Tip cells in angiogenesis

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Contents

Abstract

In angiogenesis, the process in which blood vessels sprouts grow out from a pre-existing vascular network, so-called endothelial tip cells play an essential role. Tip cells are the leading cells of the sprouts, they guide following endothelial cells and sense their environment for guidance cues. Because of this essential role, the tip cells are a potential therapeutic target for anti-angiogenic therapies, which need to be developed for diseases such as cancer and major eye diseases. The potential of anti-tip cell therapies is now widely recognized, and the surge in research this has caused has led to improved insights in the function and regulation of tip cells, as well as the development of novel *in vitro* and *in silico* models. These new models in particular will help to understand essential mechanisms in tip cell biology and may eventually lead to new or improved therapies to prevent blindness or cancer spread.

Introduction

Inhibition or stimulation of blood vessel formation from the pre-existing vascular network, a process called angiogenesis, has potential for therapeutic management of diseases ranging from myocardial infarction and stroke to cancer, and to ophthalmic diseases including diabetic retinopathy and age-related macular degeneration.

The search for targets for pro- and anti-angiogenic therapies has been ongoing for the last century and had a boost from the discovery of a specialized endothelial phenotype, the tip cells, that lead the growing vascular sprouts (Gerhardt et al. 2003). The tip cell is crucial for angiogenesis, and inhibition or induction of the phenotype would theoretically be an ideal therapeutic strategy for several diseases. By targeting the tip cell only, the mature, quiescent vascular network should not be affected and remain intact. In this way, adverse side effects of therapy may remain limited.

The main driving force behind sprouting is vascular endothelial growth factor (VEGF). Targeting this protein to inhibit angiogenesis has proven to be effective in some diseases such as intestinal cancer and age-related macular degeneration, but does not have the major beneficial effect that was anticipated. It appears that inhibition of VEGF can trigger other pathways to take over stimulating angiogenesis. Furthermore, targeting VEGF can disturb the balance between angiogenesis and fibrosis in neovascular tissue, which in ophthalmic disease can have devastating effects on the visual outcome of patients. Therefore, inhibition of a specific key process, such as the genesis of tip cells or their ability to correctly lead the growing sprout, is an attractive alternative therapeutic strategy.

The current pace of research, together with new research strategies, such as the use of *in silico* prediction models and *in vitro* cell culture experiments, enables rapid expansion of our understanding of key tip cell functions.

Here we review the main characteristics of tip cells, the history of tip cell research, the key regulatory pathways involved in the generation of the tip cell phenotype, and the established and novel suitable tip cell research models.

Characteristics

Tip cells are the leading cells of newly forming sprouts during angiogenesis (fig 1). They possess filopodia to aid migration (Phng et al. 2013; Schlingemann et al. 1990; Sabin 1917), and show a low proliferation rate (Gerhardt et al. 2003; Ausprunk & folkman 1995). Tip cells prevent the trailing cells from becoming tip cells and force them to take on the more proliferative stalk cell phenotype (Hellström et al. 2007), thus limiting the number of sprouts and allowing for more efficient angiogenesis. However, stochastic differences in concentrations of growth factors surrounding sprouting endothelial cells and intrinsic variability in the expression of regulating proteins such as Notch1, Dll4 and VEGFR2, enable stalk cells to overtake tip cells and take on this phenotype themselves. Theoretically, this elasticity in phenotype results in the selection of cells that are most suitable to lead a sprout and to become a tip cell as well as optimized directional guidance (Jakobsson et al. 2010). Detailed *in silico* experiments on lateral inhibition have been performed by Bentley et al (Bentley et al. 2008), more details about this can be found in the section on *in silico* models. Recently, it has also become clear that the metabolism of tip cells and stalk cells is different. Endothelial cells rely on glycolysis for their metabolic needs, and VEGF signalling of tip cells elevates the metabolic rate, whereas Dll4-Notch1 signalling reduces glycolysis in stalk cells (Eelen et al. 2013). Cells with known phenotypical characteristics of tip cells are also present in endothelial cell cultures**,** where they can be identified by their selective CD34 expression (Siemerink et al. 2012).

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Discovery

Formation of blood vessels has been the subject of scientific research for over 120 years. Early studies used microscopic techniques for the analysis of blood vessel formation, including live chick embryos and transparent chambers implanted in rabbit ears (Sabin 1917; Clark et al. 1931). Already then the difference between *de novo* formation of blood vessels from precursor cells and the sprouting of new blood vessels from pre-existing vessels was noticed. The former was termed vasculogenesis, whereas the latter was termed angiogenesis (Sabin 1917). Aspects of angiogenesis, such as the formation of loops by means of anastomosis of sprouts (Clark et al. 1931) were reported in the following decades.

Most information on angiogenesis came from research in oncology. In 1971, Judah Folkman introduced a novel theory concerning tumour growth (Folkman 1971). He hypothesized that the growth of tumours can be divided into two stages: stage 1, when the tumour is small (up to 2-3 mm), it receives its nutrition through diffusion from surrounding tissue, and stage 2, when the tumour becomes larger it needs its own blood vessels for its growing needs and because of the limitation of diffusion distances. According to Folkman's theory, without angiogenesis, a solid tumour no longer grows in size and cancer cells would have no access to the blood stream to metastasize. Angiogenesis was therefore presented as the promising therapeutic target (Folkman 1971). Even more so because endothelial cells in vessel walls are normal cells with genetically stable DNA, in contrast to cancer cells and therefore, unlike the cancer cells, they cannot become resistant to therapy (Van Noorden et al. 1998).

This promising effect of anti-angiogenic therapy in cancer patients induced a hype. At that time, little was known about the initiation of angiogenesis, although already in 1895, Roux et al hypothesized that an increased metabolism of tissue is the stimulus for formation of new blood vessels (Roux 1895), whereas it was also suggested that the stimulus is probably a chemical substance (Loeb 1893). Michaelson (1948) and later Ashton (1966) proposed that in the retina such a factor 'X' was induced by hypoxia(Michaelson I C 1948; Ashton 1966). A breakthrough confirming the latter hypothesis was achieved in 1974 by Folkman et al., with the discovery of the first tumour angiogenic factor (TAF, supposedly this was basic fibroblast growth factor (FGF)), which induced tumour angiogenesis (Folkman 1974). After this initial discovery, several other pro-angiogenic growth factors were isolated, the most important of which was vascular endothelial growth factor (VEGF), initially named vascular permeability factor because of its ability to induce vascular leakage. Several anti-VEGF therapies were developed and tested in various types of cancer. Unfortunately, these therapies were not or only mildly successful in a few types of cancer and a new strategy was needed. The cause of the lack of effects of anti-angiogenic therapy in cancer remained unclear for a number of years until the transcription factor hypoxia-inducible factor (HIF)-1α was discovered. It then became clear that tissue hypoxia can inhibit its breakdown and induce other mechanisms besides production of pro-angiogenic factors, such as anaerobic glycolysis, increased invasion and migration, to keep cells alive under hypoxic conditions.

An alternative strategy to growth factor-directed approaches in anti-angiogenesis therapy is the targeting of the cells leading the angiogenic sprouts, the tip cells. Tip cells have a different phenotype and functions that differ from other endothelial cell phenotypes and therefore express a different set of proteins. It should therefore be possible to target only the tip cells and leave the rest of the vascular network unaffected. Although officially named in 2003, (Gerhardt et al. 2003) a number of characteristics of tip cells, such as the filopodia (Schlingemann et al. 1990; Sabin 1917) and the difference in mitosis rate between the sprouting front and areas behind the front (Ausprunk & Folkman 1977), had already been attributed to cells at the sprouting front in angiogenesis. Furthermore, tip cell-like cells had been observed in the development of the brain (termed axonal growth cones (Sperry

1963)), and in the tracheal system of drosophila melanogaster (Zelzer & Shilo 2000). Once researchers realized that tip cells may be a therapeutic target, research accelerated and more details of the tip cell phenotype and its regulation were elucidated, including the ability of endothelial cells to switch between the phenotype of tip cells and that of stalk cells (Jakobsson et al. 2010), and the signalling pathways involved in tip cell function, such as the VEGF pathway and signalling between tip and stalk cells involving Dll4-Notch1. Unfortunately, a specific marker for tip cells *in vivo* has not been found yet, whereas the *in vitro* marker CD34 was recently described (Siemerink et al. 2012).

Important pathways

VEGF

VEGF is the major driving force behind angiogenesis. The VEGF family consists of 6 members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF). Since VEGF-A is the major angiogenesis factor, we will mainly focus on this protein. There are 3 known receptors for VEGF: VEGFR1 (also known as FLT1), VEGFR2 (also known as FLK1 or KDR) and VEGFR3 (also known as FLT4). VEGFR2 and VEGFR3 are membrane-bound receptors with a tyrosine kinase signalling domain. VEGFR1 mainly exists in a soluble form and acts as a negative regulator of signalling induced by VEGF binding to VEGFR2 which is facilitated by the higher affinity of VEGF for VEGFR1 compared to VEGFR2 (Koch & Claesson-Welsh 2012). In mice, the knock-out of each of the three VEGF receptors proved to be lethal at the embryonic stage due to vascular defects, illustrating the importance of all three VEGFRs (Koch & Claesson-Welsh 2012).

Under hypoxic conditions, VEGF production is dramatically increased by stabilization of its mRNA. It can induce angiogenesis in *in vitro* models of angiogenesis, but also in *ex vivo* models such as the aortic ring model and the chick chorioallantoic membrane (CAM) model. *In vivo* studies in mouse retinas have also shown its pro-angiogenic effect (Gerhardt et al. 2003).

The importance of VEGF in physiological angiogenesis becomes apparent when either the gene or its receptors are inactivated in mice. Mice lacking one allele of either VEGF or VEGFR1 or VEGFR2 all die around post-fertilization day 9. VEGF $^{+/-}$ mice show a poorly developed vascular network, a defective development of blood islands, the aggregations of mesenchymal cells where vasculogenesis starts, and a defective fusion with the vascular plexus of the yolk sac (Carmeliet et al. 1996; Ferrara et al. 1996). Mice lacking VEGFR1 show endothelial cell development, but the cells fail to organize themselves into normal vascular channels, whilst mice lacking VEGFR2 failed to develop blood islands and do not show any vasculogenesis (Koch & Claesson-Welsh 2012).

Both in embryogenesis as in adult life, VEGFR2 signalling is one of the most important initiators of sprouting angiogenesis. Processes characteristic for tip cells such as migration and the extension of filopodia are all mediated through VEGFR2 signalling (Gerhardt et al. 2003; Koch & Claesson-Welsh 2012). VEGFR3 is a critical regulator of lymphangiogenesis, but is also be expressed in the vasculature and is upregulated in tip cells during angiogenesis (Witmer et al. 2002), suggesting a crucial additional role for its ligands VEGF-C and VEGF-D.

The stimulating effect of VEGF on angiogenesis is mainly exerted through its effects on tip cells. VEGFR2 and VEGFR3 are hardly present on stalk cells , whereas VEGFR1 is present in equal amounts in the microenvironment of tip cells and stalk cells (Gerhardt et al. 2003)(fig 2A-C, enlargements A,B). In accordance, addition of VEGF to endothelial cell cultures increases the number of tip cells (Siemerink et al. 2012). In silico modelling by Bentley et al (2008) has also shown the importance of the VEGF signalling pathway for tip cell selection (Jakobsson et al. 2010; Bentley et al. 2008).

Notch-Dll4

Signalling through Notch receptors (Notch 1-4) by binding its corresponding ligands (Dll1,-3,-4 and Jag 1,-2) plays a role in many developmental processes by regulation of proliferation and differentiation (Aster 2014). Most important for angiogenesis are the Notch1 receptor and its ligand Dll4, which are crucial for

signalling between tip cells and stalk cells and the regulation of the number of tip cells and stalk cells, a process called lateral inhibition.

The regulation of the number of tip cells is important for the correct development of a functional vascular bed. Mice with a deletion of either Dll4 or Notch1 die in utero, despite an increase in vascular sprouting and branching. Furthermore, inhibition of Dll4 in mouse tumour models resulted in a denser vascular network, but an attenuation of tumour growth and tissue perfusion (Phng & Gerhardt 2009).

During sprouting angiogenesis, Dll4 can be found on tip cells after its induction by VEGF. When Dll4 binds to the Notch1 receptor on adjacent cells (fig 2C), it is cleaved by ADAM metalloproteinases and γ-secretase, and its intracellular domain translocates to the nucleus where it initiates transcription of stalk cell genes, and represses transcription of tip cell genes (fig 2D-E, enlargement D) (Phng & Gerhardt 2009). Inhibition of Dll4-Notch signalling during vascular development results in increased sprouting, branching and filopodia extension, and a denser, more interconnected vascular network (Hellström et al. 2007). Furthermore, expression of tip cell genes such as Unc-5B and PDGF-β is increased upon inhibition of Dll4-Notch1 signalling (Hellström et al. 2007). *In vitro* data show that the number of $CD34^+$ tip cells in endothelial cell cultures can be reduced by culturing on a Dll4 coated surface (Siemerink et al. 2012). *In silico* experiments show the complexity of the Dll4-Notch1 signalling pathway in more detail (Sprinzak et al. 2010). All these data indicate that Dll4-Notch1 signalling reduces the number of tip cells during vascular sprouting.

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Neuropilins

There are 2 known neuropilin receptors; neuropilin-1 (NRP-1) and NRP-2, each with an isoform-specific binding pattern. NRP-1 is mainly expressed on arterial endothelial cells and seems to be most crucial for angiogenesis, whereas NRP-2 is mainly expressed on venous and lymphatic endothelium and is reportedly involved in lymphangiogenesis (Djordjevic & Driscoll 2013). NRP-1 was initially identified as a receptor for semaphorin-3A (SEMA-3A) (Djordjevic & Driscoll 2013)and as a co-receptor for VEGF-A. More recently, several other proteins have shown the capability to interact with NRP-1, amongst which fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF) and integrin-β1 (Djordjevic & Driscoll 2013). Knock-out of NRP-1 in mice results in embryonic lethality associated with heart and vascular abnormalities as well as deficiencies in neuronal guidance. Mice lacking NRP-2 are viable, albeit smaller in size with minor abnormalities in lymphatic development. Moreover, targeted inhibition of NRP-1 by a monoclonal antibody results in disrupted angiogenesis in the mouse trachea. On the other hand, overexpression of NRP-1 in mice leads to increased blood vessel growth with leaky and haemorrhagic vessels (Djordjevic & Driscoll 2013).

NRPs interact with several proteins such as FGF and integrins. Both NRPs have distinct binding partners, for example NRP-1 can bind the VEGF isoforms VEGF-A, -B, -E and PlGF, whereas NRP-2 binds VEGF-A, -C and –D (Djordjevic & Driscoll 2013). Crosslinking experiments performed by Soker et al. (1998) revealed that NRP-1 can act as a co-receptor for VEGFR2, thereby enhancing VEGF-VEGFR2 signalling (Soker et al. 1998) (fig 2B, enlargement B). This is one of the possible explanations for the vital role of NRP-1 in angiogenesis. However, NRP-1 can also act in a VEGF-independent manner by promoting integrin-mediated endothelial cell adhesion to fibronectin, another essential component of angiogenesis (Valdembri et al. 2009). Therefore, the exact role(s) of NRPs in angiogenesis still need to be unravelled.

Angiopoietins and Tie receptors

The Tie (Tyr kinase with Ig and EGF homology domains) receptors, Tie 1 and Tie2, and their ligands, angiopoietin (Ang) 1 and 2 form an endothelial cell-specific signalling system involved in angiogenesis and vascular maturation. Ang1 and Ang2 have antagonistic roles in the activation of quiescent vessels and induction of tip cells, with Ang1 acting as stabilizer and Ang2 as an inducer of migration and tip cell behaviour. Since the function or even the ligands for Tie1 have not been identified, this receptor is not discussed here.

Ang1 was the first ligand of Tie2 to be identified. Mice lacking Ang1 have the same phenotype as Tie2 $^{-/-}$ mice (embryos die between E10.5 and 12.5 due to lack of vascular development after formation of the primary plexus), suggesting that Ang1 exclusively binds to Tie2 (fig 2F, enlargement F). Evidence suggests that Ang1 is essential for the establishment and maintenance of vessel integrity, since Ang1 controls endothelial permeability, and induces recruitment of vessel-supporting cells such as smooth muscle cells (SMCs) and pericytes . Ang1-Tie2 signalling can also function in migration by regulation of expression of matrix-degrading proteases. The outcome of Ang1-Tie2 signalling is context dependent: in an endothelial monolayer, a confluent situation, Ang1 binding results in translocation of Tie2 to cell-cell contacts (cadherins), whereas during cell spreading and migration, Ang1, either soluble or bound to a substrate, results in translocation of Tie2 to cell-matrix contacts (integrins). These different binding domains result in activation of different downstream targets (Augustin et al. 2009).

Ang2, the second Tie2 ligand, was initially believed to be an antagonist for Ang1, since it binds to Tie2, but without subsequent phosphorylation of Tie2 . It can also inhibit phosphorylation of Tie2 by Ang1. Mice overexpressing Ang2 have a similar phenotype as mice lacking Ang1 or Tie2, although it is more severe. Ang2$^{-/-}$ mice have a mild phenotype, except for retinal vessels where a distinct defect in sprouting can be detected: at postnatal day 10 (P10), when a wild type retina is completely vascularised, large areas of Ang2 $^{-/-}$ retinas are without vascular coverage and have hyaloid vessels, which are normally in regression in wild type retinas at this stage, and finally no penetrating vessels to the deeper layers of the retina develop. The importance of Ang2 for sprouting is shown in mice with oxygen-induced retinopathy. In this model, mouse pups are raised in a hyperoxic environment, which causes vessel regression in the retina. After having returned to room air, the mice develop ischemia in the retina and subsequent neovascularization (more details about this model can be found later in this review). Ang2$^{-/-}$ mice do not show any neovascularization under these circumstances (Augustin et al. 2009).

The exact roles of Ang1 or Ang2 in either tip cell or stalk cell function still has to be elucidated. In sprouting blood vessels, there is hardly any expression of Ang1, whereas Ang2 is expressed by tip cells at the sprouting front. Interestingly, Tie2 is almost completely absent in tip cells. Whilst Ang2 expression is upregulated by VEGF treatment of endothelial cells in culture, Tie2 expression diminishes as a consequence (Felcht et al. 2012). The lack of phosphorylation of Tie2 after binding Ang2, together with this differential expression suggests that Ang2 is able to bind receptors other than Tie2, with integrins as a promising candidate (fig 2G, enlargement G). Ang2 is able to bind $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ integrins, albeit with lower affinity than Tie2, which are all expressed in tip cells, while Tie2 is not (Felcht et al. 2012) These integrins are necessary for Ang2-induced stimulation of sprouting *in vitro* .

Bone morphogenic proteins and SMADs

Bone morphogenic proteins (BMPs) are members of the TGF-β superfamily. There are approximately 20 BMPs in total and they can either inhibit or stimulate angiogenesis. Best studied in the context of

angiogenesis are BMP 2, 4, 6, 7, 9 and 10, where BMP 2, 4, 6 and 7 are pro-angiogenic and BMP 9 and 10 are anti-angiogenic (Wiley & Jin 2011). Furthermore, BMPs can inhibit or elicit tip cell functions such as filopodial extension formation, and they are important for the induction of the stalk cell phenotype. There are 3 types of BMP receptors: type 1 receptors (activin receptor-like kinase (ALK) 1-7), type 2 receptors (ActRIIA, ActRIIB, BMPRII, TGFbRII and AMHRII) and type 3 co-receptors (betaglycan, endoglin or RMG-a, b, c) (David et al. 2009).

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The basic concept of signalling of BMPs is as follows: BMPs dimerize and bind to a tetraheteromeric receptor complex composed of 2 type 1 receptor subunits, and 2 type 2 receptor subunits, whereas affinity is modulated by type 3 receptors. The kinase domain of the type 1 receptor is phosphorylated and activates downstream signalling molecules called SMADs, more specifically, BMP receptors can activate SMAD1, 5 and 8. Activated SMADs form a complex with SMAD4 and translocate to the nucleus to initiate transcription (fig 2H, enlargement H) (Wiley & Jin 2011). Crucial downstream targets of SMAD are the Id proteins (inhibitor of DNA binding, dominant negative helix-loop-helix protein).

BMP signalling has been implicated in angiogenesis as an important inducer of the stalk cell phenotype. Mice lacking SMAD1 and 5 die at E9.5 due to severe angiogenic defects (Moya et al. 2012). The phenotype of these mice shows normal vasculogenesis, but impaired vascular remodelling and angiogenesis. In the hindbrain, more but non-functional sprouts were formed, showing decreased proliferation. Polarization of tip cells was lost, and there was an increase in filopodial extensions, both at the sprouting tip as well as further down the stalk. This phenotype suggests that BMP/SMAD signalling not only plays a role in the induction of a stalk cell phenotype, but also regulates polarization of tip cells and inhibits filopodial extensions.

The role in stalk cell formation was confirmed in *in vitro* experiments showing that endothelial cells devoid of SMAD1/5 prefer to have the tip cell position in sprouting assays, whereas endothelial cells overexpressing Id1 or Id3 do not show the tip cell phenotype (Moya et al. 2012). There is also cross-talk between BMP-SMAD signalling and Dll4-Notch signalling (Moya et al. 2012)(fig 2I, enlargement I). It seems that dynamic expression of any one of these pathways pushes endothelial cells towards either a tip cell or stalk cell phenotype (Beets et al. 2013).

In vivo, in vitro and in silico models

Most of the studies on tip cells cited above were performed using *in vivo* experiments, employing assays such as intersegmental vessel development in zebrafish and development of retinal vessels in mice. Both assays enable visualisation of tip cells and the provide the possibility to study tip cells in both physiological and pathological settings. New models for *in vitro* and *in silico* studies are emerging, and with these the possibilities for swifter and more detailed analysis of candidate genes for tip cell function and associated mechanisms have expanded. This section covers the most frequently-used *in vivo* models as well as new techniques for *in vitro* and *in silico* studies of tip cells.

In vivo

The zebrafish is a popular model to study angiogenesis, because of the short development time, the small size and the extracorporeal development. Furthermore, mechanisms involved in vascular development are well conserved between zebrafish and humans (Dooley & Zon 2000)[Dooley 2000]. Transgenic fish expressing fluorescent tags in blood vessels enable visualization of tip cells during development. Gene-specific knock downs allow researchers to study single genes and the effect of their expression on angiogenesis in fish and chemical compounds can be administered via the water to the embryos to assess their effect (Phng et al. 2013) (fig 3a).

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In the mouse eye, retinal blood vessels develop after birth as a front of sprouting vessels that grows from the optic nerve in the centre of the retina towards the periphery (Fruttiger 2002) (Fig 1A-B). Behind the sprouting front, remodelling and maturation take place, enabling the analysis of the angiogenic process from an emerging tip cell up until the formation of a mature vascular network. Furthermore, the primary retinal plexus is localised superficially in the retina, enabling visualization of sprouting vessels.

To study pathological angiogenesis, the oxygen-induced retinopathy model is frequently used. This model shows many similarities with the disease retinopathy of prematurity (ROP) and is aided by the fact that formation of the retinal vasculature in the mouse takes place after birth. Seven day-old mouse pups spend 5 days in a chamber with 75% oxygen, which causes regression of part of the retinal vasculature. At day 12, mice return to room air and the retina becomes hypoxic due to the lack of functional blood vessels. A wave of angiogenic activity occurs in response to growth factors that are produced in the hypoxic areas. The amount of neovascularization and the size of the avascular zone can be measured and quantified (Smith et al. 1994). This model can be used in mice after gene knock-out, or by the intravitreal injection of silencing RNA, or neutralizing antibodies. Other compounds, such as angiogenesis inhibitors or growth factors can also be injected into the eyes of these mice.

In vitro

Sprouting assays using either endothelial cells or stem cells have been widely used in angiogenesis studies. Recently, a subset of $CD34^+$ cells that resembles tip cells as found *in vivo* was discovered in cultures of human umbilical vein endothelial cells (HUVECs) (Fig 3B) (Siemerink et al. 2012). These cells extend filopodia, express known tip cell genes and have a less proliferative and more migratory phenotype, identical to tip cells *in vivo*. The percentage of $CD34^+$ cells can be increased by addition of VEGF to the culture and decreased by seeding cells on a Dll4-coated surface, which is in agreement with

regulation of the tip cell phenotype *in vivo*. A subpopulation of tip cells is generated by HUVECs in culture, which suggests that an equilibrium exists in endothelial cell cultures. The presence of tip cells in culture implies that endothelial cell cultures, even in monolayers with contact inhibition are not quiescent, but rather in an activated, angiogenic state.

In silico

Computational models have greatly contributed to the understanding of lateral inhibition through Dll4-Notch1 and the resulting spatial patterns of tip and stalk cell differentiation. More recently, mathematical and computational models have also been used to test hypotheses on the mechanisms of tip cell overtaking and to study the function of tip cells.

Collier et al. (1996) developed the first mathematical model of pattern formation due to Dll4-Notch1 dependent lateral inhibition (Collier et al. 1996), which they studied in a system of two coupled cells, a linear array of cells and in a two-dimensional, hexagonal lattice of cells. A set of coupled ordinary differential equations (ODEs) described the level of Notch activation and the level of Delta activity in each cell. They used a simple rule to describe lateral inhibition: "the more intense the inhibition a cell receives, the weaker its ability to deliver inhibition must become" (Collier et al. 1996). This feedback loop was represented in their model by a reduction of Dll4 production in a cell upon Notch activation. Small perturbations within a monolayer of initially equivalent cells induced an alternating pattern of cells with high and low Notch activation states (stalk and tip cells respectively), a pattern often seen in experiments. They concluded that lateral inhibition with a sufficiently strong feedback can create alternating patterns of cells with high and low Delta expression.

Sprinzak et al. (2010, 2011) developed a refined ODE model of Delta-Notch signaling to study the effect of cis-interactions, the mutual inactivation of interacting Delta and Notch of the same cell, on lateral inhibition (Sprinzak et al. 2011; Sprinzak et al. 2010). The model represents a two-dimensional array of hexagonal cells. Each cell has a concentration of Notch and Delta. Sprinzak et al. showed that cis-interaction between Delta and Notch speeds up the patterning dynamics and amplifies the feedback in lateral inhibition, since down-regulation of Dll4 simultaneously increases the number of 'cis-interaction free' Notch receptors in that cell. Cis-interaction also allows for an alternative mode of feedback in which Notch production is stimulated upon Notch activation, as was found *in vivo*. Thus cis-interaction refines the regulation of tip cell selection.

Besides in tip cell selection during angiogenesis, Dll4-Notch mediated lateral inhibition plays an important role in patterning of bristles in Drosophila epithelium (Cohen et al. 2010). Cells with high levels of Delta expression are destined to give rise to a bristle. Live imaging showed a gradual process of refinement in the late stages of lateral inhibition-mediated patterning, leading to a sparse spacing of cells with high levels of Dll4 (Cohen et al. 2010). Using a model of lateral inhibition, based on the model of Collier et al. (Collier et al. 1996), Cohen et al. (Cohen et al. 2010) were not able to reproduce the sparse spacing in a monolayer of epithelial cells that was geometrically similar to experimental images. Since live imaging showed that movement of the cell body contributed little to patterning refinement, Cohen et al. (2010) asked if cells might accomplish refinement with long, dynamic filopodia, which were observed to form a lateral web extending across several cell diameters at the basal side of the epithelial cells. Bristle spacing could be mimicked when the model was extended with dynamically forming filopodia that can contact and signal distant non-neighboring cells. After inhibiting filopodia dynamics the bristles differentiated more closely together both in the model and in the experiments.

Bentley et al. (2008, 2009) studied how filopodia contribute to tip cell selection during sprouting (Bentley et al. 2009; Bentley et al. 2008). They developed a computational model representing a single hollow, cylindrical sprout of ten endothelial cells. In this model, the membrane of each endothelial cell is composed of agents from which a filopodium can grow. Filopodia are assumed to extend towards higher concentrations of vascular endothelial growth factor (VEGF) (Bentley et al. 2008). Dll4 production is up-regulated upon VEGFR2 activation and VEGFR2 production is down-regulated by Notch1 activity (Phng & Gerhardt 2009), such that endothelial cells near higher concentrations of VEGF more likely differentiate into tip cells. Filopodial growth is stimulated by VEGF signaling, while surface extension by filopodia formation increases VEGF signaling, creating a positive feedback loop. The model predicted that tip cell patterning will stabilize faster in VEGF gradients than in uniform VEGF environments and that high VEGF levels induce oscillation of the alternating tip-stalk cell pattern. Anastomosis led by filopodia can create new cell-cell junctions with new Dll4-Notch signaling opportunities, which can make tip and stalk cells within the sprout switch fate (Bentley et al. 2009).

Detailed *in vitro* and *in vivo* imaging showed that cells in the sprout regularly overtake the tip cell, indicating competition for the tip cell position (Jakobsson et al. 2010). To study the mechanisms behind tip cell competition in more detail, Bentley et al. extended their model (Bentley et al. 2014) by using the Cellular Potts Model (CPM) (Graner & Glazier 1992) to represent the shape and movement of the cells, thereby explicitly modeling cell-cell adhesion and junctional reshuffling. Bentley et al. (Bentley et al. 2014) hypothesized that VEGF stimulates endocytosis of vascular endothelial cadherin (VE-cadherin), thus reducing the adhesion between endothelial cells. They further assumed that Notch activity decreases extension of polarized actomyosin protrusions towards the sprout tip. Tip cell competition in the model was most in line with experimental observations and perturbations when adhesion differentially depends on VEGFR2 signaling and polarized protrusions differentially depend on Notch activity.

Although it is now well established that both tip and stalk cells are present during angiogenesis and vasculogenesis, it is not clear what biophysical properties of tip cells lead sprouts and affect the morphology of vascular networks. Palm et al. (2014) used computational modeling to address these questions (Palm et al. 2014) (fig 3C). They added a model of tip cell selection to a previously published CPM model of angiogenic sprouting and vasculogenesis (Merks et al. 2008), in which sprouts form due to cell-cell contact inhibited chemotaxis towards a compound secreted by the endothelial cells themselves (Fig 3C). Palm et al. performed large parameter sweeps and morphological analyses on the resulting vascular network, to identify “tip cell behaviors’’: Tip cells move towards the sprout tip when they adhere more to the extracellular matrix than to neighboring cells, or when they are less sensitive to the secreted compound, compared to stalk cells. Tip cell selection, and the resulting switching of tip and stalk fate, localizes tip cells to the sprout tips, and thereby stabilizes the vascular network morphology at tip cell free branches.

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Concluding remarks

The tip cell has presented itself in the last decade as a promising target for pro- and anti-angiogenic therapies. Specific functions and complex mechanisms of regulation of the tip cell phenotype are unravelling. However, the lack of a marker for tip cells *in vivo* is a major obstacle for detailed research. The discovery of an *in vitro* marker such as CD34 will enable cell sorting to perform experiments with solitary tip cells and non-tip cells. *In silico* modelling provides another valuable approach by which important mechanisms can be elucidated which can then be confirmed in *in vitro* or *in vivo* experiments.

Figure legends

Figure 1.

A. Tip cells in the retina of a mouse (white arrow) at the top of newly formed capillaries during angiogenesis at 5 days after birth (P5). The capillaries are stained with Isolectin B4 (green).
B. Higher magnification of the edge of an area in the mouse retina where tip cells at the top of newly-formed capillaries are shown to have filopodia (arrow heads). Bars are 250 μm.

Figure 2. Overview of the key regulatory pathways for tip cell selection.

Tip cells are represented in red, stalk cells in blue and the phalanx cells in orange.

A. VEGF: VEGF-A is produced upon hypoxia, tip cells express VEGFR2 and -3, two receptors that exert the pro-angiogenic effects of VEGF. Soluble VEGFR1 is produced by stalk cells and acts as a sink for VEGF to prevent signaling in stalk cells
B. Neuropilins: NRP-1 acts as a co-receptor for VEGFR2 to enhance VEGF signaling
C. Notch1-Dll4: Dll4 is expressed by tip cells, Notch1 by stalk cells. Upon binding, Notch1 is cleaved, and the intracellular domain (NICD) is translocated to the nucleus where it recruits transcription factors to replace expression of tip cell genes by expression of stalk cell genes.
D. Angiopoietins and Tie2: Ang1 is expressed in mature vessels and its main function is vessel stabilization.
E. Ang2 can bind Tie2 to inhibit Ang1-mediated phosphorylation of Tie2; it also binds integrins on tip cells to enhance sprouting.
F. BMPs and SMADs: Pro- and anti-angiogenic BMPs bind to their receptor and phosphorylate SMAD1/5/8. In tip cells, the SMAD1/5/ 8 complex induces polarization and migration.
G. In stalk cells, the SMAD 1/5/8 complex forms a complex with NICD to promote the stalk cell phenotype.

Figure 3. In vivo, in vitro and in silico models for angiogenesis

A. Fli1a-eGFP transgenic zebrafish, at 24 h post fertilization. At this stage, sprouting occurs in the intersegmental vessels (white arrows) and tip cells are present on each sprouting vessel.
B. $CD34^{+}$ cell in a human umbilical vein endothelial cell (HUVEC) culture. HUVECs where grown on coverslips coated with gelatin and stained for F-actin (red), CD34 (green) and DAPI (blue).
C. In silico vascular network formed from a spheroid of endothelial cells by a mechanism of cell-cell contact-inhibited chemotaxis (Merks et al. 2008)[Merks 2008]. Tip cells are indicated in red.

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Key words

Angiogenesis, tip cell, stalk cell, anti-angiogenic therapy, VEGF

Key concepts

- Sprouting angiogenesis is led by tip cells, which can sense their environment and direct the sprouting process
- Tip cells are followed by stalk cells, which have a more proliferative phenotype.
- The number of tip cells is regulated by lateral inhibition between the tip cells and stalk cells; the tip cells prevent the adjacent cells from becoming tip cells and thereby optimize the number of tip cells.
- The tip cell and stalk cell phenotype are definite, and stalk cells can overtake tip cells through stochastic cellular and environmental differences, the stalk cells then become tip cells and force the former tip cells to become stalk cells.
- Tip cells exist in human umbilical vein endothelial cell cultures and can therefore be studied *in vitro*.

Further reading

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Figure 1

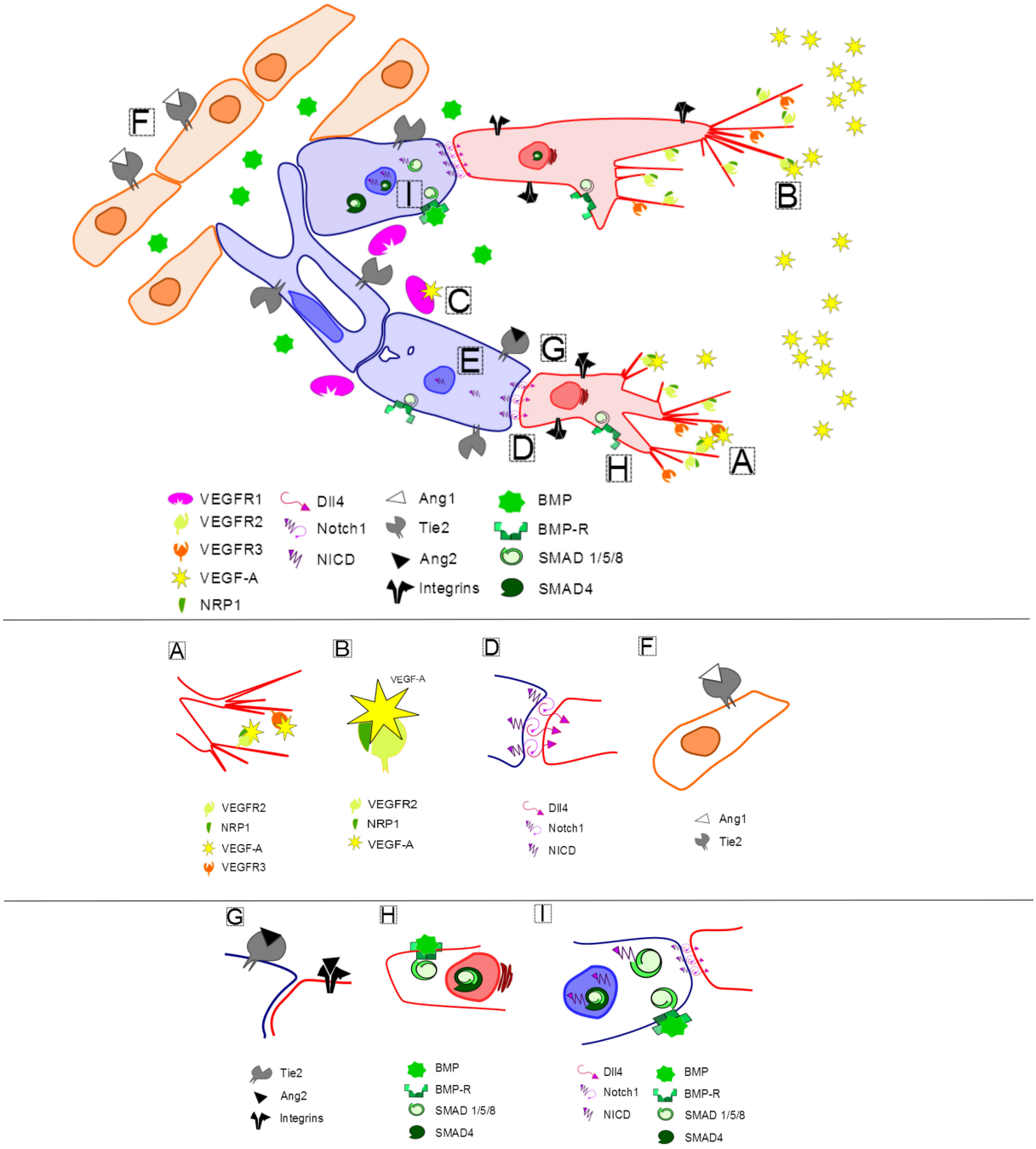
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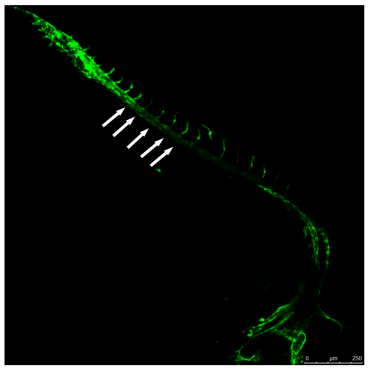
Figure 2

A



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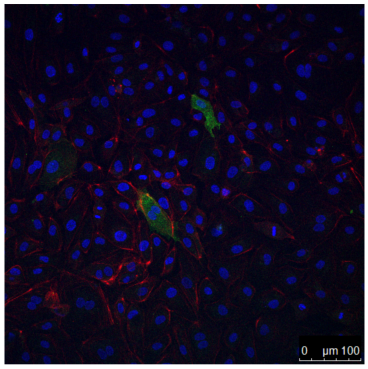

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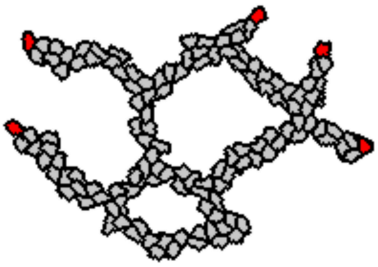
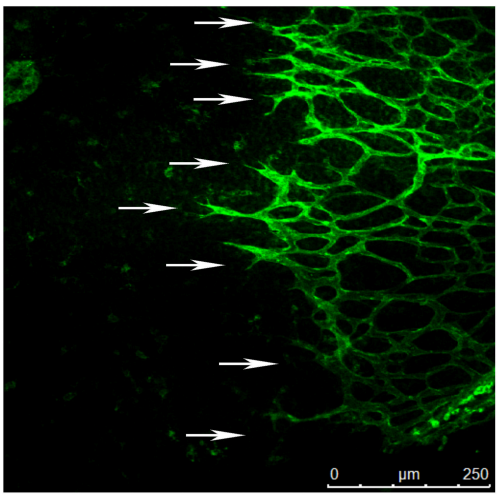


Figure 3

A



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