CHAPTER 6

VEGF and Endothelial Guidance in Angiogenic Sprouting

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Abstract

The cellular actions of VEGF need to be coordinated to guide vascular patterning during sprouting angiogenesis. Individual endothelial tip cells lead and guide the blood vessel sprout, while neighbouring stalk cells proliferate and form the vascular lumen. Recent studies illustrate how endothelial DLL4/NOTCH signalling, stimulated by VEGF, regulates the sprouting response by limiting tip cell formation in the stalk. The spatial distribution of VEGF, in turn, regulates the shape of the ensuing sprout by directing tip cell migration and determining stalk cell proliferation.

Key Messages

- Angiogenesis is a guided process.
- Endothelial tip cells lead each vascular sprout.
- VEGF induces tip cell formation.
- VEGF gradients are formed by heparin-binding isoforms.
- VEGF gradients guide tip cell migration and gauge stalk cell proliferation.
- VEGF and NOTCH signalling cooperate to select and guide endothelial tip cells in retinal development.

Introduction

The term angiogenesis summarizes a set of morphogenic events that expand and fine-tune the initial, more primitive, embryonic vascular network into a hierarchical network of arterioles, venules and highly branched capillaries to provide efficient blood supply and organ specific vascular functions.[1] These "angiogenic" events include sprouting morphogenesis, intussuseptive growth, splitting, remodelling, stabilization and differentiation into arterioles, venules and capillaries. At the cellular level, angiogenesis involves at least two distinct cell types, endothelial cells and supporting mural cells (pericytes and vascular smooth muscle cells), and requires a number of different cellular functions, such as migration, proliferation, cell survival, differentiation and specialization. A plethora of factors are involved at different levels, either stimulating or inhibiting angiogenesis. However, vascular endothelial growth factor (VEGF or VEGFA) plays a key role in most, if not all morphogenic events during angiogenesis (reviewed in ref. 2).

More than a decade of research on VEGF and angiogenesis has provided evidence that VEGF has multiple roles in endothelial cells, controlling both physiological and pathological

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angiogenesis. A major challenge is therefore to clarify how the different cellular functions of VEGF are concerted into precise morphogenic events. For example, how are the proliferative and migratory responses of endothelial cells to VEGF integrated during sprouting angiogenesis to facilitate the protrusion of a new, diameter-controlled vascular tube? Why do endothelial cells in some instances proliferate, and in others migrate? What controls the direction of the migratory response to VEGF? Which cells are susceptible to regression in the absence of VEGF as a survival signal? How does VEGF stimulate arterial identity in some endothelial cells, while inducing fenestration in others? Clearly, with every new function of VEGF that is discovered, the number of new questions is steadily increasing. Possible scenarios that may explain the diversity of the cellular responses to VEGF include the presence of specific receptors or receptor/coreceptor pairs, a possible morphogen function with concentration-dependent effects, or the context of the endothelial cells in their microenvironment. In the present chapter, I will discuss recent experimental advances that explain how some of the cellular functions in response to VEGF are orchestrated to promote guided vascular sprouting.

Mechanics of Angiogenic Sprouting

In order to extend a new cellular tube from a preexisting (quiescent) vascular network, the endothelial cells must be coordinated in their response. Endothelial cells in a culture dish proliferate and migrate in response to VEGF stimulation. When wounded in a so-called "scratch assay", the monolayer will close again by both proliferation and migration. VEGF stimulation accelerates this process. A confluent monolayer of endothelial cells may in some aspects resemble a quiescent vessel wall. Unlike the closure of an endothelial (or epithelial) monolayer, the formation of a new sprout requires selection of a distinct site on the vessel where endothelial cells start to invade the surrounding tissue or matrix, whereas other cells along the vessel stay put (Fig. 1A). In theory, if all cells begin to migrate, the vessel should disintegrate. Conversely, if all cells were to proliferate, the vessel would likely only increase in diameter. Thus the first process in angiogenic sprouting must be the selection of a distinct site on the mother vessel where sprout formation is initiated. This selection process will have to be reiterated as the new sprout elongates, branches and connects with other sprouts to form an expanding network. Proliferation will need to occur to provide more cells for sustained sprouting. These basic principles may not only apply to angiogenic sprouting in vertebrates, but more generally to different types of tubular sprouting processes, whereever they occur throughout development in the animal kingdom. For example, tubular sprouting in the *Drosophila* trachea has been studied in great detail, and we can learn by comparison.

Cell specification is one of the fundamental principles during formation of the *Drosophila* tracheal system. Each sprout is headed by a specialized tip cell, the fate of which is controlled by a number of different signalling pathways containing DPP, NOTCH and FGFR (reviewed in ref. 3). In this system, the sprouting process is induced by FGF secreted from distinct cell clusters in the vicinity of the tracheal cells. The tip cells extend dynamic filopodia towards the FGF source and migrate in a directional fashion up the FGF gradients. In contrast, the following cells do not adopt the tip cell phenotype, but form the stalk during the sprouting process. Genetic studies and mosaic analysis have clarified that the tip cell fate is inhibited in stalk cells through signals that negatively regulate the FGFR signaling pathway (sprouty) as well as bi-directional signalling between tip and stalk through the DELTA/NOTCH pathway (reviewed in ref. 3). Overexpression of the FGFR in all tracheal cells results in ectopic filopodia extension from the stalk, indicating that FGFR levels and activity underly the distinction between tip and stalk cell behaviour and fate.[4,5]

In attempts to understand the process of angiogenic sprouting, early imaging of salamander tails in the 1930s provided the first notion of dynamic protrusive behaviour at the tip of each vascular sprout; subsequently, detailed images of vascular sprouts in the developing CNS identified elaborate filopodia extension from the sprout tip, leading the authors to speculate that specific tip cells, which extend these filopodia, function to read guidance cues in the tissue

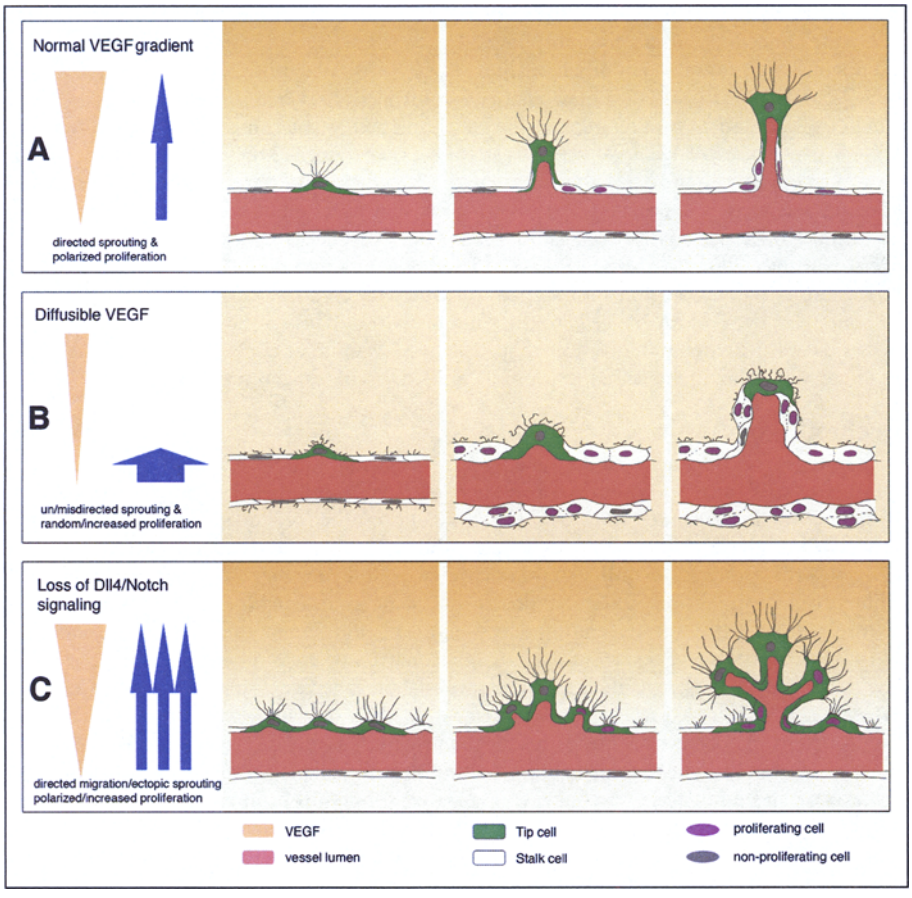

Figure 1. Schematic illustration of the cellular mechanisms that pattern vascular sprouting. A) Graded distribution of VEGF; sequential steps from left to right illustrate the induction of a tip cell (green) by VEGF (orange), polarization of a tip cell with rapid directed migration (blue arrow, left panel), and proliferation (pink nuclei) of the stalk cells (white). Polarization of the tip cell in a steep VEGF gradient leads to long, directed filopodia extension towards higher VEGF concentration. Polarized proliferation occurs with the division axis perpendicular to the long axis of the vessel. The pulling tip cell likely helps to polarize the stalk cell division. B) Diffuse distribution of VEGF, like in *Vegfa120/120* mice, leads to the undirected extension of short filopodia extension, although tip cell induction does occur. Tip cell migration is slow and many stalk cells proliferate due to widespread VEGF. Stalk cell proliferation is not polarized, causing vessel dilation/hypertrophy. C) Loss of DLL4/NOTCH signaling leads to excessive sprouting through increased tip cell numbers. NOTCH signaling normally inhibits the tip cell response in stalk cells. Tip cell numbers further increase through slightly elevated proliferation.

during the sprouting process (refs. 6-8, and references therein). Notably, these observations were made before VEGF was discovered,[9] and long before we learned how VEGF guides angiogenic sprouting by directing endothelial tip cell migration along extracellular gradients of heparin-binding isoforms.[10,11]

The ultrastructure of endothelial tip cells and their filopodia in contact with surrounding tissue was studied in the rat cerebral cortex in 1982.[7] The same team later used HRP injection

and correlative microscopy to study tip cell filopodia and vascular lumen formation in regions where two sprouts connect, leading the authors to suggest that filopodia are important in anastomosis.[12] Tip cell filopodia where also found to lead sprouts and engage in sprout anastomosis in an angiogenesis model in vitro[13] and later also observed during sprouting angiogenesis in the chicken yolk sac[1] and CNS.[14,15] Risau and colleagues suggested through studies of the developing CNS that VEGF, expressed in the ventricular zone, may function to direct the vessel tips during the sprouting process.[15] Earlier, Ausprunk and Folkman had provided evidence that proliferation is largely confined to the stalk region during angiogenic sprouting.[16] Using irradiation to block proliferation, Folkman and colleagues also discovered that sprouting can in principle progress without cell division, indicating that the driving force for sprout elongation is likely a pulling force exerted by the tip cells, rather than a pushing force originating from dividing stalk cells. However, sustained sprouting beyond two days in the cornea pocket angiogenesis assay requires that cell numbers are replenished by division. Thus the mechanism of angiogenic sprouting involves (a) the local induction and selection of tip cells, (b) the directed migration of tip cells along a sufficiently adhesive substrate to provide the pulling force, and (c) the balanced proliferation of stalk cells during sprout elongation (Fig. 1A). Finally, the migration and explorative behaviour of the tip cells must cease upon anastomosis of two sprouts to establish a stable new connection between two branches of the growing vascular plexus.

Before I discuss how VEGF might affect each of these processes, I will briefly consider the composition and architecture of the tissue attracting the new vessel sprouts. Evidently, there will be tissue specific differences, and these are important for vascular patterning, as each organ brings its own requirement for efficient nutrient supply and oxygenation, but also creates physical constraints for blood vessels to restrain their growth and maintain adequate organ function. The postnatal retina of rodents provides an excellent model system to understand the interaction of tip and stalk cells with the surrounding tissue and to illustrate how VEGF expression, deposition and signalling controls guided vascular patterning within tissues.

The Mouse Retina Model

The retina comprises a sensory outpost of the brain, and as such may be regarded as a model for angiogenesis in the developing CNS in general. However, unlike most other parts of the CNS, the retina of rodents becomes vascularized only after birth (for recent comprehensive reviews of retinal vascular development and the retina as a model for angiogenesis research, see refs. 17,18). Vessels emerge from a capillary ring at the optic disk and sprout radially just underneath the inner limiting membrane, the vitreal surface of the retina. The sprouting vessels form an elaborate network that reaches the retinal periphery about one week after birth. The vessels are guided by a network of astrocytes, which forms only days before the vessels grow. Genetic manipulation of the astrocyte density results in a similar alteration of vascular density, illustrating the close relationship of astrocytes and vessels.[10,19] In fact, astrocytic network formation is a prerequisite for retinal angiogenesis at this stage. This is further supported by the fact that animals without retinal astrocytes also lack retinal vessels throughout evolution. The speed and accuracy of primary plexus formation along the astrocytic network is truly remarkable and requires tight regulation, as delayed or impaired retinal angiogenesis leads to an invasion of hyaloid vessels from the vitreous into the retina, and the ensuing abberant retinal vasculature may cause retinal scarring and detachment.

Detailed analysis of the physical association of the vascular sprouts with the underlying astrocytic network showed that the endothelial tip cells elongated their filopodia almost exclusively along the astrocyte surface.[10,20] Indeed, filopodia without astrocyte contact failed to stretch towards the retinal periphery in a directed fashion, suggesting that the astrocyte surface has adhesive properties. Dorrell and colleagues proposed that R-cadherin may mediate at least part of this adhesive function,[20] however, unequivocal evidence hereof is lacking so far. Rather, we have found that R-cadherin deficient mice show no defects in tip cell filopodia alignment or vascular development in the retina (H. Gerhardt, J. Hakansson and H. Semb, unpublished observations).

The astrocytes themselves appear to respond to endothelial tip cell contact by undergoing maturation and remodeling.[21] In the mature retinal network, the vascular surface is completely covered by astrocytic endfeet, and their inimate relationship makes an important contribution to the induction and maintenance of the blood retina barrier.[22,23] At the time when the initial vascular plexus has reached the periphery, a wave of secondary angiogenesis is initiated from veins and capillaries in their vicinity. The new sprouts elongate along the retinal radial glia (Mueller glia) to branch and anastomose into two consecutive plexuses in the inner and outer plexiforme layers.

VEGF Gradients Guide Tip Cell Migration

The extracellular distribution of growth factors is often tightly controlled to ensure spatial patterning of the appropriate tissue response. In angiogenesis, the extracellular distribution of VEGF is controlled at the level of transcription, isoform splicing, cell surface retention and likely uptake and degradation to result in extracellular gradient formation for proper vascular patterning.

Eli Keshet and colleagues studied the mouse retina and later the human retina in detail and discovered that astrocytes produce ample amounts of VEGF.[24,25] It was also in this tissue that Keshet first identified hypoxia-dependent regulation of VEGF expression. In situ hybridisation for VEGF mRNA reveals an intriguing picture, with VEGF expression confined largely to the avascular periphery throughout retinal development (see Chapter 3 by M. Fruttiger). The avascular periphery shrinks as the retinal plexus advances, and so does the zone of high VEGF expression.[21] Thus VEGF production is spatially graded and ideally suited to provide directionality to the migratory response of the endothelial tip cells. In addition, VEGF gradient formation is supported by the distinct retention properties of the different VEGF isoforms (for details on VEGF isoforms see Chapter 1 by Y.S. Ng).

The longer splice isoforms (VEGF164 and VEGF188 in the mouse) contain C-terminal basic amino acids sequences that interact with negatively charged heparan sulfate side chains on the cell surface or in the extracellular matrix. The shorter VEGF120 lacks this C-terminal retention motif, but still binds and activates the FLT1/VEGFR1 and KDR/VEGFR2 receptors. A detailed study of mice genetically engineered to produce only single isoforms, in lieu of several alternatively spliced isoforms, confirmed that the heparin-binding isoforms are secreted, but retained close to producing cells and thereby display a graded protein distribution around the site of production.[11] Accordingly, retinal astrocytes secrete and retain VEGF protein in their environment in a graded fashion. Intriguingly, endothelial tip cells express KDR mRNA in abundance and localize the receptor protein to both their cell body as well as their filopodia.[2,10] Thus, in analogy to axonal growth cones, which carry receptors during neuronal guidance, the endothelial tip cell filopodia with their receptors are ideally suited to perform the sensing function originally postulated by Marin-Padilla.[8] Indeed, we observed in a series of gain and loss of function studies that the extracellular VEGF distribution is essential for the length and orientation of tip cell filopodia in vivo (see below).

Endothelial tip cells are most prominently found in the periphery of the developing vascular plexus, where most of the new sprouts are forming. Tip cell formation and sprouting co-distribute with areas of highest VEGF concentration, suggesting that tip cell formation and filopodia protrusion may be induced by VEGF. Indeed, we observed induction of new tip cells and excessive filopodia formation on hyaloid vessels exposed to high VEGF levels in transgenic animals overexpressing individual VEGF isoforms from the lens crystallin promoter.[10] Conversely, sequestration of endogenous VEGF by intraocular injection of soluble FLT1 rapidly leads to loss of tip cell filopodia.[10] Also, neutralizing antibodies to KDR, but not FLT1 inhibit filopodia formation, indicating that VEGF mediates tip cell induction and filopodia extension via activation of KDR.[10] However, FLT1 may help to shape the extracellular VEGF gradient, as the soluble form of FLT1 (sFLT1) might act as a VEGF sink, keeping VEGF levels low close to the vessel stalk cells.[26]

Although all VEGF isoforms appear to induce filopodia formation, the effects on filopodia morphology and vascular patterning are strictly isoform specific. For example, Ruhrberg and colleagues found that mouse hindbrains expressing solely VEGF188 contained many tip cells that extended numerous long filopodia, whereas tip cells were sparser and tip cell filopodia shorter in the presence of VEGF120 only.[11] Correlating with the tip cell phenotype, VEGF120 induced the formation of a poorly branched network of vessels with enlarged diameter. Vice versa, mice producing only VEGF188 developed a highly branched network of thin vessels. Finally, mice producing only VEGF164, which is a heparin-binding isoform that is also diffusible, developed vessels that were indistinguishable from those in wild type littermates.

Close examination of the site of VEGF expression, and the localization of the VEGF protein, had demonstrated for the first time that VEGF120 diffused over considerable distance within tissues, whereas the heparin binding isoforms build a steep extracellular VEGF gradient. Ruhrberg and colleagues therefore concluded that diffusible VEGF120 reached the endothelium over large distances and stimulated continued proliferation of endothelial cells, which in turn increased vessel diameter, whereas VEGF188 gradients were so steep that vessels branched excessively.[2,11]

A close examination of vascular patterning in the retinas of *Vegfa120/120*mice illustrated that tip-cell migration and expansion of the retinal vascular plexus is dramatically reduced as a consequence of a shallow VEGF gradient.[10] Interestingly, the length of the tip cell filopodia closely correlated with the speed of retinal plexus migration and the assumed gradient in all experimental observations. Indeed, vessels in mice that carry only one *Vegfa120* allele instead of the correctly spliced wild type allele (*Vegfa+/120* mice), already show reduced tip cell migration, filopodia shortening and perturbed vessel branching.

Further mechanistic insight into the role of VEGF gradients in tip cell migration emerged through a series of experiments involving intraocular VEGF injection. Injection of the heparin-binding VEGF164 isoform rapidly reduced tip cell migration, shortened tip cell filopodia and enlarged stalk diameter, features that were highly reminiscent of the phenotype of mice expressing VEGF120 only. Taken together, these results suggested that the key parameter for tip cell migration, tip cell polarization and directional filopodia extension was the precisely controlled extracellular localization of VEGF.

RT-PCR revealed that the heparin-binding VEGF164 is the dominant VEGF isoform in the retina. Thus the normal endogenous distribution of VEGF is likely controlled by retention of this growth factor close to the site of production to build the extracellular VEGF gradient. This gradient is required for proper tip cell polarization, directed filopodia extension and migration, following the basic principles of chemotaxis (Figs. 1A,B).

Balancing Tip Cell Migration and Stalk Cell Migration through Extracellular VEGF Gradients

The proliferation of stalk cells is also controlled by VEGF distribution (Fig. 1). Here, the local availability of VEGF within the plexus appears to determine cell division. In a normal, unperturbed retina, only a limited zone of the peripheral vascular plexus behind the sprouting front shows significant endothelial cell proliferation, as judged by BrdU incorporation, Ki67 or phospho-histone 3 labelling. Injection of VEGF, however, leads to widespread proliferation throughout the vascular plexus. This clearly shows that most endothelial cells in the growing vascular plexus, irrespective of their differentiation status and smooth muscle coverage, can in principle respond to VEGF by proliferation. During normal development, the amount of VEGF produced correlates closely with local perfusion and oxygen supply, as *Vegfa* gene transcription is controlled by hypoxia. As mentioned above, the avascular periphery in the retina is hypoxic and produces very high levels of VEGF. Within the plexus, on the other hand, VEGF production is largely downregulated. However, local differences in perfusion and oxygenation result in a residual amount of VEGF production. Low oxygenation of venous blood correlates with higher VEGF production from astrocytes around the venous area, while astrocytes close to arteries produce little or no VEGF.[27] The distribution of endothelial proliferation follows this

pattern exactly, with significant levels in veins and the surrounding plexus and essentially no proliferation in and around arteries.

Intriguingly, it is the relative difference in VEGF production around arteries and veins compared to the VEGF levels in front of the sprouting tip region that appears to regulate the patterning of the primary plexus ahead of the remodeling veins and arteries. Where the difference is greater, around the arterial regions, the implied long-range gradient of extracellular VEGF should be steep and result in the directed extension of long filopodia and rapid tip cell advancement over the retinal surface. Near veins, where this gradient is expected to be less steep, tip cell advancement and polarity should be comparably reduced. The opposite would be true for proliferation of the stalk cells, resulting low stalk cell proliferation in the former case, and higher stalk cell proliferation in the latter case. Taken together, these findings illustrate how the spatial VEGF distribution might balance and regulate the VEGF responses of tip and stalk cells and thus shapes the morphology of nascent vessels (Figs. 1A, B).

We were able to validate the concept that the spatial distibution of VEGF controls endothelial responses to VEGF further by hyperoxia and hypoxia treatment of neonatal mice. Exposure to lower than normal oxygen levels increased VEGF production and therefore raised the level of residual VEGF available to the vascular plexus, but left the avascular periphery unperturbed; in contrast, exposure to higher than normal oxygen levels had the opposite effect. Conceptually, high oxygen should thus steepen the gradient, whereas hypoxia should decrease it. Consistent with this idea, high oxygen levels enhancd tip cell migration and reduced stalk cell proliferation. Hypoxia in turn reduced tip cell migration and increased stalk cell proliferation, resulting in the formation of a vascular plexus that appeared very similar to the one observed in mice expressing VEGF120 only. Together, these data illustrate not only how important the extracellular distribution of VEGF is for vascular patterning, but also how the regulation of VEGF production and retention contributes to extracellular gradient formation to balance tip and stalk cell proliferation.

Recent data from Bautch and colleagues added another dimension to the control of stalk cell proliferation by VEGF distribution. Monitoring endothelial cell division in mouse embryonic stem cell-derived embryoid bodies, they found that stalk cells normally divided with their plane of cytokinesis perpendicular to the vessel long axis.[28] This polarized cell division promoted vessel elongation during sprouting. Interestingly, stalk cell polarization occured independently of flow, but was instead regulated by heparin-binding VEGF isoforms. Bautch and colleagues had earlier suggested that cell proliferation and VEGF gradient formation in the embryoid body system is regulated by FLT1, in particular the soluble form, which acts as an extracellular VEGF sink around established vessels.[26,29] They then showed that diffusible VEGF120 and loss of soluble FLT1 both led to randomization of the cell division axis, and thus resulted in abberant vessels with enlarged diameter.

Based on the results described above, it is tempting to speculate that the vascular abnormalities in mice expressing VEGF120 as the only VEGF isoform are caused by a combination of loss of tip cell polarization and increased stalk cell proliferation, as well as the loss of polarization of stalk cell cytokinesis (Fig. 1B).

Precisely heparin-binding isoforms polarize the division axis of stalk cells remains unclear. Many studies illustrate that endothelial cell shape, polarity and division axis can be regulated by flow through shear stress.[30] While this is highly unlikely to occur in the sprouting regions, where both flow and pressure are low, it is possible that the pulling force generated by the tip cells (see above) is transmitted to the stalk cells to polarize their division axis. Thus, the loss of tip cell polarization and directed migration in the absence of VEGF gradients could directly be responsible for a loss of stalk cell polarization. Further studies are required to investigate this link.

Tip Cell Formation following VEGF Stimulation Is Controlled by Dll4/NOTCH1 Signaling

The concept of endothelial tip and stalk cells has helped to direct current research towards identifying distinct cellular responses of different endothelial cell populations and their integration during angiogenic sprouting. Which signals select a tip cell from a given endothelial cell population, and what stops the stalk cells from responding in a similar fashion? Clearly both types of endothelial cells are stimulated by the same growth factor, VEGF, and both respond through KDR signaling, yet their behaviour is very different. Our initial characterization of the tip cell illustrated that tip and stalk cells carry a differential transcriptional signature. Today, we know only a handful of genes that are expressed preferentially in the tips, including *Kdr*, *Pdgfb* (encoding the patelet-derived growth factor B), *Apln* (encoding apelin), *Dll4* (encoding delta-like 4).

In vitro, VEGF induces a large number of genes in endothelial cells from various origins. One of the genes induced by VEGF is the NOTCH ligand DLL4. In situ hybridization suggested that *Dll4* expression is restricted to developing arteries and the tips of vascular sprouts.[31,32] A number of recent studies have now addressed the function of DLL4/NOTCH signaling in angiogenesis. Very similar to VEGF, DLL4 levels appear to require very tight regulation, as haploinsufficiency for *Dll4* causes embryonic lethality.[33-35] Interestingly, loss of DLL4/NOTCH signaling in angiogenesis assays in vitro[36] and in mouse and zebrafish in vivo leads to ectopic sprouting and increased tip cell numbers[37-41] (Fig. 1C). Monitoring NOTCH signaling activity through NOTCH target genes and a transgenic NOTCH reporter in the mouse retina, we observed strong NOTCH signaling in the sprouting zone. As observed for *Dll4* expression, NOTCH reporter activity displayed a "salt and pepper" distribution pattern among the endothelial cells in the sprouting zone. Intriguingly, *Dll4* mRNA was almost never observed to be strongly expressed in two neighboring cells at any given time, independent of their position at the tip or in the stalk of the sprouts. Loss of NOTCH activity recapitulates the phenotype caused by loss of DLL4, with strongly increased filopodia protrusions, increased tip cell numbers, excessive sprouting and fusion. These data suggested that DLL4/NOTCH signaling controls protrusive activity. The fact that VEGF induces *Dll4* expression,[39,42] and the observation of increased filopodia formation, sprouting and branching specifically in the region exposed to VEGF in mouse mutants together suggest that DLL4/NOTCH signalling functions to limit tip cell formation in the vascular zone exposed to VEGF.

DLL4, like other NOTCH ligands, activates the NOTCH receptor in a cell-cell contact dependent manner. A series of protease cleavage events of both the ligand and the receptor is required for signaling. Gamma secretase is the last cleavase during receptor activation, severing the intracellular NOTCH domain from the transmembrane region.[43,44] The NOTCH intracellular domain (NICD) is translocated to the nucleus, where it binds to RBPJ (previous names: RbpSuh and CBF1) to activate transcription of target genes, including members of the HES and HEY family of transcriptional repressors (reviewed in refs. 45,46). Short-term inhibition of gamma secretase in the retina rapidly increases filopodia formation, in particular from cells situated in the stalk, suggesting that NOTCH is required in stalk cells to suppress protrusive activity. Moreover, expression of the tip cell marker *Pdgfb* becomes widespread at the front, suggesting that the tip cell program is activated in stalk cells. Mosaic analysis in mouse retina and zebrafish showed that cells unable to receive NOTCH signaling are more likely to adopt a tip cell phenotype, whereas activation of NOTCH prohibits cells from becoming tip cells.[37,40] These data argue for a model in which individual cells that are stimulated by VEGF compete for the leadership position, i. e. the tip cell phenotype.

How the competitive advantage of one cell over the other is established remains unclear. Eichmann and coworkers suggested that KDR levels determine the tip cell response and are controlled by NOTCH signaling.[41] This hypothesis is indeed attractive, as a negative feedback loop in which VEGF induces DLL4, which in turn activates NOTCH in neighboring cell to

suppress KDR levels and thus KDR activity, would suffice to pattern the endothelial population into tip and stalk cells. In the analogous case of the *Drosophila* trachea, FGFR levels (breathless) are indeed capable of selecting the tip cells in response to FGF (branchless) stimulation.[4,5] Alternative mechanisms are possible, in particular as coreceptors for VEGF may regulate the downstream activity and specificity of the KDR signaling pathway.

Importantly, excessive numbers of tip cells subsequent to DLL4 or NOTCH inhibition increase vessel sprouting and branching, and therefore also vascular density; however, the ensuing vascular network is poorly functional. Both in the retina, and in tumour models, the tissue becomes hypoxic and undersupplied, despite the increased vascular density. In fact, two papers from Regeneron and Genentech demonstrated that tumour growth is strongly reduced as a result of poor vascular function in *Dll4* heterozygous mutant mice.[47,48] Finally, Leslie and colleagues illustrated that DLL4/NOTCH signaling also functions to terminate tip cell protrusive acitivity once two tip cells fuse in the dorsal longitudinal anastomosing vessel of zebrafish.[38]

Conclusions

VEGF stimulates endothelial cells to sprout and proliferate to form new vessel structures. VEGF induces DLL4, which functions to pattern the endothelial population into tip and stalk cells. The tip cells then migrate along the VEGF gradients, whereas stalk cell proliferate in a polarized fashion to supply further endothelial cells. The balance between migration and polarized proliferation controls the length and diameter of the stalk (Figs. 1A-C). VEGF gradients, arising through regulated retention of VEGF on the cell surface and in the extracellular matrix, govern these polarization processes. In the future, it will be interesting to examine how matrix molecules participate in these mechanisms and how VEGF retention and DLL4/NOTCH signalling influence each other in the patterning process.

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