

REVIEW

Cell behaviors and dynamics during angiogenesis

Charles Betz, Anna Lenard, Heinz-Georg Belting and Markus Affolter*

ABSTRACT

Vascular networks are formed and maintained through a multitude of angiogenic processes, such as sprouting, anastomosis and pruning. Only recently has it become possible to study the behavior of the endothelial cells that contribute to these networks at a single-cell level *in vivo*. This Review summarizes what is known about endothelial cell behavior during developmental angiogenesis, focusing on the morphogenetic changes that these cells undergo.

KEY WORDS: Anastomosis, Angiogenesis, Endothelial cells, Live imaging, Pruning, Sprouting, Vegf, Zebrafish

Introduction

Vascular networks perfuse the entire body and guarantee the adequate circulation of metabolites and evacuation of waste products. The vascular system emerges as one of the earliest organs in the embryo to support the rapid growth of all tissues while re-adjusting its own shape to adapt to the changing requirements of the embryo. The adult vasculature is generally quiescent with regard to expansion and remodeling, but retains the capacity to swiftly exit this apparent dormancy, for example, during wound healing and tumor neovascularization.

The blood vessels that comprise vascular networks contain various components. Endothelial cells (ECs) form the tunica intima, a single-cell-layered endothelium that is exposed to the vessel lumen. The intermediate layer – the tunica media – is made up of elastin, collagen and smooth muscle cells. Finally, the outermost layer – the tunica adventitia – contains connective tissue, collagen and elastic fibers. The two outer layers are added to newly developed vessels during maturation of the vasculature to provide functional specialization as well as support and stability. Since we discuss here only the early events of developmental angiogenesis, we concentrate almost exclusively on the behavior and role of ECs.

During development, blood vessels form via two (predominantly) sequential processes: vasculogenesis and angiogenesis. Vasculogenesis is the *de novo* formation of vessels from aggregating mesodermal precursors. A prominent example of vasculogenesis is the formation of the dorsal aorta, in which angioblasts, which are endothelial progenitors, coalesce and align in a chord before remodeling into a tube (Axnick and Lammert, 2012; Ellertsdóttir et al., 2010; Jin et al., 2005; Sato, 2013; Swift and Weinstein, 2009). Although primarily occurring during early development, vasculogenesis is also observed in certain pathologies such as tumor vascularization, ischemia and

endometriosis (Drake, 2003; Laschke et al., 2011; Tang et al., 2009; Tongers et al., 2010). The second process of generating new blood vessels, and the major topic of this Review, is angiogenesis – the sprouting of new vessels from pre-existing ones, followed by the formation of vascular loops through anastomosis of sprouts and the optimization of the vascular network by vessel pruning (Potente et al., 2011; Schuermann et al., 2014).

A key step during angiogenic sprouting and anastomosis is the formation of an interconnected luminal space, allowing the subsequent circulation of blood. This ability of ECs to assemble into vascular networks in a dynamic fashion is remarkable, given the multitude of individual cell behaviors that must be tightly orchestrated. Recent studies using fluorescent markers combined with time-lapse imaging have indeed demonstrated that ECs exhibit numerous, sometimes unique, cellular behaviors during sprouting angiogenesis. These cell behaviors, and their coordination, are the major subjects of this Review. In order to describe EC behavior in angiogenesis in detail, we refrain from going into much detail about the molecular control of these behaviors and instead refer the reader to other recent reviews on this aspect of angiogenesis (Blanco and Gerhardt, 2013; Geudens and Gerhardt, 2011; Herbert and Stainier, 2011; Schuermann et al., 2014; Siekmann et al., 2013; Wacker and Gerhardt, 2011). Furthermore, since most *in vivo* studies of the dynamics of cell behavior in angiogenic processes use zebrafish embryos, our Review is heavily ‘biased’ towards studies carried out in this model system, although we do describe and touch upon findings from other models.

Model systems for studying angiogenesis

Despite their anatomical differences, vertebrates share similar developmental programs that give rise to the cardiovascular system. As such, a number of model systems have been used to study angiogenesis, and they each present specific advantages and/or disadvantages. Below, we briefly outline some of the key models that have been used to study angiogenesis *in vivo*.

Mouse models

Studying angiogenesis using mouse models allows researchers to choose from a large selection of floxed genes and tissue-specific Cre driver lines, and a huge repertoire of specific antibodies for molecular analyses. Popular angiogenesis models in mice include retinal explants (Fruttiger, 2002; Stalmans et al., 2002; Uemura et al., 2006), vascularization of the hindbrain (Tata et al., 2015) and cancer xenografts (Rofstad, 1994). However, imaging angiogenesis *in vivo* in the mouse embryo is difficult due to the inaccessibility of the uterine environment during development, although progress has been made using live imaging in the yolk sac (Udan et al., 2013) and intracranial windows to follow angiogenesis in the brain (Burrell et al., 2013; Vajkoczy et al., 2000). Other *in vivo* angiogenesis models, such as the bone (Kusumbe et al., 2014; Ramasamy et al., 2014), also present a new perspective on the use of rodents in angiogenesis research.

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Quail and chick embryos

Alternative models for angiogenesis are quail and chicken embryos because they develop *ex utero* (Ribatti et al., 1996; Ghaffari et al., 2015). Recently, transgenic quails expressing enhanced green fluorescent protein (EGFP) in ECs have been described (Sato, 2013; Sato et al., 2010) and they have helped researchers to study the sprouting behavior of ECs during the formation of the dorsal aortae in detail. In order to get further insights into the cellular aspects of angiogenesis, more transgenic lines labeling different cellular structures will have to be established and studied.

Zebrafish

During the past decade, the zebrafish embryo has proved to be an excellent model for studying cell behavior during angiogenesis at high resolution *in vivo*. Whereas angiography and fluorescent markers such as cytoplasmic EGFP or cell membrane-tethered EGFP have allowed the overall process of blood vessel formation in zebrafish to be examined, more specific cell markers have provided a detailed understanding of the cellular activities underlying these processes. In most cases, these markers are transgenic reporters, which consist of fluorophores fused to specific proteins. These reporters, combined with the use of novel microscopy techniques (e.g. lightsheet microscopy), have enabled researchers to assess changes in cell polarity, cell shape and cytoskeletal dynamics during angiogenesis.

Most of the studies on blood vessel morphogenesis in zebrafish have focused on the formation of the intersegmental vessels (ISVs; see Box 1, Fig. 1). These studies have helped us to understand the basic cellular and molecular mechanisms of vascular network formation. Other zebrafish vascular beds that have attracted attention (Fig. 1) include the vasculature of the head and the brain (Gore et al., 2012; Isogai et al., 2001; Kimura et al., 2015; Lenard et al., 2013; Chen et al., 2012), the caudal vein plexus (Wiley et al., 2011) and the subintestinal veins (SIVs) (Goi and Childs, 2016; Hen et al., 2015; Koenig et al., 2016; Lenard et al., 2015; Nicoli et al., 2007). It should be noted, however, that ECs in different

vascular beds might use different molecular cues and morphogenetic mechanisms to form vascular tubes. Nonetheless, several studies have taken advantage of the fact that the same process (e.g. anastomosis and pruning) can be studied in different vascular beds; this allows the series of cellular events that occur in each context to be compared and allows the vascular bed that is best suited with regard to accessibility for high-resolution time-lapse imaging to be selected for more detailed, subsequent studies. An important additional advantage of the zebrafish embryo resides in the fact that these embryos can survive for about seven days in the absence of blood flow, thus allowing the influence of blood pressure and/or blood flow on angiogenic processes to be studied. This has been crucially important in studies of lumen formation, since it turns out that blood pressure plays a central role in this key step of angiogenesis.

Angiogenic sprouting is a multistep event

Angiogenic sprouting can be considered a multistep branching morphogenesis process (Caussinus et al., 2008; Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003) because sprouts grow out of existing vessels and keep constant contact with the parent vessel. Angiogenic sprouts were observed and described many decades ago, and in several studies, the observation was made that cells at the tip of a sprout show a rather specific morphology, characterized by numerous filopodial extensions that are reminiscent of the structure of axonal growth cones or of tip cells in the tracheal system of insects. Such angiogenic ‘tip cells’ were first reported in quail embryos (Kurz et al., 1996). The role of these cells was subsequently described and analyzed in detail in the mouse retina (Gerhardt et al., 2003). Tip cells are thought to guide the angiogenic sprout by providing a ‘read out’ of the signaling environment; in contrast, the ‘stalk cells’ that trail behind the tip are more proliferative and thus act as building blocks for the nascent sprout. However, it should be noted that the distinction between tip and stalk cells is not fixed since both cell types can switch position and function (Jakobsson et al., 2010; reviewed in Siekmann et al., 2013). Furthermore, in some vascular beds, tip and stalk cells can proliferate at similar rates (Nicoli et al., 2012).

During developmental angiogenesis, the formation of angiogenic sprouts can be divided into three major phases: (1) tip cell selection, during which a cell in a pre-existing vessel is selected to become a migratory leading cell, which suppresses neighboring cells from adopting the same fate by lateral inhibition; (2) sprout extension or elongation, in which the tip cell moves along a chemotactic or physical hindrance-based path, followed by trailing stalk cells that connect the tip cell to the original vessel; (3) lumen formation and/or expansion – a process that links the luminal space of the sprout with the parent vessel. Once the sprouts have formed, they connect with existing vessels via anastomosis and can also be remodeled via a process termed pruning. Below, based on recent findings, we detail sprout formation in the above-mentioned order, although it should be emphasized that lumen formation, for example, can occur at different steps and can be implemented before sprout elongation.

Tip cell selection and function

Zebrafish intersegmental vessels (ISVs) start sprouting shortly after the establishment of the dorsal aorta (DA), at a stage when persistent blood circulation has not yet been initiated. The emergence of these sprouts is guided by several factors (reviewed in Hasan and Siekmann, 2015), including attractive signals (e.g. Vegf-A) and repulsive signals (e.g. Semaphorin3a). Vegf signaling in sprouting

Box 1. Blood vessel development in zebrafish

As in other vertebrate clades, angioblasts in zebrafish are derived from the lateral plate mesoderm. These angioblasts differentiate into ECs, which form the bulk of the embryonic blood vessels, although it has been recently observed that some cranial zebrafish vessels form from ECs that differentiate from head angioblast clusters (Proulx et al., 2010). Shortly after, the heart starts beating, and blood flow in the dorsal artery (DA)/posterior cardinal vein (PCV) loop is observed by 28 h post fertilization (hpf). Around 24–28 hpf, the first angiogenesis processes can be observed as tip cells emerge from the dorsal part of the DA and migrate dorsally to form the intersegmental vessels (ISVs). ISV sprouts start to anastomose by around 30–32 hpf, a process that eventually gives rise to the dorsal longitudinal anastomotic vessel (DLAV). Around the same time, blood flow in the newly formed and connected vessels initiates. In a second sprouting phase, tip cells emerge and sprouts grow out of the PCV and the CV (positioned underneath the aorta) and anastomose in an approximately alternating manner with ISVs to form segmental veins (vISVs), as opposed to the segmental arteries (aISVs) that form in the first sprouting phase. The developing vISVs prune their original connection to the DA to complete the process. For details, the reader is referred to more comprehensive reviews (Ellertsdottir et al., 2010; Gore et al., 2012; Herbert and Stainier, 2011; Schuermann et al., 2014). Other vascular beds that have been studied in the zebrafish embryo (see Fig. 1) include the vasculature of the head, the caudal vein plexus and the subintestinal veins (SIVs).

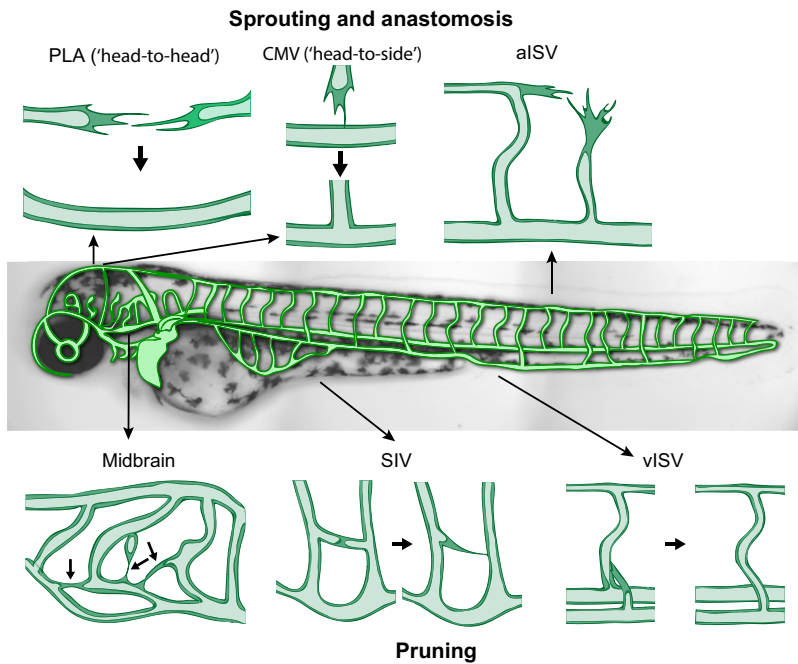


Fig. 1. Vascular beds in the developing zebrafish embryo.

The central panel shows an overview of the zebrafish vasculature at ~3 days post fertilization (dpf), highlighting the locations of different vascular beds. Sprouting and anastomosis have been studied in the head vasculature, during the formation of the palatocerebral artery (PLA), which arises through the connection of two sprouts ('head-to-head'), and during the formation of the communicating vessel (CMV) and the nasal ciliary artery (NCA, not shown), which both form through the connection of a sprout to a pre-existing vessel ('head-to-side'). Sprouting and anastomosis have also been studied in the trunk vasculature, during the formation and connection of T-shaped tip cells of neighboring segmental arteries (aISV). The pruning of blood vessels has been studied in the midbrain vasculature, in the eye (specifically in the cranial division of the internal carotid artery, CrDI not shown), in the subintestinal vein (SIV) and during the formation of segmental veins (vISV).

ECs in the segmental vessels induces intracellular calcium oscillations and these oscillations are important in the determination of the tip cells (Yokota et al., 2015). The growing ISVs follow the intersomitic fissure until they reach the myoseptum, from which the tip cells migrate towards the dorsal roof of the neural tube (Ellertsdóttir et al., 2010). Once they reach their dorsal-most position, tip cells adopt a 'T' shape by sending out cell extensions in anterior and posterior directions (Childs et al., 2002), allowing them to contact and fuse (anastomose) with tip cells from neighboring segments (Fig. 1). This T-shape morphology is quite different from that exhibited by the majority of other tip cells during angiogenesis, which are elongated along one axis (the axis of migration); the latter morphology is observed in several alternative sprouting models in zebrafish, for example, in many brain vessels, such as the communicating vessel (CMV), the posterior cerebral vein (PCeV) and the palatocerebral artery (PLA) (Fig. 1). The relevance of this morphological difference in angiogenic cell behavior is presently unclear. Either way, the characterizing hallmark of all tip cells is the presence of numerous filopodia that likely probe the surroundings for guidance cues. Interestingly, filopodia appear to be dispensable for directed tip cell migration in ISVs (Phng et al., 2013), suggesting that, rather than being strictly required, they might play a supportive role in migration efficiency. Stalk cells have fewer filopodia; here, in addition to interacting with the extracellular matrix, they might play a role in lumen formation and cell rearrangements.

Sprout extension

During ISV formation, the sprout is initially formed by the tip cell and one or two cells that are recruited from the DA. The subsequent proliferation of both tip and stalk cells supports stalk elongation (Blum et al., 2008; Leslie et al., 2007; Siekmann and Lawson, 2007). A recent study analyzed the morphological changes that occur in dividing ECs in zebrafish embryos (Aydogan et al., 2015), suggesting that sprout extension and lumen formation (discussed in detail below) are interlinked. This study showed that during the early phase of ISV sprouting, the sprout is not lumenized. Thus, division of an EC can take place without involving neighboring ECs, which is in contrast to the process in sprouts or blood vessels

that contain a lumen. Vessel architecture also influences the relationship between sprout extension and lumen formation. For example, the ISVs and the dorsal longitudinal anastomotic vessel (DLAV) can form two different types of tubes based on their cellular configuration: multicellular tubes, in which the lumen is formed between cells by a cord hollowing process and unicellular tubes, in which the lumen forms transcellularly (discussed below). In the latter case, a single cell surrounds the lumen, and such a unicellular architecture might impose limitations with respect to lumen maintenance during cell proliferation. Indeed, in unicellular tubes, the lumen collapses during mitosis and re-perfuses rapidly after the completion of cytokinesis (Aydogan et al., 2015). By contrast, the lumen in multicellular tubes is preserved during EC division, thereby maintaining blood flow.

Interestingly, the sprout does not only extend by cell proliferation; recent studies have revealed that cell elongation and cell rearrangements are important components of stalk elongation (Sauteur et al., 2014). EC elongation is driven by the transition of junctional contacts from a roundish to an elliptic form; this change requires the adhesion molecule VE-cadherin, as well as actin polymerization. The changes in junctional contacts eventually lead to cell rearrangements in the stalk; how these cell rearrangements are brought about and whether similar mechanisms are at work during later steps of angiogenesis remains to be studied. The role of physical forces during sprout outgrowth and extension is also unclear; does the tip cell exert a pulling force on the stalk cells, or is it rather that the stalk cells push the tip forward through cell rearrangements and cell division? Further studies are required to answer these questions.

Lumen formation and extension

Lumen formation is a critical step in the development of all tubular organs and occurs via a wide variety of cellular mechanisms (Baer et al., 2009; Charpentier and Conlon, 2014; Lubarsky and Krasnow, 2003; Wang et al., 2010; Sigurbjörnsdóttir et al., 2014). With respect to the vasculature, several mechanisms have been suggested. These include cord hollowing, cell hollowing, transcellular lumen formation and lumen ensheathment (Charpentier and Conlon,

2014). However, how the lumen forms in angiogenic sprouts remains a highly debated topic and there are a number of reasons for this. Firstly, although it is easy to locate cell divisions (e.g. by using markers for the mitotic spindle or condensing chromosomes), it is less easy to mark the initial steps in the formation of a luminal space and this has proved to be a technical limitation for the field. If such a luminal space were generated by vesicle transport and fusion, one could label the relevant vesicles and follow their trafficking routes; unfortunately, however, the process of lumen formation is not well understood, and in the vasculature, such live markers are not currently available. In most studies, lumen formation is thus observed by the local lack of signal, e.g. ‘black’ spaces surrounded by fluorescently labeled membrane and/or cytoplasm of endothelial cells. Once a luminal compartment is connected to the circulation, it can be labeled and unambiguously identified via angiography by injection of fluorescent particles into the blood stream. Secondly, different blood vessels may in fact use different mechanisms for lumen formation, suggesting that one common mechanism does not ‘fit all’. Finally, and as we discuss below, individual blood vessels may also choose alternative pathways in a context-dependent manner.

In the zebrafish embryo, lumen formation during angiogenic sprouting has been analyzed mostly in the developing ISVs, using high-resolution time-lapse imaging. These ISVs appear very early during embryo development when the circulatory system is just being established, when blood flow is unstable and blood pressure is low (see Box 1). Sprouts developing at later time points are often already lumenized during the very early sprouting phase and the lumen extends concomitantly with the outgrowing sprout (see Lenard et al., 2013). A similar phenomenon is also seen in the developing retinal vasculature in mice, where the lumen extends close to the front of tip cells (Gerhardt et al., 2003; Pelton et al., 2014).

In an early study, it was argued that cells in the ISVs are organized in a cord-like fashion (Childs et al., 2002) and that the lumen assembles from large intracellular vacuoles, which eventually fuse intracellularly to hollow out stalk cells and generate an interconnected luminal space (Kamei et al., 2006; Fig. 2A). The latter study also used cultured ECs as a model system and concluded that the mechanisms of lumen generation *in vivo* were very similar to those observed *in vitro*. However, due to the lack of suitable transgenic lines or antibodies, the precise cellular architecture of ISV sprouts was not known at that time. Subsequent studies showed that such sprouts often exhibit differing cellular architectures, making conclusions about individual cells and how they coordinate lumen formation rather difficult (Blum et al., 2008). Using more-specific cell markers, a recent study reinvestigated this and concluded that heterogeneous mechanisms contribute to lumen formation in ISVs *in vivo* (Yu et al., 2015). This study provides

further evidence for vesicle (vacuole) formation and the generation of an intracellular lumen, but also observed the formation of a lumen between paired ECs, that is most likely generated by a mechanism of cord hollowing, similar to that observed during anastomosis of ISVs (Herwig et al., 2011; Lenard et al., 2013; see below).

A more recent study (Gebala et al., 2016) challenges the view that sprouts expand the lumen through the generation and fusion of intracellular vacuoles (Kamei et al., 2006). Instead, this study proposes that hemodynamic forces dynamically shape the apical membrane of single or groups of ECs to form and expand new lumenized vascular tubes (Gebala et al., 2016; see Fig. 2B). The authors describe spherical deformations of the apical membrane during lumen expansion in the sprout. These ‘inverse blebs’ protrude into the surrounding EC, which reacts by local recruitment of actomyosin to counteract the local deformations, before the lumen expansion proceeds. This process of lumen expansion is similar to the mechanism of intracellular lumen formation described in tip cells during anastomosis (Herwig et al., 2011; Lenard et al., 2013).

While most blood vessels appear to lumenize via the above-mentioned mechanisms, ECs are also able to form a tube by a process called lumen ensheathment. This process is seen during the development of the zebrafish common cardinal vein; ECs migrate collectively as a sheet over the blood stream towards the heart and, eventually, ECs at the lateral edges migrate downwards and engulf the lumen (Helker et al., 2013). A similar mechanism has also been described during the formation of the DA in quail embryos (Sato et al., 2010). The formation of the luminal compartment of the DA has also been studied to some extent and appears to occur via cord hollowing (Axnick and Lammert, 2012; Jin et al., 2005; Strilić et al., 2009).

Clearly, several mechanisms might contribute to lumen formation in nascent sprouts and more studies are needed to decipher which mechanisms, if any, are observed more frequently and how or when a particular mechanism is selected. It will also be important to examine lumen formation in different vascular beds by focusing on the very early stages of sprouting, when the tip cell starts to leave the parental vessel.

Anastomosis: making new connections

Vascular anastomosis is the process that generates connections between angiogenic sprouts and blood vessels, and hence is fundamental for vascular network formation. Anastomosis can occur between two sprouts and involve two tip cells (‘head-to-head’ anastomosis), or between sprouts and a functional blood vessel, involving only one tip cell (‘head-to-side’ anastomosis) (Fig. 1). Recent live-imaging studies of developing ISVs, cerebral blood vessels and the SIV of the zebrafish embryo revealed a series of cell

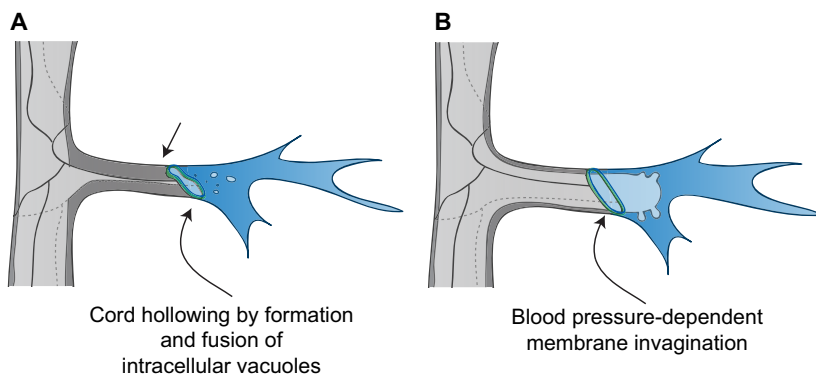


Fig. 2. Modes of lumen formation and expansion during angiogenesis. (A) Cord hollowing and vacuolar fusion. During this process, a new lumen is formed between cells by the formation of local lumina and their coalescence via cell rearrangements (cord hollowing). It has also been proposed that intracellular vacuoles grow to form larger intracellular compartments, which eventually fuse with the plasma membrane and merge with nascent extracellular luminal spaces. (B) A lumen can also form by blood pressure-induced invagination of the lumen into the tip cell. The blood pressure pushes the apical membrane that separates the stalk and the tip cell into the tip cell, like a finger poking into a balloon. The directed membrane expansion during this process is supported by ‘inverse blebbing’ (see text).

behaviors that succeed each other in a stereotypical mode, starting from initial filopodial contacts and ending with a novel, multicellular connection supporting blood flow (Herwig et al., 2011; Kochhan et al., 2013; Lenard et al., 2013, 2015). Below, we describe these cell behaviors, which are summarized in Fig. 3.

An important first step in anastomosis is the formation of a stable contact between two ECs. During initial contact formation, filopodia from neighboring tip cells make and break connections several times before one single connection is stabilized and reinforced by the deposition of adherens junction (AJ) proteins, such as Cdh5/VE-cadherin, at the contact site (Lenard et al., 2013;

Phng et al., 2013). At these adhesion sites, cells deposit de novo apical membrane, which eventually leads to the formation of ring-shaped AJs with apical membrane in between; since this process happens in both anastomosing cells, a small luminal pocket is generated at this site. Similar de novo ‘contact’ sites have also been reported in Madin-Darby canine kidney (MDCK) cells as they polarize and form a lumen, and these have been called apical membrane initiation sites (AMISs) (Bryant et al., 2010; Martín-Belmonte et al., 2008). In line with these studies, we refer to such polarized contact sites within the developing vasculature as AMISs.

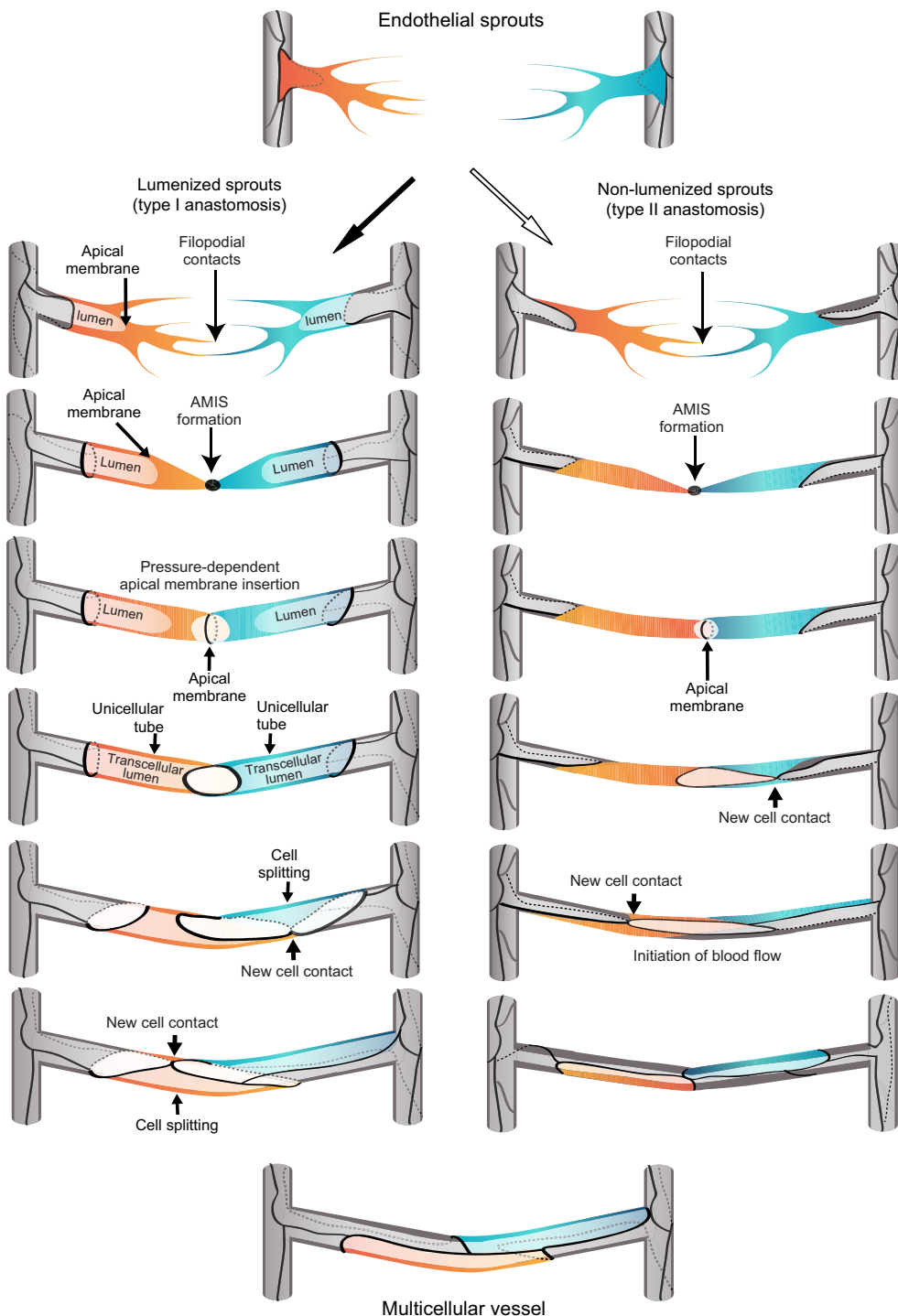


Fig. 3. Cell behaviors during anastomosis. Schematic depicting the different stages of blood vessel fusion in the case of lumenized sprouts (type I anastomosis; left) and non-lumenized sprouts (type II anastomosis; right). Both processes are initiated by the formation of filopodial contacts between sprouts, which eventually stabilize in one location. An apical membrane initiation site (AMIS) is formed at this novel contact site and apical membrane is inserted. In type I anastomosis, apical membrane invagination through blood pressure and subsequent apical membrane fusion generates a unicellular tube containing cells with a transcellular lumen. The subsequent transition from a unicellular to a multicellular tube in type I anastomosis involves cell rearrangements and cell splitting. In type II anastomosis, cell rearrangements lead to lumen coalescence and the formation of a multicellular tube. Figure adapted from Lenard et al., 2013; see the video abstract in their paper for more information.

After these initial steps of contact formation and EC polarization, two different cellular mechanisms – which we refer to here as type I and type II anastomosis (Fig. 3) – lead to the formation of an interconnected luminal space and the formation of multicellular, perfused tubes. In different vascular beds, these two mechanisms appear to occur at different frequencies, most likely depending on the degree of blood pressure and the presence or absence of a lumen in the participating tip cells.

Type I anastomosis, which is seen in the case of lumenized sprouts, occurs in the presence of blood pressure on the proximal apical membrane of the tip cell and involves the rapid growth and invagination of this apical membrane into the cell body (Fig. 3). In this case, blood pressure pushes the luminal space from the connecting stalk cells through the elongated tip cell, while being confined by a growing apical membrane (Lenard et al., 2013; see also Gebala et al., 2016). Eventually, the expanding lumen reaches the newly anastomosed site. The apical membrane of the expanding lumen then fuses with the de novo inserted apical membrane at the contact site, thereby generating a continuous intracellular lumen within this cell. Blood pressure subsequently extends the new apical membrane of the neighboring EC (a former tip cell) until its own two individual apical membranes meet and fuse, thereby generating a second cell with a transcellular lumen. At this stage, the new connection between two sprouts has been converted into a patent tube and blood flow commences. The hallmark of type I anastomosis is thus the transcellular hollowing of the two interacting tip cells, leading to the formation of two cell segments in the conformation of a unicellular tube. In the zebrafish PLA (and in other blood vessels), this newly formed unicellular tube is eventually converted into a multicellular tube via complex cellular rearrangements involving cell splitting. During these cell rearrangements, the cell with a transcellular lumen splits up on one side of the tube in order to allow the establishment of cell contacts between two neighbors of a former tip cell – a process that has been visualized by labeling individual cells in the developing vasculature with junctional markers (Lenard et al., 2013). Since in this particular scenario a single cell encompassing a lumen splits on one side, cell splitting does not lead to two cell bodies (one with a nucleus and one without), but rather transforms a doughnut-shaped cell into a flatter endothelial cell (see Lenard et al., 2013 for a more detailed description of endothelial cell splitting; see also Fig. 3 and Fig. 5F).

Type II anastomosis, which is seen in the case of a non-lumenized sprout, occurs in the absence of blood pressure (e.g. in a vessel in which the stalk is not lumenized up to the tip cell) and also initiates with contact or AMIS formation and the subsequent generation of an apical membrane pocket (Herwig et al., 2011). Because blood pressure does not push against the proximal apical membranes of the tip cells, these membranes do not invaginate and only expand slowly. Concomitantly with the insertion of new apical material, cell rearrangements similar to those seen in the second phase of type I anastomosis occur and eventually lead to the formation of new cell contacts between the anastomosing tip cells and stalk cells. The establishment of novel contacts between non-lumenized, neighboring ECs does not require cell spitting, but leads to the coalescence of the isolated luminal pockets into a single luminal space, which can subsequently be opened for blood flow (see Herwig et al., 2011). Thus, type II anastomosis is characterized by the formation of a multicellular tube omitting a unicellular intermediate stage. Interestingly, a rather similar process of lumen formation has been reported in *Ciona intestinalis* during the formation of the notochord (Denker and Di Jiang, 2012; Dong et al.,

2009), suggesting that the connection of serially arranged luminal pockets via cell rearrangements may be a common mechanism of tube formation. Although it has not been shown in detail, it is possible that this process is also involved in the formation of a patent lumen in the dorsal aorta.

The two modes of anastomosis mentioned above describe the behavior of individual cells during the anastomosis of two sprouts led by tip cells. However, during embryonic development, there are also many instances in which a sprout connects and fuses with a patent vessel, as seen during the fusion of the CMV with the PLA in the zebrafish head (Lenard et al., 2013), or the fusion of the nasal ciliary artery (NCA) with the cranial division of the internal carotid artery (CrDI) in the zebrafish eye (Kochhan et al., 2013). Even though these events are conceptually different from an anastomosis event involving two tip cells, the underlying processes share many similarities. For example, in all cases the process starts with the formation of an adhesive site between filopodia from the approaching sprout and the target vessel itself. This is followed by the formation of an AMIS and the rapid insertion of apical membrane, resulting in membrane invagination from the lumenized vessel into the former tip cell, fusion of individual apical membranes and subsequent cell rearrangements to generate a multicellular tube. Since one of the contact partners (the perfused vessel) is lumenized and under pressure, the process is analogous to type I anastomosis (Lenard et al., 2013).

Pruning: removing vessels

After functional vascular networks have been established, they often remodel in order to optimize flow or to adapt to changing demands in blood flow. The cellular aspects of this process have been summarized recently (Korn and Augustin, 2015; Ricard and Simons, 2015). In short, although apoptosis has been implicated in the regression of larger blood vessels, it turns out that smaller vessels are pruned by the reabsorption of ECs into the remaining vasculature. Interestingly, this pruning event is regulated by blood flow. In a pioneering study involving long-term time-lapse imaging of the developing zebrafish midbrain (from 1.5 to 7.5 days of embryonic development), it was shown that the developing vasculature undergoes extensive vessel pruning, regulated by blood flow changes (Chen et al., 2012). Vessel pruning occurs preferentially at loop-forming segments in a process involving lateral migration of ECs from the pruning to the stable vessel. Based on a wealth of experimental interventions and supported by hemodynamics-based simulations, it was further shown that changes in blood flow drive this vessel pruning via lateral migration of ECs. Similar results were obtained in the zebrafish eye, where a segment of the CrDI regresses upon fusion with the NCA (Kochhan et al., 2013). It was also demonstrated that it is not the absence of perfusion per se that leads to pruning, but rather the difference in blood flow between different branches of the loop. The role of blood flow in vessel pruning was also examined more recently using an elegant combination of experimental systems, namely mouse retinal explants and developing zebrafish ISVs (Franco et al., 2015). Using a Golgi marker as readout for EC axial polarity, it was shown that local differences in blood flow drive ECs to orient and migrate against the direction of flow. Pruning resulted from the dynamic and polarized migration of ECs in segments with low flow, accompanied by the stabilization of segments with high flow. The idea that emerges from these studies is that strong blood flow acts as an ‘attractor’ for cells, whereas poorly perfused vessels are less ‘attractive’ and thus promote regression of less-functional vessel segments.

Recently, the zebrafish SIV has also been used to study the cellular mechanisms of blood vessel regression in detail. Similar to what has been observed in anastomosis, pruning occurs via two different modes – type I and type II (Fig. 4) – depending on the state of vessel perfusion during the process (Lenard et al., 2015).

Type I pruning occurs in vessels that remain perfused until late in the pruning process. Such vessels undergo a transition from a multicellular to a unicellular tube by cell rearrangements; cells in the vessel to be pruned migrate towards the neighboring vessels that will be maintained. These cell rearrangements conceptually look like

anastomosis in ‘reverse mode’ (Kochhan et al., 2013). During formation of the unicellular tube, the last bridging cell starts to wrap itself around the lumen, eventually contacting and fusing with its own contralateral side as the two neighboring cells disconnect from each other. This endothelial cell self-fusion (see also below) generates a doughnut-like cell; since the cell fuses with itself, it contains only one nucleus (see Fig. 5G for a schematic illustration of the process). Subsequently, the lumen collapses, often after multiple rounds of lumen separation and reconnection. As a last step in pruning, the remaining two cells of the pruning branch, which still adhere to each

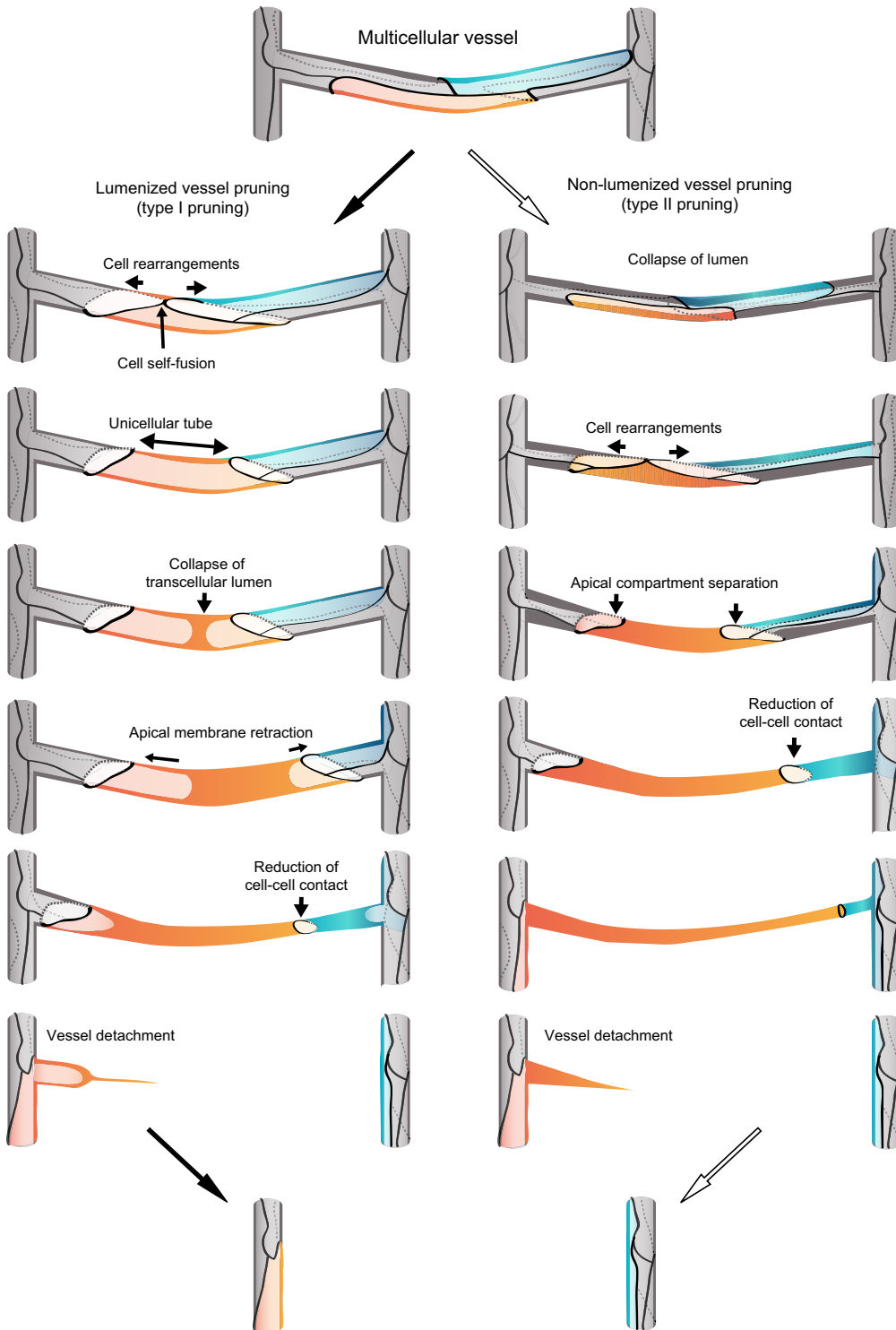


Fig. 4. Cell behavior during pruning. Blood vessel pruning can occur by two different modes, depending on the presence or the absence of a lumen during this process. Type I pruning, which occurs for vessels that maintain a lumen until the later stages of pruning, involves cell self-fusion in which the remaining endothelial cell in a pruning vessel wraps itself around the lumen, fuses with itself and forms a unicellular tube. Type II pruning involves the early collapse of the lumen in the multicellular tube, followed by cell rearrangements that lead to a unicellular, non-lumenized bridge. Finally, the remaining cell-cell contact is reduced, culminating in detachment of the vessel. Superficially, pruning resembles anastomosis in reverse.

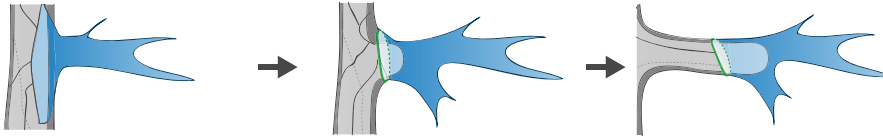
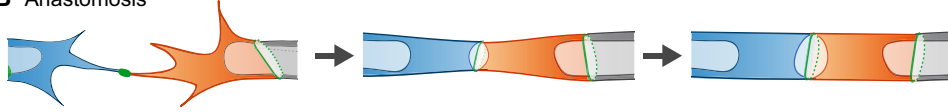
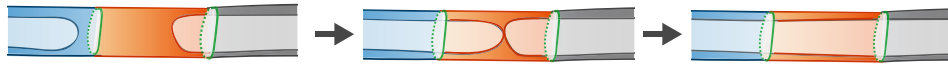
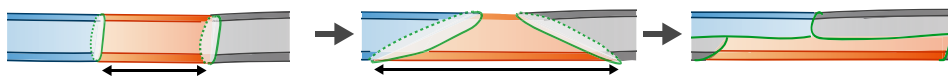
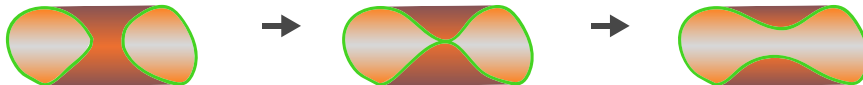
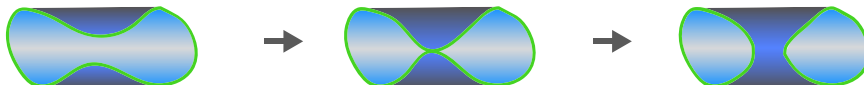
A Cell migration**B Anastomosis****C Apical membrane invagination/fusion****D Cell elongation and rearrangements****E Cell division****F Cell splitting****G Cell self-fusion**

Fig. 5. Summary of the morphogenetic cellular behaviors involved in angiogenesis. (A) Cell migration occurs during the formation of a new sprout. (B) New cell-cell contact formation occurs during anastomosis and involves the deposition of new junctional material (i.e. AMIS formation) and the subsequent addition of apical membrane. (C) Blood pressure-dependent apical membrane invagination and subsequent apical membrane fusion generate a continuous lumen. (D) Cell rearrangements within an existing vessel allow for a change in vessel architecture, from unicellular to multicellular tubes. Similar cell rearrangements also occur during sprout elongation. (E) During cell division in unicellular tubes, the lumen collapses temporarily to preserve vascular integrity. (F) Cell splitting occurs during the transformation of a unicellular tube (formed by an endothelial cell with a transcellular lumen) into a multicellular tube during type I anastomosis. (G) Cell self-fusion accompanies the cell rearrangements that occur during pruning. The last cell in the pruning vessel wraps around the lumen and fuses with its contralateral part to generate a unicellular tube.

other, disconnect and fully retract into the parental vessel. Thus, while ECs rearrange by ‘migrating’ towards each other during vessel anastomosis, ECs ‘migrate’ away from each other during pruning. In addition, although cell splitting allows new neighbors to establish cell contacts during anastomosis, EC self-fusion allows neighboring cells to terminate contacts during pruning.

Type II pruning is initiated when the lumen collapses at an early step of the pruning process, thereby generating a multicellular tube without a continuous lumen. In such a configuration, the ECs in the branch undergoing pruning move away from each other and integrate into neighboring vessels, leaving only a unicellular bridge between the connecting vessels. Further stretching of this connection gives rise to a very fine cytoplasmic connection and, finally, to the loss of contact, resulting in complete retraction of the cell into the recipient vessel. The EC self-fusion process, which occurs in type I pruning in a cell with a transcellular lumen, does not occur in type II pruning since the lumen already collapses at an early stage.

Linking cell behaviors to molecular pathways: open questions

As we have highlighted above, recent high-resolution time-lapse analyses of angiogenic processes have resulted in a wealth of information concerning the distinct cell behaviors occurring during sprouting, anastomosis and pruning (summarized in Fig. 5).

However, only in some cases have aspects of the molecular basis underlying these cellular behaviors been elucidated. Nonetheless, these recent advances provide us with a foundation on which we can build; forward genetic analyses should eventually result in the isolation and characterization of genes/proteins affecting these distinct cellular activities, while knowledge about the cell behaviors involved in angiogenesis allows us to take a hypothesis-driven reverse genetic approach. Next, we briefly comment on some of the molecular aspects involved in regulating cellular behaviors during angiogenesis. These comments are not meant to be comprehensive, but rather represent a selection of issues and open questions that we find particularly interesting.

Endothelial cell migration

Cell migration is certainly one of the processes that is studied most intensively in angiogenesis. EC migration is triggered by a multitude of signaling pathways that can act in a particular manner on different blood vessels, and this selectivity is thought to be important for coordinating the simultaneous formation of separate blood vessels (Vanhollebeke et al., 2015; Wiley et al., 2011; Ulrich et al., 2016). In addition to activating signals, angiogenic sprouts encounter numerous guidance cues that ensure proper pathway finding (reviewed by Carmeliet and Tessier-Lavigne, 2005; Hasan and Siekmann, 2015; Larrivee et al., 2009). Since the vasculature

invades all organs, it will be essential to dissect the relevant migratory cues in each case individually and identify attractive as well as repulsive cues in order to get a coherent and comprehensive picture of the mechanisms that regulate EC migration during angiogenesis (for examples, see Cha et al., 2012; Harrison et al., 2015; Tata et al., 2015; Thomas et al., 2013; Xu et al., 2014).

Endothelial cell elongation and cell rearrangements

Sprout outgrowth has been viewed as a simple form of collective cell migration, whereby the tip cell interprets guidance cues while stalk cells trail behind (for a review, see Haeger et al., 2015). However, recent analyses of the dynamics of sprouting ECs have shown that stalk cells are quite active and compete with other ECs for the tip cell position. This behavior is regulated by inter-endothelial Delta-Notch signaling and possibly by different VE-cadherin dynamics (Bentley et al., 2014; Jakobsson et al., 2010). Furthermore, at least in developing ISVs, sprout elongation is not just driven by EC proliferation and migration, but also by extensive cell shape changes, i.e. cell elongation. This EC elongation is reflected and driven by the remodeling of EC junctions and requires the adhesion molecule Cdh5/VE-cadherin as well as F-actin polymerization (Sauteur et al., 2014). Cell shape changes based on junctional remodeling are not only associated with EC elongation during sprout outgrowth, but also with EC rearrangements observed during anastomosis and pruning; cell rearrangements thus not only tightly accompany the angiogenesis process, they represent a major aspect all three steps of angiogenesis. A high degree of EC migration/rearrangement has also been observed in other systems [e.g. in avian embryos and in embryoid bodies (Jakobsson et al., 2010; Sato et al., 2010)]. In the developing zebrafish vasculature, cell rearrangements still occur in the absence of blood flow during anastomosis and pruning, demonstrating that flow or pressure are not essential in these cases. However, during the pruning process, the directionality of migration appears to be determined by differences in blood flow.

One noteworthy property of cell rearrangements in the vasculature is that they appear to lead to the formation of multicellular tubes by default. In the DLAV, cell rearrangements occur in the absence of blood flow (Herwig et al., 2011) and in the PLA, the experimentally induced transient loss of blood pressure leads to type II anastomosis (lumen formation via cell rearrangements; Lenard et al., 2013). In addition to the question of why this is the case, a number of issues regarding cell rearrangements remain unsolved. For example, how do ECs rearrange within the endothelium? Do they use the neighboring ECs or the extracellular matrix as substrate for migration? And how do cells coordinate their rearrangement/migration? Further imaging studies in wild-type and mutant situations will provide more insights into these issues.

Cell contact and AMIS formation

Blood vessel anastomosis is initiated by the establishment of filopodial contacts between two tip cells. However, contacts between tip cells can also occur in the absence of filopodia, when actin polymerization is blocked by latrunculin B (Phng et al., 2013). It is therefore thought that while filopodia are not essential for anastomosis, their contact may facilitate or fine-tune the process. Cdh5/VE-cadherin plays an important role early in the anastomosis process and is required for interactions between filopodia and tip cells and for the efficient formation of a single contact site (Lenard et al., 2015; Montero-Balaguer et al., 2009). Upon the deposition of Cdh5/VE-cadherin and presumably other junctional proteins at the

contact site, an AMIS is generated. Many molecules involved in contact formation (Ebnet et al., 2008) and apical membrane insertion (Caviglia and Luschnig, 2014; Datta et al., 2011; Roinot et al., 2013; Sigurbjörnsdóttir et al., 2014) have been identified in several models of epithelial morphogenesis, and it is very likely that similar molecules and processes regulate the formation of initial contacts between ECs and the formation of an AMIS.

Rapid apical membrane expansion and fusion

One important step in generating a continuous luminal space during anastomosis is the expansion and fusion of the two apical membrane compartments generated in a tip cell during type I anastomosis (Fig. 3). This process might be similar to the luminal membrane expansion and fusion events occurring during tube fusion in the *Drosophila* tracheae (Caviglia and Luschnig, 2014). In fusing tracheal cells, tracks containing F-actin and microtubules are assembled and serve to transport membrane vesicles towards the growing apical domains. Specialized components are then required for the fusion of the two independent apical compartments (Caviglia et al., 2016). It will be interesting to find out whether similar molecular players are involved during vessel anastomosis. The emerging role of blood pressure in the generation of a transcellular lumen (Lenard et al., 2013) and in lumen formation in tip cells of ISVs (Gebala et al., 2016) also requires further investigation. While it has been shown that inverse blebbing plays a role in generating an axial extension of the luminal compartment in ISVs (Gebala et al., 2016), it is not clear how blood pressure translates at the molecular level into the rapid growth of apical membranes. Most importantly, it is unknown from which membranous compartment the apical membrane derives, and how it is brought to the right place (Caviglia and Luschnig, 2014; Sigurbjörnsdóttir et al., 2014). The analysis of vesicle trafficking in anastomosing tip cells should provide insight into this issue.

Endothelial cell splitting

Cell splitting, which is observed during the transformation of a unicellular tube into a multicellular tube (see Fig. 3 and Fig. 5F for schematic representations), is a rather peculiar cell behavior that has not been seen before as an integral part of other morphogenetic processes. Since in this particular scenario, a single, doughnut-shaped cell encompassing a lumen splits on one side, cell splitting does not lead to two cell bodies (one with a nucleus and one without), but rather transforms this doughnut-shaped cell into a flatter endothelial cell. The molecular players involved in cell splitting might be similar to those involved in the final stages of cytokinesis (abscission) during mitosis (reviewed in Fededa and Gerlich, 2012). Alternatively, and more likely, splitting of the cell membrane might result in small lesions that are sealed by generic membrane healing processes (Jimenez and Perez, 2015). Using available molecular markers for each of these processes and analyzing them during angiogenesis might provide some initial insights into the possible molecular mechanisms involved in cell splitting. It should also be noted that, like individual ECs, entire blood vessels can also split (through the process of intussusceptive angiogenesis; see Box 2) but whether and when this occurs during developmental angiogenesis *in vivo* remains unclear.

Endothelial cell self-fusion

While cell-cell fusion is a prominent process during development and occurs during the fusion of gametes, the generation of multinucleate muscle fibers and during placenta formation

Box 2. Intussusception: the splitting of vessels

Intussusceptive angiogenesis (also referred to as intussusceptive sprouting) is the process in which a blood vessel splits longitudinally into two (Styp-Rekowska et al., 2011). This event was first reported during rat lung development (Burri et al., 1974). A hallmark of intussusception is the formation of intraluminal ‘pillars’, which are diagonal cellular connections that span the lumen within a vessel from one side to the other. The first step in pillar formation involves the involution of the endothelial layer on opposing sides of the intussuscepting vessel. These indentations then grow towards each other and finally connect, forming a hollow pillar within the tube that is later invaded by pericytes and myofibroblasts. Finally, neighboring pillars fuse, resulting in longitudinal splitting of the entire vessel (Gianni-Barrera et al., 2014; Mentzer and Konerding, 2014). Intussusception is influenced by blood flow (Djonov et al., 2002), stretching (Belle et al., 2014) and signaling (Baum et al., 2010). However, it should be noted that most of the studies describing intussusception have used cast formation or EM analyses, and the available models are thus inferred from snapshots. At present, it is not clear to what extent and where intussusception occurs *in vivo* during developmental angiogenesis. High-resolution time-lapse analyses, together with further comparisons of the intussusception process with other angiogenic cellular behaviors, are needed to provide further insights into the cellular aspects of this process.

(Aguilar et al., 2013; Demonbreun et al., 2015; Kim et al., 2015; Pérez-Vargas et al., 2014; Podbilewicz, 2014), cell self-fusion is a much rarer phenomenon, but has been well described in *C. elegans* (Rasmussen et al., 2008). Since, in these particular cases, cells fuse with themselves (as illustrated in Fig. 5G), these fusion events do not lead to multinucleate cells. It is worth noting that during tracheal branching morphogenesis in flies, cells rearrange during stalk elongation in a manner similar to that during pruning in the vasculature and contact themselves during the process (Jaźwińska et al., 2003; Ribeiro et al., 2004); however, in the developing tracheal system, the cells establish stable autocellular junctions and do not eliminate these self-contacts (i.e. they do not self-fuse).

Interestingly, recent experiments with MDCK cells have shown that mammalian cells tend to eliminate self-contacts (Sumida and Yamada, 2013), a process that might be similar or identical to the EC self-fusion event occurring during pruning. E-cadherin is strictly required for self-contact elimination in MDCK cells, and further studies have shown that Rho GTPases and the downstream effectors Actin-related protein 2/3 (Arp2/3) complex and Myosin II induce membrane fusion at self-contacts of MDCK cells (Sumida and Yamada, 2015). It will be very interesting to see whether EC self-fusion and the elimination of self-contacts by mammalian cells are regulated by similar molecular pathways, and whether VE-cadherin is required for self-contact elimination in HUVEC cells.

Conclusions

During the past decade, much progress has been made in understanding how the behavior of individual ECs is coordinated to generate an interconnected and functional vascular network. However, many additional questions can be raised. How do individual sprouts know where to connect? Do they simply connect to any ECs they contact? Or are there specific molecular cues involved in selected contact formation? How is a branch selected to undergo pruning? In particular, how are local differences in blood flow sensed and converted to initiate pruning? Finally, how do mural cells such as pericytes contribute to different angiogenic cell behaviors? While partial answers to several of these questions have already been proposed, we still have only a limited

understanding of these distinct cell behaviors and the molecular circuits underlying them. It is clear that straightforward genetic analyses of the above-mentioned processes in live animals, combined with high-resolution time-lapse imaging, will not be sufficient to elucidate the details of all the cell biological processes involved. In order to move forward, it will be important to bridge fluorescent microscopy with electron microscopy (EM), and use correlated light microscopy and EM (CLEM) (de Boer et al., 2015) to better understand, for example, what happens during lumen formation and expansion (e.g. how are vesicles involved?) and during other angiogenic processes. Furthermore, since many of these cellular processes (i.e. vesicle trafficking, cytoskeletal rearrangements, etc.) play essential functions in other organs, they are not easy to dissect using mutational analyses in animals, and novel approaches using more acute spatial and temporal protein manipulations will also have to be developed and used in the future (see Bieli et al., 2016; Strickland et al., 2012). However, with ever-improving microscopes and methods, it is likely that much progress will be made in the next few years, and it will be fascinating to see how the dynamic assembly of ECs into patent vascular networks is controlled and executed at the molecular level.

Competing interests

The authors declare no competing or financial interests.

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