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Review

"Sprouting angiogenesis", a reappraisal

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ABSTRACT

Angiogenesis is defined as a new blood vessel sprouting from pre-existing vessels. This highly regulated process take place through two non-exclusive events, the so-called endothelial sprouting or non-sprouting (intussusceptive) microvascular growth. This review article will provide a brief overview of some relevant topics defining sprouting angiogenesis and including: (i) The concept of functional specialization of endothelial cells during different phases of this process, involving the specification of endothelial cells into tip cells, stalk cells, and phalanx cells bearing different morphologies and functional properties; (ii) The interplay between numerous signaling pathways, including Notch and Notch ligands, VEGF and VEGFRs, semaphorins, and netrins, in the regulation and modulation of the phenotypic characteristics of these cells; (iii) Some fundamental and consecutive morphological processes, including lumen formation and perfusion, network formation, remodeling, pruning, leading to the final vessel maturation and stabilization.

Contents

Introduction

Vascular development and homeostasis are underpinned by two fundamental features: the generation of new vessels to meet the metabolic demands of under-perfused regions and the elimination of vessels that do not sustain flow.

Research in angiogenesis has greatly improved allowing the characterization of different molecular mechanisms and the identification of several angiogenesis agonists and antagonists.

Angiogenesis is defined as a new blood vessel sprouting from pre-existing vessels. This can be accomplished through endothelial sprouting or non-sprouting (intussusceptive) microvascular growth (IMG) (Ribatti, 2006). This latter postulated that the capillary network increases its complexity and vascular surface by insertion of a multitude of transcapillary pillars, a process called 'intussusception' (Djonov et al., 2000a).

The first description of sprouting angiogenesis in tumor growth was reported by Ausprunk and Folkman, 1977, which indicated the following stages: (1) The basement membrane is locally degraded on the side of the dilated peritumoral post-capillary venule situated closed to the angiogenic stimulus; (2) Interendothelial contacts are weakened and endothelial cells

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migrate into the connective tissue; (3) A solid cord of endothelial cells form; (4) Lumen formation occurs proximal to the migrating front, contiguous tubular sprouts anastomose to form functionally capillary loops, parallel with the synthesis of the new basement membrane and the recruitment of pericytes. Ausprunk and Folkman identified that blood vessel sprouting can initially progress without cell division, while sustained sprouting and further outgrowth require proliferation.

A variety of in vivo and in vitro models of angiogenesis have been developed which have greatly contributed to the remarkable advance in this field. Among the in vitro assays, the three most used include rabbit cornea (Muthukkaruppan and Auerbach, 1979), the developing mouse retina (Fruttiger, 2007), and the intersegmental vessel growth in zebrafish (Lawson and Weinstein, 2002). In vitro models with isolated endothelial cell lines have allowed to study selected aspects of the angiogenic process, including endothelial migration, proliferation, proteolytic digestion of the extracellular matrix and capillary tube formation (Cimpean et al., 2010).

Human endothelial cells were first placed on dishes coated with a thin layer of extracellular matrix proteins by Folkman and Haudenschild (1980), and organized into tube-like structures. Under both light and electron microscope, Grant et al. (1991) showed that these capillary-like networks had lumens and the endothelial cells possessed membrane specialization similar to those observed in vessels in vivo. In vitro assays were further developed independently by Montesano and Madri (Montesano et al., 1983; Madri and Williams, 1983) by using a three-dimensional collagen gel. Montesano et al. (1983) demonstrated that when a monolayer of microvascular endothelial cells on the surface of a collagen gel is covered with a second layer of collagen, it reorganizes within a few days into a network of branching and anastomosing tubules.

In Paku and Paweletz, 1991 integrated the schema by Ausprunk and Folkman by means of ultrastructural observations, as follows: (1) A structural alteration of the basement membrane occurs, characterized by the loss of electron density over the entire circumference of the dilated mother vessel, followed by a partial degradation of the basement membrane at places where endothelial cell processes are projecting into the connective tissue; (2) Endothelial cells migrate arranged in parallel, maintaining their basal-luminal polarity a forming a slit-like lumen and sealed by intact interendothelial junctions; (3) Basement membrane is deposited continuously by the polarized endothelial cell while only the tip of the growing capillary bud is devoid of basement membrane; (4) Proliferating pericytes migrate along the basement membrane of the capillary bud, resulting in a complete coverage of the new vessel.

Endothelial tip, stalk, and phalanx cells

Initiation of sprouting requires the specification of endothelial cells into tip and stalk cells bearing different morphologies and functional properties. Endothelial tip cells primarily migrate but proliferate only minimally, in contrast to endothelial stalk cells, which do proliferate. In 2003, the concept of "tip" and "stalk" cell phenotype was described for emerging sprouts (Gerhardt et al., 2003), even though filopodia studded cells at the front were already described before this work by Kurz et al. (1996) and, later, by Ruhrberg et al. (2002). Accordingly to this "new" model, two principal cells are involved in sprouting angiogenesis, namely "tip cell" and "stalk cell". Tip cell is migratory and polarized, while stalk cell proliferates during sprout extension and forms the nascent vascular lumen cell. The phenotypic specialization of endothelial cells as tip or stalk cells is very transient and reversible, depending on the balance between pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and Jagged-1 (JAG-1), and suppressors of endothelial cell proliferation, such as delta-like ligand 4 (Dll4)-Notch activity(Eilken and Adams, 2010; Geudens and Gerhardt, 2011; Wacker and Gerhardt, 2011; Tung et al., 2012)

Tip cells express high levels of Dll-4, platelet derived growth factor-b (PDGF-b), unc-5 homolog b (UNC5b), VEGF receptor-2 (VEGFR-2), and VEGFR-3/Flt-4, and have low levels of Notch signaling activity (Gerhardt et al., 2003; Claxton and Fruttiger, 2004; Lu et al., 2004; Siekmann and Lawson, 2007; Suchting et al., 2007). Zebrafish tip cell is a highly branched structure (Siekmann and Lawson, 2007), while in other mouse angiogenesis models, such as brain and retina (Gerhardt et al., 2003, 2004; Dorrell and Friedlander, 2006), the tip cell extend numerous filopodia, that serve to guide the new blood vessel in a certain direction toward an angiogenic stimulus, proliferates minimally, and adopts a highly branched shape while moving (Isogai et al., 2003). Bentley et al. (2009) developed a new physics-based model tightly coupled to in vivo data and proposed a novel filopodia-adhesion driven migration mechanism, demonstrating that cell–cell junction size is a key factor in establishing a stable tip/stalk pattern.

Stalk cells produces fewer filopodia, are more proliferative, form tubes and branches, and form a vascular lumen (Thurston and Kitajewski, 2008). They also establish junctions with neighboring cells and synthesize basement membrane components (Phng and Gerhardt, 2009). Only a regulate balance between both processes establishes adequately shaped nascent sprouts. (Eilken and Adams, 2010; Geudens and Gerhardt, 2011; Wacker and Gerhardt, 2011; Tung et al., 2012). During the transition from active sprouting to quiescence endothelial cells tip cell adopts a "phalanx" phenotype, resembling a phalanx formation of ancient Greek soldiers, that is lumenized, non-proliferating, and immobile cells, which promotes vessel integrity and stabilizes the vasculature through increased cell adhesion and dampened response to VEGF (Bautch, 2009; Mazzone et al., 2009).

Role of VEGF and VEGFRs

VEGF signaling pathway has been established as the master regulator of angiogenesis. Endothelial cells express VEGFR-2, a tyrosine kinase receptor that positively drives the mitogenic and chemotactic responses of endothelial cells to VEGF. Tip cell migration depends on a gradient of VEGF, whereas stalk cell proliferation is regulated by VEGF concentration (Gerhardt et al., 2003). The leading tip cell responds to a VEGF gradient by migrating outward from the parent vessel up the gradient (Ruhrberg et al., 2002). VEGF induces the formation and extension of filopodia as well as the expression of Dll4 protein in the tip cells, and filopodia engage with those of a nearby tip cell to form a "bridge" and the formation of a new vessel (Bentley et al., 2009). As the vessel elongates, the stalk cell proliferate in response to VEGF, creates a lumen, synthesizes a basement membrane and associates with pericytes, and increases the mass and surface of the growing vessel.

VEGF stimulates tip cell induction and filopodia formation via VEGFR-2 which is abundant on filopodia, whereas VEGFR-2 blockade is associated with sprouting defects (Phng and Gerhardt, 2009). Moreover, activation of Cdc 42 by VEGF triggers filopodia formation (De Smet et al., 2009). VEGFR-1 expression is induced by Notch signaling to reduce VEGF ligand availability, preventing tip cell outward migration. VEGFR-1 is predominantly expressed in stalk cells, is involved in guidance and limiting tip cell formation, and loss of VEGFR-1 increases sprouting and vascularization (Chappell et al., 2009; Chappell and Bautch, 2010).

During both mouse and zebrafish angiogenesis, VEGFR-3 is most strongly expressed in the leading tip cell and is down-regulated by Notch signaling in the stalk cell (Shawber et al., 2007; Siekmann and Lawson, 2007).

Role of notch and notch ligands

Notch receptor are large transmembrane proteins that when are activated by their ligands expressed on adjacent cells, regulate cell fate in multiple lineage (Bray, 2006). The Notch signaling pathway is essential for vascular development. The necessity of Dll-4/Notch-1 signaling in the endothelium has been well established as loss of a single copy of Dll-4 or deletion of Notch-1 causes vascular defects and embryonic lethality (Gale et al., 2004; Limbourg et al., 2005). Notch-1 and Notch-4 and three Notch ligands, JAG-1, Dll-1, and Dll-4 are expressed in endothelial cells for the induction of arterial cells fate and for the selection of endothelial tip and stalk cell during sprouting angiogenesis (Kume, 2009). A loss of Notch signaling induces while its activation reduces sprouting. Notch-1 deficient endothelial cells adopt tip cell characteristics (Hellstrom et al., 2007), while Notch signaling activity is greater in stalk cells, in which activation of Notch by Dll4 leads to a down-regulation of VEGFR-2 and -3 in these cells (Hofmann and Iruela-Arispe, 2007). Cells dynamically compete for the tip position utilizing differential VEGFR levels, as cells with higher VEGFR signaling produces more Dll4 and therefore inhibit their neighboring cells. In this context, a fine tuned feed-back loop between VEGF and Notch/Dll4 signaling pathways is established and a cross-talk between these pathways is essential for proper patterning of the vasculature (Holderfield and Hughes, 2008).

Blockade of Notch leads to widespread Flt4 expression, increases filopodia and sprouting, and promotes tip cell activity (Thurston et al., 2007; Siekmann and Lawson, 2007). Tip cells with low Notch activity have high VEGFR-2 and low VEGFR-1 expression, which results in higher levels of Dll4 expression and, hence through a higher production of Dll4 than in neighboring cells, an increased ability to suppress its neighboring cells from becoming tip cells (Jakobbson et al., 2010). Endothelial cells with higher levels of VEGF increase Dll4 expression which further increases the cell sensitivity to VEGF, and this cell becomes the tip cell selected for outward migration for the parent vessel (Hellstrom et al., 2007; Lobov et al., 2007; Sainson et al., 2005; Siekmann and Lawson, 2007; Tammela et al., 2008). Stalk cells have high levels of Notch signaling activity and elevated expression of JAG-1 (Benedito et al., 2009). Stalk cell JAG-1 antagonizes Dll4 activity, reducing the induction of Notch signaling in the adjacent tip cell, which therefore maintains its responsiveness to VEGF stimulation and migrates outward to establish a new branch (Benedito et al., 2009). However, JAG-1 poorly activates Notch-1, as modification of Notch by FRINGE glycosyltransferase favors activation by Dll4 (Eilken and Adams, 2010). Notch-regulated ankyrin-repeat protein (Nrarp) is a down-stream of Notch that counteracts Notch signaling and is expressed in stalk cells at branch points (Phng et al., 2009). Silencing of Nrarp is responsible of a reduction in vessel density, due to a lowered endothelial cell proliferation as a consequence of an up-regulation of Notch and VEGFR-1, of poorly luminized vessels, remodeling of endothelial junctions and vessel regression. By inducing Wnt signal in stalk cells, Nrarp is responsible of vascular stabilization and lowering Wnt signal induces vessel regression (Phng et al., 2009).

Semaphorin 3E (SEMA3E) and netrin-UNC5B signaling

Semaphorins are secreted or membrane-bound guidance cues that interact with receptor complexes, formed by neuropilins (NRPs) alone or NRP/plexin family proteins (Carmeliet and Tessier-Lavigne, 2005). SEMA3A is expressed by endothelial cells of developing vessels and inhibits endothelial migration by interfering with integrin function (Bates et al., 2003; Serini et al., 2003). Indeed, SEMA4D induces endothelial cell migration and tubulogenesis in vitro and stimulates blood vessel formation in vivo (Basile et al., 2004). Loss of plexin-D1 expressed by endothelial cells is responsible of an aberrant sprouting into SEMA3E-expressing tissues in zebrafish embryos (Adams and Eichmann, 2010). In the mouse retina, SEMA3E activates plexin-D1 on tip cells to fine-tune the balance on tip and stalk cells necessary for even-growing vascular fronts by coordinating VEGF's activity in a negative feed-back (Kim et al., 2011).

Netrins are laminin-related secreted bifunctional guidance cues, which also bind to extracellular matrix components: attraction and repulsion are mediated by binding to deleted in colorectal cancer (DCC) and UNC5 family receptors, respectively (Dickson and Keleman, 2002). Lu et al. (2004) demonstrated that UNC5B is selectively expressed in the vascular system by arteries, a subset of capillaries and endothelial tip cells. UNC5B inactivation results in enhanced sprouting, whereas netrin-1 prompts filopodia retraction of endothelial cells (Adams and Eichmann, 2010). These results suggest that SEMA3E-plexin-D1 and netrin-UNC5B might act at specific guide-posts for developing vessels, suggesting that different repulsive signals might act at distinct vessel branching sites.

Lumen formation and perfusion

Many organs, including blood vessels, are composed of epithelial tubes with an epithelial surface lining the lumen, that transport vital fluids (Lubarsky and Krasnow, 2003). The lumen of a blood vessel is essential for providing blood to any given tissues. In the vascular system, lumen formation involves a complex molecular mechanism composed of endothelial cell repulsion at the cell–cell contacts within the endothelial cell cords, junctional rearrangement, and endothelial cell shape change (Iruela-Arispe and Davis, 2009). After the vascular lumen has been established, blood initiates to flow through the newly formed vessel.

Early observation in the eighteen century demonstrated that capillaries send out sprouts, which extend until they meet and anastomose with other sprouts or capillaries and into which a lumen advances. Two different ways of lumen formation have been discussed: cord hollowing (Billroth, 1856) and cell hollowing (Sabin, 1920). In the former, slit-like lumen formation takes place between facing endothelial cells. In the latter, the ensuing lumen is the result of coalescence of intracytoplasmic vesicles. Clark and Clark also contributed other details to our knowledge of angiogenesis including the development of capillary lumina and fusion of sprouts (Clark and Clark, 1939).

During the final steps of capillary development, endothelial cell migration comes to a halt and endothelial cells form a lumen and re-establish functional adherens junctions (Dejana, 1996). Adherens junction formation was shown to be associated with the inhibition of endothelial cell migration in monolayers. This process was shown to be mediated by vascular endothelial cell cadherin (VE-cadherin) (Lampugnani et al., 1995; Dejana, 1996), a cell adhesion molecule belonging to the cadherin protein family, exclusively expressed in endothelial cells and facilitating their homotypic interaction (Cavallaro et al., 2006; Labelle et al., 2008). VE-cadherin is strictly required for the polarization of endothelial cells in vitro and in vivo, and VE-cadherin-based junctions are subjected to continuous reorganization, which renders them highly dynamic and sensitive to extracellular stimuli (Strilic et al., 2009; Lampugnani et al., 2010).

The lumen of the mouse dorsal aorta forms by organized changes in endothelial cell shape (Strilic et al., 2009), while an alternative mode has been proposed in which intercellular and intracellular vacuoles forms and fuse along connected endothelial cells (Kamei et al., 2006; Blum et al., 2008). The intracellular vacuolization is a rapid way to create endothelial cell luminal spaces (Bayless and Davis, 2002; Bayless et al., 2000; Davis and Camarillo, 1996; Koh et al., 2008). During this process collagen (Davis and Senger, 2005) represents a promorphogenic factor, whereas basement membrane proteins are inhibitory for vascular morphogenesis (Davis and Senger, 2005, 2008). In accordance with ultrastructural observations in the rat mesentery lumen formation occurs relatively late in endothelial cords, and first develop at the proximal parts of sprouts, whereas the tips of long and pointed sprouts of a considerable length do not contain detectable lumina (Rhodin and Fujita, 1989).

During lumen formation, two functionally different phenotypes of endothelial cells are recognized. The first phenotype, represented by endothelial cells in mature blood vessels, is characterized by an apico-basal polarity and junction-mediated contact inhibition. The second phenotype is found in activated tissues and is characterized by the loss of apico-basal polarity and adherents junctions, a spindle-shaped morphology, and the ability for guided migration. Endothelial cell polarization often starts with the delivery via exocytosis of de-adhesive apical glycoproteins, including CD34-sialomucins, such as CD34 and podocalyxin (PODXL), to the cell–cell contact (Martin-Belmonte et al., 2007; Ferrari et al., 2008). During delivery of apical glycoproteins, the adherens junctions translocate to lateral positions, and the new junctions have properties of adherens and tight junctions, as they contain both VE-cadherin (characteristic of adherens junctions) and zonula occludens-1 (ZO-1) protein (characteristic of tight junctions) (Bazzoni and Dejana, 2004).

Formation of apical cell surfaces and electrostatic repulsion of negatively charged apical glycoproteins are sufficient for the initial de-adhesion of adjacent endothelial cells and for slit formation, but are not sufficient for the development of a patent vascular lumen. VEGF-A therefore induces cell shape changes that further separate the apical cell surfaces from each other (Strilic et al., 2009). After lumen formation, the lumen diameter of vessel sprouting increases, and hemodynamic stimuli caused by shear stress significantly contribute to increased diameter. Vascular lumen expansion is force-dependent and involves F-actin cytoskeleton and/or blood flow. Conversely, genetic factors prevail during early embryogenesis and, despite lack of blood flow and mural cell coverage (Strilic et al., 2009; Wiegreffe et al., 2009), endothelial cell proliferation directly affects lumen diameter (Graupera et al., 2008; Stenman et al., 2008). A key extracellular matrix protein required for vascular lumen formation and remodeling is fibronectin (Bazigou et al., 2009; Stenzel et al., 2011), required for proper aortic lumen formation in the early mouse embryo and for retinal angiogenesis in the newborn mouse (Stenzel et al., 2011; Yang et al., 1993).

It has been recognized that endothelial cells use other cells as migration tracks during capillary network remodeling. In addition to sprouting, Nehls et al. (1998) indeed defined a new type of endothelial cell migration, namely guided migration of endothelial cells along performed capillary-like structures. By fluorescent labeling of living endothelial cells, these authors observed that endothelial cells in immature microvascular networks migrated along preformed capillary-like structures. By guided migration, endothelial cells increase on preexisting capillary-like structures which consequently led to thickening and to increased capillary diameters. Endothelial cell-derived fibroblast growth factor-2 (FGF-2) is involved in the regulation of guided migration.

Network formation

Following assembly of primitive vessels in the early embryo, remodeling transforms the plexus into an organized network of arteries, capillaries, and veins. Network formation represents a crucial step in the angiogenic process and provides the growing tissue with a newly-constituted apparatus of immature and rudimentary vascular channels. The development of a complex, interconnected meshwork of crude capillary tubules is the structural substrate upon which acts the fine-tuning process of vascular remodeling. Network formation originates from coalescence of sprouts or by IMG. IMG is an alternative or additional mechanism of capillary growth whereby the vascular network expands by insertion of newly formed columns of interstitial tissue (interstitial tissue structures) into the vascular lumen called tissue pillars or posts. They are initially of small dimensions, showing a diameter between 0.5 and 2.5 mm, and will consecutively grow to larger size (Patan et al., 1996). Interestingly, transluminal pillar formation is one particular way of expanding vessels (Djonov et al., 2000b; Patan et al., 2001). Thus, it represents a crucial mechanism for vascular network formation. According to Burri's group, this mechanism proceeds through four steps: (1) Protrusion of opposing capillary walls into the lumen and the creation of a contact zone between facing endothelial cells; (2) Reorganization of their intercellular junctions and central perforation of the endothelial bilayer; (3) Formation of an interstitial pillar core by invading supporting cells (myofibroblasts, pericytes) and deposition of matrix, such pillars ranging in diameter from 1 to 2.5 μm; and (4) Enlargement in thickness of the pillars without additional qualitative alteration (Djonov et al., 2000a,b, 2002).

The sequence of pillar formation was observed during the initial remodeling of the capillary plexus into immediate pre- and post-capillary feeding vessels. This process was termed intussusceptive arborization (IAR) and provides a mechanism by which well-perfused capillary segments are transformed into terminal arterioles and veins. As a result of this process, a complex arterial and venous vascular tree arises from the primitive capillary plexus, forming a second layer.

Other mechanisms involving sprout fusion, tip cell filopodia, and macrophages, participate to the vascular network formation. A sprouting vessel, by fusing with a target sprout acquire a functional lumen and become stable (Iruela-Arispe and Davis, 2009). Tip cell filopodia interact to initiate junction formation and reinforce sprout formation (Bentley et al., 2009). Macrophages may also facilitate sprout fusion by bridging sprouts to their potential target (Fantin et al., 2010).

Remodeling

The initial endothelial plexus generated by vascular sprouting consists of a homogenous web of endothelial cell tubes and sacs. This plexus is created in excess and the final adjustment of vascular density involves the regression of unnecessary vessels through a process of vascular remodeling and pruning which creates a more differentiated vascular network (Risau, 1997). Remodeling involves the growth of new vessels and the regression of others as well as changes in the diameter of vessel lumens and vascular wall thickening. Examples of regression include the regression of capillaries in prechondrogenic regions to allow differentiation of cartilage (Hallmann et al., 1987), and the regression of the hyaloids vasculature to allow the development of the vitreous body in the eye (Latker and Kuwabara, 1981).

Remodeling determines the formation of large and small vessels, the establishment of directional flow, the association

with mural cells (pericytes and smooth muscle cells) and the adjustment of vascular density to meet the nutritional requirements of the surrounding tissue. An interesting event which precedes vascular remodeling is the phenomenon of capillary retraction, described for the first time by Clark (1918), and Clark and Clark (1939). According to these authors, retraction of sprouts is observable during the rapid growth phase of capillaries and usually associates with elongation of other capillaries in the immediate vicinity. Retraction of capillary sprouts, which is not synonymous with regression of capillaries, could be defined as an event by which endothelial cells are withdrawn from one capillary sprout to become incorporated either into the mother vessel or into adjacent sprouts which are elongating. Remodeling is a complex phenomenon which requires a vast array of molecular signaling. One of the earliest identified events involved in vascular remodeling was the interaction between the receptor tyrosine kinase tie-2 (Schnürch and Risau, 1993; Sato et al., 1993) and its ligand, Angiopoietin (Ang) (Davis et al., 1996). Mice lacking tie-2 or Ang die between embryonic days 9.5 and 12.5, and the embryos show a persisting capillary plexus, reminiscent of a defect in vascular remodeling and angiogenesis (Sato et al., 1993; Suri et al. 1996). Researchers have constantly looked for identification of useful models for studying vascular remodeling. The developing vitreal vasculature can be viewed as a good in vivo model of vascular remodeling, maturation, and regression since it has a finite lifespan encompassing all of these processes. Indeed, formation and maturation of the vitreal vasculature involves extensive initial vasculogenesis followed by combinations of vasculogenesis and angiogenesis events which are accompanied by pruning and remodeling of the maturing vitreal vessel network (Lang and Bishop, 1993; Yang and Cepko, 1996, Mitchell et al.,1998; Ash and Overbeek, 2000). Tubedown-1 (tbdn-1) expression in endothelial cells becomes down-regulated during the formation of capillary-like structures in vitro and is regulated in vivo in a manner which suggests a functional role in dampening blood vessel development. Paradis et al. (2002) showed that tbdn-1 was expressed highly in the vitreal vascular network (tunica vasculosa lentis and vasa hyaloidea propria) during the pruning and remodeling phases of this transient structure. Overall, these data indicate that the developing vasculature responds dynamically to the growing needs of the embryo by remodeling vessels as required.

Pruning

Pruning was first described in the embryonic retina and involves the removal of excess of endothelial cells which form redundant channels (Ashton, 1966). Simulations suggest how flow might affect vessel pruning, which due to low wall shear stress, is highly sensitive to the pressure drop across a vascular network. In fact, the degree of pruning increases as the pressure drop increases (Skalak and Price, 1996). Pruning and remodeling of the vascular network may be stimulated by tissue-derived signaling molecules and blood flow conditions (e.g. wall-shear stress and pressure). Although the morphological hallmarks of IMG and IAR, crucial mechanisms for generating and expanding a capillary network, were frequently observed in capillaries and terminal microvessels with diameters <25 μm, the formation of transluminal pillars and folds in arteries and veins with diameters of up to 110 μm was a surprise. Such pillars were closely associated with bifurcations and were shown to be involved in remodeling and pruning of larger microvessels (Djonov et al., 2002). This process hence was termed intussusceptive branching remodeling (IBR).

An interesting model of vascular pruning is provided by modification of pulmonary vascularization under certain conditions (Howell et al., 2003). Chronic hypoxia caused by migration of native sea-level dwellers to high altitude or chronic lung disease leads to the development of increased pulmonary vascular resistance and pulmonary hypertension (Meyrick and Reid, 1978; Hopkins and McLoughlin, 2002). The structural changes that are thought to underlie the increased vascular resistance can be broadly classified into two processes: firstly, remodeling of the walls of the pulmonary resistance vessels and, secondly, pruning in the total number of blood vessels in the lung (Meyrick and Reid, 1978; Rabinovitch et al., 1979). The structural changes include muscularisation of non-muscular arterioles, increased medial thickness of muscular arterioles, adventitial hypertrophy and deposition of additional matrix components, including collagen and elastin, in the vascular walls (Rabinovitch et al., 1979; Stenmark and Mecham, 1977). The second major structural alteration caused by chronic hypoxia is loss of small blood vessels, which is said to increase vascular resistance by reducing the extent of parallel vascular pathways (Hislop and Reid, 1976, 1977; Rabinovitch et al., 1979).

In another vascular system, the retina, oxygen supply is regarded as a key factor in blood vessel pruning. Earlier studies have highlighted the key role of oxygen and VEGF in the formation and remodeling of the retina vasculature (Stone and Maslim, 1997; Dorrell and Friedlander, 2006). In addition to preventing new vessel growth, hyperoxia suppresses VEGF production and leads to obliteration by apoptosis of already formed vessels (Stone et al., 1995). This suggests that vascular pruning accompanying natural remodeling is caused by hyperoxia upon the onset of flow through the newly formed vascular system. Exposure to hyperoxia leads to excessive regression of capillaries, while arteries become refractory to this insult (Benjamin et al., 1998). Excessive vascular pruning is the initiating event in the pathogenesis of retinopathy of prematurity (ROP), a blindness-causing disease induced in premature infants placed in oxygen chambers, and VEGF protects retinal vessel form hyperoxia-induced obliteration in an experimental model of ROP (Alon et al., 1995). Activated leukocytes, extravasate in the retina and are involved in mediate endothelial cell apoptosis (Ishida et al., 2003).

Maturation and stabilization (pericyte recruitment)

The stabilization of the newly formed vessel and the maintenance of the existing vasculature are late events in the angiogenic process. Blood flow is critically important for determining the vessel fate [vessels with high flow widen, while vessels with low flow regress (Le Noble et al., 2004)], although recent advance of vascular biology strongly argues for the autonomous fate control achieved by blood vessels (Jones, 2011). More recently, Chen et al. (2012) have demonstrated that lymphatic endothelial and vessel phenotype are negatively regulated by blood flow and that changes in hemodynamic forces can reprogram lymphatic vessels to blood vessels in postnatal life.

Several cellular and non-cellular components in the blood vessel, including endothelial cells, pericytes, smooth muscle cells, fibroblasts, glial cells, inflammatory cells, and the extracellular matrix, coordinately regulate the maintenance of vessel integrity at varying degrees in different vascular beds (Xu and Cleaver, 2011; Patel-Hett and D'Amore, 2012; Stratman and Davis, 2012). Pericyte adhesion to native capillaries and endothelial cell wrapping by surrounding pericytes are basic events in blood vessel stabilization and maturation. It has been hypothesized that concomitant with sprouting, endothelial cells direct the differentiation of mural cell precursors from the adjacent tissue by the secretion of soluble factors (Beck and D'Amore, 1997; Nehls et al., 1992). A different view is that mural cells become associated with

endothelial cell by migrating along newly made vascular sprouts (Nicosia and Villaschi, 1995). A role for mural cells in maintaining vascular integrity was suggested by a number of gene knock-out studies. This includes disruption of the genes encoding the endothelial specific receptors tie-1 and tie-2 (Dumont et al., 1994; Puri et al., 1995), the tie-2 ligand, (Ang-1) (Suri et al., 1996), tissue Factor (TF) system (Carmeliet et al., 1996) and the PDGF-b/PDGF-b receptor system (PDGFb-R) (Leveen et al., 1994; Lindahl et al., 1997). Mice deficient in these genes show a hemorrhaging phenotype often associated with a reduced number of α- smooth muscle actin (SMA)-positive perivascular cells and microaneurysm formation. Ang-1- and TF-deficient mice also show defects in the remodeling of the homogenous yolk-sac vascular plexus.

In the ocular model, for instance, changes in the state of ocular pericytes are known to be associated with vasculopathy in eye diseases in humans. Pericyte death, thickening of the basement membrane, and changes in extracellular matrix expression pattern in proliferative diabetic retinopathy have been described (Yanoff, 1966; Bloodworth and Epstein, 1967; Cogan and Kuwabara, 1967; Addison et al., 1970; Ashton, 1966; Speiser et al., 1968; Podestà et al., 2000). Studying postnatal remodeling of the retina vasculature, it has been showed that pericyte recruitment represented a critical step in vascular maturation (Benjamin et al., 1998). It proceeded by outmigration of cells positive for α–SMA from arterioles, and coverage of primary and smaller branches lagged many days behind formation of the endothelial plexus. The transient existence of a pericyte free endothelial plexus coincided temporally and spatially with the process of hyperoxia-induced vascular pruning, which is a mechanism for fine tuning of vascular density according to available oxygen (Benjamin et al., 1998).

Concluding remarks

Angiogenesis, describing the development of microvessels from pre-existing ones, was recognized as early as 1787, when John Hunter noted that it occurred during the development of reindeer antlers (Hunter, 1787). Angiogenesis requires integrated activities of heterogeneous endothelial cell populations and perturbations of any of these relationships compromises vessel development and function. More recent advances have introduced the concept of functional specialization of endothelial cells during the sprouting process in the so-called endothelial tip cells distinct from the endothelial stalk cells, and have highlighted important roles for components of the Notch and Wnt signaling in the regulation of sprouting angiogenesis. Pro-angiogenic signals, such as VEGF, promote endothelial motility, filopodia extension and proliferation, and under the control of Notch signaling, whether endothelial cells become tip cells or stalk cells (Fig. 1).

Modern experimental approaches can provide further insights in elucidating mechanisms of angiogenesis and can provide powerful tools for examining this process that govern the new formation of vessels in both physiological and pathological conditions. Among the in vitro models of sprouting angiogenesis, it is to note the sprouting model in which HUVEC-coated beads in a fibrin matrix sprout and form lumenized vessels (Nakatsu et al., 2007). Another model allows a programmed differentiation of mouse embryonic stem cells to form lumenized vessels (Kearney and Bautch, 2003). Embryoid bodies are clusters of embryonic stem cells able to differentiate into multiple cell lineages, including endothelial cells, which can be used to investigate quantitative and cell gene functions in sprouting angiogenesis (Jakobsson et al., 2010).

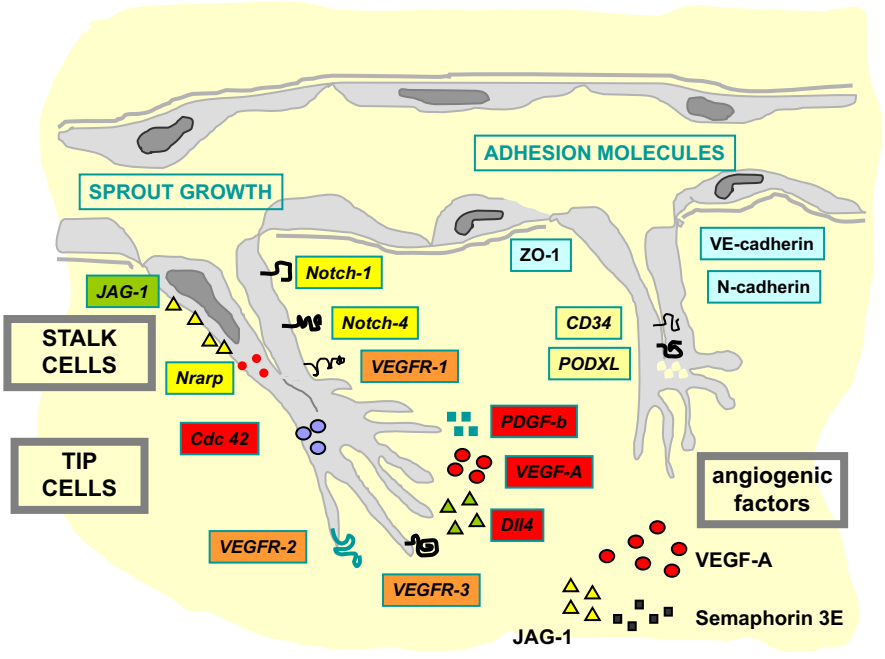

Fig. 1. The functional specialization of endothelial cells during the sprouting process. VEGF-A induces the formation and extension of filopodia as well as the expression of Dll4 protein in the tip cells. Tip cells express high levels of Dll-4, PDGF-b, UNC5b, VEGFR-2, and VEGFR-3/Flt-4, and have low levels of Notch signaling activity. During both mouse and zebrafish angiogenesis, VEGFR-3 is most strongly expressed in the leading tip cell and is down-regulated by Notch signaling in the stalk cell. Stalk cells produces fewer filopodia, are more proliferative, form tubes and branches, and form a vascular lumen. They also establish junctions with neighboring cells and synthesize basement membrane components. Stalk cells have high levels of Notch signaling activity and elevated expression of JAG-1. Nrarp is a down-stream of Notch that counteracts Notch signaling and is expressed in stalk cells at branch points. Adherens junction formation is associated with the inhibition of endothelial cell migration in monolayers. This process is mediated by VE-cadherin. Another cell adhesion molecule expressed in endothelial cells is the N-cadherin. VE-cadherin is strictly required for the polarization of endothelial cells. Endothelial cell polarization starts with the delivery of de-adhesive apical glycoproteins to the cell–cell contact via exocytosis. De-adhesive molecules include CD34-sialomucins, such as CD34 and PODXL.

Computational modeling is a powerful predictive tool for the understanding of vascular development. In this context, in silico and in vitro studies highlight the importance of cell shape (elongated vs round) during vascular development (Merks et al., 2006). The initial sprouting process could be replicated in silico, indication that VEGF/Notch regulation is sufficient to pattern this process (Bentley et al., 2009). This model also predicted that the balance between VEGFR-1 and VEGFR-2 expression in endothelial cells affects their potential to become the tip cell in a growing sprout (Jakobbson et al., 2010). Another modeling approach that has been used to study cell behaviors and cell-cell communication in sprouting angiogenesis is the so called "agent-based modeling" (ABM) (Qutub and Popel, 2009). Recently, Artel et al. (2011) developed an ABM that included sprout initiation, guidance, extension, and anastomosis to study the relationship between pore size of an ex-vivo scaffold and the rate of invasive angiogenesis. Arima et al. (2011), using time-lapse imaging in the aortic ring assay and a combination of labeling techniques that allows nuclear tracking of endothelial cells, performed a comprehensive analysis of cell movements and their relative contribution to sprout elongation. These models have provided new information about cell behaviors and their molecular regulation during blood vessel sprouting, even if a full understanding if how angiogenic sprouting and vessel network formation are regulated and several open questions regarding the spatial organization of signals and responses will require the integration of data from different model systems.

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