Control of Blood Vessel Formation by Notch Signaling

Fabian Tetzlaff and Andreas Fischer

Abstract

Blood vessels span throughout the body to nourish tissue cells and to provide gateways for immune surveillance. Endothelial cells that line capillaries have the remarkable capacity to be quiescent for years but to switch rapidly into the activated state once new blood vessels need to be formed. In addition, endothelial cells generate niches for progenitor and tumor cells and provide organ-specific paracrine (angiocrine) factors that control organ development and regeneration, maintenance of homeostasis and tumor progression. Recent data indicate a pivotal role for blood vessels in responding to metabolic changes and that endothelial cell metabolism is a novel regulator of angiogenesis. The Notch pathway is the central signaling mode that cooperates with VEGF, WNT, BMP, TGF-β, angiopoietin signaling and cell metabolism to orchestrate angiogenesis, tip/stalk cell selection and arteriovenous specification. Here, we summarize the current knowledge and implications regarding the complex roles of Notch signaling during physiological and tumor angiogenesis, the dynamic nature of tip/stalk cell selection in the nascent vessel sprout and arteriovenous differentiation. Furthermore, we shed light on recent work on endothelial cell metabolism, perfusion-independent angiocrine functions of endothelial cells in organ-specific vascular beds and how manipulation of Notch signaling may be used to target the tumor vasculature.

Keywords

Angiogenesis · Notch signaling · Arteriovenous differentiation · Tumor angiogenesis · Angiocrine signaling · Endothelial metabolism · Endothelial cells

F. Tetzlaff
Vascular Signaling and Cancer (A270), German Cancer Research Center (DKFZ), Heidelberg, Germany

Vascular Biology, CBTM, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

A. Fischer (✉)
Vascular Signaling and Cancer (A270), German Cancer Research Center (DKFZ), Heidelberg, Germany

Vascular Biology, CBTM, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

Department of Medicine I and Clinical Chemistry, Heidelberg University Hospital, Heidelberg, Germany
e-mail: a.fischer@dkfz.de

Abbreviations

ADAM	A disintegrin and metalloprotease
ALK	Activin receptor-like kinase
BMP	Bone morphogenetic protein

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CADASIL	Cerebral Autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
DLL	Delta-like
EC	Endothelial cell
FGF	Fibroblast growth factor
FOX	Forkhead box protein
HES	Hairy and enhancer of split
HEY	Hairy/enhancer-of-split related with YRPW motif
IL	Interleukin
NICD	Notch intracellular domain
NRARP	Notch-regulated ankyrin repeat-containing protein
NRP	Neuropilin
PFKFB3	6-Phosphofructo-2-kinase
PI3K	Phosphatidylinositol 4,5-Bisphosphate 3-kinase
RBPJ	Recombining binding protein suppressor of hairless
SHH	Sonic hedgehog
SMAD	Mothers against decapentaplegic
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 Introduction

The vasculature comprises one of the largest organs in mammals. Blood vessels nourish all tissues in the body and provide gateways for immune surveillance. In addition, vascular cells provide organ-specific paracrine factors, also termed angiocrine factors, which instruct the behavior of neighboring cells. Angiocrine signaling is essential for the maintenance of homeostasis and metabolism, stem cell differentiation, organ regeneration and tumor progression (Rafii et al. 2016). The importance of the vasculature becomes apparent by studying vascular dysfunction, which is the major contributor to human mortality. Abnormalities in vessel functionality are causative for heart infarction, stroke, neurodegenerative diseases, dementia, diabetic complications and obesity-associated disorders, while excessive blood vessel formation is a hallmark of cancer, chronic inflammation and eye diseases such as wet macular degeneration (Folkman 2007). Drugs that inhibit blood vessel growth have recently become first-line therapies for certain eye and tumor diseases (Carmeliet and Jain 2011) .

Blood vessels are formed by endothelial cells (ECs), which provide an anti-thrombotic surface, and by mural cells (vascular smooth muscle cells and pericytes). In mature vessels, ECs are in a quiescent state, divide rarely and form barriers between blood and surrounding tissues. ECs have the remarkable capacity to switch between the quiescent and the activated state during injuries, hypoxia, inflammation or tissue growth, when the formation of new blood vessels is required (Potente et al. 2011).

The *de novo* formation of blood vessels from mesodermal-derived endothelial precursor cells is called vasculogenesis (Risau and Flamme 1995). It occurs predominantly during early development to generate a primordial vascular plexus and the first large vessels such as the dorsal aorta. The vascular plexus is further remodeled and new vessels are formed from the pre-existing ones in a process called angiogenesis (Herbert and Stainier 2011). Similarly to new branches growing on a tree, angiogenesis occurs primarily by sprouting of new branches from existing microvessels. Angiogenesis occurs throughout life as capillaries grow and regress accordingly to functional demands. For example, physical exercise stimulates angiogenesis in skeletal muscle (Hellsten and Hoier 2014) and expansion of adipose tissue is also associated with the formation of new blood vessels (Cao 2010). Intussusception (vessel splitting) is another way of generating new vessels. During this process blood vessels develop transluminal tissue pillars which subsequently fuse resulting in new vascular entities (Makanya et al. 2009). Once the new vessels establish nutrient and oxygen supplies that meet the metabolic tissue demand, the ECs will turn quiescent (Risau 1997).

Notch signaling is of utmost importance for vessel morphogenesis and function. Based on a series of previously published excellent review articles (Blanco and Gerhardt 2013; Carmeliet

and Jain 2011; Eilken and Adams 2010; Gridley 2010; Siekmann et al. 2013; Potente et al. 2011), this chapter will summarize the current view about Notch signaling in the vasculature with a focus on vessel sprouting, arteriovenous differentiation, EC metabolism and tumor angiogenesis. We will also highlight recent work showing the tight interconnections of the Notch pathway with other core signaling pathways and its roles for organ-specific angiocrine signaling.

2 Notch Signaling in Endothelial Cells

Canonical Notch signaling requires the interaction of membrane-bound Notch ligands on the signal-sending cell with Notch receptors on the signal-receiving cell to trigger proteolytic cleavages of the Notch receptors. γ-secretase releases the active Notch intracellular domain (NICD) from the cell membrane, which translocates to the nucleus, binds to the transcription factor Rbpj [also known as CSL, CBF1, Su(H) or Lag2] and activates gene expression (Kopan and Ilagan 2009). In principle, expression of the Notch ligands Dll1, Dll4, Jag1 and Jag2 and the Notch receptors Notch1 and Notch4 on ECs has been reported (Hofmann and Luisa Iruela-Arispe 2007). However, one needs to keep in mind that the individual endothelial expression patterns are quite variable in different vascular beds (e.g. Notch signaling is much higher in arterial than venous ECs), and are depending on the developmental state. Compared to the normal, quiescent vasculature in tissues of the adult, the expression of Notch ligands is typically stronger in tumor blood vessels (Patel et al. 2005; Lu et al. 2007; Jubb et al. 2012; Gale et al. 2004; Mailhos et al. 2001; Scehnet et al. 2007). Prototypical Notch1 target genes in ECs are *Hey1*, *Hey2*, *Hes1*, *Nrarp*, *EphrinB2*, but also the Notch ligand-encoding gene *Dll4* (Dou et al. 2008; Fischer et al. 2004; Taylor et al. 2002; Liu et al. 2006; Krebs et al. 2001; Phng et al. 2009; Lawson et al. 2002; Ridgway et al. 2006; Lobov et al. 2007; Iso et al. 2006; Patel et al. 2005). The latter is quite unusual and suggests a positive Dll4-Notch1 feedback loop in ECs (Diez et al. 2007; Lanner et al. 2013). Notch ligands are also cleaved by the γ-secretase and their intracellular domain enters the nucleus. However, no functional role for a potential "Notch reverse signaling" during angiogenesis could be detected (Liebler et al. 2012; Redeker et al. 2013).

Gene targeting studies in mice revealed that deletion of *Dll4* (Duarte et al. 2004; Krebs et al. 2004; Gale et al. 2004), *Jag1* (Xue et al. 1999), *Notch1* (Huppert et al. 2000; Krebs et al. 2000; Limbourg et al. 2005), *Notch1*/*Notch4* (Krebs et al. 2000), the Notch S2 cleavage enzyme *Adam10* (Glomski et al. 2011), components of the γ-secretase complex (Herreman et al. 1999; Li et al. 2003), *Rbpj* (Krebs et al. 2004), *Hey1*/*Hey2* (Kokubo et al. 2005; Fischer et al. 2004), or a constitutive endothelium-specific expression of activated alleles for *Notch1* (Krebs et al. 2010) or *Notch4* (Uyttendaele et al. 2001) lead to embryonic lethality with severe vascular remodeling abnormalities and defects in arteriovenous specification. Besides embryonic development, Notch signaling coordinates vascular remodeling also in the adult (Limbourg et al. 2007; Takeshita et al. 2007). Interestingly, the loss of a single *Dll4* allele already results in severe angiogenesis defects (Duarte et al. 2004; Gale et al. 2004; Krebs et al. 2004). *Dll4* and *Vegf-a* belong to the very few genes, of which heterozygosity results in a lethal embryonic phenotype.

One could assume that endothelial Notch ligands act in a redundant manner. However, it was shown that they play distinct roles in blood vessel morphogenesis and do not act redundantly (Preuße et al. 2015). Expression of Dll1 on ECs begins later than that of Dll4 during fetal mouse development. While Dll4 is needed to establish arterial cell fate (*see below*), Dll1 is required for maintenance of arterial cell fate (Sörensen et al. 2009). On the other hand, Jag1 can even antagonize Dll4/Notch1 signaling in ECs during tip/stalk cell selection depending on the glycosylation pattern of Notch1 receptor (Benedito et al. 2009).

3 Sprouting Angiogenesis

The outgrowth of a new vessel branch is stimulated by proangiogenic growth factors, which are released during hypoxia, inflammation, nutrient starvation or from oncogene-transformed cells. These shift the balance between proangiogenic (e.g. VEGF, FGF) and antiangiogenic (e.g. endostatin, angiostatin, tumstatin, soluble VEGFR1) factors towards a proangiogenic outcome, an event termed the "angiogenic switch" (Folkman 1995; Folkman 2007). The most important proangiogenic protein is vascular endothelial growth factor (VEGF-A; hereafter called VEGF). The complex signaling biology of VEGF family members [VEGF-A, -B, -C, −D, −E and placenta growth factor (PlGF)] and VEGF-A splice isoforms has been reviewed elsewhere (Simons et al. 2016). Deletion of *Vegf* or its receptors in mice leads to embryonic death as consequence of abnormal vascular development (Fong et al. 1995; Dumont et al. 1998; Shalaby et al. 1995; Carmeliet et al. 1996; Ferrara et al. 1996). In the postnatal mouse retina, a Vegf gradient is generated by the already existing astrocyte network that serves as a guiding scaffold for the developing blood vessels (Ruhrberg et al. 2002; Gerhardt et al. 2003).

Angiogenesis is induced by VEGF, which signals through VEGFR2 and VEGFR3 to activate quiescent ECs. Activated ECs protrude filopodia, secrete matrix metalloproteinases to degrade the basement membrane and become invasive (Arroyo and Iruela-Arispe 2010). The breakdown of basement membrane is in particular mediated by EC podosome rosettes (Seano et al. 2014). Podosomes are specialized actin-based structures that degrade extracellular matrix and promote invasive cell migration (Murphy and Courtneidge 2011). The formation of EC podosomes is controlled by VEGF and Notch signaling (Spuul et al. 2016). Furthermore, stimulated ECs release angiopoietin-2 leading to detachment of pericytes. This further allows ECs to invade the surrounding tissue (Augustin et al. 2009). During invasion ECs usually remain connected to the vessel network (Blanco and Gerhardt 2013).

The nascent sprout contains two different cell phenotypes: tip and stalk cells (Fig. 1). The leading tip cell is characterized by its position, its long and dynamic filopodia and its pro-invasive and migratory behavior (Gerhardt et al. 2003), but also its highly glycolytic metabolic activity (De Bock et al. 2013). Similar to axonal growth cones, tip cells integrate attractive and repellent guidance cues (e.g. Semaphorin, Netrin, VEGF or Slit proteins) to define the route in which the new sprout grows (Adams and Eichmann 2010). Guidance is facilitated by actin-rich filopodia on the tip cells, whose formation is driven by VEGF via RhoGTPase signaling. Interestingly, filopodia are not absolutely necessary for migration of ECs as lamellipodia can partially compensate for their function (Phng et al. 2013). It was reported that there can be two cells that extend filopodia and have significant overlap in space and time at the tip of angiogenic sprouts (Pelton et al. 2014). This surprising observation challenges the model of a single EC at the sprout tip. The trailing stalk cells are proliferative, less migratory than tip cells and form the nascent vascular lumen (Gerhardt et al. 2003). Furthermore, tip and stalk cells possess distinct gene expression profiles (e.g. higher expression of *Dll4*, *Vegfr2*, *Vegfr3*, *Pdgfb*, *Unc5b*, *Cxcr4*, *Nidogen-2*, *Esm1*, *Angiopoietin-2*, *Apelin* in tip cells) (Del Toro et al. 2010; Blanco and Gerhardt 2013). For cell proliferation, stalk cells have to generate biomass (nucleotides, protein, lipids). Therefore, cell metabolism differs between tip and stalk cells (see 3.4). Stalk cells produce extracellular matrix and recruit pericytes that attach to the new vessel sprout (Fig. 1). ECs in new vessel loops that are well covered by mural cells and have again become quiescent were named "phalanx cells" (Mazzone et al. 2009).

3.1 VEGF and Notch Signaling Control Tip/Stalk Cell Selection

The ability of ECs to lead a nascent sprout is strongly dependent on their VEGF receptor expression profile and their competence to

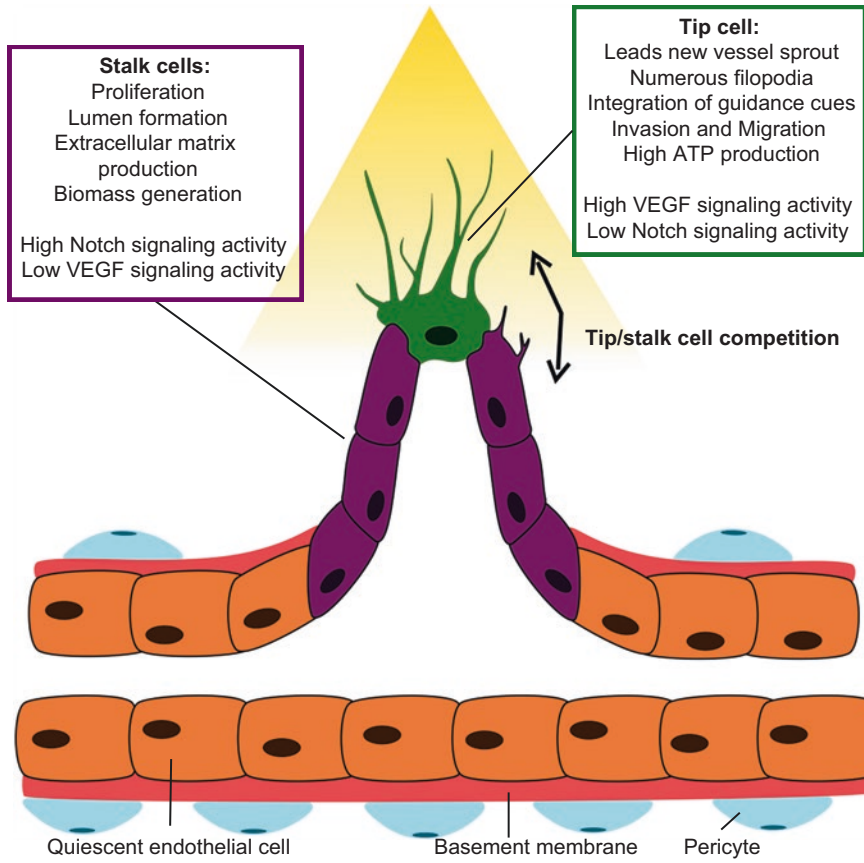

Fig. 1 Model of tip/stalk cell phenotypes. The leading tip cell protrudes many filopodia and guides the new vessel sprout towards the VEGF gradient. Tip cells are highly invasive and migratory and require high ATP amounts, which are predominantly generated by glycolysis. The trailing stalk cells proliferate and form a new vessel lumen. The newly formed vessel sprout gets covered by extracellular matrix proteins and by pericytes. However, this is a dynamic process and stalk cells battle for the tip position to take over the lead

respond to VEGF (Jakobsson et al. 2010; Gerhardt et al. 2003). While tip cells are characterized by high expression levels of Vegfr2 and also Vegfr3 (Tammela et al. 2008; Tammela et al. 2011; Zarkada et al. 2015; Blanco and Gerhardt 2013), the role of the Vegfr1, which acts as a VEGF trap, is less clear (Siekmann et al. 2013). In zebrafish, Notch-driven Vegfr1 expression acts as a negative regulator of tip cell differentiation (Krueger et al. 2011) and neuronal-derived soluble Vegfr1 is critical for guiding the direction of vessel growth (Wild et al. 2017).

VEGF signaling acts upstream of the Notch pathway and induces Dll4 expression (Lawson et al. 2002; Ridgway et al. 2006; Lobov et al. 2007; Patel et al. 2005). It has been suggested that Vegf acts via the PI3K pathway activating the Forkhead family transcription factors Foxc1 and Foxc2, which then bind to a *Dll4* enhancer element, or alternatively via the disassembly of a repressor complex at the *Dll4* promoter (Seo et al. 2006; Hayashi and Kume 2008). Subsequently, Dll4 binds and signals to Notch1 receptors on adjacent ECs. The Notch-induced transcription factors Hey1 and Hey2 decrease expression of Vegfr2/3 and thereby reduce responsiveness to VEGF. Such cells will most likely behave as stalk cells (Blanco and Gerhardt 2013). Therefore the nascent sprout is guided by a tip cell with high Dll4 expression and low Notch signaling activity followed by stalk cells with high Notch signaling output (Fig. 2).

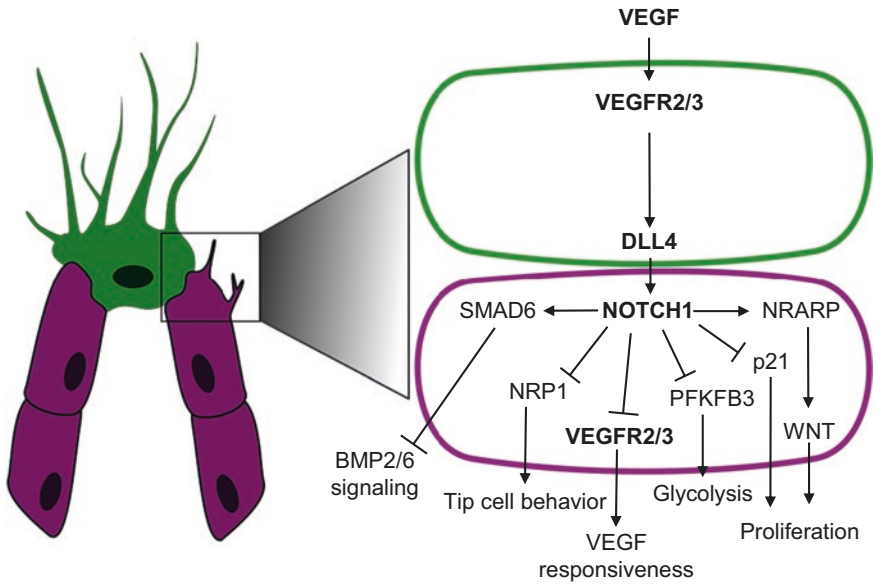

Fig. 2 Core signaling pathways during tip/stalk cell selection. VEGF induces tip cell behavior and expression of the Notch ligand DLL4. This leads to NOTCH1 activation in adjacent cells which adopt the stalk cell phenotype. In stalk cells, Notch signaling represses expression of tip cell-enriched genes like VEGFR2/3 and thereby suppress responsiveness to the pro-angiogenic VEGF. Notch inhibits expression of PFKFB3, an activator of glycolysis, which is required to adopt the tip cell phenotype. Moreover, Notch inhibits proliferation via inhibition of p21 but this is counteracted via WNT signaling since stalk cells need to proliferate. In addition, Notch activates expression of the inhibitory SMAD6 proteins to counteract pro-angiogenic BMP2/6 signaling. Notch inhibits NRP1 expression, which suppresses the stalk cell phenotype by limiting SMAD2/3 activation

Studies with genetic or pharmacologic inhibition of Notch signaling underlined the importance of this pathway during sprouting angiogenesis and tip/stalk cell selection. Notch inhibition leads to the formation of excessive tip cell numbers and vessel branches, a process called hypersprouting (Noguera-Troise et al. 2007; Ridgway et al. 2006; Hellström et al. 2007; Lobov et al. 2007; Siekmann and Lawson 2007; Suchting et al. 2007; Sainson et al. 2005; Leslie et al. 2007). Accordingly, ECs with low Notch signaling activity dominate at the tip cell position, whereas Notch-active ECs are mostly excluded (Jakobsson et al. 2010; Hellström et al. 2007; Siekmann and Lawson 2007; Benedito et al. 2009).

Dll4/Notch1 is the most important ligand and receptor pair in coordinating angiogenesis. However, the situation is more complex. For example, stalk cells also express few Dll4 ligands on their membrane and this could potentially lead to signaling back to Notch receptors on tip cells. This is antagonized by the Notch ligand Jag1, which is strongly expressed on stalk cells (Hofmann and Luisa Iruela-Arispe 2007; Benedito et al. 2009) and inhibits Dll4/Notch1 signaling. Thereby, Jag1 antagonizes signaling from the stalk back to the tip cell (Benedito et al. 2009) and it may also prevent Notch overactivation in the stalk cell plexus.

3.2 Crosstalk Between Notch and Other Signaling Pathways to Control Tip/Stalk Cell Selection

Numerous additional molecules influence tip or stalk cell fate selection through interactions with Notch signaling. In brief, WNT/β-catenin signaling promotes transcription of *Dll4* by binding to an enhancer element (Corada et al. 2010) or through protein interaction of β-catenin with Rbpj (Yamamizu et al. 2010). Furthermore,

WNT signaling induces expression of the transcription factor Sox17, which can activate Notch signaling and promote expression of tip cell-enriched genes (Lee et al. 2014; Corada et al. 2013). On the other hand, Sox17 expression is repressed by Notch signaling in stalk cells (Lee et al. 2014). It was demonstrated, that the mRNA level of *Sox17* is not altered by Notch whereas the protein level of Sox17 is. This shows that Sox17 is post-transcriptionally regulated by the Notch pathway. Taken together this indicates that through a negative feedback loop, hypersprouting is prevented. Similarly, Notch and WNT signaling are linked via Nrarp to control the stability of new vessels. Notch induces Nrarp expression, which in turn limits Notch signaling and promotes WNT signaling in stalk cells (Phng et al. 2009).

The competence of ECs to become a tip cell is also influenced by bone morphogenetic proteins (BMPs) and TGF-β signaling. Bmp9 signals through Alk1 in stalk cells to induce Smad1/5/8 phosphorylation. These Smads synergize with activated Notch receptors to induce expression of Notch targets Hey1 and Hey2, which inhibit VEGF receptor expression (Larrivée et al. 2012; Moya et al. 2012). This is further promoted by Smad1/5-mediated induction of Id proteins which augment Hes1 protein levels (Moya et al. 2012). However, the roles of BMP signaling for tip/stalk selection and angiogenesis are not fully defined yet and still controversial. Very recently, it was reported that Notch promotes expression of the inhibitory Smad6 protein and thereby limits the responsiveness of stalk cells towards the proangiogenic Bmp2 and Bmp6 (Mouillesseaux et al. 2016). Lastly, it was reported that the stalk cell phenotype has to be actively repressed to allow tip cell formation. Neuropilin-1 (Nrp1) plays a key role in suppressing the stalk cell phenotype through limiting Smad2/3 activation. Nrp1 promotes tip cell behavior and the formation of filopodia (Fantin et al. 2013; Fantin et al. 2015). Notch downregulates Nrp1 expression and thus promotes stalk cell behavior (Aspalter et al. 2015).

The Notch-dependent acquisition of the stalk cell phenotype also requires the phosphatase Pten (Serra et al. 2015). Furthermore, Dll4 expression in tip cells is regulated via laminin/integrin signaling (Stenzel et al. 2011). Besides crosstalk of Notch signaling with other signaling pathways, direct protein-protein interactions influence tip-stalk-cell selection. Synaptojanin-2-binding protein (Synj2bp) stabilizes Delta-like protein expression in stalk cells to allow continuous Notch signaling within the stalk cell plexus and to prevent formation of ectopic vessel branches (Adam et al. 2013).

3.3 The Dynamic Nature of Tip/Stalk Cell Differentiation

EC tip and stalk cell specification does not represent permanent cell fate decisions but rather dynamic fluctuations in cell phenotypes (Blanco and Gerhardt 2013). The Gerhardt laboratory has shown that stalk cells compete in a highly dynamic manner for the tip position leading to frequent exchange of the tip cells (Jakobsson et al. 2010). Such EC shuffling occurs every few hours (Ubezio et al. 2016). Mechanistically, the VEGF-Dll4/Notch feedback system drives the competition for the tip/stalk cell selection. This is facilitated by the oscillatory output strength of Notch signaling (Kageyama et al. 2007). As such, the expression of Dll4 fluctuates in individual ECs within sprouting vessels (Ubezio et al. 2016). Therefore, one can assume that concomitantly the levels of Vegfrs, Dll4 and Notch target genes change constantly as ECs interact with each other. As a result, the competence of acting as a tip cell changes constantly, certain stalk cells are relieved from tip cell inhibition and overtake the lead position (Blanco and Gerhardt 2013). This leads to a dynamic position shuffle in the growing sprout.

The tip cell competence concept is further strengthened by the finding that the continual flux in Notch signaling output strength in individual ECs results in differential VE-cadherin turnover to generate spatial differentials in cell-cell adhesions and polarized junctional protrusions. These permanent switches between active and inactive cell junctions allow EC rearrangements during sprout elongation (Bentley et al. 2014).

3.4 Control of Angiogenesis by Metabolism

The vasculature contributes to systemic metabolism control. On the one hand the endothelium controls the shuttling of nutrients from blood to tissue cells in an organ-specific manner (Robciuc et al. 2016; Jais et al. 2016; Hagberg et al. 2010; Corvera and Gealekman 2014) and therefore plays a critical, but poorly understood role, for organ homeostasis. On the other hand, metabolism controls angiogenesis. For example, the expansion of adipose tissue requires angiogenesis, which is stimulated by proangiogenic factors released from adipocytes (Corvera and Gealekman 2014). ECs contain metabolic sensors and their effectors (Sirtuins, mTOR, Pgc1α, Lkb1, Ampk, Foxos and Sirt1) (Potente and Carmeliet 2017) and respond to alteration in nutrient supply. To understand how cellular metabolism affects angiogenesis, one needs to consider how ECs generate ATP. Research from the Carmeliet laboratory revealed that ECs are very glycolytic and produce the majority of ATP by metabolizing glucose into lactate rather than by oxidative phosphorylation, even if plenty of oxygen is available (De Bock et al. 2013). As such, ECs behave similar to cancer cells, which consume high amount of glucose for aerobic glycolysis (Schulze and Harris 2012). Although much less ATP is gained compared to oxidative phosphorylation, glycolysis has the advantage of generating ATP in a very rapid manner and glycolysis allows energy production in hypoxic areas, into which angiogenic ECs need to migrate (Potente and Carmeliet 2017).

Activated ECs require in particular high glycolytic flux for migration and invasion (De Bock et al. 2013; Cruys et al. 2016). This is facilitated by VEGF and hypoxia signaling that together increase the uptake and breakdown of glucose by up-regulating glucose transporter type-1 and glycolytic enzymes, such as 6-Phosphofructo-2-kinase (Pfkfb3) and lactate dehydrogenase-A (Yeh et al. 2008; Peters et al. 2009; Nakazawa et al. 2016; De Bock et al. 2013). Even in ECs with constitutive Notch1 signaling, which are genetically determined to become stalk cells, enhanced glycolysis by Pfkfb3 activation induces tip cell behavior (De Bock et al. 2013). This indicates that EC metabolism can exert control over genetic circuits (Potente and Carmeliet 2017).

In stalk cells, Notch signaling reduces but not eliminates the expression of Pfkfb3 and Pfkfbp3-driven glycolysis, as it is also essential for stalk cells (De Bock et al. 2013). Moreover, stalk cells must synthesize all cellular components (e.g. nucleotides, proteins and lipids) for cell division and cell growth. Therefore, ECs also break down fatty acids to generate carbons for the *de novo* nucleotide synthesis and not only for energy production (Schoors et al. 2015).

It will have to be determined how exactly the metabolic status influences the EC genetic program and vice versa. Fluctuations of Notch and VEGF signaling outputs alter glycolysis rates and ATP production in ECs and thereby change the fitness of ECs to battle for the tip position (Spuul et al. 2016; Potente and Carmeliet 2017; De Bock et al. 2013). The energy status also controls the activity of Foxo1 by Sirt1 and the latter inhibits Notch signaling through deacetylation of the Nicd1 resulting in increased angiogenesis (Guarani et al. 2011). Latest research showed that Foxo1 is an essential regulator of vascular growth by coupling metabolic and proliferative activities in ECs via inhibition of Myc, which fuels glycolysis and mitochondrial metabolism (Wilhelm et al. 2016). In addition, the Notch signaling activity in ECs is influenced by plasma glucose levels (Yoon et al. 2014) and by the presence of certain pro-inflammatory fatty acids (Briot et al. 2015). Taken together, these reports show that Notch signaling integrates angiogenic signaling with the metabolic status.

3.5 Anastomosis of Vessel Sprouts and Remodeling of the New Vessel Network

Newly formed sprouts need to connect with other sprouts or existing vessels to generate a new circulatory loop. Anastomosis is a complex process that has not yet been fully resolved (Betz et al. 2016). Tip cells contact other tip cells to initiate fusing of two sprouts (Isogai et al. 2003),

which is supported by tissue-resident macrophages (Tammela et al. 2011; Fantin et al. 2010; Outtz et al. 2011). Anastomosis requires the formation of new VE-Cadherin-containing EC junctions to consolidate the connection (Bentley et al. 2014). Such junctions are essential for EC polarization and lumen formation. After formation of a patent lumen, blood flow contributes to stabilize the new vascular loop. Increasing oxygen tension decreases VEGF production and helps to switch the activated EC status into a quiescent one. Further vessel maturation includes production of extracellular matrix, recruitment of mural cells, remodeling into a hierarchical network and the pruning of excessive vessel branches (Potente et al. 2011). Notch signaling is critically involved in the recruitment and the tight interactions of ECs with pericytes and smooth muscle cells (Fouillade et al. 2012). Further research is required to elucidate the detailed mechanisms of how Notch signaling is involved in vessel pruning.

4 Arteriovenous Differentiation

After the assembly of the first primitive vessels in the embryo or in a growing tissue of the adult (e.g. muscle or adipose tissue) a rapid differentiation into a hierarchically organized network of arteries, capillaries, veins and lymphatic vessels occurs. The specification of lymphatics has been reviewed elsewhere (Yang and Oliver 2014). Arteries transport blood away from the heart towards the capillaries. As such, arterial vessels are subjected to high blood pressure and pulsatile shear stress, whereas veins face low-pressure gradients can contain valves to prevent backflow and are more distensible than arteries (Corada et al. 2014).

Several studies indicated that vascular progenitor cells, which form the first large vessels in the embryo, are already committed for arterial or venous cell fate (Quillien et al. 2014; Kohli et al. 2013). On the other hand, it was shown that venous-fated EphB4-positive ECs migrate away from arterial-fated EphrinB2-positive ECs in mixed vessels to establish the first artery and vein (Lindskog et al. 2014; Herbert et al. 2009). Subsequently, new branches sprout out of the first arteries and veins. Time-lapse movies of zebrafish embryos demonstrated that vessel sprouts can disconnect from the originating vein and reconnect with the adjacent artery (Betz et al. 2016). Also tip cells from venous sprouts can migrate backwards and incorporate into newly formed arteries in mice and fish (Xu et al. 2014). This suggests that the arteriovenous cell fate is not terminally defined in the early stage of development.

4.1 Arterial Differentiation

Vascular remodeling can occur in absence of blood flow and is largely determined by genetic factors whereby the VEGF and Notch pathways play key roles. Arterial and venous ECs possess specific molecular identities such as EphrinB2 expression exclusively in arterial and EphB4 exclusively in venous beds (Wang et al. 1998). Notch pathway components are expressed at much higher levels in arterial than venous ECs (Villa et al. 2001; Claxton and Fruttiger 2004) and are major players during embryonic arterial differentiation (Gridley 2010; Swift and Weinstein 2009). This was demonstrated by gene targeting approaches in mouse and zebrafish, which revealed that disruption of the Notch pathway does not only lead to impaired vessel sprouting but also to poorly formed arterial vessels, loss of arterial markers (e.g. EphrinB2, Hey2, Cxcr4, Cx40, Nrp1) and/or ectopic expression of venous markers (e.g. EphB4, COUP-TFII (Nr2f2), Nrp2) (Lawson et al. 2001; Zhong et al. 2001; Zhong et al. 2000; Fischer et al. 2004; Lawson et al. 2002; Duarte et al. 2004; Krebs et al. 2004; Sörensen et al. 2009).

Dll4-mediated Notch signaling induces expression of arterial-specific genes (Kim et al. 2008; Iso et al. 2006) and suppresses the expression of the master regulator of venous specification, COUP-TFII (Swift et al. 2014). Dll1 plays a distinct role. Dll1 is expressed selectively on fetal mouse arteries and is not required for the establishment but for the

maintenance of arterial identity and VEGF receptor expression (Sörensen et al. 2009). It should be taken into account that blood pressure, blood flow dynamics and hypoxia are also important for the proper differentiation and the maintenance of arteriovenous identity (Le Noble et al. 2005; Lanner et al. 2013; Diez et al. 2007).

Once the circulatory system is formed and fully functional, the arteriovenous fate needs to be actively maintained to prevent the formation of arteriovenous shunts. Arteriovenous malformations in the brain are an important cause of intracerebral hemorrhage in young adults (Lawton et al. 2015). Increased NOTCH1 activity has been observed in human arteriovenous malformations (Murphy et al. 2009; Zhuge et al. 2009). Based on gene targeting approaches, Notch signaling appears to be involved in its pathogenesis. Interestingly, both endothelial-specific inhibition and over-activation of Notch signaling can lead to the formation of arteriovenous malformations at least in certain vascular beds (Trindade et al. 2008; Carlson et al. 2005; Miniati et al. 2010; Murphy et al. 2012; Murphy et al. 2014; Murphy et al. 2008; Gale et al. 2004; Murphy et al. 2009).

Besides maintaining arterio-venous identity, Notch signaling is required to maintain integrity of vascular smooth muscle cells. Neomorphic mutations in *NOTCH3*, which often lead to unequal numbers of cysteine residues in the extracellular domain, cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). This leads to degeneration of vascular smooth muscle cells in small-sized arteries, changes in brain blood perfusion that cause migraine attacks, stroke and dementia. Gene targeting experiments have shown that mice carrying a CADASIL-causing *Notch3* point mutation displayed attenuated myogenic responses and reduced caliber of brain arteries as well as impaired cerebrovascular autoregulation and functional hyperemia (Chabriat et al. 2009; Joutel et al. 2010).

4.2 Venous Specification

It was previously believed that venous differentiation is the default differentiation pathway in the absence of Notch activation. However, mouse knockout studies revealed a pivotal role for the transcription factor COUP-TFII, which is exclusively expressed in venous and lymphatic ECs to establish venous fate (You et al. 2005). Interestingly, Notch signaling suppresses COUP-TFII expression, most likely via Hey transcriptional repressors, and thereby allows arterial fate specification (Swift et al. 2014). In summary, it appears likely that Notch and COUP-TFII repress each other to allow the establishment of the arterial or venous gene expression programs, respectively.

4.3 Upstream Regulators of Notch During Arteriovenous Differentiation

It still remains unclear what mechanisms act upstream of Notch signaling in early phases of arteriovenous differentiation. Hypoxia might play an important role by inducing DLL4 expression (Diez et al. 2007; Patel et al. 2005). In zebrafish, Shh and Vegf-a act upstream of Notch to promote arterial differentiation. Alternatively, Shh might promote arterial differentiation independently of VEGF signaling via the calcitonin receptor-like receptor (Wilkinson et al. 2012). In mammals, neurons or glial cells release VEGF to support arterial differentiation. VEGF signaling via Erk induces transcription of Dll4 and arterial-specific genes (Deng et al. 2013; Ren et al. 2010). However, VEGF signaling can also induce Pi3k activity, which has an opposite effect on arterial morphogenesis (Hong et al. 2008; Ren et al. 2010), indicating that other factors are needed to fine-tune VEGF signaling branches. Neuropilin-1, which is more abundantly expressed on arterial than venous ECs, could be one of these factors as it promotes Vegfr2 trafficking and Erk signaling (Lanahan et al. 2013).

Besides VEGF signaling, the expression of Dll4 during arterial differentiation is also promoted by SoxF transcription factors (Corada et al. 2013; Sacilotto et al. 2013), WNT/β-catenin signaling (Corada et al. 2010; Yamamizu et al. 2010), angiopoietin-1 (Zhang et al. 2011) and the transcription factors Foxc1 and Foxc2 (Seo et al. 2006; Hayashi and Kume 2008). Lastly, it should be noted that also blood flow dynamics induce the expression of Notch pathway components and other arterial-specific genes in cultured ECs (Lehoux and Jones 2016) and endothelial cells in mice (Ramasamy et al. 2016). Furthermore, studies using cultured cells have shown that such physiologic forces can sensitize the negative regulatory region of Notch1 to ADAM-mediated cleavage (Gordon et al. 2015). As such, a large amount of genetic and environmental factors promote EC Notch signaling to enable and maintain arterial morphogenesis.

5 Organ-Specific Vascular Beds and Angiocrine Signaling

A major challenge for the research field will be the analysis of organ-specific vascular beds. Blood vessel anatomy and function differs dramatically between organs and even within the same organ (e.g. the fenestrated endothelium in kidney glomeruli vs. the continuous endothelium in peritubular capillaries). The tightness of vessels is adapted to the organ-specific requirements with e.g. tight EC connections in the central nervous system and gaps (fenestrations) in the sinusoidal endothelium of liver, endocrine organs or bone marrow (Aird 2007). Little is known so far regarding how these differences are established during development and maintained throughout life. This is, however, of utmost importance. For example, treatment of mice with tyrosine kinase inhibitors targeting VEGF receptors led to pronounced regression of fenestrated capillaries, that are typically present in endocrine organs and that under normal conditions express high levels of Vegfr2/3 (Kamba et al. 2006). Similar data were obtained in pancreatic islets by a genetic approach (Lammert et al. 2003), indicating that VEGF acts as a survival factor for fenestrated capillaries in endocrine organs.

Several angiocrine functions have recently been described in which ECs control organ development and regeneration by secreting e.g. growth factors or by providing niches and cell surface molecules for hematopoietic stem cells or tumor cells (Rafii et al. 2016). Here we focus on such examples in which the Notch pathway is critically involved.

Work from the Adams laboratory gave fascinating insights on how blood vessels orchestrate the formation, function and remodeling of bone (Kusumbe et al. 2014). In contrast to other organs, active Notch signaling in bone ECs promotes blood vessel growth. Furthermore, Notch regulates the angiocrine release of Noggin, which is involved in bone growth, mineralization and chrondrocyte maturation (Ramasamy et al. 2014). It is known that many diseases lead to impaired skeletal blood flow. Interestingly, flow-responsive genes induce endothelial Notch signaling in bone. Therefore, impaired blood flow hampers osteogenesis and rejuvenation of bone through impaired EC Notch signaling and decreased angiogenesis (Ramasamy et al. 2016).

In the liver, Notch1 is important to maintain quiescence and morphology of the specialized sinusoidal vasculature. Disruption of *Notch1* using the rather tissue-unspecific Mx-Cre line led to de-differentiation of sinusoidal ECs, vascular remodeling, detachment of mural cells and intussusceptive angiogenesis (Dill et al. 2012; Dimova et al. 2013). In the bone marrow, Jag1 expression on ECs is important for hematopoietic stem cell differentiation (Poulos et al. 2013) and niche-forming vessels can be restored by activation of EC Notch signaling (Kusumbe et al. 2016). In the lung, Jag1 expressed on pulmonary capillary ECs induces Notch signaling in perivascular fibroblasts and thereby enhances lung fibrosis (Cao et al. 2016).

Lastly, we want to emphasize that aside from their role in angiogenesis, tumor ECs possess additional roles. ECs within a solid tumor mass are in close contact with tumor cells and many immune cells and their released cytokines. As

such, tumor ECs often do not form tight barriers any more, exhibit altered gene expression programs and also actively alter the behavior of adjacent cells in the tumor microenvironment. In this regard, ECs can provide several membrane-bound and secreted factors that promote tumor progression (Butler et al. 2010). Notch ligands of the Delta-like and Jagged families are frequently present on tumor ECs and can promote Notch signaling in adjacent tumor cells. This increased aggressiveness of lymphoma cells (Cao et al. 2014), promotes the cancer stem cell phenotype (Lu et al. 2013; Zhu et al. 2011), increases tumor cell survival (Pedrosa et al. 2015) and facilitates metastasis (Sonoshita et al. 2011). Interestingly, Notch ligands can also be secreted by tumor cells via exosomes and be incorporated in EC membranes at distant sites to either activate or inhibit Notch signaling (Sharghi-Namini et al. 2014; Sheldon et al. 2010). Furthermore, Notch activation in ECs can be driven by inflammation and this in turn contributes to increased expression of leukocyte adhesion molecules (Liu et al. 2012; Verginelli et al. 2014). Work from our group showed that sustained NOTCH1 activation in ECs leads to senescence, expression of adhesion molecules and weakening of cell junctions that promote transmigration and homing of circulating tumor cells (Wieland et al. 2017).

6 Tumor Angiogenesis and Notch Targeting Agents

Angiogenesis is a hallmark of cancer (Hanahan and Weinberg 2011). The growth of small tumor cell clumps into a clinically relevant tumor is only possible by the induction of blood vessel growth into the tumor mass. Tumor vessels have an abnormal structure and often function poorly. The endothelial lining contains gaps and disorganized cell-cell junction integrity. Also the coverage with pericytes is frequently impaired making vessels leaky. This increases interstitial pressure what impairs the transport of nutrients and drugs towards tumor cells. Moreover, vascular leakiness facilitates intravasation of tumor cells and dissemination (Goel et al. 2011). The tumor vasculature lacks a strict hierarchical structure, arteriovenous identity is poorly defined, vessels have irregular lumen sizes, are often tortuous shaped and thin-walled. Both hyper-vascularized and poorly vascularized tumor areas accompany tumor heterogeneity. Irregular vessel branches, shunts, blind-ended branches, weak vessel contractility and irregular lumen sizes together lead to abnormal and very heterogeneous perfusion rates. Irregular perfusion impairs oxygen, nutrient and drug delivery, thereby limiting the efficiency of chemotherapy and radiation. Impaired perfusion causes aggravation, as hypoxic tumor areas secrete even higher amounts of proangiogenic factors leading to the formation of even more chaotic vessel structures with increased permeability (Carmeliet and Jain 2011; Potente et al. 2011).

VEGF targeting substances are in clinical use but show limited efficiency (Carmeliet and Jain 2011; Potente et al. 2011). Anti-VEGF drugs inhibit the formation of new vessel sprouts and also induce regression of pre-existing tumor vessels, in particular immature vessels. It is assumed that the mode of action is not starving the tumor to death but rather to normalize the tumor vasculature by regression of immature vessels and maturation of the remaining ones. The normalized tumor vasculature is better perfused and enables better delivery of cytotoxic agents to tumor cells (Goel et al. 2011). It is assumed that many initially sensitive tumors develop resistance against VEGF-targeting drugs by secretion of other proangiogenic proteins (e.g. FGF2, PDFG, PlGF, IL-8, ANG2) and by other means of vessel formation (e.g. cooption of already existing vessels) (Bergers and Hanahan 2008). This indicates that better combination therapies are required to target the tumor vasculature.

Besides VEGF, Notch signaling is an interesting target. As in physiological angiogenesis, Notch signaling is involved in tumor angiogenesis (Noguera-Troise et al. 2006; Ridgway et al. 2006; Lobov et al. 2007). However, the pathological high VEGF concentrations may disrupt oscillatory Notch signaling outputs and thereby impair the formation of proper cell junctions and promote vessel expansion (Bentley et al. 2014; Ubezio et al. 2016). Dll4 and Jag1 are abundantly

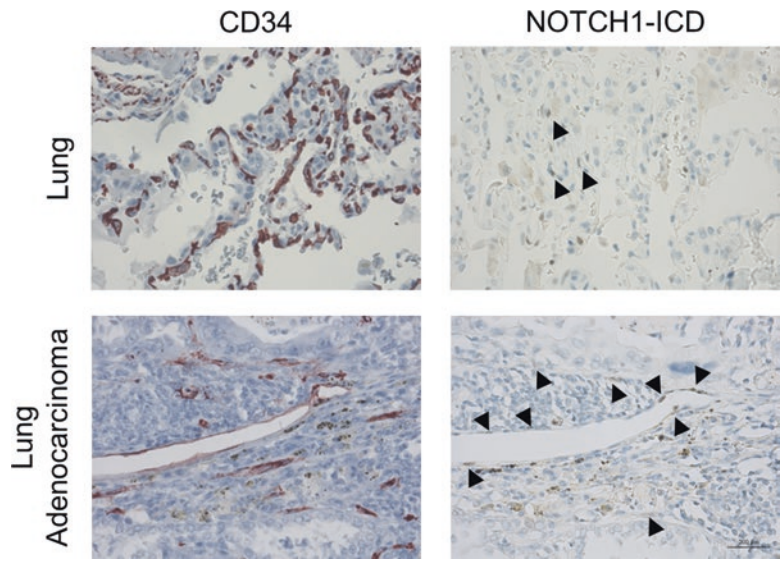

Fig. 3 Notch signaling is active in blood vessels of adult and tumor blood vessels. Sections of normal lung and lung adenocarcinoma were stained against the endothelial marker CD34 (brown color) or the cleaved NOTCH1 receptor (NOTCH1-ICD). Cell nuclei were counterstained with hematoxylin (blue color). Magnification 400-fold

expressed on tumor vessels (Patel et al. 2005; Lu et al. 2007; Jubb et al. 2012; Gale et al. 2004; Mailhos et al. 2001; Scehnet et al. 2007) and tumor vessels often exhibit strong Notch1 activity (Fig. 3). By computational modeling, it was suggested that the higher production levels of the antagonistic ligand Jag1 give rise to a hybrid tip/stalk phenotype that leads to poorly perfused vessels (Boareto et al. 2015).

Manipulation of EC Notch signaling appears to be an attractive target to interfere with tumor progression. Notch signaling is often hyperactive in cancer cells (in particular in the cancer stem cells) and acts as an oncogene in many tumor entities. Therefore, Notch inhibition could target tumor cells and tumor vessels simultaneously. Many academic groups and pharmaceutical companies have developed Notch inhibiting substances and several ones are in phase I/II trials (Andersson and Lendahl 2014). In rodent models, blockade of Dll4, Notch1 or γ-secretase leads to a non-productive hypersprouting phenotype resulting in central tumor necrosis (Noguera-Troise et al. 2006; Ridgway et al. 2006; Scehnet et al. 2007). This may sound paradoxical, but the excessive vessel branches generate such a chaotic network that dramatically diminishes tumor perfusion. Whether this can also be achieved in human cancer patients is not clear yet. γ-secretase inhibitors, which block the activity of all four Notch receptors, have quite profound adverse effects (e.g. gastrointestinal toxicity) in clinical trials (Andersson and Lendahl 2014) but neutralizing antibodies against individual Notch receptors might be able to overcome this (Wu et al. 2010). In addition, antibodies targeting individual Notch ligands have also been developed (Andersson and Lendahl 2014). Nevertheless, DLL4-neutralizing antibody can also cause severe adverse effects (Yan et al. 2010), e.g. development of congestive heart failure was observed in clinical phase I studies (Chiorean et al. 2015; Falchook et al. 2015; Smith et al. 2014). It will be important to study the underlying mechanisms to overcome this problem.

As outlined above, it appears to be more reasonable to induce tumor vessel normalization instead of tumor vessel regression. A novel approach to achieve this might be targeting EC metabolism. ECs are highly glycolytic and high rates of glucose breakdown are instrumental for adopting the tip cell phenotype during sprouting. A rather mild inhibition of glycolysis can be achieved by targeting its activator Pfkfb3. In mouse cancer models, Pfkfb3 inhibition tightened the vascular barrier, improved adhesion of pericytes and reduced the pro-inflammatory phenotype of tumor ECs that facilitates metastasis (Cantelmo et al. 2016). Another option to normalize the tumor vasculature could be the acti-

vation of EC Notch signaling. As shown by genetic approaches in mice, this reduced tumor angiogenesis, but increased vessel diameter and improved perfusion and oxygenation (Li et al. 2007). Notch activation could also help to reduce glycolysis in ECs as Notch signaling reduces expression of Pfkfb3 (De Bock et al. 2013). While Notch-inhibiting substances are in clinical trials, we still lack fully validated drugs to activate Notch signaling in a therapeutic manner. The Kitajewski laboratory has generated soluble Notch1 extracellular domain proteins fused to IgG-Fc (Notch decoys) that bind and inhibit selectively either the stimulatory Delta-like or the inhibitory Jagged ligands (Funahashi et al. 2008; Kangsamaksin et al. 2015). These Notch decoys inhibit sprouting angiogenesis and also target pericytes in the vessel wall (Klose et al. 2015; Funahashi et al. 2008; Kangsamaksin et al. 2015). Future experiments will determine whether Notch-activating substances can be used successfully in combination with chemotherapy to better target tumor cells.

7 Perspectives

In recent years there has been a significant progress in the understanding of Notch signaling during sprouting angiogenesis. However, much remains to be learned. As the tip/stalk cell selection is tightly dependent on subtle fluctuations in Notch signal output strengths, it is necessary to determine multiple genetic and environmental factors, such as hemodynamics and metabolites that fine-tune ligand expression and localization at the cell surface, receptor glycosylation, NICD protein stability, nuclear NICD complex formation and the dynamic control of Notch target gene expression.

Inducible tissue-specific transgene models and therapeutic antibodies will be key to determine how VEGF and Notch signaling are involved in organ-specific angiogenesis, maintenance of EC quiescence, as well as barrier and transport functions throughout life. There is already solid evidence that VEGF does not only control blood vessel formation, but also acts as a survival factor for ECs (Domigan et al. 2015) and non-vascular cells (Mackenzie and Ruhrberg 2012). Similar to this, basal Dll4/Notch activity has been detected in quiescent ECs (Zhang et al. 2011) and is important to maintain vascular integrity and function (Liu et al. 2011; Yan et al. 2010). Lastly, angiocrine functions of ECs have attracted enormous attention (Rafii et al. 2016). It will be fascinating to see how ECs control the function of parenchymal cells through the secretion of signaling molecules or through providing membrane-bound factors that orchestrate the behavior of its neighboring cells in organ-specific vascular beds.

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