsky and Krasnow, 2003). After angioblasts have aggregated into a cord-like structure, they ultimately have to assemble a tube. This could in principal occur by different morphogenetic processes, including (i) cell hollowing, where cells form vacuoles that fuse between cells to form a continuous intracellular lumen; (ii) wrapping, where cells migrate in a polarized state and surround the future lumen; (iii) cord hollowing, where cells within the cord attain apical–basal polarity and the lumen is formed by membrane separation and fluid influx; or by (iv) cavitation, where cells in the middle of the rod undergo apoptosis leaving a luminal space behind (Hogan and Kolodziej, 2002; Kucera et al., 2007; Lubarsky and Krasnow, 2003).

Tube formation of the DA has been studied at the cellular level by transgenic and immunofluorescent analyses in zebrafish embryos (Jin et al., 2005). Shortly after angioblasts have formed a cord, the DA begins to lumenize (21 hpf; Figs. 2B, C). Analysis of proteins involved in apical–basal polarization, such as fibronectin or β-catenin, has shown that this process is preceded by endothelial polarization and the formation of junctions between ECs, suggesting that a cord hollowing process forms the lumen of the DA. At the 20-somite stage (19 hpf), cell junctions, as visualized by ZO-1 and Claudin5, are discernable between the cells forming the dorsal aorta (Jin et al., 2005). The mechanisms of lumen formation in the zebrafish dorsal aorta and caudal vein have recently been examined by in vivo time-lapse analyses (Herbert et al., 2009). These studies confirmed a cord hollowing mechanism in the DA, whereas the lumen of the CV is formed by ventrally sprouting venous angioblasts that coalesce around resident blood cells. This mode of lumen formation has not been described before and it is likely to be different from cell wrapping as described above because the sprouting angioblast do not seem to migrate as an epithelial sheet of cells.

In a recent, comprehensive study, lumen formation of the paired dorsal aortae has been examined in the mouse embryo (Strilić et al., 2009). By anatomical and immunofluorescent analyses, Strilić and colleagues show that the lumen of the dorsal aortae forms in discrete steps similar to those observed in the zebrafish and, for the first time, they were able to decipher the molecular mechanisms involved in this process. Consistent with observations in zebrafish, the first steps of lumen formation are initiated upon formation of intercellular adherens junctions between angioblasts. Junctional remodeling then leads to an apical interface between adjacent ECs, followed by an accumulation of anti-adhesive CD34-sialomucins, such as CD34 and Podocalyxin (PODXL). Proper localization of these proteins to the apical surface depends on the presence of VE-cadherin. The subsequent formation of the aortic lumen is driven by a VEGF-A-dependent constriction of the apical surfaces as well as EC elongation induced by the F-actin cytoskeleton. These cell shape changes apparently rely on interactions between Moesin with CD34-sialomucins, which lead to an apical localization of F-actin. This view is supported by the analysis of Moesin and of *Podxl* mutants which both exhibit reduced levels of apical F-actin and a delay in aortic lumen formation. Furthermore, loss of *Podxl* leads to a reduction of Moesin at the sites of endothelial contacts, suggesting that PODXL connects with Moesin in order to recruit F-actin.

In summary, these studies indicate that the morphogenetic mechanisms that drive lumen formation in the dorsal aorta/aortae have been conserved between teleosts and mammals. In either case, coalescence of ECs and subsequent apical–basal polarization of a vascular cord appear to be the primary steps. The lumen is then formed between apical surfaces of apposing ECs by a cord hollowing process. Whether the molecular mechanisms that underlie lumen formation are conserved between fish and mouse remains to be determined. Furthermore, it will be interesting to learn about the morphogenetic processes that may be required to further inflate the lumen.

Angiogenesis

While the primary axial vessels are formed by vasculogenesis, elaboration of the vasculature, i.e. the formation of secondary blood vessels, occurs via angiogenesis, a process by which new blood vessels are generated from a pre-existing one. However, with respect to morphogenetic cell behaviors, angiogenesis can occur in quite different ways. Originally, it has been described as a sprouting process, by which a new vessel is branching off a primary vessel (reviewed by Patan, 2000). In addition to sprouting, a considerably different mode of angiogenesis called intussusception has been described in mammals (reviewed by Makanya et al., 2009). During intussusceptive angiogenesis, a vessel splits along its longitudinal axis into two new branches, thus effectively enlarging the vascular surface area. This process plays an important role in vascular remodeling during plexus formation. In zebrafish, angiogenesis by intussusception has not yet been described and therefore we focus our discussion on sprouting angiogenesis.

Sprouting angiogenesis

Sprouting angiogenesis was described as a general mechanism of microvascular growth during the 1970s, and its relevance for tumor growth and metastasis was soon recognized (Folkman, 1982). Early on angiogenesis was studied in a variety of in vivo and tissue culture systems such as the chorion allantoic membrane of the chick or the corneal pocket (reviewed by Patan, 2000). As an outcome of these studies, sprouting angiogenesis was described as a sequence of events that include (i) migration of ECs toward the angiogenic stimulus, (ii) alignment of ECs in a bipolar mode, (iii) lumen formation and cell divisions distant to the tip of the sprout and (iv) connection of individual sprouts to initiate circulation (Ausprunk and Folkman, 1977; Patan, 2000).

In recent years, much progress has been made in establishing systems in which angiogenic processes can be followed in detail. These include the retinal vasculature of the mouse, which develops postnatally, and the zebrafish embryo, in which all aspects of angiogenesis can be followed in vivo. Embryonic vasculogenesis and angiogenesis in zebrafish occur in ways very similar to those in mammals. In contrast to the latter, zebrafish embryos do not require extra-embryonic vasculogenesis due to their extrauterine development. This greatly facilitates the analysis of embryonic blood vessel formation, as it is not influenced by prior extra-embryonic events. Although all major blood vessels are easily accessible in the zebrafish embryo, the intersegmental blood vessels (ISV) have been most thoroughly studied because of their metameric organization and relatively simple anatomy.

ISV formation in the zebrafish embryo

Formation of ISVs in the zebrafish embryos involves two waves of angiogenic sprouting (Isogai et al., 2003). ECs of the primary wave form the segmental arteries (SA). During the primary wave, ECs sprout from the DA at ~22 hpf. These sprouts grow dorsally and – once they have reached the level of the dorsal neural tube – connect with their neighbors from anterior and posterior segments to form the future dorsal longitudinal anastomotic vessel (DLAV). The second wave, which starts at 32 hpf, involves ECs from the PCV (Yaniv et al., 2006). These sprouts will either connect to an existing SA, thereby transforming it into a vein (SV), or, alternatively, they will grow up to the level of the horizontal myoseptum and form a population of cells

named parachordal lymphangioblasts (PLs) (Hogan et al., 2009; Isogai, 2003). The majority of these cells eventually migrates away from the horizontal myoseptum and contributes to the lymphatic vasculature (Hogan et al., 2009).

Formation of the SA has been described in detail by in vivo time-lapse and immunofluorescent analyses (Blum et al., 2008; Childs et al., 2002; Isogai et al., 2003; Lawson and Weinstein, 2002b). These studies have led to several models of SA morphogenesis. While earlier studies suggested that the SA is made up by 3 cells that are arranged serially in a head to tail fashion (Childs et al., 2002), mosaic analyses and the analysis of endothelial cell junctions showed that SAs are composed of 4–6 cells that extensively overlap along the proximodistal axis of the vessel (Blum et al., 2008). Taken together, these findings suggest a model of SA formation, as shown in Fig. 3. Initially, one or two cells migrate out of the epithelium of the DA forming the sprout (Fig. 3A). During dorsal outgrowth, this sprout consists usually of 3–4 cells, one tip cell and two or three stalk cells (Fig. 3B). When the tip cell has made contacts with its anterior and posterior neighbors, the basic scaffold of the SA is formed (Fig. 3C). Because of cell divisions that occur at varying time points, the stalk can consist of a variable number of cells generating a large degree of morphological heterogeneity, which is illustrated by the variation of junctional patterns (Blum et al., 2008). However, further cell divisions and cellular rearrangements during vessel assembly lead to a paired configuration of cells along the proximodistal extent of the SA, which then forms a lumen. In the following section, we discuss some of the morphogenetic and molecular mechanisms that govern the different aspects of SA formation. Since much progress has also been made in other angiogenesis models, we will discuss them in comparison with SA formation.

Sprouting of SA and SV appears to be triggered by different signals. For example, VEGF-A is critical for SA formation, while it appears dispensable for SV formation, since SV sprout normally in embryos that are mutant for phospholipase C-γ (*plc-γ* , which is a downstream mediator of VEGF-A/VEGFR-2 signaling; Bahary et al., 2007; Habeck et al., 2002; Lawson et al., 2003; Nasevicius et al., 2000; Covassin et al., 2009; Covassin et al., 2006). Here, we will focus on the morphogenesis of the SA. The regulation of angiogenesis by VEGFs and their receptors has been studied in many previous publications in great detail (reviewed by Cébe-Suarez et al., 2006; Matsumoto and Mugishima, 2006; Olsson et al., 2006; Shibuya and Claesson-Welsh, 2006; Yamazaki and Morita, 2006). While early studies focused on general pro-angiogenic functions of VEGF signaling, more specific roles for VEGF signaling in patterning of angiogenic sprouts have recently been revealed (Covassin et al., 2006; Gerhardt et al., 2003; Ruhrberg et al., 2002). Upon VEGF-A/VEGFR-2 signals, ECs initiate the angiogenic program, which entails the loosening of junctional connections with neighboring cells, migratory behavior towards the angiogenic stimulus and cell division (reviewed by Lampugnani and Dejana, 2007). Cells within the nascent sprout respond in different ways to the *VEGF-A*. Whereas cells located at the base (termed stalk cells) show increased rates of proliferation, the leading cell (termed tip cell) sends long and dynamic filopodia into the surrounding environment to guide the growing sprout towards the stimulus (Gerhardt et al., 2003). Endothelial tip and stalk cells do not only have different functions and behaviors (discussed below), they also show differences in gene expression. For example, Platelet derived growth factor B (*Pdgfb*) and *Flt4* are expressed at higher levels in the tip cell than in the stalk cell (Gerhardt et al., 2003; Siekmann and Lawson, 2007).

Patterning and angiogenic behavior of sprouts are regulated by the cooperation of the Notch and VEGFR-2 signaling pathways (Hellström et al., 2007; Siekmann et al., 2008; Siekmann and Lawson, 2007; Suchting et al., 2007; reviewed by Phng and Gerhardt, 2009; Siekmann et al., 2008). The tip cell, receiving the highest level of VEGFR-2 signal, responds with an upregulation of the Notch ligand Delta-like-4 (DLL4), which leads to increased intracellular Notch signaling in the neighboring stalk cells (Hellström et al., 2007). In zebrafish, loss of DLL4 function leads to prolonged angiogenic activity in the ISVs, whereas over-activation of *Notch* signaling leads to a quiescent phenotype (Leslie et al., 2007; Siekmann and Lawson, 2007). DLL4 also regulates angiogenesis by suppressing VEGF-C dependent FLT4 (VEGFR-3) signaling in endothelial cells in mouse and fish (Hogan et al., 2009; Tammela et al., 2008). In zebrafish, VEGF-C/FLT4 signaling is required for venous and lymphatic development (Covassin et al., 2006; Küchler et al., 2006). However, a “kinase-dead” allele of FLT4 is able to rescue the hyperbranching phenotype caused by the loss of *DLL4* function (Hogan et al., 2009). Furthermore, in the absence of DLL4, arterial cells become more sensitive to varying levels of VEGF-C in the embryo (Hogan et al., 2009). These experiments point out a mechanism for how different endothelial lineages can respond specifically to sources of VEGF-C in the trunk. Venous and lymphatic cells, which do not express *dll4*, are able to respond to VEGF-C/FLT4, whereas in arterial cells this pathway is inhibited by DLL4 (Hogan et al., 2009).

Although the molecular mechanisms that control angiogenic behavior in the sprout may not be identical in fish and mouse, there is a common theme in that differences in intracellular Notch signaling confer different cell behaviors along the proximodistal axis of the sprout. Tip cells (low Notch) extend numerous filopodia, are highly migratory and thus display the strongest angiogenic behavior while the proximal stalk cells (high Notch) appear less migratory. It has

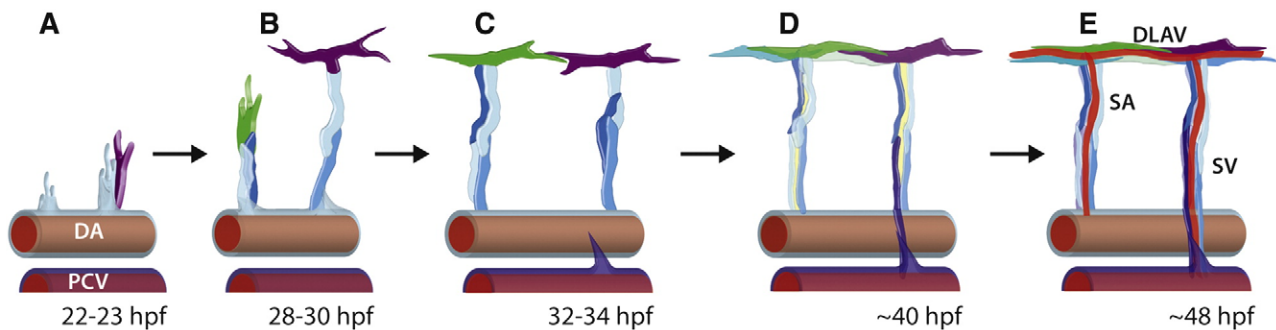

Fig. 3. A model for the morphogenetic events that lead to the formation of ISV and DLAV in the Trunk. Two neighboring sprouts are depicted as representative examples. The leading cells are indicated in green and purple, respectively. At 22 hpf ECs of the DA form sprouts (A) that grow along the somite boundaries up to the dorsal roof of the neural tube (B). During these stages, the sprout consists of 2 to 4 cells that are stabilized by interendothelial junctions (not indicated). At the dorsal side of the embryo, the tip cells send extensions toward their anterior and posterior neighbors to establish connections. During this phase, the ECs establish a scaffold consisting of a vascular cord that is not yet lumenized (B, C). Further cell rearrangements and cell divisions lead to formation of a continuous apical surface that may surround initial luminal spaces (yellow) (D). At around 32 hpf, a secondary wave of angiogenic sprouts emerges from the PCV. These sprouts either generate a group of lymphatic cells, called parachordal lymphangioblasts (not shown), or connect with the adjacent primary vessel (D, on the right), which will become a segmental vein. Blood flow in ISVs commences after SA, SV and DLAV have been established (E).

been proposed that the reduced migratory behavior of stalk cells is important to maintain sprout integrity and connection to the DA (Siekmann et al., 2008).

While the general aspects of angiogenesis in the ISVs of the fish and the postnatal vasculature of the mouse retina are very similar, there appear to be differences in gene expression and pattern of cell proliferation. While in the mouse retina cell proliferation is largely restricted to the stalk (Gerhardt et al., 2003), in fish similar rates can be observed in stalk and tip cells (Blum et al., 2008). Furthermore, based on gene expression—intersegmental sprouts in the fish appear not as polarized as those in the mouse retina. In contrast to the mouse retina, the tip cell markers *dll4* and *flt4* are quite uniformly expressed in tip and stalk cells of the sprouting SA (Hogan et al., 2009; Leslie et al., 2007; Siekmann and Lawson, 2007). Similar to zebrafish, ISV sprouts in the mouse embryo show a relatively even distribution of DLL4 protein (Tammela et al., 2008). These differences between distinct types of blood vessels raise the possibility that, compared to the mouse retina, the state of the tip cell in ISV is less defined. It will be interesting to see whether these differences are due to different interpretations of VEGF/Notch signals or whether they are connected to different morphogenetic processes occurring in the two systems (see below).

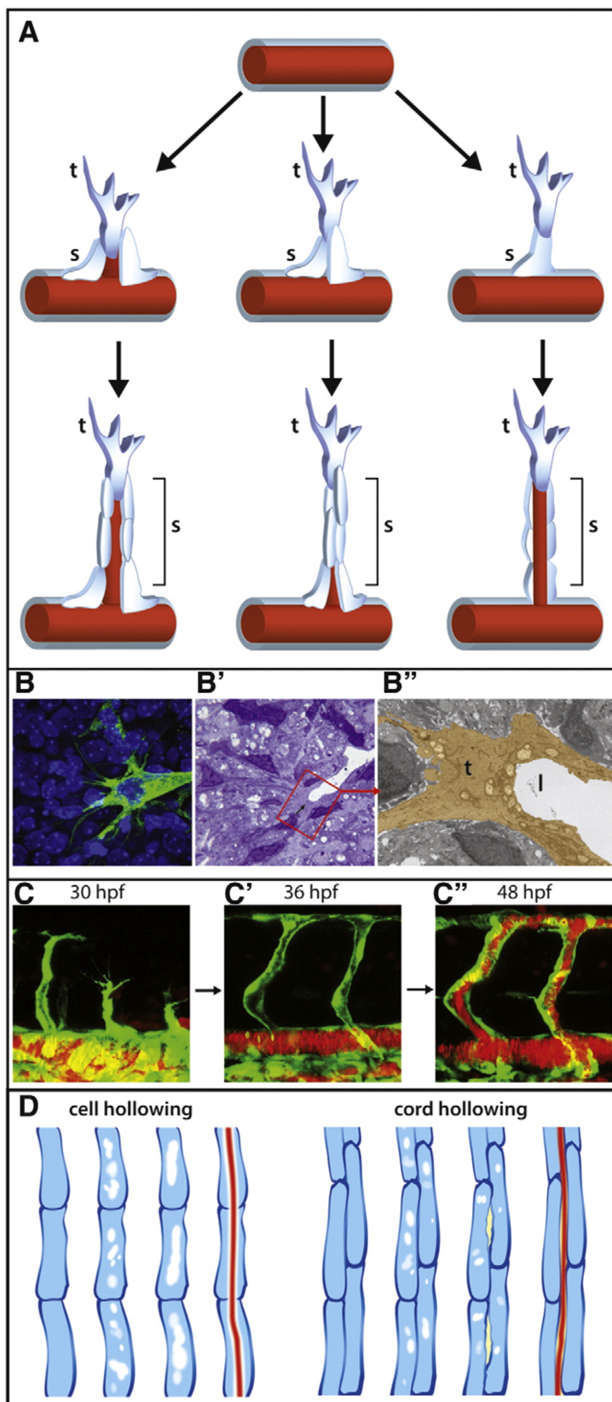

Fig. 4. Different morphogenetic mechanisms that underlie sprouting angiogenesis. (A) Three examples for the cellular organization of an angiogenic sprout. Depending on how the cells are arranged in the sprout, different types of vessels may form. Left: Branching morphogenesis by budding. ECs remain epithelial, while the sprout grows via cell division within the stalk. The lumen remains open and continuous at all times. Middle: Formation of a multicellular tube by cord hollowing. This shows an example, where cells grow in a paired configuration maintaining an apical surface in between. The lumen remains open at the base but is closed in distal region. Close to the tip, cells may be of a more mesenchymal character and undergo cell divisions. Cellular rearrangements will then lead to a continuous apical surface and open up the luminal space. Right: Formation of a unicellular tube with an intracellular lumen. At the tip of a capillary, the lumen may also form within a string of cells. The cells hollow out by vacuole formation followed by exocytosis. This mode of lumen formation will generate a so-called intracellular lumen. (B) Lumen formation behind the leading tip cell in the mouse retina. The lumen of the nascent sprout extends to the tip of the growing sprout. This situation is in agreement with the configuration shown in panel A. Isolectin B4 labeling (green) of an endothelial tip cell projecting long filopodia. Nuclei, Dapi, blue (B). Semithin (B′) and close up of ultrathin (B″) en face section of the sprouting front in the retina illustrating continuous lumen formation (l) just behind the tip cell (t). The endothelial tip cell in panel C is pseudocolored brown. Figure courtesy of Denise Stenzel and Holger Gerhardt, London Research Institute—Cancer Research UK. © Gerhardt et al., 2003. Originally published in J. Cell Biol. doi:10.1083/jcb.200302047. (C) Lumen formation in the ISV and DLAV of a zebrafish embryo. During scaffold formation, neither ISV nor DLAV are perfused, suggesting that the lumen is formed subsequently. Blood flow is initiated after subsequent remodeling and establishment of the intersegmental veins (compare to Fig. 3). Confocal still pictures from an in vivo time-lapse movie of a transgenic zebrafish embryo (*TG:fli1a:EGFP^{y1};gata1:DsRed^{sd2}*). ECs are labeled in green; erythrocytes are labeled in red. (D) Alternate models of lumen formation in the zebrafish ISV. Depending on the cellular arrangement of cells in an angiogenic sprout, de novo lumen formation can occur in at least two different ways. If cells are arranged in a serial fashion, the lumen may be generated by cell hollowing (left, see also A). In this process, ECs pinocytose solutes from extracellular space and form vacuoles that coalesce and fuse to give rise to an intracellular lumen. Eventually intracellular vacuoles of neighboring cells will fuse by exocytosis and form a patent lumen (see Kamei et al., 2006). Alternatively, if cells are arranged in a paired fashion, they may form a lumen by cord hollowing (right, see also A). This process requires establishment of a continuous apical surface that is bounded by at least two ECs. Vacuoles can then be exocytosed into this intercellular space, which will eventually become the vascular lumen.

How the angiogenic sprout lumenizes is still controversial. Different morphogenetic behaviors and cellular configurations in the sprout can have significant impact on how a vessel is formed. In principle, there are at least 3 different morphogenetic processes of tubulogenesis that may occur in an angiogenic sprout: budding, cord hollowing or cell hollowing (see Fig. 4A). When a novel tube is formed by budding, the ECs that follow the tip cells maintain their epithelial character with a defined apical–basal polarity. During budding, the luminal space of the sprout remains continuous with that of the parent vessel, extending up to the tip cell. Tube branching by budding has been described in many experimental systems including the tracheal system in *Drosophila* and several branched organs in mammals (Baer et al., 2009). Vessel branching by budding appears to occur in larger caliber capillaries that are constantly perfused. In the mouse retina, the lumen is located immediately adjacent to the tip cell (see Fig. 4B). In addition, in vivo time-lapse recording of blood vessels in the zebrafish brain appears consistent with such morphogenetic mode of tubular branching (Huisken and Stainier, 2009).

Formation of ISV does not occur by budding since the initial sprouts do not contain a lumen continuous with the DA (Fig. 4C). Rather, the lumen becomes patent at the time when SA, DLAV and SV have formed proper connections. After labeling circulating blood with fluorescent tracer dyes, it was observed that (from the perspective of the DA) the lumen opens up in a stepwise manner from proximal to

distal (Kamei et al., 2006). Because vacuole formation and fusion has long been considered an important component of lumen formation, the stepwise expansion of luminal space was interpreted as a succession of vacuolar fusion events that generate a unicellular tube containing an intracellular lumen (Kamei et al., 2006; Fig. 4D). Capillaries that contain intracellular lumens have indeed been described (Bar et al., 1984) and have been called "seamless tubes" because they are characterized by the absence of cell junctions along the longitudinal axis of the vessel. More recently, analyses of cell junctions within nascent sprouts and patent ISVs showed that the cells in the stalk overlap extensively along the proximodistal axis and that ISVs are multicellular tubes containing an extracellular lumen (Blum et al., 2008). This cellular configuration is more consistent with a lumen formation process by cord hollowing (Fig. 4D). In this model, cells in the stalk rearrange to form a continuous apical surface. The lumen is then formed by a process, in which small pre-luminal spaces (rather than vacuoles) are formed by exocytosis and/or paracellular influx of liquids. The stepwise opening of the lumen from the direction of the aorta would then be consistent with the completion of cell rearrangements in the stalk. The events described here would be quite comparable to those described above for the lumen formation in the dorsal aorta.

The three mechanisms discussed here are not necessarily mutually exclusive. For example, it is possible that sprouting angiogenesis contains aspects of both, budding and chord hollowing, depending on the extent to which apical–basal polarity is maintained in the stalk cells. Likewise, it is possible that a single blood vessel contains regions of intracellular as well as regions of extracellular lumen. Taken together, it is clear that different vessels can form by various morphogenetic mechanisms. It remains to be explored what the decisive factors are that determine which mechanism is used. It is likely that parameters such as vessel caliber, blood pressure of the parental vessel as well as cell number within the sprout play important roles.

Guidance cues along the way—endothelial pathfinding

To effectively oxygenate a given organ, blood vessels have to be evenly distributed within this tissue. This can be achieved in different ways, for example by controlling the number of angiogenic sprouts that are generated (e.g. ISV) or by the formation of a plexus (e.g. retinal vasculature in the mouse), which is then remodeled by a pruning process. In recent years, a number of ligands (and their respective receptors) that provide endothelial guidance cues have been described, including the Semaphorin, Netrin, Ephrin and Slit systems (reviewed by Larrivée et al., 2009). Interestingly, these signals were originally described as cues for axonal growth cones (reviewed by Eichmann et al., 2005). It has become clear since that many of the signaling pathways that act during axonal pathfinding are also employed for guidance of angiogenic sprouts.

In the zebrafish, the guidance of angiogenic sprouts is best studied during SA formation. Segmental arteries sprout from the DA at the intersomitic boundary; as they grow out, they follow the intersomitic fissure up to the horizontal myoseptum, from where they change their path to grow more or less straight to the dorsal roof of the neural tube. The exit point of intersegmental sprouts is regulated by molecular guidance cues. In *out of bounds* (*obd*) mutants, angiogenic sprouts form ectopically along the ventral somite border (Childs et al., 2002). Furthermore, these sprouts no longer avoid the ventral somite and the ISVs take on a plexus-like organization. Molecular analyses showed that *obd* encodes the receptor PlexinD1 that is expressed in ECs and interacts with the ligands SEMA3A1/2, which is expressed in ventral somites. Upon ligand binding, a repulsive signal is activated in the ECs prohibiting them from moving into the somite region (Torres-Vazquez, 2004). Recently, it has been shown that the interaction of different Plexins and Semaphorins is also important for the timing of SA sprout formation (Lamont et al., 2009).

Analyses in mouse have uncovered an additional and quite different molecular mechanism that limits the number of sprouts. Bautch and coworkers have shown that a soluble form of FLT-1 (VEGFR-1), sFLT-1, is secreted from ECs adjacent to the forming sprout (Chappell et al., 2009). This isoform is able to bind VEGF, thereby removing it from the environment surrounding the sprout. It is thought that sFLT-1 serves two purposes: it ensures the ordered formation of sprouts from an activated endothelium and it prevents the early sprout to connect back to its original vessel.

The sharing of attracting and repulsive signaling pathways by neurons and ECs appears to be a common theme. In fact, it has been shown that growing neurons and nascent capillaries can walk the same tracks (Mukouyama et al., 2002). It will be interesting to see whether in these instances neurons and ECs simply use the same cues provided by the stromal cells or whether they also navigate by direct cell–cell interactions. It is noteworthy that not only the tip cell but also stalk cells appear to express guidance receptors albeit at lower levels (Larrivée et al., 2009). In zebrafish, for example, the guidance receptor *PlexinD1* is expressed at comparable levels in the tip and the stalk of nascent SA (Torres-Vázquez et al., 2004). This raises the possibility that also stalk cells are involved in angiogenic pathfinding. Indeed, it appears that the tip cell fate is not fixed and that cells at the stalk can become tip cells and vice versa. In murine allantoic explant cultures, migratory ECs are passing each other at the tip (Perryn et al., 2008). In a similar fashion, ECs are changing lead during the outgrowth of vascular cords that sprout from differentiating murine embryonic stem cells (Holger Gerhardt, personal communication). It has been suggested that these tip cell turnovers are regulated by oscillations in Notch signaling along the vascular sprout (Phng and Gerhardt, 2009). It should be noted that these vascular cords are not perfused during early outgrowth and are, in that respect, similar to developing SAs rather than vessels in the postnatal mouse retina.

Cell–cell adhesion during sprouting angiogenesis

The above observations indicate that the angiogenic sprout is highly plastic with regard to signaling events and cell–cell interactions and that cellular rearrangements play an important role in blood vessel morphogenesis. It is generally believed that cellular rearrangements involve remodeling of intercellular junctions (Baer et al., 2009). In *Drosophila*, adherens junctions play an important role for cellular rearrangement during various processes, such as border cell migration or tracheal morphogenesis (Pacquelet and Rørth, 2005; Ribeiro et al., 2004). In the case of border cell migration very different cell behaviors, invasive cell migration and cell adhesion require DE-cadherin. These distinct cellular activities are mediated by homophilic interactions of DE-cadherin between different cell types: interactions among border cells maintain cohesion of the migratory cells while interactions between border cells and nurse cells allow invasive migration into the substratum (Niewiadomska et al., 1999). The role of VE-cadherin during vertebrate angiogenesis is less clear. Mice that are mutant for VE-cadherin die at mid-gestation exhibiting vascular defects that are consistent with a role for VE-cadherin in maintaining vascular integrity (Carmeliet et al., 1999; Crosby, 2005). Further in vitro analyses have also emphasized a role of VE-cadherin in endothelial cell survival and stabilizing the endothelium, in part by antagonizing VEGFR-2 signaling (reviewed by Lampugnani and Dejana, 2007; Vestweber et al., 2009). Recent organotypic cell culture and knockdown experiments in zebrafish point at a role of VE-cadherin in angiogenic sprouting (Abraham et al., 2009). In these studies, quiescent endothelial tubes did not respond to VEGF stimulation unless VE-cadherin function was reduced. They further showed that VE-cadherin suppresses sprouting by inhibiting VEGF-R2/RAC1 signaling. In the zebrafish ISV, knockdown of VE-cadherin led to the formation of ectopic "branch points" along the proximo-

distal axis of the ISV, which were interpreted as prolonged angiogenic behavior of the ECs within the ISV.

While the above results suggest an anti-angiogenic function, other studies indicate a more pro-angiogenic role for VE-cadherin. As discussed above, blood vessel formation is a very dynamic process involving cell rearrangements and cell migration. These dynamics have been studied using murine allantoic explant cultures, which allow to measure the migration of individual and groups of ECs in a process called vasculogenic sprouting, which involves the outgrowth of multicellular sprouts from a primary plexus (Perryn et al., 2008; Rupp et al., 2004). ECs actively migrate over the substrate, frequently passing each other during outgrowth of the vascular rod. These rearrangements are effectively inhibited by addition of a VE-cadherin blocking antibody (Perryn et al., 2008), indicating that VE-cadherin is required for cellular rearrangements as they occur during vasculogenic sprouting. In zebrafish, we have observed that VE-cadherin is also essential for cellular rearrangements during SA formation (H.-G. Belting and M. Affolter, unpublished observation). Therefore, it appears that in addition to functions in vascular integrity and cell survival, VE-cadherin is also involved in sprouting angiogenesis. One can easily imagine that, analogous to the role of DE-cadherin in border cell migration, VE-cadherin may fulfill dual functions at the same time: maintenance of cell–cell contacts and cellular rearrangements.

Conclusions and perspectives

Blood vessel formation includes a spectrum of different morphogenetic processes such as budding, cord hollowing, cell hollowing, cell wrapping and intussusception. The genetic and molecular bases, which initiate and control these different processes is not known. It is clear, however, that ECs of different vascular beds are different in their gene expression profile and that blood vessels are anatomically highly diverse (Rocha and Adams, 2009). Morphogenesis may also be influenced by extraneous factors such as diverse extracellular matrices and signals, shear stress due to blood pressure or differences in cell number (Aird, 2007; Nguyen et al., 2006; Sottile, 2004).

The morphogenetic events that underlie blood vessel formation are likely to determine the way a vessel ultimately lumenizes. During sprouting angiogenesis, larger vessels appear to extend their lumens in conjunction with the outgrowing sprout, while small capillaries may form their lumens de novo by cell hollowing or cord hollowing. The respective contribution to lumen formation in different vessels remains to be determined.

Elaboration of the vascular tree requires additional processes, such as vessel fusion and pruning. How these processes occur at the morphogenetic level has not yet been described in much detail. With regard to vessel fusion, one can envisage that cellular remodeling plays a major role. Furthermore, it is likely that the fusion process occurs differently depending on vessel type. In the zebrafish, the DLAVs form in a non-perfused state and blood circulation commences subsequently. Recently, VE-cadherin localization during DLAV formation revealed that cells from adjacent sprouts undergo extensive rearrangements (Blum et al., 2008). Initial contacts between neighboring sprouts require VE-cadherin, as knockdown of VE-cadherin function leads to slowed-down formation of cell–cell contacts (Montero-Balaguer et al., 2009). It will be interesting to examine the exact cellular mechanisms that drive vessel fusion during DLAV formation and compare them with those occurring in perfused vessels.

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