

# Coordinating cell behaviour during blood vessel formation

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## Summary

The correct development of blood vessels is crucial for all aspects of tissue growth and physiology in vertebrates. The formation of an elaborate hierarchically branched network of endothelial tubes, through either angiogenesis or vasculogenesis, relies on a series of coordinated morphogenic events, but how individual endothelial cells adopt specific phenotypes and how they coordinate their behaviour during vascular patterning is unclear. Recent progress in our understanding of blood vessel formation has been driven by advanced imaging techniques and detailed analyses that have used a combination of powerful *in vitro*, *in vivo* and *in silico* model systems. Here, we summarise these models and discuss their advantages and disadvantages. We then review the different stages of blood vessel development, highlighting the cellular mechanisms and molecular players involved at each step and focusing on cell specification and coordination within the network.

**Key words:** Angiogenic sprouting, Model systems, Vascular network, Angiogenesis

## Introduction

Blood vessel networks arise either through an assembly process (vasculogenesis; see Glossary, Box 1) or through the coordinated expansion of a pre-existing network (angiogenesis; see Glossary, Box 1) (Risau, 1997). Vasculogenesis relies on the local differentiation of mesoderm-derived angioblasts (see Glossary, Box 1) into endothelial cells (ECs) that coalesce into a primitive network (Swift and Weinstein, 2009). Angiogenesis, by contrast, comprises several morphogenic events during which pre-existing ECs coordinately sprout, branch, form lumen, make new connections (anastomose) and rearrange themselves (remodel), resulting in a functional network that supports and regulates effective blood flow (for a review, see Eilken and Adams, 2010). During this sprouting process, the ECs migrate, proliferate, establish junctions and apical-basal polarity, and deposit a stabilising basement membrane. Some of the most fundamental questions in vascular biology concern how the ECs coordinate their individual behaviours during angiogenic sprouting, and how tissue-level signals regulate collective EC behaviour to build branched, tubular networks that exhibit tissue-specific patterning and functionality.

The deregulation of blood vessel formation generally has major consequences for normal development, as organogenesis is critically dependent on blood supply (for a review, see Carmeliet, 2003). The inactivation or mutation of genes involved in blood vessel development often results in embryonic lethality. Prime

examples are the key angiogenic regulators, vascular endothelial growth factor (VEGF) and the notch pathway ligand delta-like 4 (DLL4), for which even the loss of a single allele is lethal, illustrating dose-sensitivity and crucial importance for angiogenic regulation during development (Carmeliet et al., 1996; Ferrara et al., 1996; Gale et al., 2004).

Later in life, angiogenesis plays a key role in many diseases (Carmeliet, 2003). Excess or paucity of blood vessels and their impaired function contribute to the progression of cancer growth and metastasis, ischemic retinopathies and stroke, as well as many metabolic disorders.

Here, we review the individual steps involved in sprouting angiogenesis, focusing on the molecular players and cellular mechanisms that govern individual and coordinated EC behaviour at each step. We also summarise several *in vivo*, *in vitro* and *in silico* model systems that have delivered key insights and discuss their major advantages and limitations as models for angiogenic sprouting.

## Angiogenic sprouting models

### In vivo models

#### The mouse retina model

The mouse retina has contributed significantly to our understanding of mechanisms of angiogenic sprouting (for reviews, see Gariano and Gardner, 2005; Uemura et al., 2006; Fruttiger, 2007; Stahl et

### Box 1. Glossary of terms

**Angioblasts.** Mesoderm-derived precursors of endothelial cells.

**Angiogenesis.** The formation of new blood vessels from pre-existing vessels via sprouting.

**Astrocytes (or astrocytic glial cells).** Non-neuronal star-shaped cells in the nervous system that provide support and protection for ECs and nerve cells.

**Diabetic retinopathy.** Eye disease associated with diabetes, caused by damage to the blood vessels in the retina.

**Femoral artery.** Large artery in the thigh, the main artery providing blood to the lower limbs.

**Mural cells.** Cells covering the endothelial cell tube, subdivided into pericytes in the microvasculature and vascular smooth muscle cells in larger vessels. Their function is to stabilise nascent vessels, provide support and guide remodelling.

**Optic disc.** The location in the retina where axons come together and leave the eye as the optic nerve and where the blood vessels enter the eye. Also called the 'blind spot' because there are no photoreceptor cells in this region.

**Pericytes.** Supportive cells forming a discontinuous layer around small diameter blood vessels. A type of mural cell.

**Retinopathy of prematurity (ROP).** Eye disease in premature babies, caused by a temporal arrest in development of the retinal vasculature, followed by outgrowth of abnormal vessels.

**Vasculogenesis.** The formation of new blood vessels from precursor cells.

**Vitreous surface.** The surface of the retina in contact with the vitreous, the gel-like substance that fills the eye.

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al., 2010). Shortly after birth, blood vessels emerge at the optic disc (see Glossary, Box 1) and sprout radially just below the vitreal surface (see Glossary, Box 1) of the retina until they reach the peripheral margin. The growing vessels are guided by a network of astrocytes (see Glossary, Box 1), which produce VEGF (Stone et al., 1995; Dorrell et al., 2002; Ruhrberg et al., 2002; Gerhardt et al., 2003). In a second phase commencing around postnatal day (P) 6, vascular branches also sprout downwards into the retina to form additional plexuses (Gerhardt et al., 2003) (for reviews, see Gariano and Gardner, 2005; Uemura et al., 2006; Fruttiger, 2007; Stahl et al., 2010). The fact that vascularisation occurs postnatally in this model makes it accessible to manipulation, drug delivery, and alterations in oxygen tension. Imaging capabilities are enhanced by the planar outgrowth of the plexus, which, upon flat-mounting and immunostaining, can be investigated at high resolution with excellent contrast (Fig. 1A,B). In addition, the different stages of angiogenic network formation are visible in a single sample owing to the spatiotemporal sequence of sprouting, guidance, remodelling and maturation. However, live imaging of sprouting retinal vessels *in vivo* has not been achieved to date, and we lack information on the true dynamics of vascular development in this model.

#### The mouse hindbrain model

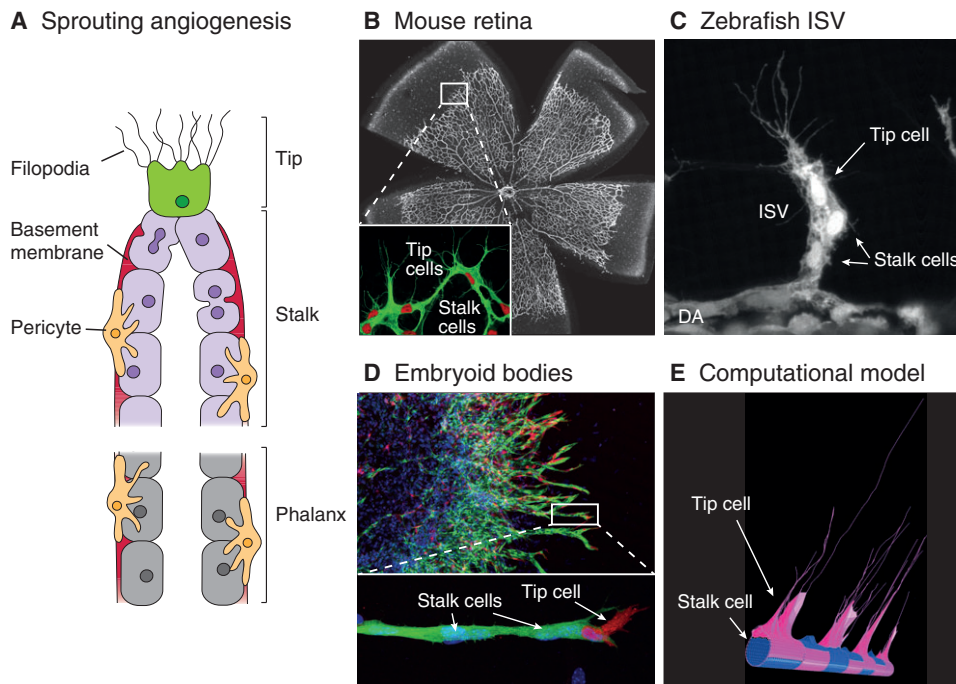
Studies on angiogenic sprouting and vascular patterning in the embryonic mouse hindbrain have provided us with key insights into angiogenic guidance functions of neuropilin 1 (NRP1), VEGF

(Ruhrberg et al., 2002; Gerhardt et al., 2004), netrin and UNC5B (Lu et al., 2004). This vascular network forms between E10.5 and E13.5, and can be visualised by opening up the roofplate and flat-mounting the neural tube like an open book. The hindbrain vascular network is ideal for imaging and is used to evaluate the morphology and density of radial sprouts that emerge from the perineural plexus, and the branching frequency and vessel diameter in the subventricular plexus. A major advantage of this model over the retinal model is the fact that it can be used to study the details of angiogenic sprouting defects in embryonic lethal mutants, such as *Nrp1* knockouts (Gerhardt et al., 2004). However, live imaging has also not been achieved in the mouse hindbrain.

#### Zebrafish as a model for angiogenic sprouting

The zebrafish (*Danio rerio*) is used as a model for vertebrate development in many research fields, including vascular biology. Zebrafish embryos are easy to manipulate, have a rapid generation time (precursors to all major organs are established within 36 hours) and, because of their transparency, the development of organs and tissues can be easily imaged and analysed.

Blood vessel development in zebrafish follows a spatiotemporally conserved pattern (Isogai et al., 2003). The transgenic zebrafish line *Fli1:eGFP<sup>h1</sup>*, expressing green fluorescent protein (GFP) in all ECs (Lawson and Weinstein, 2002), has been particularly useful for analysing the dynamics of this process. For example, the formation of intersegmental vessels (ISVs), which form by sprouting from the dorsal aorta (DA) and grow along the somite boundaries to connect



**Fig. 1. Cell analysis in sprouting angiogenesis models.** (A) Schematic illustration of a growing sprout. The sprout is guided by a tip cell (green), which uses filopodia to scan the environment for attractive and repulsive cues. Stalk cells (purple) proliferate, form a lumen, deposit a basement membrane (red) and attract pericytes (orange). Both tip and stalk cells are activated endothelial cells (ECs). By contrast, phalanx ECs (grey) represent quiescent cells that do not proliferate. (B–D) Representative images of vessel networks and sprouts in different model systems, highlighting tip and stalk cells. (B) The mouse retina at post-natal day (P) 5. Inset shows higher magnification of sprouting front showing tip cells with filopodia and stalk cells. Red, endothelial nuclei (Erg); green, Isolectin-B4. (C) A growing intersegmental vessel (ISV) in a 28 hours post-fertilisation (hpf) old transgenic *Fli1:eGFP<sup>h1</sup>* zebrafish embryo. (D) Sprouts growing from a mosaic embryoid body *in vitro*, which comprises DsRed-expressing wild-type (WT) cells (red) and *VEGFR2<sup>EGFP/+</sup>* cells (green). Nuclei are also counterstained (blue). (E) Tip (pink) versus stalk (blue) cell selection simulated in a computational model.

at the dorsal side of the embryo and form the dorsal longitudinal anastomosing vessel (DLAV), has been well characterised (Childs et al., 2002; Isogai et al., 2003; Blum et al., 2008) (Fig. 1C). In contrast to the mouse retina and hindbrain model systems, zebrafish embryos allow live time-lapse imaging of blood vessel development over long periods. Furthermore, as the young embryos can survive several days without blood flow (Pelster and Burggren, 1996; Stainier et al., 1996), the flow dependency of angiogenic processes can be studied in mutants that have no blood flow. One disadvantage of the zebrafish model is that the species is less closely related to human, and the duplicated genome sometimes poses problems of redundancy upon gene silencing (Taylor et al., 2003). However, in rare cases, knockdown strategies in zebrafish have resulted in more severe phenotypic effects than knockout of the same gene in mouse (e.g. *synectin*; *Gipc1* – Mouse Genome Informatics) (Chittenden et al., 2006).

#### The cornea pocket assay

The cornea pocket assay is an *in vivo* angiogenesis assay that is based on the induced vascularisation of the normally avascular cornea in adult mice (Kenyon et al., 1996). A slow-release pellet containing angiogenic growth factors, such as VEGF, is placed into a surgically prepared micropocket in the cornea of adult mice. After about 5 days, sprouting blood vessels reach the micropellet and the angiogenic response can be quantified without dissection of the eye. The exclusive analysis of newly formed blood vessels in a previously avascular tissue enables the specific effects of individual growth factors to be studied (Kubo et al., 2002; Kisucka et al., 2006; Kuhnert et al., 2008; Koch et al., 2011). However, obtaining a standardised angiogenic response can be difficult, owing to the sensitivity of the angiogenic response to pellet size, the location of the micropocket and the released levels of growth factors.

#### Disease models

Several disease models have been developed to mimic aspects of ischemia (inadequate blood supply) and tumour angiogenesis, enabling experimental studies of the mechanisms of pathological angiogenesis and the evaluation of anti- or pro-angiogenic treatments.

#### The hindlimb ischemia model

In the hindlimb ischemia model, ligation of the femoral artery (see Glossary, Box 1) induces tissue ischemia in the leg muscle and the concomitant neovascularisation response can be assessed (Baffour et al., 1992; Emanuelli et al., 2001; Limbourg et al., 2009). This ligation method is relatively easy to perform because the femoral artery is readily accessible. The neovascular response includes formation of parallel collateral vessels and induction of sprouting angiogenesis. State-of-the-art microCT (microcomputed tomography) imaging, which recreates a 3D model based on X-ray-created cross-sections, can deliver detailed information about the perfused network in three dimensions (Osés et al., 2009).

#### Retinal ischemia models

The mouse retinal vasculature is also frequently used as an ischemia disease model to mimic aspects of human retinopathy of prematurity (ROP; see Glossary, Box 1) and diabetic retinopathy (see Glossary, Box 1) (Smith et al., 1994; Stahl et al., 2010). In the ROP model, mouse pups are exposed to a 75% oxygen-enriched atmosphere between P7 and P12. The elevated oxygenation causes vessel regression and suppresses normal vessel formation, leaving large parts of the central retina avascular. Upon return to normal

atmospheric oxygen levels, the avascular regions become ischemic, triggering a neovascular response. The new outgrowth of vessels is poorly organised, causing leakiness and ectopic vessel formation at the vitreal surface of the retina. Whereas most studies in the past used histological transverse sections to quantify neo-vessels, recent studies use retinal flat-mounts and FITC-dextran perfusion, vessel labelling or Evans Blue to analyse neo-vessel formation and vascular integrity (Jones et al., 2008; Stahl et al., 2010).

#### Tumour angiogenesis models

Tumour growth is critically dependent on neovascularisation of the growing tumour, and several anti-cancer treatment strategies thus aim to interfere with the tumour-induced angiogenesis process (reviewed by Carmeliet and Jain, 2011). Tumours can be formed spontaneously in mice carrying mutations in oncogenes or tumour suppressor genes, or they can be induced by carcinogen or radiation treatment. Alternatively, cancerous cells can be transplanted into different tissues in mice. The transplantation approach has the advantage that tumour induction is controllable. Furthermore, the recent development of tumour cells that express luciferase, together with advanced *in vivo* imaging techniques, allows the detection of even small tumour nodules. By contrast, tumour induction by genetic or chemical approaches, or by radiation, requires close monitoring of the animals because the unlabelled tumours are only detectable once they reach a certain size.

The development of intravital imaging techniques has also enabled the study of dynamic processes between tumour cells, tumour cells and stroma, and tumour cells and blood vessels during disease progression. Implantation of specialised imaging ‘windows’ further allows prolonged analysis of tumour growth and vascularisation without the need for repeated surgery (for reviews, see Fukumura and Jain, 2008; Lohela and Werb, 2010). Advances in microscopy techniques, such as the use of infrared two-photon and second harmonic generation microscopy, allow for deeper tissue imaging and decreased phototoxicity during intravital imaging (Andresen et al., 2009).

Tumour models have also been developed in zebrafish during the last decade. Both chemically induced (Mizgirev and Revskoy, 2006) and spontaneous zebrafish tumour models (Patton et al., 2005) as well as transplantation models (Haldi et al., 2006; Nicoli et al., 2007; Stoletov et al., 2007) have been developed in adult fish or in embryos. In these models, stimulation of angiogenic sprouting towards and into tumour masses has been shown (Haldi et al., 2006; Mizgirev and Revskoy, 2006; Nicoli et al., 2007), as has the interaction of tumour cells with blood vessels to induce metastasis (Stoletov et al., 2007).

#### Ex vivo and in vitro models

##### Retinal explant cultures

Recent *ex vivo* studies of retinal explants in short-term culture suggest that the dynamic analysis of ongoing retinal vascular sprouting might be feasible (Murakami et al., 2006; Sawamiphak et al., 2010; Unoki et al., 2010b). In this approach, retinas are dissected, placed on a filter and covered with a collagen matrix. VEGF is then used to induce sprouting of the retinal vessels (Murakami et al., 2006). In this model, the normal endogenous tissue gradient of VEGF is likely to be disrupted, and directional guidance and balanced EC proliferation might not be comparable with the *in vivo* situation. Furthermore, because these explant models lack vascular perfusion, flow- and shear stress-dependent remodelling, as well as any tissue response feedback mechanisms, cannot be studied.

### The aortic ring assay

In this assay, rings are cut from mouse or rat aorta and embedded in a collagen matrix. They begin forming angiogenic sprouts in response to the injury induced by the dissection procedure (Nicosia and Ottinetti, 1990; Masson et al., 2002). Within a few days, sprouts emerging from the ring form a microvascular network forms that undergoes maturation and recruits mural cells (see Glossary, Box 1) from the aortic wall (Iurlaro et al., 2003). This model has been used to study dynamic sprout outgrowth, tissue invasion and mural cell recruitment (Gerhardt et al., 2003; Chun et al., 2004; Franco et al., 2008; Graupera et al., 2008; Aplin et al., 2010).

Such *ex vivo* vascular explants reproduce the natural environment of outgrowing vessel branches more accurately than isolated ECs; however, influences of blood flow and blood pressure are not present. Rat aortic rings, unlike mouse explants, can be cultured without addition of serum to the medium (Nicosia and Ottinetti, 1990; Masson et al., 2002), enabling the evaluation of pro- or anti-angiogenic compounds in a chemically defined environment. By using transgenic animals as the source of tissue for the aortic ring assay, the effects of gene knockout, mutations and overexpression of genes of interest can be studied.

### Embryoid body sprouting assay

Embryoid bodies (EBs) are clusters of embryonic stem cells (ESCs) cultured *in vitro* to differentiate into multiple cell lineages, including ECs. When embedded in a collagen matrix and stimulated with VEGF, EBs form capillary-like structures in a manner comparable with vasculogenesis and angiogenesis (Jakobsson et al., 2007). A branched 3D network of sprouts forms after one week (Fig. 1D). The EC sprouts are surrounded by mural cells and a basement membrane, and become lumenised (Jakobsson et al., 2007). The EB endothelium also undergoes arterial/venous differentiation (Muller-Ehmsen et al., 2006), but the lack of blood flow precludes shear stress-dependent remodelling (Nguyen et al., 2006).

EBs are ideal for studying gene function as they can be constructed using genetically manipulated ESCs that overexpress or lack a gene of interest. Indeed, the inactivation of angiogenesis-regulating genes, such as those encoding vascular endothelial growth factor receptor 2 (VEGFR2; KDR – Mouse Genome Informatics) or VE-cadherin (CDH5 – Mouse Genome Informatics), gives rise to phenotypes that are similar to those observed in *in vivo* models (Vittet et al., 1997; Jakobsson et al., 2006). Furthermore, by mixing different genotypes of ESCs, EBs can be used as a cell competition assay to investigate quantitative and cell-autonomous gene function in sprouting (Fig. 1D) (Jakobsson et al., 2010). Time-lapse imaging of EBs delivers dynamic information on EC behaviour during the sprouting process.

### Other *in vitro* models of angiogenesis

The *in vitro* models described above are best suited to studying the process of angiogenic sprout outgrowth. However, numerous other methods have been used to induce *in vitro* tubule formation by ECs that have not been described here owing to space limitations. These many examples have been discussed previously by Staton et al. (Staton et al., 2004).

### *In silico* models

Increasingly, computational models are being used to predict the outcome of biological processes. The predictions can then be tested in the laboratory and, based on discrepancies or new observations, the computational model can be refined, leading to a deeper understanding of the process.

Computational and mathematical models of angiogenesis have focused mainly on tumour angiogenesis, addressing the qualitative migratory response of individual ECs to angiogenic factors, and quantitatively predicting sprout densities during vascular network formation (for a review, see Chaplain et al., 2006). Recent studies have simulated various signalling, mechanical and flow-related aspects of tumour angiogenesis, utilising a variety of modelling approaches (for reviews, see Peirce, 2008; Mac Gabhann et al., 2010).

Recently, computational modelling has also entered the field of developmental angiogenesis. Based on simulations of the feedback loop between VEGF and notch signalling, the initial sprouting process could be accurately replicated *in silico*, indicating that VEGF/notch regulation is sufficient to pattern this process (Bentley et al., 2008; Bentley et al., 2009) (Fig. 1E). This computational model also predicted that the balance between VEGFR1 (FLT1 – Mouse Genome Informatics) and VEGFR2 expression levels in ECs affects their potential to become the tip cell in a growing sprout (Jakobsson et al., 2010), and illustrated the effects of notch signalling dynamics on tip cell selection and branching (Guarani et al., 2011). As a caveat, it should be noted that the value of computational modelling crucially depends on the availability of quantitative biology data that can inform the process, and that the value will greatly increase with iterations of predictions and experimental testing. Evidence is mounting that better integration of computational models, and their testing in parallel with biological models, will lead to a much better understanding of developmental angiogenesis.

### Sprouting angiogenesis: the key cell types

The concept of behavioural coordination during angiogenesis rests on the observation that not all ECs behave the same; functional specification and heterogeneity allows for effective job-share and teamwork. The prototypical example is the ‘tip-stalk’ concept in angiogenic sprouting, which describes two distinct cell phenotypes based on their gene expression profiles and the functional specifications of ECs within a newly formed sprout. The leading cell, called the tip cell, is migratory and polarised. This cell extends long filopodia that scan the environment for attractant or repellent signals, and hence serves to guide the new blood vessel in a certain direction (Fig. 1A) (Ruhrberg et al., 2002; Gerhardt et al., 2003; Lu et al., 2004; De Smet et al., 2009). Adjacent to the tip cell, the following cells, termed stalk cells, proliferate during sprout extension and form the nascent vascular lumen (Fig. 1A) (Gerhardt et al., 2003). Tip cell migration can occur without stalk proliferation and vice versa. However, only a regulated balance between both processes establishes adequately shaped nascent sprouts (Ruhrberg et al., 2002; Gerhardt et al., 2003; Ruhrberg, 2003).

The tip and stalk cell phenotypes characterise the ‘activated endothelium’. Once lumenised connections and blood flow have been established, the migratory activity and proliferation of ECs ceases (Bussmann et al., 2011) and they eventually adopt a ‘quiescent phenotype’. Quiescent ECs are characterised by a more regular ‘cobblestone’ appearance, which resembles a phalanx formation of ancient Greek soldiers, leading Mazzone and Carmeliet to coin the term ‘phalanx cells’ (Fig. 1A) (Mazzone et al., 2009). Where, when and how the activated endothelium transits to quiescence is poorly understood. Although tip and stalk cells are defined by neighbourhood relationships, no clear positional relationship can be assigned to stalk and phalanx cells in the developing plexus. Both the proliferative stalk cells and the quiescent phalanx cells are covered by supporting pericytes (see

Glossary, Box 1) (Betsholtz et al., 2005; Mazzone et al., 2009), connected by adhesion and tight junctions, and embedded in basement membrane (Fig. 1A).

The distinct tip and stalk EC phenotypes display differential gene expression profiles, suggesting that their specification is determined genetically. Several recent studies have analysed and compared the expression profiles of tip and stalk cells (del Toro et al., 2010; Strasser et al., 2010). These studies have identified a list of genes with which tip cells are enriched, which includes VEGFR2, platelet-derived growth factor B (PDGFB), the netrin receptor unc-5 homolog B (UNC5B), the notch ligand DLL4, EC-specific molecule 1 (ESM1), the peptide ligand apelin (APLN) and the membrane-inserted matrix metalloprotease 14 (MMP14) (Gerhardt et al., 2003; Lu et al., 2004; Hellstrom et al., 2007; Yana et al., 2007; del Toro et al., 2010; Strasser et al., 2010). However, a single unique gene or protein that can be used reliably and unambiguously as a molecular marker for tip cells has not been identified.

A key pathway regulating the specification of tip and stalk cells is the notch signalling pathway. Notch signalling is an evolutionarily conserved cell-cell contact-dependent communication mechanism best known for its role in instructing differential fate decisions between neighbouring cells during development (Chitnis, 1995; Lewis, 1998) (Box 2). Furthermore, in epithelial cells, presentation on filopodia of notch ligands has been shown to extend the effective range of notch-signal induction from a single cell to beyond its immediate neighbours (De Jossineau et al., 2003). During angiogenesis, activated notch signalling inhibits tip cell formation and promotes the stalk cell phenotype (Hellstrom et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). Genetic and pharmacological inactivation of notch in mouse and zebrafish illustrated that tip cell formation is the default response of the activated endothelium, whereas the lumen-forming stalk cell phenotype is acquired through notch activation (Hellstrom et al., 2007; Leslie et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). Recent observations challenge the idea that tip and stalk cells resemble fully differentiated cell fates. Instead, their specification is surprisingly dynamic and transient, relying on continued competition between the cells (Jakobsson et al., 2010). As a consequence, tip cells are overtaken, and exchanged, as neighbouring cells take their place.

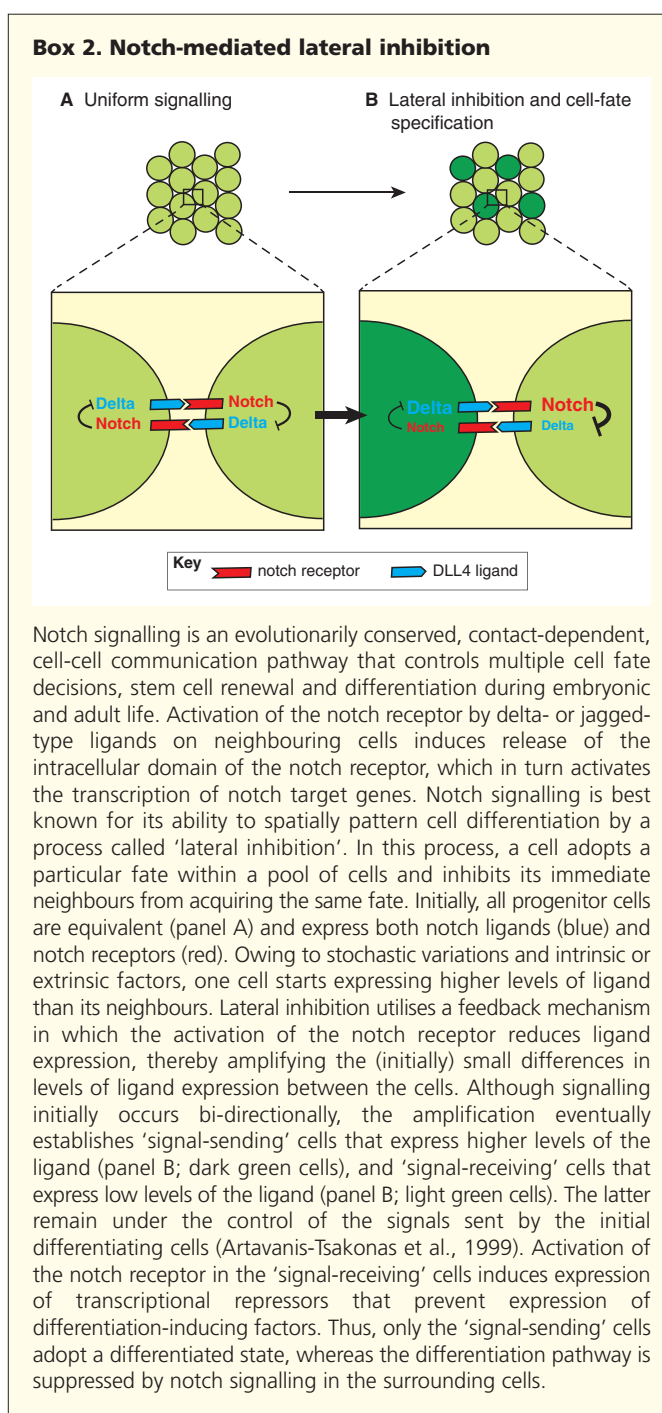
### Sprouting angiogenesis: a multistep process

Upon induction of angiogenic sprouting, ECs exposed to angiogenic stimuli become activated and compete for the tip cell position (Jakobsson et al., 2010). This process limits the number of outgrowing sprouts. Networks are then formed by fusion (anastomosis) of sprouts, stabilisation and remodelling of newly formed connections.

It has been noted that vascular sprouts morphologically resemble the growth cones of developing axons and the tip cells of growing tracheal tubes in *Drosophila* (Kater and Rehder, 1995; Ribeiro et al., 2002). In addition, the functional similarities between blood vessel development and pulmonary tubulogenesis, kidney ureteric duct formation, mammary gland branching and neuronal wiring indicate a remarkable degree of evolutionary conservation in the process of branching morphogenesis (see Box 3).

### Sprout initiation

When nutritional and oxygen demands within a tissue exceed the supply provided by existing blood vessels, the tissue sends out signals that stimulate the formation of new blood vessels (for



reviews, see Germain et al., 2010; Carmeliet and Jain, 2011). A key angiogenesis-promoting signal is VEGF. VEGF activates the endothelium through stimulation of VEGFR2. This leads to the selection of a tip cell, tip cell branching, vessel sprouting, EC proliferation and lumen formation (Fig. 2).

### Tip cell selection

Although apparently all ECs exposed to VEGF become activated, not all ECs respond by directed migration (Gerhardt et al., 2003). Instead, a fine-tuned feedback loop between VEGF and notch/DLL4 signalling establishes a 'salt and pepper' distribution of tip and stalk cells within the activated endothelium (Hellstrom et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Siekmann and

### Box 3. Sprouting and cellular competition in other branching systems

Branched tubular networks in many organs and organisms show mechanistic similarities and a remarkable degree of evolutionary conservation. Cellular competition for the lead position during branching morphogenesis has been discovered in several systems.

As in the vertebrate vascular system, a newly formed airway tracheal branch in *Drosophila* is guided by a leading tip cell, and extension of the sprout involves cell intercalation and proliferation of stalk cells. Tracheal branching in *Drosophila* is regulated by FGF signalling, and the FGF ligand Branchless (BNL) forms the tracheal counterpart of VEGF in angiogenic sprouting (Sutherland et al., 1996). BNL signalling through the FGF receptor Breathless (BTL) induces filopodia formation and initiation of sprouting. Cells with the highest BTL levels competitively acquire the lead position and form the tracheal tip cell (Ghabrial and Krasnow, 2006).

Branching processes in the vertebrate lung, mammary gland and kidney ureteric duct are similarly regulated by FGF signalling, with additional roles played by TGF- $\beta$  signalling and other pathways (Horowitz and Simons, 2008; Lu et al., 2008). Whereas competition in *Drosophila* tracheal branching and angiogenesis establishes one or two leading cells, competition in branching of lung, ureteric epithelium and mammary gland leads to clustering of cells that collectively form the bud tip. In all cases, competition involves differential growth factor receptor levels. However, Notch-mediated lateral inhibition appears to be integrated only in the tracheal and angiogenic competition mechanisms, in which selection of single tip cells is crucial for sprouting (Ikeya and Hayashi, 1999; Ghabrial and Krasnow, 2006; Jakobsson et al., 2010).

Lawson, 2007; Suchting et al., 2007; Bentley et al., 2008). The resulting cell-fate specification mechanism is reminiscent of a classical notch-mediated lateral inhibition process (Muskavitch, 1994; Lewis, 1998), in which a cell adopts a particular fate and prevents (by lateral inhibition) its immediate neighbours from acquiring the same fate (Box 2). Although activated ECs would be assumed to initially express similar levels of notch and DLL4, and thus undergo a balanced reciprocal notch activation, stochastic differences in local VEGF concentrations, in filopodia elongation (and thus VEGF exposure) or in transcription rate lead to small imbalances: one cell will express slightly higher DLL4 levels and, thus, will dominate its neighbours by activating more notch signalling. The cell with more DLL4, and less notch activity, will be selected as the tip cell. Activation of notch inhibits VEGFR2, indirectly inhibiting DLL4 expression levels, thereby reinforcing the dominance of the selected tip cell and limiting the number of tip cells induced by VEGF (Fig. 2A,B) (Williams et al., 2006; Hellstrom et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Suchting et al., 2007). Currently, an open question in the field is whether and how DLL4 might activate notch in cells that are not in direct contact with a tip cell. Given that the precise shape of tip and stalk cells is poorly defined, it is unclear whether long-range lateral inhibition, possibly mediated by long cellular processes (De Jossineau et al., 2003), is active and necessary to explain tip cell spacing. A recent study suggests that, at least in tumour angiogenesis, DLL4 exosomes might affect long-range signalling, but signalling between ECs via exosomes has not been demonstrated (Sheldon et al., 2010).

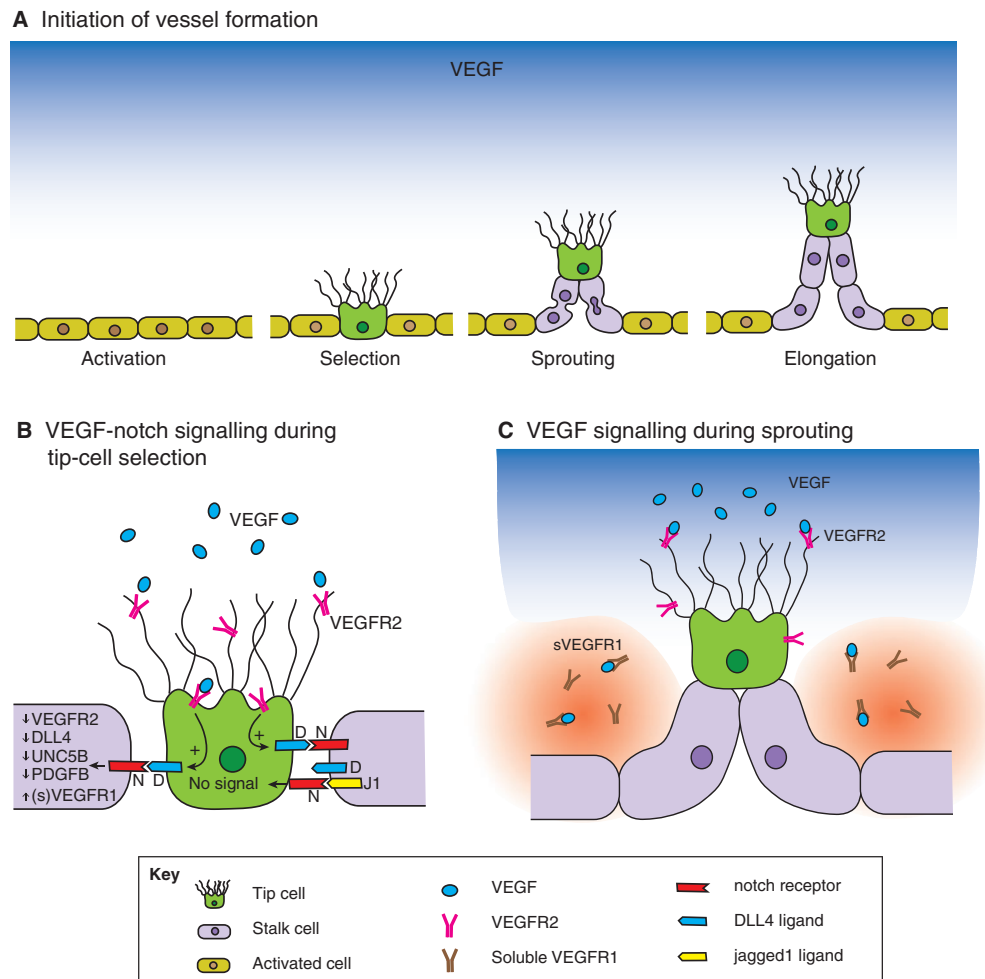
The dynamic interaction between VEGF and notch signalling was unravelled independently by several groups while studying the process of angiogenic sprouting in the postnatal mouse retina (Hellstrom et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). They all observed DLL4 expression in

ECs at the leading front of the vascular plexus and found that inhibition of DLL4/notch signalling results in increased vascular density due to excessive sprouting. Similar findings were described in the zebrafish ISV sprouting model. Inhibition of the Notch pathway induces hyperbranching of the ISVs and leads to an increased number of ECs in the ISVs (Leslie et al., 2007; Siekmann and Lawson, 2007). By contrast, overexpression of the activated Notch receptor blocks sprouting of the ISVs (Siekmann and Lawson, 2007). Besides downregulating VEGFR2, notch signalling also affects VEGFR1 and VEGFR3 (FLT4 – Mouse Genome Informatics) expression. Notch activation leads to increased levels of VEGFR1 and soluble (s)VEGFR1 (Fig. 2B,C) (Harrington et al., 2008; Funahashi et al., 2010; Krueger et al., 2011). This dampens the angiogenic sprouting response to VEGF, as both receptor variants act as a decoy for the VEGF ligand and limit VEGFR2 activation (Fong et al., 1995; Hiratsuka et al., 1998). During both mouse and zebrafish angiogenesis, VEGFR3 is most strongly expressed in the leading tip cells and is downregulated by notch signalling in the stalk cells (Shawber et al., 2007; Siekmann and Lawson, 2007). Blocking VEGFR3 signalling counteracts the hyperbranching phenotype upon notch inhibition (Tammela et al., 2008). Surprisingly, a recent study suggests that, during angiogenesis, VEGFR3 acts independently of its kinase activity and ligand-binding capabilities, and modulates VEGFR2-mediated signalling by forming VEGFR2-VEGFR3 heterodimers (Nilsson et al., 2010). Notch signalling also downregulates the expression of NR1, which is a positive regulator of VEGFR2 signalling (Williams et al., 2006). Collectively, these molecular regulatory processes lead to increased responsiveness of the tip cell to VEGF and decreased sensitivity of stalk cells to VEGF.

Computational modelling illustrated that the feedback loop between VEGF and DLL4/notch signalling is, in principle, sufficient to pattern ECs into tip and stalk cells, but only in a narrow window of VEGF concentration. This lack of robustness of a simple VEGF/DLL4 feedback loop was confirmed by *in vivo* studies, which identified that Jagged 1, an additional notch ligand, also participates in specification by antagonising notch activation through DLL4 (Benedito et al., 2009). As Jagged 1 is expressed predominantly in stalk cells, it can effectively prevent DLL4-mediated reciprocal activation of notch in tip cells, thereby greatly enhancing the efficacy of the lateral-inhibition process (Fig. 2B). Thus, DLL4 and Jagged 1 coordinately control the process of angiogenic sprouting and confer robustness to the crucial event of tip-stalk specification.

### Tip cell branching

Tip ECs extend branch-like filopodia through remodelling of the actomyosin and microtubule cytoskeleton, similar to the way in which neurite extensions protrude from neuronal cell bodies (Kater and Rehder, 1995; Gerhardt et al., 2003). The cytoskeletal dynamics that regulate cellular branching are dependent on myosin II contractility, which is partly determined by the physical properties, and hence stiffness, of the extracellular matrix (ECM) (Fischer et al., 2009; Myers et al., 2011). Local downregulation of myosin II contraction within a cell allows lamellipodia formation and initiation of EC branching (Fischer et al., 2009). EC polarisation, and hence the directionality of filopodia extension, during migration or upon exposure to shear stress is dependent on CDC42 activity, a small GTPase of the Rho family (Etienne-Manneville, 2004). The directionality of filopodia extension is further determined by the distribution of heparin-binding VEGF isoforms (VEGF<sup>165</sup> and VEGF<sup>188</sup>) in the local environment (Ruhrberg et al., 2002). Recent work showed that VEGF-induced



**Fig. 2. Sprout induction.** (A) The initiation of blood vessel formation. The presence of VEGF (blue gradient) activates the endothelium (yellow cells) of existing blood vessels. A VEGF/notch-dependent regulatory mechanism ensures the selection of a limited number of tip cells (green) by blocking tip cell formation in the immediate neighbours (via lateral inhibition). Tip cells sprout towards the VEGF gradient, and the adjacent stalk cells follow the guiding tip cell and proliferate to support sprout elongation. (B) VEGF/notch regulatory feedback during tip cell selection. The activation of VEGFR2 (pink) by VEGF (blue circles) induces the expression of the notch ligand DLL4 (D; blue). The subsequent activation of notch (N; red) by DLL4 in contacting cells reduces their expression of VEGFR2 and DLL4, thereby making them less sensitive to VEGF-mediated activation and limiting their ability to activate notch signalling in neighbouring cells. The expression of other tip cell-enriched genes, such as UNC5B and PDGFB is reduced in stalk cells, whereas the expression of the non-signalling VEGF decoy receptors VEGFR1 and soluble (s) VEGFR1 is increased, further reducing the likelihood of VEGFR2 activation in these cells. Furthermore, jagged1 (J1; yellow), which is selectively expressed in stalk cells, competes with DLL4 in cis for binding to notch receptors on tip cells. Jagged1 binds, but does not activate the notch receptor, thereby preventing notch activation in the tip cells. (C) VEGF signalling during sprouting. Soluble VEGFR1 (sVEGFR1; brown) produced by the cells immediately next to the outgrowing vessel branch sequesters VEGF molecules, thereby creating a corridor of higher VEGF levels perpendicular to the parent vessel. This corridor might act to optimise spreading of the vascular network and to avoid contact with nearby emerging sprouts.

filopodial extension is dependent on ephrinB2-mediated internalisation of VEGFR2 (Sawamiphak et al., 2010), but whether and how localised VEGFR activation might lead to localised filopodia formation remains unclear.

#### Sprout elongation: orchestrating tubular morphogenesis

Once the tip cell has been selected, it guides the growing sprout towards a gradient of VEGF and other attractive guidance cues (Gerhardt et al., 2003). The stalk cells that form the body of the sprout then proliferate to deliver the necessary building blocks for the growing sprout (Fig. 2A) (Gerhardt et al., 2003). As the vessel elongates, the stalk cells create a lumen, produce a basement membrane and associate with pericytes.

#### Sprout guidance

Over the past few years, a growing number of ligand-receptor interactions involved in neuronal guidance (e.g. Eph-ephrin, netrins-Unc, Robo-Slit, semaphorins-plexins, semaphorins-neuropilins) have also been found to regulate vascular patterning and EC guidance (for reviews, see Carmeliet and Tessier-Lavigne, 2005; Larrivee et al., 2009). Given that the major blood vessels and nerve fibres within the body are strikingly aligned, questions of co-patterning by common pathways and mutual interactions between ECs and nerve cells have received increasing attention.

The initial outgrowth of the sprout from the parent vessel is regulated by a combination of extrinsic guidance cues (Eichmann et al., 2005; Holderfield and Hughes, 2008) and a local vessel-derived

and VEGFR1-dependent mechanism of guidance (Chappell et al., 2009). The production of soluble VEGFR1 (sVEGFR1) by the cells immediately adjacent to the emerging sprout leads to a local depletion of VEGF and the formation of a 'corridor' of higher VEGF levels ahead of the newly forming sprout (Fig. 2C). The purpose of this local integration of guidance regulations is probably to optimise efficient spreading of the vascular network and to avoid premature contact with nearby emerging sprouts.

Recent time-lapse imaging studies have revealed a dynamic shuffling of tip and stalk cells at the leading front of growing sprouts (Jakobsson et al., 2010). This was shown both *in vitro* (in EB sprouting assays) and *in vivo* (in mice and zebrafish), challenging the idea of stable tip and stalk cell selection. The positional exchange also suggests that cells constantly have to re-evaluate the VEGF/notch signalling loop when they meet new neighbours. ECs compete for the tip cell position based on their relative levels of VEGFR1 and VEGFR2, the activities of which gauge the expression level of the DLL4 ligand; lower expression levels of VEGFR1 or higher levels of VEGFR2, compared with neighbouring cells, result in higher levels of DLL4 expression and, hence, an increased ability of a cell to suppress its neighbouring cells from becoming tip cells (Jakobsson et al., 2010). Conceptually, this suggests that the leader of the team, the tip cell, is constantly challenged by cells within the stalk region to demonstrate its dominance in terms of VEGFR levels. If the challenging stalk cell is 'better equipped', it takes over the leadership and becomes the new tip cell. The surprisingly competitive behaviour of ECs ensures that the cell with the best guiding capacities will lead the sprout. Accordingly, the guidance of a growing sprout is not entirely determined at the level of a single tip cell, as was originally hypothesised (Gerhardt et al., 2003), but rather is the consequence of team play in a population of cells. By coupling competition to the relative sensitivity to environmental VEGF, this collective cell behaviour should, theoretically, enhance the ability of ECs to determine the direction of the VEGF gradient and, thus, might provide robustness to the patterning process. Similar cellular competition mechanisms have also been described in other branching processes (Box 3).

#### Stalk cell proliferation drives sustained elongation

Proliferation contributes to the sustained growth of a newly forming vessel. Tip cells rarely proliferate (Gerhardt et al., 2003), although division of the leading cell of ISV sprouts in zebrafish has been observed (Siekmann and Lawson, 2007; Blum et al., 2008). By contrast, stalk cells are strongly proliferative and, thus, support sustained elongation of the growing sprout (Fig. 2A) (Gerhardt et al., 2003). However, experimental observations indicate that stalk cell proliferation does not push the tip cell forward, but rather that the tip cells themselves interact with the surrounding substrate to pull the growing sprout further in the direction of growth (Ausprunk and Folkman, 1977; Caussinus et al., 2008; Phng et al., 2009). In *Drosophila* tracheal tubes, sprouting tip cells were also shown to exert a pulling force that allows new stalk cells to intercalate into the stem of the growing sprout (Caussinus et al., 2008). Furthermore, Ausprunk and Folkman identified that blood vessel sprouting can initially progress without cell division (Ausprunk and Folkman, 1977), indicating that a similar pulling force of the vascular tip cell is likely to be present. However, sustained sprouting and further outgrowth of the vessel branch requires proliferation of the stalk cells (Ausprunk and Folkman, 1977), and decreased stalk proliferation correlates with branch regression (Phng et al., 2009).

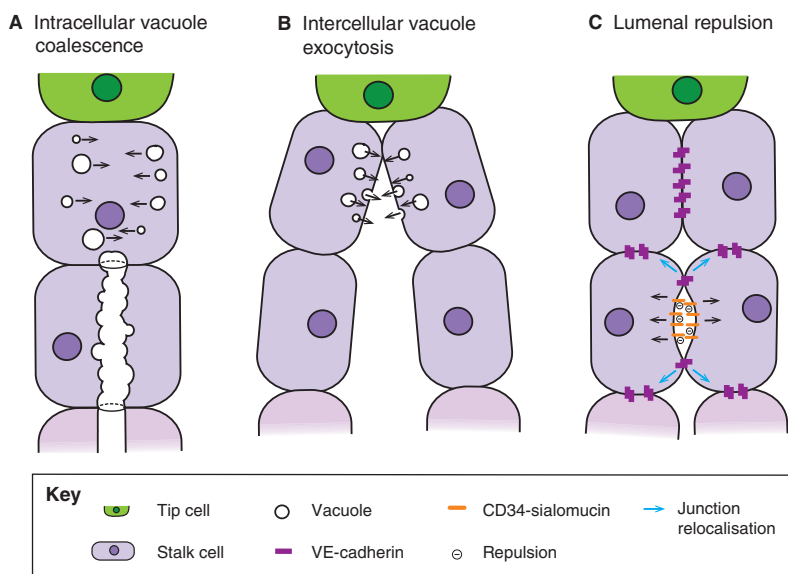
#### The orientation of stalk cell divisions

In addition to the frequency of cell division, the orientation of the division plane is important for tubular morphogenesis during sprout outgrowth. De-regulation or randomisation of division orientation leads to abnormal vessel morphologies, because the orientation of cell division determines whether a vessel becomes longer or wider (Zeng et al., 2007). Similar to findings in other tubular structures, the plane of EC divisions in a growing sprout is mostly perpendicular to the long axis of the vessel, supporting lengthening of the tube (Fig. 2A) (Fischer et al., 2006; Zeng et al., 2007). Intriguingly, this regular behaviour of dividing ECs is also influenced by VEGF gradients, adding another level of complexity to the multiple regulatory roles of this angiogenic factor (Zeng et al., 2007). It is noteworthy that VEGF-dependent determination of the EC division plane occurs in the absence of blood flow. In perfused vessels, EC polarity is linked to the direction of shear stress produced by blood flow, which is sensed by the transmembrane proteins PECAM1, VE-cadherin and VEGFR2 and transduced by CDC42-dependent signalling (Tzima et al., 2003; Noria et al., 2004; Gomes et al., 2005; Tzima et al., 2005). It is tempting to speculate that ECs within blind-ended and non-perfused sprouts are able to orientate their division in the direction of sprout elongation through pulling forces exerted by the tip cell in a VEGF gradient.

#### Lumen formation

A second important role of the stalk cells within a newly forming blood vessel is to establish a vascular lumen. Various mechanisms have been proposed to exist in different model systems and even in different vascular beds (Ellertsdottir et al., 2010; Wang et al., 2010). In larger capillaries that remain constantly perfused, the lumen of a newly forming sprout remains continuous with that of the parent vessel. This suggests that the stalk cells retain their apical-basal polarity when they bud off from the parent vessel. This mechanism has also been described in the zebrafish brain vasculature (Huisken and Stainier, 2009; Ellertsdottir et al., 2010). By contrast, zebrafish ISVs appear to be formed by another mechanism as the initial primary ISVs are not lumenised when they sprout from the dorsal aorta. To explain how lumens form in this situation, Kamei et al. first proposed a model (Fig. 3A) in which the vascular lumen of ISVs is formed by fusion of intracellular vacuoles, which ultimately fuse across the ECs of a newly formed sprout to form a continuous lumen (Kamei et al., 2006). Later, Blum et al. analysed the arrangement of EC junctions in the ISVs and DLAV and found that cell-cell junctions are present over the entire length of the vessel, even across the presumptive lumen-forming cells. This is inconsistent with the model of simple vacuole fusion, which would result in a head-to-tail arrangement of ECs and seamless vessels. Therefore, it was proposed that the luminal space of ISVs is formed between cells by exocytosis of vacuoles to form an intercellular space (Fig. 3B) (Blum et al., 2008; Wang et al., 2010). More recently, aortic lumenisation in mouse and zebrafish was shown to similarly occur extracellularly. However, in these studies, vacuoles were not observed and lumen formation was associated with relocalisation of junctional proteins and cell-shape changes (Fig. 3C) (Strilic et al., 2009; Wang et al., 2010). Cell-cell adhesion between ECs is mediated via tight junctions and adherens junctions (Dejana et al., 2009). These intercellular junctions are formed by homophilic interactions of adhesive proteins that further interact with intracellular partners and with the actin cytoskeleton. At tight junctions, adhesion can be mediated by claudins, occludin, members of the junctional adhesion molecule





**Fig. 3. Models of lumen formation during sprout outgrowth.** (A) Intracellular vacuole coalescence.

Endothelial cells (ECs) can form a lumen by forming intracellular vacuoles that coalesce and connect with each other and with vacuoles in neighbouring cells.

(B) Intercellular vacuole exocytosis. ECs can form a lumen by producing exocytotic vacuoles that are released into the intercellular space. (C) Luminal repulsion. Alternatively, an intercellular lumen can be created by apical membrane (luminal) repulsion. VE-cadherin (purple) establishes the initial apical-basal polarity in the ECs and localises CD34-sialomucins (orange) to the cell-cell contact sites. The negative charge of the sialomucins induces electrostatic repulsion and initial separation of the apical membranes, thereby relocalising the junctional proteins to the lateral membranes. Further separation and establishment of the lumen is based on F-actin-mediated cell-shape changes (not shown).

(JAM) family, or by EC selective adhesion molecule (ESAM). EC adherens junctions are predominantly formed by vascular endothelial (VE)-cadherin (for a review, see Dejana et al., 2009). During lumen formation in the mouse aorta, VE-cadherin is required for establishing the initial apical-basal cell polarity of aortic ECs and localising CD34-sialomucins to the cell-cell contact site (Fig. 3C) (Strilic et al., 2009). The negative charge of the sialic acids in CD34-sialomucin in turn induces electrostatic repulsion of the apposing EC surfaces, leading to the initiation of lumen formation (Fig. 3C) (Strilic et al., 2010). During cell separation and extension of the lumen, the VE-cadherin-expressing junctions are relocalised to the lateral cell contact sites (Fig. 3C). CD34-sialomucins further recruit F-actin to the luminal cell surface (Strilic et al., 2009). VEGF-dependent localisation of non-muscle myosin II to this apically enriched F-actin cytoskeleton induces EC shape changes and further separation of the adjacent ECs, fully establishing the aortic lumen (Strilic et al., 2009).

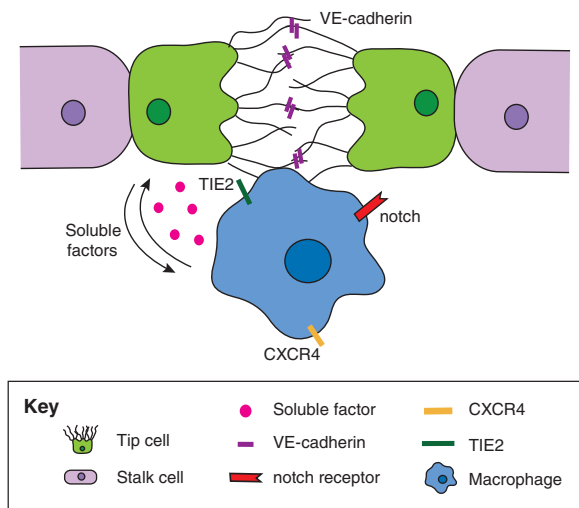
Because the stalk cells proliferate even when a lumen is already present, it is important that proliferation is tightly coordinated with EC junction formation in order to maintain patent and sealed vessels; however, the mechanism by which these two processes are coordinated remains unclear.

### Sprout anastomosis: connecting the vascular network

When the tip cell of a growing sprout contacts other sprouts, new cell-cell junctions are established and the sprouts become connected in a process called anastomosis. Recently, it was found that VE-cadherin is present not only at cell-cell junctions but also at the tips of EC filopodia (Fig. 4) (Almagro et al., 2010). Its presence there probably facilitates the early establishment of new cell-cell junctions. Anastomosis can also be observed in zebrafish as the ISVs reach the dorsal roof, branch horizontally and make contact with neighbouring sprouts to form the DLAV. During this event, VE-cadherin in tip cells was shown to localise to tip-tip contact sites immediately upon contact with neighbouring sprouts (Blum et al., 2008). As the tip cells of the two sprouts crawled over each other, the VE-cadherin contacts expanded.

How the tip cells meet to establish new contacts is not fully understood. Recent descriptions of macrophage-tip cell interactions, in particular at sites where two tip cells make

contact via their filopodia, raised the hypothesis that macrophages might act as 'bridge cells' that facilitate the contact and possibly stabilise nascent connections (Fig. 4) (Checchin et al., 2006; Fantin et al., 2010; Schmidt and Carmeliet, 2010; Rymo et al., 2011). It is important to note, however, that anastomosis in the complete absence of macrophages is normal, albeit less frequent (Checchin et al., 2006; Kubota et al., 2009; Fantin et al., 2010; Rymo et al., 2011), illustrating that the basic mechanism is likely to be EC-autonomous and that the macrophages might be involved in modulating and refining the connection process. A similar phenomenon has been described in the *Drosophila* tracheal system in which tracheal tip cells, which are similar to vascular tip cells, extend filopodia to explore their environment (Wolf et al., 2002; Schmidt and Carmeliet, 2010). During branch fusion, the tracheal tip cells extend their filopodia along the surface of specialised mesodermal bridge cells to help them to position correctly (Wolf et al., 2002). This tip-bridge cell interaction in *Drosophila* is dependent on FGF signalling, whereas the molecular basis for macrophage-tip cell interactions remains to be elucidated. Possible receptor-ligand candidates that mediate this interaction are notch-DLL4, TIE2 (TEK – Mouse Genome Informatics)-angiopoietin-2 (ANG2) or chemokine receptor CXCR4-stromal cell-derived factor-1 (SDF1; CXCL12 – Mouse Genome Informatics) (Fig. 4). The notch, TIE2 and CXCR4 receptors are expressed in macrophages (De Palma et al., 2007; Fung et al., 2007; Lima e Silva et al., 2007; Fantin et al., 2010), whereas their respective ligands DLL4, ANG2 and SDF1 are expressed in tip cells (Ridgway et al., 2006; Hellstrom et al., 2007; Strasser et al., 2010; Unoki et al., 2010a), although CXCR4 has also been described as a tip cell enriched gene (Strasser et al., 2010). Several recent publications illustrate that macrophages also promote angiogenic branching in the retina and in aortic ring cultures (Checchin et al., 2006; Kubota et al., 2009; Fantin et al., 2010; Rymo et al., 2011). Surprisingly, in the deeper retinal vessel plexus, macrophages negatively regulate vessel density by producing sVEGFR1 (Stefater et al., 2011). Observations in the aortic ring assay, hint towards a two-way communication between blood vessels and macrophages (Fig. 4), whereby blood vessels attract macrophages, which in turn secrete soluble pro-angiogenic factors (Rymo et al., 2011). Considering



**Fig. 4. Current concepts of anastomosis.** Schematic illustration of tip cell fusion. For simplicity, the vascular lumen is not illustrated. The formation of new connections between growing vessels is facilitated by vessel interactions with macrophages (blue) that can act as bridge cells that promote filopodia contact between tip cells (green). Upon contact, adhesion junctions are formed by VE-cadherin, first at the tips of filopodia and later also along the extending interface of the contacting cells. The precise role of macrophages and the molecular regulation of anastomosis are not understood. Possible candidate pathways involved are the notch, TIE2 and CXCR4 signalling pathways; the notch receptors (red), the TIE2 receptor (green) and the CXCR4 receptor (yellow) are expressed on macrophages and their cognate ligands are expressed on tip cells (not shown). A two-way interaction between ECs and macrophages through (unknown) soluble factors (pink) has been also described.

the breadth of literature on various influences of macrophages on the vasculature during both developmental and pathological angiogenesis, it is clear that ECs and macrophages engage in multiple interactions, the outcome of which will probably be highly context dependent.

#### Network formation: remodelling and maturation

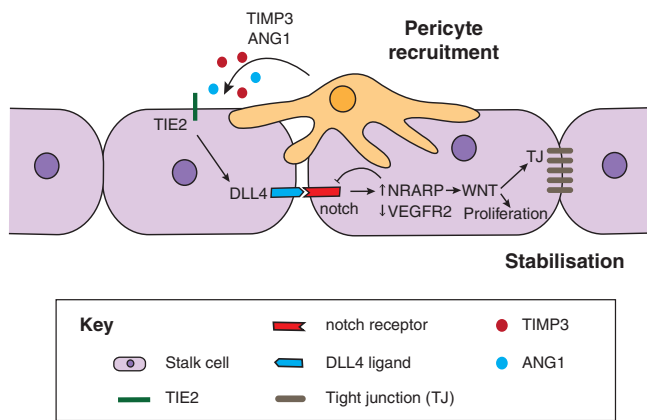
After the establishment of new connections within the vascular network, significant remodelling occurs; some branches are stabilised whereas others regress. One important factor regulating remodelling is oxygen, as elevated oxygen levels induce vascular pruning, ensuring that vascular density is correctly adapted to tissue oxygenation (Claxton and Fruttiger, 2005). Nevertheless, the processes of vascular remodelling, regression and stabilisation are far from being completely understood. Pericytes play an important part in vascular stabilisation. Whether the actual presence of pericytes protects vessels from regression is controversial (von Tell et al., 2006). Early studies suggested that recruitment of smooth-muscle actin-positive pericytes marks the end of a plasticity time-window in vascular development during which pruning can occur (Benjamin et al., 1998). In addition, pericyte coverage correlated with vessel protection against regression in the retina during development and disease (Chan-Ling et al., 2004). By contrast, other studies showed that tumour vessels abundantly covered by pericytes were not protected from regression upon VEGF inhibition (Inai et al., 2004). Thus, the exact mechanisms and conditions of vascular stabilisation by pericytes remain to be unravelled. The factors that are involved

in pericyte recruitment include angiopoietin-1 (ANG1)-TIE2, platelet-derived growth factor B (PDGFB)-PDBFRB, transforming growth factor- $\beta$ 1 (TGFB1)-activin receptor-like kinase 5 (ALK5; TGFBR1 – Mouse Genome Informatics) and notch signalling components (Jain, 2003; Schemet et al., 2007). Endothelial production of ANG1 initially regulates pericyte recruitment through induction of expression of the chemokine MCP-1 (monocyte chemoattractant protein-1; CCL2 – Mouse Genome Informatics) (Aplin et al., 2010). The recruited pericytes prevent regression of newly formed blood vessels by delivering vascular stabilisation factors, such as tissue inhibitor of metalloproteinase 3 (TIMP3) and, again, ANG1 (Fig. 5) (Sundberg et al., 2002; Saunders et al., 2006). Thus, the current literature collectively suggests that the process of vascular remodelling and maturation is strongly dependent on the ANG-ligand-TIE-receptor signalling mechanism.

ANG1 induces vessel stabilisation via signalling through the TIE2 receptor (Suri et al., 1996). ANG2 was initially considered to be an antagonist for ANG1/TIE2 signalling that inhibited vessel stabilisation and favoured vascular regression (Maisonpierre et al., 1997). However, later findings have shown that ANG2 can also activate the TIE2 receptor, albeit weakly and in a context-dependent manner (Kim et al., 2000; Teichert-Kuliszewska et al., 2001; Daly et al., 2006). Furthermore, ANG1 can have opposing roles either by inducing vascular quiescence or stimulating angiogenesis; differential gene expression profiles are induced in TIE2-expressing ECs depending on whether ANG1 stimulation occurs in the presence or absence of cell-cell contact (Fukuhara et al., 2008; Saharinen et al., 2008).

DLL4/notch signalling also plays a crucial role in the vascular stabilisation process by affecting both ECs and pericytes. The activation of DLL4/notch signalling in ECs has a vessel stabilising effect via inhibition of angiogenic sprouting (Hellstrom et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). Notch signalling also promotes vascular stabilisation more directly through the induction of notch-regulated ankyrin repeat protein (NRARP) expression (Phng et al., 2009) and via the production of ECM components (Benedito et al., 2008; Trindade et al., 2008; Zhang et al., 2011). NRARP limits notch signalling and promotes WNT/CTNBN1 signalling in stalk cells, which supports vascular stability and prevents EC retraction by inducing proliferation and improving intercellular junctions (Fig. 5) (Phng et al., 2009). Additionally, it has been suggested that notch signalling can stabilise newly formed blood vessels by affecting pericytes. For example, pericyte marker expression in cells cultured from bone marrow increases with notch signalling (Stewart et al., 2011), and a recent study suggested that the stabilising effect of ANG1/TIE2 signalling between pericytes and ECs is mediated via induction of DLL4 expression in ECs (Fig. 5) (Zhang et al., 2011). However, the full relevance of these observations for pericyte-mediated stability in vivo remains unclear, as previous studies reported no defects in pericyte recruitment in DLL4-deficient mice.

In addition to the establishment of a quiescent and stable vasculature, DLL4/notch signalling also appears to be important for further maintenance of the quiescent state. EC-specific inducible deletion of RBPJ, the downstream transcriptional regulator of all canonical notch signalling, triggers spontaneous angiogenesis in multiple tissues in adult mice (Dou et al., 2008). Also, specific blockade of DLL4-mediated notch signalling in mice using a DLL4-specific antibody resulted in liver pathologies characterised by abnormal activation of ECs (Yan et al., 2010).



**Fig. 5. Vessel stabilisation.** Stalk cells (purple) recruit pericytes (orange) to stabilise the vasculature, possibly through the production of stabilising factors such as TIMP3 and ANG1. ANG1 signalling through the TIE2 receptor stabilises the vasculature, in part via inducing DLL4 expression in the endothelial cells (ECs) and activating notch signalling. Notch activation then plays a dual role in vascular stabilisation: first, it downregulates VEGFR2 expression, thereby preventing further sprouting through activation of the VEGF/notch signalling pathway; second, it induces the expression of NRARP, which promotes WNT signalling leading to increased proliferation and tight junction (TJ) stabilisation.

These findings indicate that DLL4/notch signalling is essential for repressing angiogenesis in established blood vessels and for maintaining quiescence in the endothelium.

## Conclusion

Angiogenesis research has moved into an exciting phase of *in vivo* cell biology, with numerous new tools and methods enabling researchers to address questions of vascular patterning at the single-cell level. Dynamic high resolution imaging, sophisticated genetic tools for single cell labelling and cell-autonomous gene-function analysis have established new concepts of EC competition and cooperation and will continue to define the precise molecular control of the individual steps in the angiogenic sprouting process.

Despite exciting progress, many basic questions remain unanswered. For example, what are the specific roles of newly identified tip and stalk cell genes and how do they integrate with the fundamental VEGF/notch regulatory feedback loop? What is the biological function of the dynamic tip and stalk cell exchange? How does this fit with the concept of functional specialisation? How do stalk cells that take over the tip cell position overcome the notch-mediated inhibitory instructions of the tip cell that initially prevented them from becoming a tip cell? What is the molecular identity of the phalanx cell phenotype, and how different are phalanx cells from stalk cells? What is the cellular mechanism of vessel remodelling and how do intrinsic forces (blood flow) and tissue response (hypoxia/VEGF) integrate into coordinated EC behaviour? Finally, what determines the abnormal development of blood vessels in pathologies, and how different is EC behaviour and its regulation in disease?

We anticipate that, in the coming years, detailed combinatorial analyses that bring together the knowledge of cell biologists, developmental biologists, computational biologists and clinicians will fuel progress in this field. These combined efforts, using genetics, quantitative biology, cell culture models and pre-clinical

models, aided by rapidly developing imaging technology methods and approaches to analysis of signalling *in vivo*, will greatly advance our understanding of the key processes in angiogenesis.

## Note added in proof

A paper appearing in this issue (Arima et al., 2011) confirms the recently discovered dynamic shuffling of endothelial cells along the extending sprout and frequent exchange of cells at the tip position (Jakobsson et al., 2010). Using time-lapse imaging in the aortic ring assay, and a combination of labelling techniques that allow nuclear tracking of EC and stochastic mosaic labeling of individual cells by low titre viral transduction, Arima and colleagues were able to undertake a comprehensive analysis of cell movements and their relative contribution to sprout elongation. Accordingly, effective elongation of the sprout appears to be driven by the velocity, orientation and directionality of endothelial cell migration under VEGF stimulation. Dll4/Notch signalling counteracts several of these parameters. Interestingly, the authors observe that mural cell recruitment to the advancing sprout promotes collective EC cell behavior that culminates in the 'elongation drive'. Whether all of these observations hold true *in vivo* remains to be shown. However, the systemic analysis of single and collective cell behavior to extract critical parameters will certainly continue to drive insights into the fundamental principals of blood vessel formation.

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## Competing interests statement

The authors declare no competing financial interests.

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