Angiogenic tip cells in endothelial cell cultures

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CHAPTER 2

Mechanisms of ocular angiogenesis and its molecular mediators

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ABSTRACT

Angiogenesis is defined as the formation of new blood vessels from the existing vasculature. It is a highly coordinated process occurring during development of the retinal vasculature, ocular wound healing, and in pathological conditions. Complex interactions are involved between non-vascular and microvascular cells, such as endothelial cells and pericytes, via several angiogenic growth factors and inhibitors. Of these growth factors, vascular endothelial growth factor (VEGF) has emerged as the single most important causal agent of angiogenesis in health and disease in the eye. During the angiogenic process, endothelial cells shift from a homogeneous quiescent population into a population of heterogeneous phenotypes, each with a distinct cellular fate. So far, three angiogenic specialized phenotypes have been identified: (1) 'tip cells', which pick up guidance signals and migrate through the extracellular matrix; (2) 'stalk cells', which proliferate, form junctions, produce extracellular matrix, and form a lumen, and (3) 'phalanx cells', which do not proliferate, but align and form a smooth monolayer. Eventually, a robust mature new blood vessel is formed which is capable of supplying blood and oxygen to tissues. Pathological angiogenesis is a key component of several irreversible causes of blindness. In most of these conditions, angiogenesis is part of a wound healing response culminating, via an angiofibrotic switch, in fibrosis and scar formation which leads to blindness. Currently, VEGF-A antagonists are standard care in the treatment of exudative age-related macular degeneration, and have been found to be a valuable additional treatment strategy in several other vascular retinal diseases.

INTRODUCTION

Blood vessels form an intricate hollow network of arteries, capillaries, and veins for the transport of liquids, solutes, gases, macromolecules, and cells throughout the vertebrate body. The vascular network is formed during early stages of development, and its correct and early function is absolutely critical for survival of the embryo. New blood vessels originate from endothelial precursor cells (angioblasts) by a process called vasculogenesis or from pre-existing blood vessels by angiogenesis.[1,2] Once a functional adult vascular system has been formed completely, blood vessels become quiescent. The growth potential of smaller blood vessels, however, is retained and is employed during wound healing and tissue regeneration.

Beyond its physiological roles, angiogenesis is also a hallmark of many pathological conditions, including neovascular diseases in the eye.[3-5] Excessive angiogenesis occurs when diseased cells produce abnormal amounts of angiogenic factors, overwhelming the effects of natural angiogenesis inhibitors. As the newly formed vessels mainly serve a role in a wound healing response, they usually do not restore the tissue integrity, but rather cause visual impairment when they are located in normally avascular, transparent tissues such as the cornea and vitreous. Strategies for inhibition of angiogenesis include approaches that can block the angiogenesis cascade at several steps.[4,6]

ANGIOGENESIS: MECHANISMS AND MOLECULAR MEDIATORS

Endothelial cell differentiation. All blood vessels are lined by endothelial cells (ECs), which form the interface between circulating blood in the lumen and the rest of the vessel wall. Under normal conditions, ECs are a remarkably quiescent cell type, undergoing division approximately once every 1000 days, but when activated, cell division can occur every 1-2 days.[7] Sprouting angiogenesis requires selection of ECs from an existing blood vessel which will be activated to form the new vessel, while at the same time surrounding ECs remain quiescent in their current position. From recent studies a model has emerged in which ECs differentiate into 3 specialized cell types with distinct phenotypes during angiogenesis (fig. 1).[8-10] First, a single 'tip cell' develops. This EC breaks down the basal lamina, emerges from its parent blood vessel and becomes the leading cell of the sprouting vessel. The tip cell migrates into the extracellular matrix and senses micro-environmental attractive and repulsive signals for guidance. Secondly, following directly behind the migrating tip cell, other ECs differentiate under the influence of the adjacent tip cell into 'stalk cells' that proliferate and bridge the gap between the tip cell and the parent vasculature. Stalk cells generate the blood vessel lumen through the formation of intra-cellular vacuoles, a process called 'lumenogenesis'. Thirdly, ECs behind the stalk cells differentiate into 'phalanx cells', and align in a smooth cobblestone monolayer, becoming the most inner cell layer in the new blood vessel. Phalanx cells no longer proliferate, express tight junctions and make contact with mural cells.

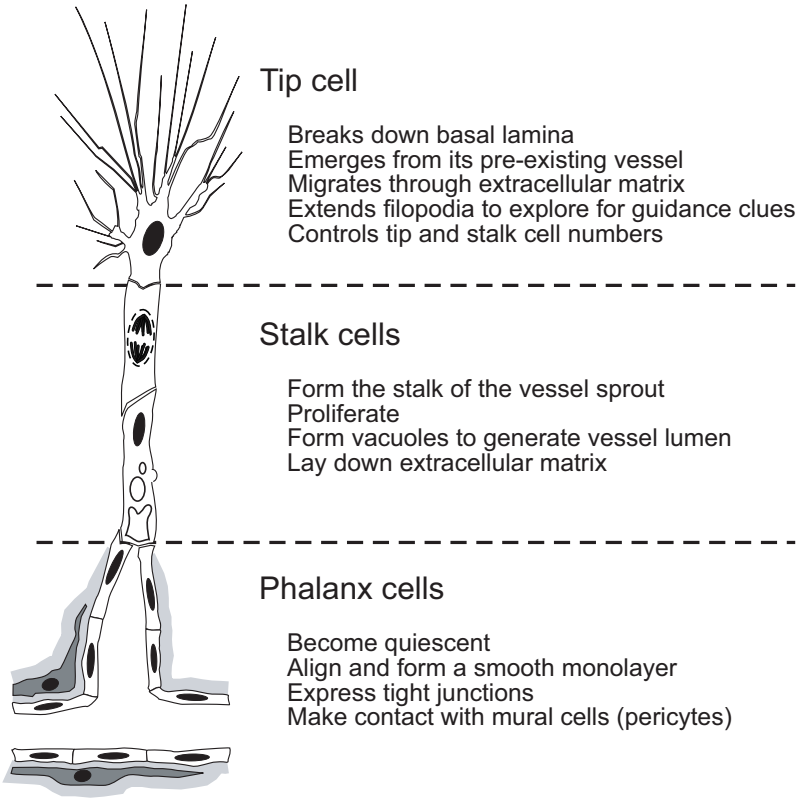

Figure 1. Representative model of sprouting angiogenesis. At least three different angiogenic specialized endothelial cells (white) are required, each with a distinct cellular fate. In addition, the new blood vessel becomes surrounded by pericytes (dark grey) and a new basal lamina (light grey).

Angiogenesis inducers and inhibitors. Angiogenesis is tightly controlled by closely interacting angiogenic and angiostatic factors, and their balance ultimately determines if, where and when the "angiogenic switch" is turned on with angiogenesis as the result.[2,9] Over the past decades, numerous inducers of angiogenesis have been identified, including the members of the VEGF family, angiopoietins, transforming growth factors (TGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF-α), insulin like growth factor (IGF), vascular endothelial-cadherin (VE-cadherin), interleukins and the members of the fibroblast growth factor (FGF) family. In addition, there is a plethora of growth factors, hormones and metabolites that have been reported to directly or indirectly stimulate physiological and pathological angiogenesis (table 1).[11,12] Not all of these factors are specific for ECs. Consistent with a major role for hypoxia in the overall process of angiogenesis, a large number of angiogenic factors involved in various stages of angiogenesis are independently responsive to hypoxia. [13] The VEGF family of proteins is the most important family of angiogenic factors that controls blood vessel formation.

Table 1. Major angiogenic factors. EC=endothelial cell, PC=pericyte, ECM=extracellular matrix, BL=basal lamina.

Protein (family)	Angiogenic members	Function(s)
Angiogenin		EC proliferation
Angiopoietins	Ang1	PC recruitment, vessel maturation
	Ang2	EC sprouting and migration, only in the presence of VEGF
Chemokine (C-C motif) ligands	CCL1 (I-309)	EC chemotaxis and differentiation
Chemokine (C-X-C motif) ligands	CXCL6, CXCL12	EC proliferation, guidance
Eph/Ephrins	EphB4/ephrinB2	Arterial/venous differentiation, tip cell guidance
Epidermal growth factor	EGF	EC proliferation and migration
Erythropoietin	EPO	EC proliferation
Fibroblast growth factor-family	aFGF, bFGF	EC proliferation and migration, ECM remodeling
Granulocyte-macrophage colony-stimulating factor	GM-CSF	EC proliferation and migration
Hepatocyte growth factor	HGF	EC proliferation and migration, PC proliferation
Hypoxia-inducible factor	HIF-1α, HIF-1β, HIF-2α	VEGF ↑
Insulin-like growth factor	IGF-1	EC proliferation, VEGF ↑
Integrins	Integrin αvβ3, Integrin αvβ5	Acquired for FGF induced angiogenesis, EC migration
Interleukins	IL-1, IL-6, IL-8, IL-13	EC proliferation, MMPs ↑
Matrix metalloproteinases	MMP-1, MMP-2, MMP-9	BL degradation, ECM remodeling
Monocyte chemotactic protein	MCP-1	Mediates TGFβ stimulated angiogenesis
Notch/delta-like ligand	Notch-1/Dll4	Tip/stalk cell regulation, arterial/venous differentiation
Plasminogen Activator	PA1	EC migration
Platelet endothelial cell adhesion molecule	PECAM-1	EC tube formation and adhesion, tip cell filopodia formation
Platelet-activating factor	PAF	EC sprouting
Platelet-derived endothelial cell growth factor	PD-ECGF	EC proliferation
Platelet-derived growth factor	PDGF-BB	PC recruitment
Prostaglandins	PGE-1, PGE-2	EC proliferation
Stromal cell-derived factor	SDF-1	Angioblast migration, tip cell guidance
Thrombin		PDGF and PAF ↑, ECM remodeling
Transforming growth factor-family	TGFα, TGFβ	at low doses: EC proliferation and migration, ECM remodeling
Tumor necrosis factor	TNFα	at low doses: EC proliferation and tube formation, tip cell 'priming'
Vascular endothelial cadherin	VE-cadherin	EC adhesion and proliferation
Vascular endothelial growth factor-family	VEGF-A, VEGF-C, VEGF-D, PlGF	Permeability ↑, EC sprouting, migration and proliferation, tip cell activation and guidance

Endogenous inhibitors of angiogenesis are defined as proteins or fragments of proteins that can inhibit the formation of blood vessels.[14] Angiogenesis inhibitors can be detected in circulating blood, suggesting that they function in the angiogenic switch as endogenous angiostatic regulators under physiological conditions. Various inhibitors of angiogenesis have been found in the body, including thrombospondin, angiostatin, endostatin and pigment epithelium-derived factor (PEDF) (table 2).[12,14]

Table 2. Major endogenous angiostatic factors. EC=endothelial cell, PC=pericyte, ECM=extracellular matrix, BL=basal lamina.

Protein (family)	Angiostatic members	Function(s)
Angiopoietins	Ang2	antagonist of Ang1, vessel destabilization only in the absence of Ang1/VEGF
Angiostatin		EC proliferation ↓ and apoptosis ↑
Chemokine (C-C motif) ligand	CCL21	EC migration ↓
Chemokine (C-X-C motif) ligands	CXCL9, CXCL10, CXCL11, CXCL13	EC migration ↓, FGF ↓
	CXCL4	inhibits VEGF and FGF binding
Endostatin		EC proliferation, migration and survival ↓, MMPs ↓
Interferons	IFNα, IFNβ, IFNγ	EC migration ↓, FGF ↓
Interleukins	IL-4, IL-10, IL-12, IL-18	EC migration ↓
Osteopontin		Integrins ↓
Pigment epithelium-derived factor	PEDF	EC migration and proliferation ↓
Plasminogen activator inhibitors	PAI-1, PAI-2	ECM remodeling ↓
Soluble neuropilin receptor	sNRP1	decoy receptor for VEGFs
Soluble vascular endothelial growth factor-receptor	sVEGFR-1	decoy receptor for VEGFs
Thrombospondins	TSP1, TSP2	EC migration and proliferation ↓
Tissue inhibitor of metalloproteinases	TIMP-1, TIMP-2, TIMP-3, TIMP-4	EC migration ↓, ECM remodeling ↓
Transforming growth factor-family	TGFβ	at high doses: EC proliferation and migration ↓, TIMPs ↑
Vascular endothelial growth inhibitor	VEGI	EC proliferation ↓
Vasculostatin		EC migration ↓
Vasostatin		EC proliferation ↓

The VEGF family and their receptors. In mammals, the VEGF family includes VEGF-A (also referred to in this review as VEGF), VEGF-B, placenta growth factor (PlGF), VEGF-C, VEGF-D, and the viral VEGF homologue VEGF-E. VEGFs bind selectively with different affinities to at least 5 distinct receptors: VEGF receptor-1 (VEGFR-1), also called Flt-1; VEGFR-2, also called Flk-1; VEGFR-3, also called Flt-4; neuropilin-1 (NRP-1); and NRP-2.[5,15,16] The VEGFRs are members of the tyrosine-kinase receptor superfamily. Ligand binding to the extracellular immunoglobulin-like domain induces receptor dimerization. VEGFR-2 is considered to be the major receptor responsible for mediating the angiogenic effects of VEGF-A. The role of VEGFR-1 in angiogenesis remains controversial as its activation has been shown to both stimulate and suppress angiogenesis. However, soluble VEGFR-1 (sVEGFR-1) inhibits retinal angiogenesis *in vivo*.[17] VEGFR-3 is highly expressed in angiogenic sprouts *in vivo* and, like VEGFR-2, its signaling mediates angiogenesis.[18] NRPs are VEGF-A_{165}, PlGF, and VEGF-B specific receptors, and form receptor complexes with VEGFRs: NRP-1 partners with VEGFR-2, whereas NRP-2 can form a complex with VEGFR-2 and VEGFR-3.[16]

VEGF-A, the best characterized and most studied of the VEGF family members, was originally described as a permeability factor, as it increases permeability of the endothelium through the formation of intercellular gaps and fenestrations. At least six human VEGF-A mRNA species, encoding VEGF-A isoforms of 121, 145, 165, 183, 189 and 206 amino acids, are produced by alternative splicing of the VEGF-A mRNA.[15,16] In mouse, the VEGF-A isoforms are one amino acid shorter i.e. VEGF-A_{120} etc. It is widely accepted that VEGF-A is crucial for both vasculogenesis and angiogenesis: loss of only a single allele in mice or zebrafish is lethal, resulting in severe vascular defects and cardiovascular abnormalities.[19] VEGF-A exerts its biologic effect through interaction with VEGFR-1 and VEGFR-2, and the neuropilin receptors NRP-1 and NRP-2.[15]

VEGF-B yields two isoforms, VEGF-B_{167} and VEGF-B_{186} by alternative splicing, which signal through VEGFR-1 and NRP-1.[16] VEGF-B is widely expressed in various tissues, including retina, but it is particularly abundant in the heart and skeletal muscle.[15] VEGF-B is able to directly stimulate EC growth and migration *in vitro* and *in vivo*.[15] However, the precise role of VEGF-B is not known, and genetic studies have revealed that VEGF-B-deficient mice are healthy and fertile, and do not display vascular defects, which indicate that VEGF-B is not involved or redundant in angiogenesis.[15,16]

PlGF is predominantly expressed in the placenta, heart and lungs, and binds VEGFR-1 and NRP-1.[16]. The binding of PlGF to VEGFR-1 leads to complex forming between VEGFR-1 and -2, which enhances VEGF-A signaling and stimulates angiogenesis.[15] PlGF upregulates the expression of VEGF-A, FGF-2, PDGF-B, matrix metalloproteinases and other angiogenic factors, suggesting that ECs are able to enhance their own responsiveness to VEGF-A by producing PlGF. Furthermore, PlGF can promote blood vessel maturation via the recruitment of mural cells.[15]

VEGF-C and VEGF-D both bind VEGFR-2, but with a lower affinity than they bind to VEGFR-3. Like VEGF-A, both VEGF-C and VEGF-D are able to stimulate the migration and proliferation

of ECs *in vitro* and *in vivo*.[15] VEGFR-3 expression is more abundant on tip cells than on stalk cells[18], whereas VEGFR-3 expression is absent on phalanx cells. It has been suggested that VEGF-C may cooperate with VEGF-A to activate ECs for angiogenic sprouting via VEGFR-2/VEGFR-3 receptor complex.

The viral VEGF homologue VEGF-E is a potent angiogenic factor as well.[16]

Matrix degradation. Before ECs can grow out from pre-existing vessels, the EC basal lamina must be degraded and the extracellular matrix needs to be remodelled.[8,9,12] This is achieved by a complex interplay of angiogenic growth factors, mural cells, and ECs. Acidic and basic fibroblast growth factors (aFGF and bFGF, respectively) and VEGF stimulate the production of collagenase and matrix metalloproteinases, and upregulate urokinase-type plasminogen activator in ECs.[20] Collagenases are enzymes that break the peptide bonds in collagens; urokinase-type plasminogen activator converts plasminogen into plasmin, leading to fibrinolysis; and matrix metalloproteinases (MMPs) are capable of degrading all kinds of extracellular matrix proteins. Furthermore, low dose-stimulation by TGF-β upregulates proteases in ECs.[21] At the same time, FGFs and VEGF downregulate endogenous inhibitors of proteolytic enzymes such as tissue inhibitors of metalloproteinases (TIMPs).[20]

Tip and stalk cell regulation. The selection of an endothelial tip cell from a population of quiescent ECs has to be tightly regulated since excessive tip cell formation would result in a poorly-patterned, hyperdense vessel network that may not be functional. Clearly both tip and stalk cells are stimulated by the same growth factor, VEGF, and both respond through VEGFR-2 signaling.[9,10,22] However, their behavior is very different and *in vivo* studies show that tip and stalk cells carry a differential transcriptional signature.[9,10,22] In tip cells, VEGFR-2 signaling induces the expression of the Notch ligand delta-like 4 (DLL4), which is transported to the cell membrane and binds to Notch receptors on adjacent ECs.[22-26] After ligand binding, Notch is cleaved in these future stalk cells, generating the Notch intracellular domain that acts as a transcriptional regulator. In these stalk cells, notch activation downregulates the expression of VEGFR-2, VEGFR-3 and NRP-1, while inducing the transcription of VEGFR-1 and its soluble splice variant sVEGFR-1.[24-26] Experimental inhibition of Dll4-Notch1 signaling raised the number of tip cells during early postembryonic angiogenesis, leading to increased sprout densities and change in vascular patterning.[22] Over-activation of Notch signaling, on the other hand, reduced the migratory behavior of ECs.[22] Other Notch ligands expressed by sprouting vessels are Jagged1 and Dll1, and loss of each of these also results in vascular defects. However, Dll4 is the only ligand expressed in tip cells, whereas Jagged1 and Dll1 are present in stalk cells.[27] These data indicate that the graded distribution of VEGF together with Dll-Notch signaling regulates angiogenic behavior of ECs by limiting the number of cells that become tip cell.

Endothelial proliferation. The stimulatory effects of VEGFs on EC proliferation have been well reported *in vitro* and *in vivo*.[5,16] Interestingly, during angiogenesis, adjacent ECs exhibit distinct cellular behavior patterns, even when exposed to a similar degree of VEGF-A, indicating that several other key molecules are involved in EC differentiation into tip cells, stalk cells or phalanx cells.[22] Co-expression of NRPs with VEGFR-2 is typical for endothelial tip cells, where it enhances VEGF-A binding to VEGFR-2, VEGFR-2 phosphorylation and VEGF-induced signaling, all of which are required for migration. In stalk cells, where NRP expression is absent, VEGF-A signaling via VEGFR-2 promotes proliferation but not migration.[8,10,22]

At low doses, TGF-β contributes to the angiogenic switch by upregulating angiogenic factors in ECs, but it has inhibitory effects at higher concentrations.[21] TGF-β family ligands stimulate type II receptors that phosphorylate type I receptors (such as activin receptor-like kinase (ALK)) and activate the downstream signaling Smads. Endoglin is a type III receptor, which facilitates ALK1/TGF-β signaling in ECs, and ALK1/Endoglin/TGFβ signaling also promotes EC proliferation and migration. Addition of a neutralizing antibody against TGF-β strongly inhibited angiogenesis *in vitro* and *in vivo*.[21] The angiogenic effects of TNF-α are similar to those of TGF-β, as it promotes EC proliferation and tube formation in lower doses, but inhibits angiogenesis in higher doses.[28]

Angiopoietin-2 (Ang-2) can act as an angiogenic factor depending on the presence of co-stimulatory molecules. For example, in the presence of VEGF, Ang-2 induces migration and proliferation of ECs by binding to the Tie2 receptor an thereby blocking Tie2 signaling of Angiopoietin-1 (Ang-1). In the absence of VEGF, however, Ang-2 causes apoptosis of ECs and regression of blood vessels. Ang-1 has an antagonizing effect on Tie2 and inhibits EC proliferation. Ang-1 secreted by pericytes binds to Tie2 on ECs, and is important for maintenance of vessel integrity and quiescence.

Several other molecules have been reported to stimulate EC proliferation, including FGFs, epidermal growth factor(EGF), CXC-chemocines and insuline-like growth factor-1 (IGF-1).[12,29]

Endothelial cell-cell interaction. EC junctions are composed of a complex network of adhesion proteins that are linked to the intracellular cytoskeletal network and signalling molecules. VE-cadherin is specifically localized to the inter-EC junction, and is known to be required for maintaining a restrictive endothelial barrier. VE-cadherin is critical for proper vascular development: VE-cadherin-null mice die in early embryonic stages because of vascular defects. [30] The functions of cadherins are modulated by catenins, which bind with the intracellular tail of the cadherins. After activation of VEGFR-2 by VEGF, catenins become highly phosphorylated, leading to loss of cell-cell junctions, allowing EC to differentiate and move from their current position. Later on during angiogenesis, the phosphorylation of catenins decreases, allowing restabilization of EC cell-cell junctions and the differentiation into quiescent phalanx cells.

Platelet EC adhesion molecule-1 (PECAM-1) is expressed on ECs, and like VE-cadherin, it is enriched in intercellular junctions. PECAM-1 mediated cell-cell junctions are necessary for the

organization of ECs in to tubular networks *in vitro*, and PECAM-1 has been shown to stimulate the formation of tip cell filopodia *in vivo*.[31]

Blood vessel guidance. Endothelial tip cells pick up attractive or repulsive signals from the tissue environment, and translate them into a dynamic process of adhesion and de-adhesion, leading to migration. In this process the tip cell forms lamellipodia (short cytoskeletal projection) and filopodia (long finger-like plasma membrane extensions).[8] Lamellipodia are located on the mobile edge of the cell. They adhere and connect the intracellular cytoskeleton to the extracellular matrix, allowing stress fibres of actin/myosin filaments to pull the cell forward. Filopodia protrude from the lamellipodial actin network and function as antennae with which tip cells probe their environment. The main regulators of filopodia and lamellipodia formation are members of the Rho small GTPases, which are induced by VEGF.[32]

An extracellular VEGF-A gradient appears to be a strong attractant for migrating ECs via binding to VEGFR-2 and NRPs, which are prominent on tip cell filopodia. An important biological property of the different VEGF-A isoforms is their heparin and heparan-sulphate-binding ability. The larger VEGF-A isoforms bind very tightly to heparin and, thus, remain sequestered in the extracellular matrix, whereas the shorter VEGF-A isoforms are freely diffusible. It is well established that VEGF-A_{189} and VEGF-A_{165} function as a chemoattractive signal that promote the polarized extension of tip cell filopodia, whereas VEGF-A_{121} can support EC proliferation but not tip cell guidance.[22,33]

Furthermore, the function of endothelial tip cells bears remarkable similarity to that of axonal growth cones. Blood vessels and nerve fibers course throughout the body alongside one another and it has been reported that during embryogenesis, their patterning is guided in large part by similar attractive and repulsive guidance cues. Thus far, four major families of receptors have been shown to regulate guidance events during axonal and vascular morphogenesis: Plexin/NRP complexes with their ligands class 3 semaphorins; ´uncoordinated-5´ (UNC5) family and ´deleted in colorectal cancer´ (DCC) with their ligands netrins; ´Roundabout´ (Robo) with their ligands Slits; and Eph and their ligands ephrins.[9,33,34]

Lumen formation. While migrating, the leading tip cell creates a tunnel throughout the extracellular matrix space. Behind the tip cell, stalk cells flatten onto the wall of this tube-like space in the extracellular matrix, resulting in an apical and basal face of the endothelium. Stalk cells form large intracellular vacuoles by fusion of intracellular vesicles, mediated by integrins, which fuse together to form a lumen.[9,35] Multiple integrins as well as the transcription factor myocyte enhancer binding factor 2C (MEF2C) are able to participate in vesicle formation and fusion *in vitro*.[35] EC interactions with the extracellular matrix establish signaling cascades downstream of integrin ligation leading to activation of the Rho family of GTPases. Inhibition of Rho GTPases results in complete blockade of EC vacuole and lumen formation *in vitro*.[35]

Recruitment of mural cells and maturation. After the initial vessel formation through angiogenesis, determination of artery or vein identity is regulated by a variety of molecular factors which specify EC fate. Distinct arterial and venous molecular markers are evident even before the initiation of circulatory flow, suggesting that molecular determinants play a critical role in arterial/venous differentiation. Several relevant genes have now been identified *in vivo*, including the Hedgehog family of secreted morphogens, notch signaling, NRPs, EphB4, ephrinB2, and VEGF. [36]

The two major classes of mural cells are the vascular smooth muscle cells, which coat veins and arteries, and the pericytes, which are present in variable amounts around capillaries. The mural cells are indispensable to provide survival and anti-proliferative factors that stabilize the newly-formed vessel. However, the hypothesis that pericyte loss initializes the first steps of angiogenesis whereas pericyte recruitment only occurs at the completion of angiogenesis is controversial, since many pericytes are found to be present in endothelial sprouts in vivo.[37]

The development and the recruitment of vascular mural cells require the function of PDGF signaling, Ang-1 and its receptor Tie-2, and Ephrin–Eph interaction. PDGFs exist as heterodimers (PDGF-AB) or homodimers composed of chains A and B. Endothelial tip cells from growing vascular sprouts generate a PDGF-B concentration gradient that promotes the recruitment of pericytes expressing the PDGF-B receptor.[37,38] This in turn activates TGF-β in pericytes, which introduces the production of basal lamina components that are required for final blood vessel maturation and stabilization.[21]

Ang-1, expressed by perivascular cells binds to and activates the Tie2 receptor, thereby stimulating mural cell attachment. In agreement, a poor association between ECs and surrounding mural cells was seen in Ang-1 and Tie2 knockouts. Ang-2 was shown to have an antagonizing effect on Tie2 inducing pericyte loss and capillary degeneration in the retina. However, endothelial expression of Tie2 has been observed on newly formed vessels that are still immature.[37]

RETINAL CIRCULATION

Retinal vascular system. The retina has a dual vascular supply: the outer one-third of the retina is supplied from the choroidal circulation; the inner two-third by the central retinal artery. The choroidal arteries pierce the sclera around the optic nerve and fan out to form the choriocapillaris, providing nutrients and oxygen to the retinal pigment epithelium and the photoreceptors in the outer part of retina, which exhibit high oxygen consumption. The corresponding venous lobules drain into the venules and veins that run anterior towards the equator of the eyeball to enter the vortex veins. The vortex veins penetrate the sclera and ultimately merge into the ophthalmic vein. The central retinal artery pierces the optic nerve close to the eyeball, emerges at the optic disk, and sends 4 main branches over the human retina, lying close to the inner limiting membrane. Each of the 4 branches of the central retinal artery supplies one quadrant of the retina. The venous equivalent of the central retinal artery is the retinal vein. The anatomy of the veins of the

orbit of the eye varies between individuals, and in some the central retinal vein drains into the superior ophthalmic vein, and in some it drains directly into the cavernous sinus.[39,40]

Unique characteristics of retinal circulation. Regulation of the microenvironment of the retina, e.g. the controlled fluid and molecular movement between the ocular vascular bed and the retinal tissues, is fundamental for appropriate retinal function and vision. Therefore, the retina has a unique blood-retinal barrier that separates the retina from the circulating blood.[41] The blood-retinal barrier is formed by complex tight junctions of retinal capillary ECs (the inner barrier) and retinal pigment epithelial cells (the outer barrier), corresponding to the two main circulations. The choroidal capillaries themselves are fenestrated, like most of the highly permeable capillaries throughout the human body. Retinal capillary ECs express a variety of unique transporters which play a key role in the influx transport of essential molecules and the efflux transport of neurotransmitter metabolites, toxins, and drugs.[41] Therefore, systemic drug administration is not suitable for the treatment of retinal diseases because of poor drug permeability across the blood-retinal barrier.

Retinal circulation is characterized by a low blood flow and a high level of oxygen extraction; arteriovenous difference in pO_2 is about 40%.[42] Autonomic nerve endings extend to the uvea and the extraocular part of the retinal blood vessels, but not to the intraocular segments of the retinal blood vessels. Therefore, retinal arterial tone is largely regulated by local factors such as local variations in perfusion pressure and in pO_2, pCO_2 and pH.[42] The presence of mechanisms that autoregulate retinal circulation may well reflect important survival strategies for the retina which are not yet fully understood.

Development of the retinal vasculature. During embryogenesis, the vascular network that supplies the retina undergoes dramatic changes and reorganization.[39] The choroidal vasculature develops in an early stage and is preceded by a peak of VEGF-A production by the retinal pigment epithelium, suggesting that VEGF-A is involved in the development of the choroidal vasculature.[43] Initially, the inner part of the eye is metabolically supported by the hyaloidal vasculature, an arterial network in the vitreous. Blood enters through the central hyaloid artery in the optic nerve, runs through hyaloid vessels in the vitreous and then exits through an annular collection vessel at the front of the eye. The hyaloid vessel system is a dense, but transient intraocular circulatory system that undergoes progressive and nearly complete regression during the latest stage of ocular development as the lens, the vitreous and the retina mature. Due to the natural regression of the hyaloidal vasculature, as well as increasing metabolic demands of maturing neurons, the retina becomes hypoxic, and therefore the formation of the retinal vasculature is induced.

Retinal angioblasts. Recent observations have suggested that the initial human retinal vasculature develops by differentiation, and organization of vascular precursor cells that are $CD39^+$ (angioblast marker) and $CD34^-/CD31^-$ (EC markers) at around 14 weeks of gestation. [44] These cells seem to emerge from a pool of precursor cells that are $CXCR4^+/c\text{-}Kit^+$ (angioblast receptors), and were found in the neuroblastic layer of human embryonic retina at 7 weeks of gestation. $CD39^+/CXCR4^+/c\text{-}Kit^+$ cells start to migrate anteriorly into the retinal nerve fiber layer where stroma derived factor-1 (SDF-1, the ligand for CXCR4) and stem cell factor (SCF, the ligand for c-Kit) levels are at their highest. With apparent migration of these vascular precursors, the expression of c-Kit declines and differentiation into angioblasts and alignment with nerve fibers occurs. A gradient of SDF-1 towards the ora serrata suggests that the angioblasts migrate towards the higher concentration. CXCR4 expression is regulated by SCF, FGF-2 and VEGF, and angioblasts continue to express CXCR4 until they become ECs that are $CD34^+/CD31^+$. These results suggest, at least in part, that vasculogenesis might contribute to growth of the primordial vessels in the central retina.[44,45] However, the identification and lineage of angioblasts within the developing retina is still controversial.[46]

Retinal angiogenesis. After formation of primordial vessels, new blood vessels sprout into the retina by means of angiogenesis, forming the vasculature of the inner retina. Retinal angiogenesis begins in the most superficial retinal layer at the optic nerve head, and radiates outwards from this central point.[3,39,45] The superficial plexus develops in a centrifugal fashion across the inner surface of the retina, with the exception of the primate fovea from which blood vessels are excluded. Retinal angiogenesis is closely regulated by supply and demand of oxygen. High oxygen tension suppresses hypoxia-induced VEGF production, and less VEGF results in less blood vessel growth.[3,39] Additional capillary networks in deeper retinal layers then arise by sprouting from the superficial arteries to form the deeper vascular plexus. Vascular pruning in the developing retina results from EC migration from retracting vessels into the surrounding newly developing vessels. The process of natural pruning can be accelerated by experimental exposure to hyperoxia.[3] The process of retinal vascular development is completed shortly before birth in humans, and a few weeks after birth in several other mammalian species including rodents. With development of the capillary plexuses and the resulting increase in oxygen tension, a capillary-free zone develops around the major blood vessels, followed by vessel retraction in the superficial plexus.[40]

Vascular patterning. The process of sprouting angiogenesis during development of the retinal vasculature is preceded by an invasion of migrating astrocytes in a centrifugal fashion across the inner surface of the retina.[3,22,40,46,47] Ganglion cells secrete PDGF-A to stimulate proliferation of astrocytes.[48,49] The retinal vascular plexus initially forms superimposed on the astrocyte network. Astrocytes at the leading edge and immediately ahead of the vascular

plexus secrete high levels of VEGF-A compared to more distally located astorcytes that already have established contact with ECs. During this burst of angiogenesis, all endothelial tip cells are closely attached to astrocytes and their filopodia orientate along the astrocyte cell bodies and processes. Experimental overexpression of PDGF-A in ganglion cells resulted in a large increase in the number of retinal astrocytes and subsequent overgrowth of the retinal vasculature *in vivo*. [49] However, blocking PDGF-A receptor reduced astrocyte network formation but showed only small changes in blood vessel formation.[49]

PATHOLOGICAL OCULAR ANGIOGENESIS

What is unique in ocular angiogenesis? Several ocular diseases are hallmarked by angiogenesis, including diabetic retinopathy, age-related macular degeneration, and retinopathy of prematurity.[3,5,46,50] In all these conditions, angiogenesis is probably stimulated by local tissue hypoxia resulting from neuronal metabolism, with varying contributions from inflammatory signals and oxidative stress. In retinal neovascularization, VEGF plays a central role.[3,5,51] At least five retinal cell types have the capacity to produce and secrete VEGF. These include the retinal pigmented epithelium, astrocytes, Müller cells, ECs and ganglion cells. However, they differ widely in their responses to hypoxia; *in vitro* studies show that Müller cells and astrocytes generally produce the greatest amounts of VEGF under hypoxic conditions.[1,22,43] The two most important forms of ocular angiogenesis are pre-retinal angiogenesis, originating from the retinal vasculature, and subretinal (or choroidal) neovascularization.

Preretinal angiogenesis. Pre-retinal angiogenesis occurs as a final common pathway in several diseases associated with capillary nonperfusion and local retinal ischemia, including diabetic retinopathy. Angiogenesis is induced by the ischemic retinal areas, and ultimately results in the formation of large contractile fibrovascular membranes within the vitreous cavity. These membranes and the associated hemorrhages cause blindness by obscuration of the visual axis and retinal detachment. When the retinal ischemia is widespread, angiogenesis and scarring can also occur on the iris and cause an untreatable form of glaucoma. Destruction of the ischemic retinal areas with laser can be effective in inducing regression and fibrosis of the newly formed vessels.

Subretinal angiogenesis. Subretinal, or choroidal neovascularisation, results from a series of pathological events affecting the retinal pigment epithelium, Bruch's membrane, and the choroid. Typically, subretinal angiogenesis is a wound healing response that occurs only when an anatomical discontinuation of Bruch's membrane is present, in combination with a driving force such as inflammation, hypoxia, and oxidative stress. For most conditions it is unknown to what extent these three mechanisms contribute to the initiation of subretinal angiogenesis. Subretinal angiogenesis is a hallmark of age-related macular degeneratioin, occuring either between the

retinal pigment epithelium and Bruch's membrane (occult choroidal neovascularisation), or between the retinal pigment epithelium and the neuroretina (classic choroidal neovascularisation).

New vessels formed by subretinal angiogenesis can later regress, leaving an atrophic retinal area, or the wound healing can progress with formation of a fibrotic scar. In both cases, the overlying neuroretina will slowly degenerate, leading to loss of sharp sight, contrast sensitivity, and color vision.

Ocular angiogenesis and wound healing responses. In most instances, pathological ocular angiogenesis is a wound healing-like response in which the formation of blood vessels is accompanied by influx of inflammatory cells, followed by myofibroblast formation.[52] Therefore, during disease progression, the angiogenic phase can be followed by a fibrotic phase. It has been shown that VEGF driven angiogenesis upregulates pro-fibrotic factors such as TGF-β1 and connective tissue growth factor (CTGF).[53] CTGF levels strongly correlate with degree of fibrosis in vitreo-retinal conditions.[52] When the balance between the angiogenic (VEGF) and fibrotic (CTGF) factors shifts to a certain threshold ratio in favour of fibrosis, the 'angio-fibrotic switch' occurs and fibrosis and scarring develop.[52] Administration of anti-VEGF drugs to patients as a therapy to regress neovascularisation could therefore lead to a temporary increase in fibrosis, a phenomenon that is indeed observed in the clinic.

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