

Regulation of angiogenesis by homotypic and heterotypic notch signalling in endothelial cells and pericytes: from basic research to potential therapies

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Received: 25 January 2008 / Accepted: 28 January 2008 / Published online: 7 February 2008
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Abstract The notch-signalling pathway regulates cell fate and differentiation through cell–cell communication. In recent years, several *in vitro* and *in vivo* studies have demonstrated that notch-signalling functions as a negative feedback mechanism downstream of the VEGF-signalling pathway that acts to finely shape the vascular network. Notch activation by the Jagged-1 and Delta-like 4 ligands regulates different steps of blood vessel development ranging from proliferation and survival of endothelial cells, to vessel branching and arterial–venous differentiation. In addition, heterotypic notch signalling from endothelial cells to pericytes is critical for vessel stabilization and maturation. Interestingly, several studies have demonstrated that blocking the notch pathway can delay tumour growth. Unexpectedly however, tumour growth inhibition by Notch was caused by an increased number of non-functional vessels, which resulted in poor tumour perfusion. This approach of modulating notch signalling, combined with the extended knowledge acquired on the basic vascular role of notch signalling, will aid the development of treatments targetting human pathologies such as tissue ischaemia and solid tumour formation.

Keywords Angiogenesis · Notch

Introduction

The formation of a fully functional vascular network occurs early during embryogenesis and is driven by two main mechanisms, termed vasculogenesis and angiogenesis [1]. While vasculogenesis gives rise to the primitive vascular plexus and the heart from mesodermal-derived haemangioblasts (which are multipotent cells that can differentiate in both endothelial cells (EC) and haematopoietic cells [2]), angiogenesis occurs as a second step and involves remodelling of the existing vasculature through sprouting and intussusceptive vessel growth. The later stages of vascularization require vessel stabilization, a process that is strongly associated with the production of the basement membrane and the recruitment and differentiation of mural cells [3]. Both vasculogenesis and angiogenesis are highly regulated processes driven by both attractive and repulsive cues that are necessary for the correct temporal and spatial location of newly formed vessels. These two processes are not restricted to embryonic development but are also observed in the adult organism in both physiological and pathological conditions such as wound healing and tumour vascularization, respectively. Due to their central role in a variety of pathological conditions, both vasculogenesis and angiogenesis have been studied intensively in recent years with the aim of developing therapeutics that can target these crucial processes. With the extensive use of *in vitro* angiogenesis assays and the use of knock-in and knock-out *in vivo* approaches, studies from the last 30 years have led to the identification of several signalling pathways involved in the cellular and molecular mechanisms regulating new vessel formation (some of which are reviewed elsewhere within this issue). The present review will concentrate on one of them, namely the notch-signalling pathway.

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Notch signalling

The Notch-signalling pathway is a cell–cell communication pathway that is evolutionary conserved from *Drosophila* (where it was first described) to human [4]. To date, four different notch receptors (Notch1, Notch2, Notch3 and Notch4) and five different ligands (Jagged-1 and -2 and Delta like-1 -3 and -4) have been identified in mammalian cells (Fig. 1). These transmembrane receptors and ligands are expressed in different combinations in most, if not all, cell types. The notch pathway regulates cell fate determination, proliferation and survival of neighbouring cells through lateral inhibition, depending on their ability to express either the receptors or the ligands (Fig. 2). The binding of a notch ligand, through its Delta-Serrate-Lag2 (*DSL*) domain, to the EGF11–12 motifs of a notch receptor triggers the proteolytic activation of the receptor, which leads to the release of its notch intracellular domain (NICD). This processing requires the activity of two proteases, namely tumour necrosis factor α -converting enzyme (TACE) and presenilin/ γ -secretase (a large protease complex made of presenilin1 or 2 as well as nicastrin, Pen-2 and Aph-1). The subsequent nuclear translocation of NICD results in transcriptional activation of genes of the Hes/E(spl) and Hey/Hesr families via the interaction of NICD with a member of the CSL (CBF1, Suppressor of hairless, and Lag-1) family of transcription factors also known as the recombination signal sequence-binding protein (RBP- $\text{j}\kappa$). In turn, the Hes and Hey proteins [5], which are transcriptional repressors, inhibit the expression of genes that drive cells to adopt a differentiated fate. Interestingly, it was shown that endocytosis of the ligand in the

signal sending cell is required for activation of notch signalling in the signal-receiving cell [6–9]. It has been proposed that this endocytosis may act as a “pulling” mechanism to separate the notch extracellular from the rest of the receptor, thereby allowing cleavage of the NICD in the receptor cell. Finally, it has also been suggested that endocytosis of the ligand may have consequences in terms of signalling in the ligand cells and thus would constitute an early step in a reverse signalling mechanism [10–12].

In addition to the above pathway known as the CSL-dependent pathway, studies in *Drosophila* have shown that Notch can also signal through other mechanisms [13]. The phenotype triggered by mutations in CSL/Su(h) was shown to be less pronounced than the one observed by deletion of the notch receptor [14], suggesting the presence of alternative CSL-independent downstream pathways (Fig. 2). Although the molecular mechanisms of such notch CSL-independent pathways are not fully characterized, they have been shown to be mediated through interactions of the NICD with additional binding partners ranging from other transcription factors (Lef1 and HIF) to cytoplasmic proteins [15–17]. For instance NICD has been shown to repress cell differentiation through inhibition of the JNK pathway by interacting with the cytoplasmic protein Deltex [18]. In addition, a recent study elegantly demonstrated that NICD activates β 1 integrin in a CSL-independent manner through the activation of the Ras pathway without activating ERK. If extended to EC, this mode of action could have strong implications in processes such as angiogenesis via the regulation of cell adhesion [19]. Finally it is possible that these additional NICD binding partners modulate the CSL-dependent pathway and explain why Notch signalling can

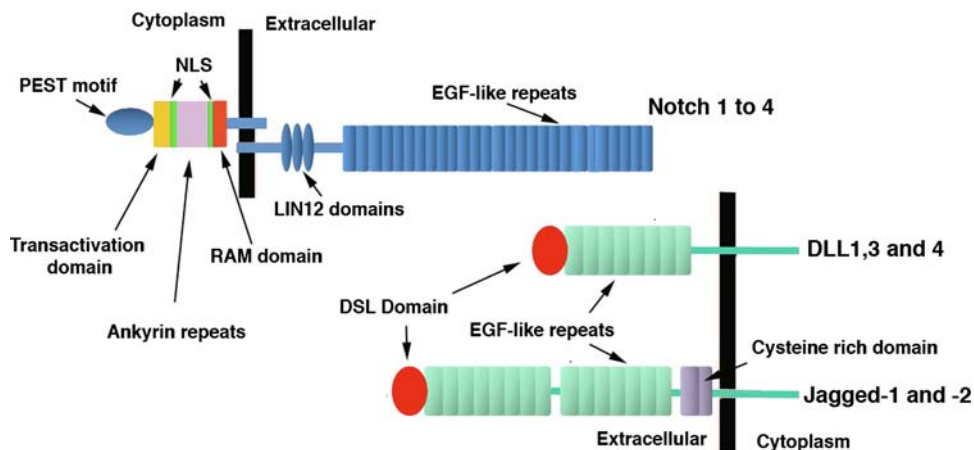


Fig. 1 Mammalian notch receptors and ligands. The extracellular domain of a notch receptor is connected to the transmembrane domain non-covalently and contains EGF-like repeats and Lin12/Notch domains. Of importance are the EGF-like repeats 11 and 12, which have been shown to directly interact with the DSL domain of notch ligands. The Lin12/notch motif is required to maintain the receptor in its resting state prior to ligand-induced activation. The intracellular

domain, which gets cleaved following ligand binding, contains a RAM domain (necessary to bind to CSL), six ankyrin repeats flanked by two nuclear localization sequences (NLS), and a transactivation domain. Finally, notch receptors contain a conserved PEST sequence near the C-terminus also known as the degradation motif. Both the Delta-like and Jagged ligands contain a DSL domain followed by EGF-like repeats. Jagged ligands also contain a cysteine-rich domain

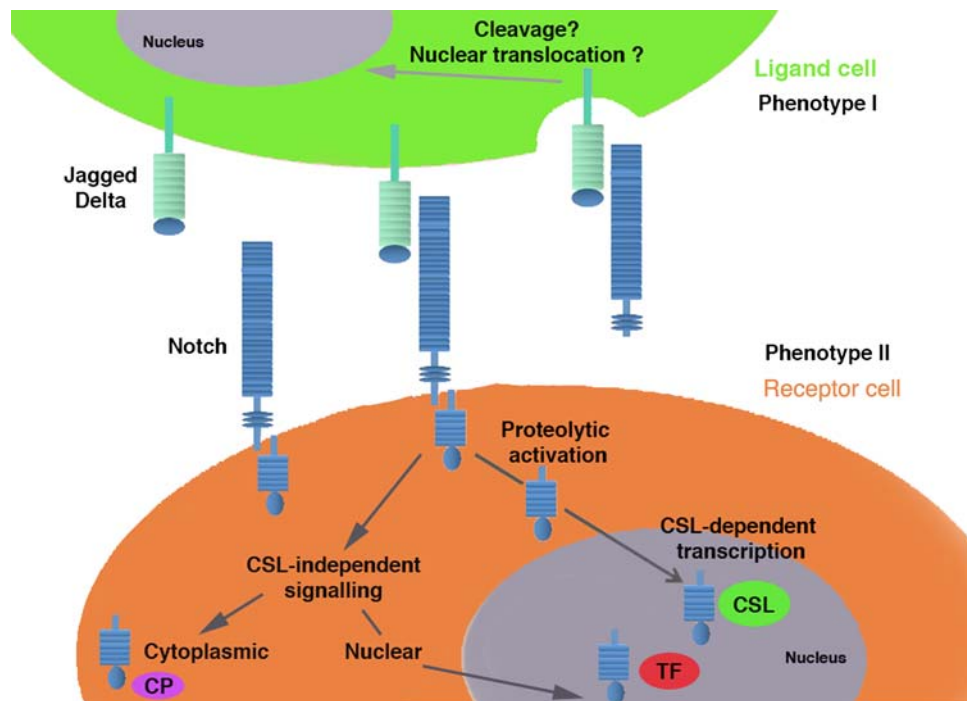


Fig. 2 Notch signalling regulates cell fate through CSL-dependent and CSL-independent pathways. Following ligand binding, a conformational change is induced in the notch extracellular domain which allows the subsequent cleavage of the receptor and release of the intra-cellular domain of notch (NICD) into the cytoplasm. The NICD translocates to the nucleus where it binds CSL to activate the transcription of genes such as Hey and Hes. NICD can also interact with other transcription factors (TF) to regulate the transcription of

additional target genes in a CSL-independent pathway. Finally, NICD can also regulate the activity of cytoplasmic proteins (CP). Endocytosis and cleavage of the ligand may also result in the generation of a ligand-ICD that could translocate to the nucleus to regulate transcription, thus triggering a bi-directional signalling pathway. Overall, when activated, notch signalling allows neighbouring cells to acquire distinct phenotypes, through a process named lateral inhibition

have opposing effects with regards to proliferation and survival depending on the cell type examined.

The fact that one receptor can be activated by multiple ligands and that one ligand can activate multiple receptors clearly highlights the complexity of this signalling pathway. Some crucial studies to address these points have shown that Notch interaction with its ligand is regulated by the level of glycosylation of the receptor [20, 21]. Both the presence and the type of *O*-fucose glycans present on the Notch receptor influence the binding to either Jagged or Delta ligands, suggesting a means by which one ligand can preferentially bind to a receptor in the presence of others. One such enzyme that can modify the receptor is the protein *O*-fucosyltransferase 1 (POFUT1), which transfers *O*-linked fucose to EGF-like repeats [22]. Additionally, Fringe enzymes add *N*-acetyl-glucosamines to the *O*-linked fucose and favour the binding of Delta-1 over Jagged-1 to Notch receptor [23, 24]. However, the mechanisms that allow the activation of ligand-specific pathways by a single receptor remain to be identified. In particular, the downstream transcriptional targets for each ligand–receptor combination are unknown and are now at the centre of current investigations [25]. Another crucial field of investigation should address if and how each ligand regulates CSL-independent pathways differently.

Notch in physiological angiogenesis

Since it was first described as a regulator of neurogenesis, Notch signalling has been shown to influence the morphogenesis of most organs. Studies in the last decade have clearly demonstrated that Notch signalling is an important regulator of several types of tubular structures, such as the *Drosophila* tracheal system and the Malpighian tubules of insects, as well as the mammalian vascular system [26–28].

The first evidence that Notch is implicated in the formation and stabilization of blood vessels came from the fact that several Notch ligands and receptors are expressed either in the endothelium or in the surrounding mural cells [29–31]. In addition, expression of the Notch4 receptor and the DLL4 ligand is mainly restricted to the vascular system [32–35]. Further evidence came from a variety of *in vivo* studies manipulating the expression levels of Notch pathway components. Homozygous deletion of the Notch1 receptor in mice, either globally or within the endothelium, caused severe vascular remodelling defects in the yolk sac, placenta and embryo proper, which lead to embryonic lethality as early as day E10.5 [36, 37]. As mentioned above, Notch4 is specifically expressed in arterial EC [34]; however, *Notch4*-null mice developed normally and were

fertile. It is only when homozygous deletions of both *Notch1* and *Notch4* were performed that a synergistic effect on the embryonic vasculature was observed, as double mutant mice exhibited more extreme vascular defects than those observed in *Notch1*-null mice [36]. Interestingly, the overexpression of an activated form of Notch4 in the mouse endothelium also led to angiogenic defects similar to that of the *Notch1/4* double knockout mice [38]. Taken together, these results indicate that either abrogation or forced activation of Notch signalling disrupts blood vessel development, suggesting that this signalling pathway needs to be finely tuned to trigger proper angiogenesis.

Similarly, mice lacking some of the Notch ligands are embryonically lethal. Mice mutant for either Jagged-1 or Dll1 perish early during gestation due to vascular defects and intense degree of haemorrhage [39, 40]. Among the Notch-signalling pathway mutants, the Delta-like 4 (DLL4) mutant has the most severe vascular phenotype potentially due to its selective expression in arteries and capillaries. Three independent studies have demonstrated that single allele deletion of DLL4 resulted in embryonic lethality (around E10.5) in most genetic backgrounds tested [41–43]. Anatomical analysis of the embryos demonstrated that lethality in DLL4 +/- embryo was due to abnormal narrowing and constriction of the aorta, defective branching and occasional arterial regression. In addition, enlargement of the pericardial sac and extra-embryonic vascular defects in the yolk sac vasculature were also described. Interestingly, heterozygote lethality due to vascularization defects has only previously been described for VEGF-A haploinsufficient mice [44, 45]. Mutations in downstream Notch effectors also triggered vascular defects. Disruption by homologous recombination of RBP-J κ resulted in growth retardation as early as E8.5 and death by E10.5 due to abnormal placental development [46]. Finally deletions of downstream Notch targets have also been investigated. Although Hey1 knockout mice are viable, combined deletion of Hey1 and Hey2 results in vessel malformation [47].

Interestingly, these *in vivo* studies showed no defects in primary vascular plexus formation, therefore suggesting that Notch signalling is not involved during embryonic vasculogenesis but is more specific for vascular remodeling through angiogenesis. Whether or not Notch signalling regulates adult vasculogenesis through the recruitment of endothelial and bone marrow progenitor cells, however, still remains to be determined.

Mode of action

The above studies clearly demonstrate that Notch signalling is involved in angiogenesis, however the molecular and cellular mechanisms by which this

signalling pathway regulate blood vessel formation have been described more recently. These experiments have collectively shown that Notch signalling involves both homotypic and heterotypic communication between EC, pericytes and stromal cells.

Notch signalling blocks EC proliferation and promotes EC survival

Normal adult vasculature is mostly quiescent with regards to cell proliferation. VEGF-A, through the activation of the VEGFR2 receptor, is the most characterized mitogenic EC growth factor, and activates cell proliferation through the MAPK/ERK and PI3K/Akt pathways [48]. Several studies have suggested that Notch signalling may antagonize cell proliferation through at least two mechanisms. Firstly Notch signalling, which is induced by VEGF-dependent up-regulation of DLL4 expression [49–51], acts as a negative feedback loop to block VEGF-A-dependent proliferation. This increased DLL4/Notch signalling results in transcriptional inhibition of both VEGFR2 and its co-receptor NRP-1 [51–53]. In addition, Notch signalling induces the expression of both the soluble and full-length forms of the VEGF decoy receptor, VEGFR1 [25]. The diffusible properties of soluble VEGFR1 suggest that DLL4 Notch signalling can inhibit EC proliferation away from the site of signalling. Secondly, Notch signalling can also influence cell proliferation by directly affecting cell division. For example, the NICD reduces phosphorylation of Erk1/2 and Akt after VEGF stimulation [54]. Activation of Notch signalling by DLL4 or by expression of Notch4 ICD induces cell cycle arrest in the G₀/G₁ phases of the cell cycle [51, 55]. It has also been suggested that Notch activation promotes cell cycle arrest through down-regulation of the minichromosome maintenance proteins MCM-2 and MCM-6 and through up-regulation of p21^{CIP} [55, 56].

In addition to its role in regulating cell proliferation, Notch-signalling functions to favour EC survival. A recent study has shown that Notch activates the expression of VEGFR3, which responds to VEGF-C to protect EC from apoptosis [57]. In addition, programmed cell death is inhibited in EC by Notch signalling through the induction of the anti-apoptotic protein BCL2 [58].

Notch regulates vessel branching through tip cell versus stalk cell differentiation

Newly forming vessels are made of two different endothelial cell types, namely the tip and the trunk (or stalk) cells [59]. The tip cell is located at the leading edge of the growing vessel where, through the presence of filopodia, it

senses the environment for angiogenic factors. In contrast, the stalk cells situated behind the tip cells are highly proliferative cells and allow the vessel to elongate towards angiogenic stimuli [60]. Blocking notch signalling in vitro in a 3D tubulogenesis assay has been shown previously to increase the number of vessel tips thus suggesting that notch signalling may have a role in tip cell versus trunk cell differentiation [28]. In the postnatal mouse retina, the notch ligand DLL4 is predominantly expressed within the vessel tips where it is induced by VEGF secreted by surrounding astrocytes [60–63]. An elegant series of experiments in the postnatal mouse retina and in zebrafish intersomitic angiogenesis models showed that loss of DLL4/Notch signalling results in a VEGF-dependent elevation of tip cell numbers and vessel branching [61–65]. These studies confirmed the in vitro observation and are consistent with a role of DLL4-induced notch signalling in lateral inhibition of the tip cell phenotype (Fig. 3). These studies demonstrated that VEGF-A induces the formation of the migratory tip cells and also induces the expression of notch ligands such as DLL4 in the same cells. This in turn actively represses the neighbouring cells from becoming tip cells and allows them to acquire stalk cell properties necessary for proper vessel elongation. As suggested by Roca and Adams [66], this model would explain why large arteries, which express high levels of the notch ligand, also have a lower level of branching compared to veins.

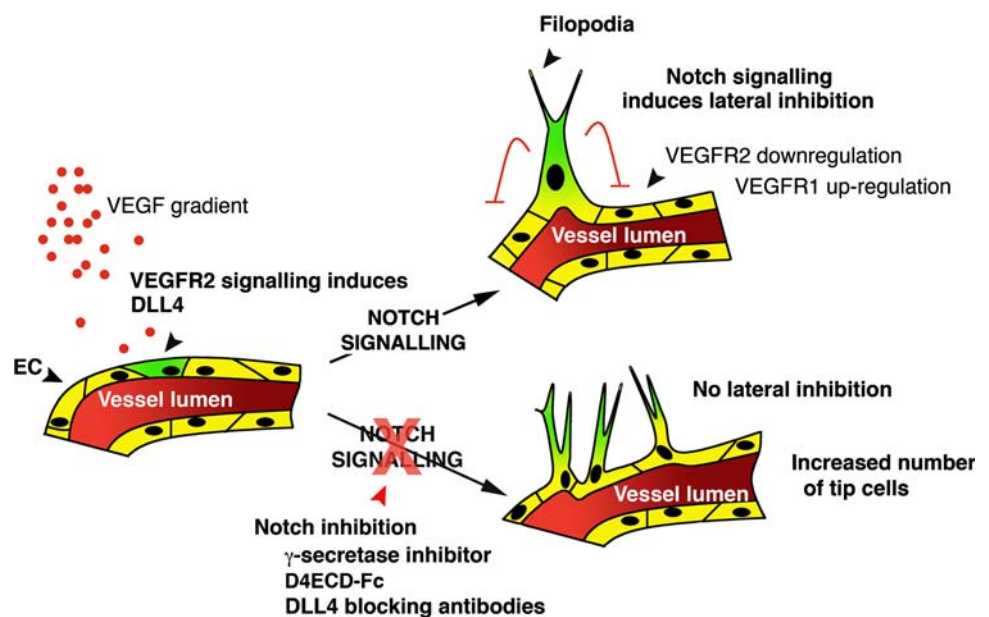
Interestingly, the above studies argue against the idea that notch signalling has a role in the original acquisition of the tip cell phenotype, which appears to be the default phenotype in response to VEGF-A. However, several lines of evidence may suggest otherwise. Tip cells, due to their ability to migrate away from the quiescent endothelium,

are believed to go through a partial epithelial-to-mesenchymal (EMT) transition. Expression of the transcription factors Snail and Slug, which are major regulators of EMT, results in transcriptional repression of cell adhesion molecules (such as cadherins) thus allowing the cells to become more invasive [67]. Interestingly, notch signalling has been shown to regulate EMT processes in physiological and pathological events [68–70]. Recent evidence from the Karsan group showed that Slug was required for notch-mediated repression of E-cadherin, resulting in β -catenin activation and resistance to anoikis [69]. This, combined with the fact that Slug was up-regulated and VE-Cadherin-2 down-regulated in cells with activated notch [25], suggests that notch signalling may in fact be actively involved in this process and thus should be investigated further to determine its precise role in vessel cell differentiation.

Arterial–venous differentiation

Although originally thought to be determined by environmental cues such as blood pressure, recent studies demonstrated that arterial–venous differentiation is specified before the initiation of blood flow and is regulated by genetic predisposition involving notch signalling. Transgenic animals in which the DLL4 coding region was replaced by a reporter gene showed that DLL4 expression is restricted to large arteries [42]. Consistent with this arterial-specific expression pattern, DLL4 heterozygous knockout mice showed prominent defects in arterial development. However, single allele deletion of DLL4 also resulted in malformation in major veins, suggesting that notch signalling also indirectly influences vein formation [41]. Arterial–

Fig. 3 Schematic representation showing how DLL4/Notch signalling prevents excessive branching by inhibiting tip cell phenotype. A gradient of VEGF-A locally activates EC to become tip cells and induces local DLL4 expression. This in turn will activate notch signalling in the neighbouring cells and prevent these cells from becoming tip cells (lateral inhibition) through the induction of VEGFR1 and the down-regulation of VEGFR2. Subsequently, these cells will become trunk/stalk cells and adopt the proliferative behaviour necessary for vessel elongation. In the absence of notch signalling, lateral inhibition is not induced and multiple branching occurs



venous malformations are not restricted to DLL4 mutant animals as they are also observed in Mindbomb (a E3 ubiquitin ligase that target Delta ligands) mutants [71] and in notch target mutants, which have been shown to cause arterial–venous shunts incompatible with embryonic development [47, 72]. During both mouse and zebrafish embryonic development, notch signalling appears to play a key role in the specification of the arterial and venous compartment by modulating the expression of arterial- and venous-specific markers [49, 71, 72]. For example, up-regulation of DLL4 led to an increase in the expression of arterial markers (such as Hey2 and Ephrin B2) and a decrease in expression of venous markers (such as COUP-TFII). In contrast, inhibition of notch signalling had the opposite effect [73, 74], suggesting that the venous phenotype is the default pathway that is actively repressed by notch signalling to induce arteries. Finally, repression of notch signalling by COUP-TFII in the venous compartment phenotype clearly demonstrates that vein formation is also programmed genetically [75]. Interestingly, the angiogenic role of notch signalling in arterial–venous differentiation extends beyond embryonic development since activated Notch4 in adult mice also led to hepatic vascular shunting, arterialization and induction of other notch pathway genes, resulting in lethality within weeks [76]. In addition, the induction of adult arteriogenesis following ischaemia has been shown to be dependent on DLL1 and Notch 1 [77, 78].

Notch signalling regulates angiogenesis by pericyte recruitment and differentiation

Pericytes, also known as Rouget cells or vascular mural cells, are derived from the mesenchyme and are present within the basement membrane of blood vessels. Pericytes are found in a variety of vascular structures such as venules, arterioles and capillaries. The ratio of pericytes to EC varies depending on the type and location of the vessels. Whereas capillaries from the retina and the central nervous system have a high density of pericytes, those located in cardiac muscles show low coverage [79]. Although, the role of pericytes is not fully understood, it appears that they regulate vascular tone, vessel maturation and mediate EC quiescence [80]. Pericytes are also thought to be precursors of vascular smooth muscle cells (VSMC), and recent studies have suggested that, additionally, pericytes could possess stem cell-like properties that allow them to be involved in tissue regeneration [81].

Although, ultrastructural studies suggest that pericytes are embedded in the EC basement membrane, direct contact with the EC also exists through the so-called “peg-socket” contacts, allowing direct cell–cell communication. Several signalling pathways such as TGF- β ,

Tie2/Angiopoietin-1, PDGF-B/PDGFB-R, EphrinB2 and sphingosine-1-phosphate signalling have been involved in vessel pericyte recruitment and heterotypic communication [80]. A number of studies clearly show that heterotypic notch signalling can also directly affect pericyte coverage. The Notch-3 receptor is highly expressed in pericytes and VSMC and disruption of Notch-3 signalling seen in Notch3 $-/-$ mutant mice results in enlarged vessels due to the lack of pericytes [82]. Similarly, patients suffering from CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) syndrome, a pathology associated with mutations of *Notch3*, also present vessels lacking pericytes [83]. The studies related in these reviews demonstrate that Notch-3 is necessary for the formation of fully functional arteries but is not required for viability and fertility. Interestingly, these deficiencies in arterial formation are not due to defects in EC but to deficiencies in VSMC maturation [84]. Two recent papers demonstrate that modulation of DLL4 signalling in tumour models results in both altered EC and pericyte phenotypes [85, 86]. Using the murine sarcoma cell line S180 implanted into adult DLL4 $+/-$ mice, Schemet and co-workers demonstrated that vessels in these tumours have a deficiency in pericyte coverage. However, as previously shown for other aspects of Notch signalling where both high and low signalling result in similar phenotypes, over-activation of DLL4 signalling in a model of U87 glioblastoma also resulted in the formation of large vessels lacking pericytes [85]. Interestingly, mutant PDGFR $-/-$ and PDGFB $-/-$ mice which have impaired pericyte coverage also show enlarged vessels [87], suggesting that pericytes function to restrict lumen size. Although the molecular mechanisms by which notch signalling regulates pericyte recruitment are not yet characterized, recent publications may give some insights. A lack of Ephrin B2 expression in pericytes results in defects in their subsequent recruitment to vessels [88]. Since DLL4/Notch signalling downregulates Ephrin B2 expression in EC [25, 74], it could be interesting to extend this finding to analyse DLL4 signalling in pericytes as it could explain the lack of pericytes observed in tumours overexpressing DLL4 [85].

Notch in vascular pathologies

The multiple roles of notch signalling as a regulator of physiological angiogenesis explain why deregulated notch signalling is often associated with human pathologies. For instance, mutations leading to decreased notch signalling have been linked with cardiovascular and neurovascular related diseases such as CADASIL syndrome, which is

caused by missense mutations in Notch-3. As mentioned previously, affected patients suffer from neurological symptoms resulting from the loss of VSMCs surrounding cerebral arteries [82]. Similarly, haploinsufficiency in Jagged-1 results in cardiac malformation, a complication observed in 60–70% of patients suffering from the Alagille syndrome (AGS) [89]. Finally, ventricular septal defects and tetralogy of Fallot, which account for 15% of all congenital heart disease in newborns, could be caused in some cases by mutations in the notch target Hey-2 [47, 90].

Another complication associated with deregulated notch signalling is preeclampsia. This medical condition occurs during pregnancy and during postpartum and affects both the mother and the unborn baby. Affecting at least 5–8% of all pregnancies, it is one of leading causes of maternal and infant illness and death [91]. A recent publication demonstrated that Notch-1, Notch-4 and Jagged-1 are down-regulated in the placenta vasculature of patients suffering from preeclampsia [92].

Aberrant regulation of the notch pathway has also been observed in various types of cancers such as T-cell acute lymphoblastic leukaemia (T-ALL), breast cancer and ovarian cancer. The tumourigenic property of notch in cancer cells is usually caused by an excess in notch signalling through processes including ligand up-regulation and expression of a truncated and active form of the notch receptor. These high levels of notch activation usually favour cancer cells to survive and proliferate [93]. In addition to this role in the tumour cells, reactivation of notch signalling in the tumour endothelium is strongly associated with vascularization of the cancer. We have shown previously that DLL4 expression in the adult can be detected in the tumour endothelium in clear cell–renal cell carcinomas (CC–RCC), breast tumours and bladder cancer, but was absent from nearby normal blood vessels [32, 50, 94]. Induction of DLL4 in the tumour vasculature is due to increased VEGF expression and/or HIF1 α stabilization, two hallmarks of tumour formation [32, 50]. In turn, increased DLL4 signalling results in faster tumour growth in some but not all tumour models tested by affecting vessel number and functionality [85, 86, 95]. Indeed, the increase in tumour growth observed in a glioblastoma model correlated with a decrease in the number of vessels. However the resulting vessels harboured clear lumens and were more perfused than those observed in control tumours, which would account for the observed increase in tumour growth. Finally, high perfusion also correlated with decreased hypoxia [85].

As shown in a model of prostate cancer [96], pericyte coverage is often chaotic within tumour vasculature and this could allow aberrant notch signalling between EC, stromal and tumour cells [97]. For example, EC notch signalling triggered by Jagged-1 located on head and neck squamous cell carcinoma cells favours tumour growth by

increasing microvessel formation [98]. Interestingly, this study highlighted the opposing effects of Jagged-1 and DLL4 as regulators of vessel number, however their respective modes of action are still not characterized and will require further investigation.

Therapeutic potential

Two alternative therapeutic approaches have been investigated with the aim of blocking notch signalling in vivo. Whereas the first targets notch receptor processing through the use of γ -secretase inhibitors [99], more recent approaches aim to target DLL4 by using either recombinant proteins or specific blocking antibodies. Treatment with the γ -secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) has been shown to inhibit the progression of T-ALL and that of solid tumours such as medulloblastoma [100, 101]. Similarly dibenzazepine (DBZ), which is more effective than DAPT (lower IC50), has been shown to be successful in the treatment of colon cancer in mice [102]. Although, anti-cancer therapies using γ -secretase inhibitors have been effective, they are limited due to the role of γ -secretase in the regulation of other signalling pathways. In addition, the side effects triggered by notch-signalling inhibition in normal tissues may have strong incidence, especially in tissues where notch may act as a tumour suppressor (such as keratinocytes).

The crucial role of DLL4 signalling in tumour development and its vascular specificity has led several groups to selectively target this notch ligand. Two different strategies have been used, including a monoclonal anti-DLL4 antibody and a soluble DLL4 fused to the Fc domain of IgG, both of which can effectively block DLL4/Notch signalling. Remarkably, systemic administration of these DLL4 blockers delayed tumour growth in mice resulting in a better prognosis [85, 86, 95, 103]. Interestingly, blocking DLL4 in these mice resulted in increased blood vessel density within the tumour, which was potentially due to increased branching and EC proliferation. However, the resulting vessels were poorly functional, lacked lumen and resulted in the tumour being hypoxic. Thus, blocking DLL4 results in non-productive angiogenesis that leads to decreased tumour growth and hence constitutes a novel mode of action for targeting angiogenesis [104].

Finally, because of the role of notch signalling in pericyte recruitment, the above therapies may also prove to be beneficial with regards to limiting vessel coverage and therefore stability. Such a mode of action will most likely result in a win-win situation in terms of therapy. Indeed, a treatment increasing pericyte coverage could stabilize the vessels and prevent new blood vessel formation, whereas a decrease in vessel coverage could sensitize the vessels to conventional treatments such as VEGF starvation [105].

Concluding remarks

Publications from the last 10 years have implicated notch signalling in multiple aspects of angiogenesis, such as EC proliferation and survival, regulation of tip versus trunk cells, arterial–venous differentiation and pericyte recruitment. These studies have recently led to the discovery of several types of inhibitors that show exciting responses for blocking tumour growth. Although promising, these inhibitors will need to be studied further in order to determine the dose and scheduling of these inhibitors in mono- and combined-therapies with conventional anti-cancer treatments, as previously performed for anti-VEGF treatments [106]. In addition, it is crucial that we strive to understand the specific role and molecular mechanisms by which each notch ligand regulates angiogenesis. Special interest should be placed on understanding the specificity of Jagged-1 and DLL4 in order to determine their downstream signalling pathways. The vast amount of knowledge acquired in recent years on notch signalling in vascular development should also be applied to treat pathologies in which more functional vessels are required, for example during wound healing and in ischaemic tissues. Finally, highlighting the complexity with which notch signalling regulates vessel formation, a recent study suggested that DLL4 signalling influences macrophage differentiation [107]. Considering the role that tumour-associated macrophages have during tumour vascularization in low oxygen tension [108], this latest study could imply a novel and additional mode of action for notch signalling as a regulator of angiogenesis.

Acknowledgements Research in the laboratory of A.L.H is supported by the Cancer Research-UK. We thank Dr. Seema Grewal for comments on this manuscript.

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