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Notch Signaling in Blood Vessels: Who Is Talking to Whom About What?

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Forkhead Factors in Cardiovascular Biology

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Notch Signaling in Blood Vessels
Who Is Talking to Whom About What?

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***Abstract*—**It has become increasingly clear that the Notch signaling pathway plays a critical role in the development and homeostasis of the cardiovascular system. This notion has emerged from loss- and gain-of-function analysis and from the realization that several hereditary cardiovascular disorders originate from gene mutations that have a direct impact on Notch signaling. Current research efforts are focused on determining the specific cellular and molecular effects of Notch signaling. The rationale for this has stemmed from the clinical importance and therapeutic potential of modulating vascular formation during various disease states. A more complete appreciation of Notch signaling, as it relates to vascular morphogenesis, requires an in-depth knowledge of expression patterns of the various signaling components and a comprehensive understanding of downstream targets. The goal of this review is to summarize current knowledge regarding Notch signaling during vascular development and within the adult vascular wall. Our focus is on the genetic analysis and cellular experiments that have been performed with Notch ligands, receptors, and downstream targets. We also highlight questions and controversies regarding the contribution of this pathway to vascular development. **(*Circ Res*. 2007;100:1556-1568.)**

Key Words: Alagille syndrome ■ CADASIL ■ endothelium ■ smooth muscle ■ vascular development

A common belief in the Notch field is that there are 2 types of biologists: those who work on Notch and those who do not yet realize they work on Notch. Although a priori this may sound pedantic, a close evaluation of the literature quickly provides an observer a vast array of evidence for the significance of Notch signaling in the development, homeostasis, and pathology of all 3 germ layers and their derivatives. Notch signaling has been implicated in cell-fate decisions and differentiation of epithelial, neuronal, bone, blood, muscle, and, more recently, endothelial cells.[1–4] Most frequently, Notch has been shown to impact cell fate either by initiating differentiation processes or by maintaining the undifferentiated state of progenitor cells.[5] In the vascular system, deregulation of this pathway can lead hereditary vascular disorders such as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), Alagille syndrome (AGS), and to tumor development (T-cell acute lymphoblastic leukemias).[6–8] What is particularly remarkable about Notch signal transduction is its "context-dependent" effects. This feature allows for tremendous versatility in the signaling outcome, such that reutilization of Notch at 2 different developmental stages

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within the same cell can result in clearly distinct outcomes. Here, the word "context" relates to the array of extracellular proteins that surround the cell or the relative levels/activity of the proteases that cleave Notch and lead to its activation; it also encompasses the availability and nature of the cytoplasmic and nuclear Notch interactors. Thus, experiments in vivo and in vitro have been difficult to interpret, as the identity of the cell receiving the Notch signal and its specific environment have a direct impact on the consequences of Notch activation. Naturally, this makes our job all the more complicated, but keeps it interesting. Although the contribution of Notch to vascular cells has been appreciated only recently, the efforts of many laboratories have contributed to the following base of knowledge:

- Notch signaling is essential for vascular development.
- Alterations in Notch signaling lead to abnormal vascular development at multiple stages and to various degrees.
- The Notch pathway is tightly regulated, and positive or negative modulation results in vascular pathology.
- Of the 4 receptors, Notch1 and Notch4 are predominant in the endothelium, whereas Notch1 and Notch3 are present in smooth muscle cells. Of the 5 ligands, Delta-like1 (Dll1), Delta-like4 (Dll4), and Jagged1 (Jag1) are the most prevalent in the endothelium, with Jag1 and Jag2 and to some degree Dll1, also being found on smooth muscle cells.
- The Notch pathway is involved in a feedback loop with vascular endothelial growth factor (VEGF), where Notch lies downstream of VEGF and the activation of Notch signaling can downregulate the expression of VEGF receptor 2.
- Notch has an important role in arterial/venous specification and is upstream of EphB4/ephrinB2 signaling.

In this review, we dissect these statements and discuss the implications of more recent findings in Notch signal transduction within the vasculature. In particular, we focus on the possibility for distinct roles of specific ligands in Notch signaling and the contribution of this signaling pathway to the initiation of vascular sprouts.

Notch Signaling Basics

In mammals, there are 4 distinct Notch receptors (Notch1 to 4) and 5 ligands (Jag1 and 2; Dll1, 3, and 4). Unlike most signaling pathways, Notch receptors and their ligands are type 1 transmembrane proteins, and consequently, activation requires cell–cell contact. In general, association between Notch ligands and receptors occurs between cells (homotypic or heterotypic) resulting in *trans*-signaling events. However, binding to receptors in *cis* (ie, within the context of the plasma membrane of the same cell) can also occur.[9,10] Specificity between the ligands and receptors has not been reported. Thus, all 4 vertebrate Notch receptors interact with all 5 ligands. Nonetheless, recent experimental evidence suggests that not all receptor/ligand interactions are productive (ie, result in signaling).[11] This new information opens up the possibility that some ligands might act as negative modulators of Notch signaling.

The activation of Notch requires a series of proteolytic events that are trigged by binding to cell surface receptors. The enzymes implicated in processing include members of the ADAM family (A Disintegrin And Metalloproteinase): ADAM10 (or Kuzbanian) in *Drosophila* and ADAM17 (TACE) in mammals. In addition, a final intramembrane cleavage of Notch receptors is accomplished by γ-secretase. A number of excellent reviews have been written on this subject.[12–14]

Once released, the intracellular domain of Notch (or NICD) translocates to the nucleus where it binds to the transcription factor CSL (for CBF1/Su(H)/Lag-1) (also known as Rpbsuh or RBP-Jκ).[15,16] This interaction converts CSL from a transcriptional repressor to an activator by displacing corepressors and recruiting coactivators. The above series of events describe the classic or CSL-dependent Notch signaling pathway. In addition, several lines of experimental evidence have indicated an alternative, CSL-independent pathway. Support for this pathway comes from genetic work in flies and differentiation assays in mammalian cells. In particular, (1) analysis of Notch and Su(H) mutant phenotypes in Drosophila has shown that the Notch phenotype is slightly stronger than that of Su(H) mutant embryos,[17,18] indicating that not all Notch functions require Su(H); (2) a series of genetic reconstitution studies in Notch mutants showed that a variety of Notch phenotypes in flies cannot be rescued by a complementary approach with Su(H)[19,20]; (3) different regions of Notch appear to be responsible for some, but not other, phenotypes, particularly the region between epidermal growth factor–like repeats 23 to 26 and within the ankyrin repeats[21,22]; (4) in mammalian (C2C12) differentiation assays, truncated forms of NICD (which are unable to activate CSL-dependent promoters) show activity even in the presence of a dominant negative CSL[23,24]; and (5) in Bergmann glia cells, a Deltex-dependent, but CSL-independent, pathway has been recently identified.[25] A more detailed molecular understanding of how Notch selects between CSL-dependent (canonical) and -independent (noncanonical) is lacking. It has been shown that Notch exists at the cell surface either as a heterodimeric form (cleaved by furin in the *trans*-Golgi) or as an intact (colinear) protein. It appears that canonical and noncanonical signaling pathways are activated downstream of these 2 physically distinct Notch receptors in response to ligand binding.[26]

Lessons from Loss- and Gain-of-Function

Morphogenesis of the vascular system requires a highly structured sequence of events that relies on the correct spatial and temporal expression of specific gene networks, leading to the development and remodeling of a primary vascular network.[27]

Gene inactivation strategies in mice have shown that Notch signaling is critical for the reorganization of vessels that derive from the primitive vascular plexus (Figure 1). Several Notch pathway mutants arrest following development of the plexus, indicating that Notch becomes critical at the stage of vascular remodeling during which the primitive plexus evolves into a hierarchic network. Notch may also contribute to events before this stage. Indeed, some results support the

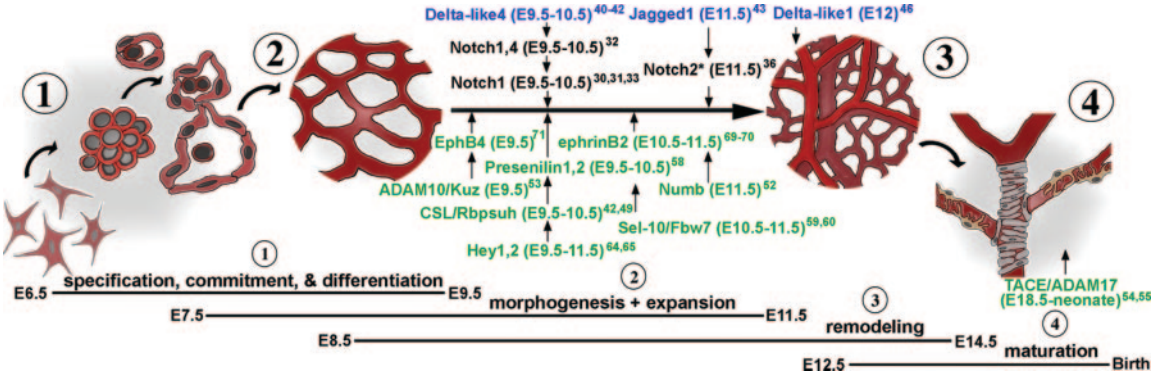

Figure 1. Genetic inactivation of Notch ligands receptors, downstream effectors, and modulators leads to embryonic lethality as a result of vascular defects. The vascular system develops from mesenchymal progenitor cells that differentiate into hemangioblasts (1) and subsequently form the primitive vascular plexus. (2) Later, this uniform network remodels into a hierarchical vascular system. (3) It is at this stage that the functional consequences of Notch signaling are most notable. As indicated, inactivation of several Notch receptors, ligands, and genes associated with Notch signaling result in embryonic lethality at the developmental stages indicated in parenthesis. Loss-of-function experiments have not been done during vascular maturation. (4) The 4 stages in vascular morphogenesis are indicated at the bottom of the figure, where they are temporally correlated with the embryonic stage (in the mouse). These stages are: (1) specification, commitment, and differentiation of endothelial cells (E6.5 to E9.5); (2) morphogenesis and expansion of the vasculature (E7.5 to 11.5); (3) remodeling (E8.5 to E14.5); and (4) maturation (E12.5 to birth). Note that the stages overlap, as developmental progression varies depending on the tissue/organ examined. *Mice with mutations of Notch2 at the ankyrin repeats were not observed to have overt vascular defects.

participation of Notch in cell-fate decisions in the hemangioblast stage, affecting the assignment of endothelial and hematopoietic fate.[28,29]

Embryos homozygous for a null allele of Notch1 die by embryonic day 9.5 (E9.5) with defects in somitogenesis and severe cardiovascular anomalies.[30–32] Cre-mediated deletion of Notch1 in the endothelium results in embryonic lethality at a similar time in development, demonstrating that arrested growth is tied to loss of this gene in vessels/endocardium.[33] In addition, Notch is an essential contributor to vascular integrity and homeostasis, as removal of Notch at later stages of development results in vascular rupture with systemic hemorrhage (J.A. Alva, J.J. Hofmann, A.C. Zovein, M. Gillufo, F. Radtke, D. Bachiller, G. Weinmaster, M.L. Iruela-Arispe, unpublished results, 2007). The involvement of the Notch pathway in vascular disorders such as CADASIL and AGS also hint at this role.[4,34] Later in this review, we expand on the contribution of Notch in mature vascular beds.

Expression of Notch2 has not been reported in vascular cells; however, the development of a hypomorphic allele for this gene revealed its participation in the hyaloid vasculature of the eye and glomerular capillary tuft formation.[35] Hypomorphs for Notch2 showed a bulbous structure in the region where the hyaloid artery branches into the surface of the lens capsule. In addition, Notch2 mutant mice exhibit anomalies in glomerular capillaries. Approximately 25% of these glomeruli have distinct aneurisms instead of capillary tufts.[35] Notch2 hypomorphic mice also display generalized edema, although it is not clear whether this is a consequence of a role for Notch2 in lymphatics or a result of its effects in the heart.[35] Together these findings indicate that Notch2 is critical for the vascular morphogenesis of a more selective group of vascular beds. A second mouse mutant of Notch2 with insertion of LacZ at the ankyrin repeats is lethal by E11.5.[36] Although these investigators did not report vascular phenotypes, the combination of an early lethality and the fact that the mutant mice displayed widespread apoptotic cell death is suggestive of vascular anomalies, at least within extraembryonic tissues such as the yolk sac and the placenta.

Although the Notch3-null mouse is viable and fertile,[37] a detailed analysis revealed that this gene is necessary for the differentiation and acquisition of arterial identity of vascular smooth muscle cells.[38] Absence of Notch3 results in enlarged arteries with abnormal distribution of elastic laminae.[38]

Loss-of-function experiments for Notch4 showed no defects in homozygous mutant mice, but the compound homozygous loss of Notch1 and 4 had a more pronounced vascular phenotype than embryos homozygous for the Notch1 receptor alone.[32]

Overexpression of constitutively active Notch4 in an endothelial-specific manner has also been reported.[39] Specifically, the investigators inserted an active form of Notch under the regulation of the VEGF receptor 2 promoter. Mutant embryos displayed disorganized vascular networks and produced dilated vessels, suggesting sensitivity to elevated levels of Notch expression.

Several Notch ligands have been inactivated in mice. A null mutation of Dll4 results in haploinsuficiency with lethality at E9.5. The degree of haploinsuficiency is background-dependent, indicating the contribution of modifier genes. As with Notch1 mutants, Dll4 mice fail to remodel the primitive vascular plexus, a finding that was evident in both the yolk sac and in the embryo proper.[40–42] In addition, the phenotype had several other similarities with Notch1-null mice, including stenosis of the large arteries, defective arterial branching, and enlargement of the pericardial sac.[40–42] These findings suggest that Dll4 is an early and critical ligand for Notch1 signaling in the vasculature. These studies also highlight the importance of dosage in Notch signaling.

The other Notch ligand to display embryonic lethality with significant vascular defects is Jag1.[43] Embryos lacking a functional Jag1 gene die at E10.5, with vascular anomalies

that include lack of remodeling and absent vitelline vessels.[43] Although the lethality of the Jag1 knockout occurs 1 to 2 days after that of Notch1 knockout, defects in vessel hierarchy in the vascular plexus and collapsed vessels in the head are reminiscent of the Notch1-null phenotype and implies that Jag1 also contributes to the remodeling of the primitive plexus.[43] The differences between the time of lethality and the nature of the vascular defects would suggest that Dll4 and Jag1 are not functionally overlapping, nor are these genes redundant, at least early in development.

Although expression of Dll1 in the vasculature is not predominant, this ligand is expressed in the endothelium of both arteries and veins during development.[44,45] More importantly, targeted disruption of Dll1 also leads to early lethality (by E12) with generalized hemorrhagic events.[46] In adult mice, Dll1 regulates the expression of ephrinB2 and plays an important role in arteriogenesis associated with vascular growth in postischemic events.[47] It is likely that Dll1 activates Notch1 during this process, as reduction in Notch1 also has been shown to minimize the vascular response in limb ischemia models.[48]

Mice lacking CSL displayed defects similar to the Notch1/Notch4 double knockout with severe growth retardation[49] and a primitive vascular plexus lacking vessel remodeling.[42] No embryonic vessels were seen penetrating the placental labyrinthine layer, and arterial marker expression was lost.[42] Additionally, an endothelial-specific knockout of CSL also resembled the Notch1, Notch1/Notch4, and Dll4 knockout phenotypes with arteriovenous malformations, pericardial effusion, and the absence of vascular remodeling, demonstrating that regulation of specific levels of Notch activity is necessary for proper vascular development.[42]

A negative regulator of Notch signaling, Numb (m-numb in mouse), is known to be involved in progenitor cell division during neurogenesis. The manner in which Numb inhibits Notch is still unclear, although m-numb–deficient mice, as well as double-knockout mice for m-numb and its homolog Numblike (numbl), displayed neural defects similar to Notch mutants.[50–52] In the vascular system, however, only the m-numb–null mice showed abnormalities. These mice died around E11.5 and had severe hemorrhaging, which was thought to be the cause of death.[52]

Inactivation of genes required for Notch cleavage also results in developmental arrest at similar times as null mutations in receptors and ligands. As mentioned previously, a role for the ADAM family in ligand-induced proteolytic cleavage of the Notch receptor has been suggested.[4,12] Mice deficient in ADAM10 (Kuzbanian) are embryonic lethal at E9.5 and also have a variety of defects in the cardiovascular system, central nervous system, and in the somites. The vascular defects are reminiscent of other perturbations in Notch signaling molecules.[53] Unlike its contributions in *Drosophila*, the role of ADAM10 in mammalian Notch signaling has not been fully explored; however, these genetic inactivation experiments support a function for ADAM10 in Notch cleavage.

Tumor necrosis factor-α–converting enzyme (known as TACE or ADAM17) has also been implicated in the processing of the Notch extracellular domain.[14] Although the initial analysis of the TACE knockout mice, which die between E18.5 and birth, did not demonstrate a phenotype similar to Notch mutants,[54] a subsequent investigation revealed cardiovascular defects, such as an enlarged heart with thicker trabecular layers and increased cell proliferation.[55]

In addition to ADAM10/ADAM17, the release of the NICD requires an additional cleavage by γ-secretase, which is mediated by the presenilin genes (PS1 and PS2).[56,57] This proteolytic event occurs within the plasma membrane, and it is critical for Notch activation.[14] Inactivation of PS1 did result in embryonic lethality, but at a later embryonic stage than Notch1 deficient mice. In contrast, PS2 null mice were viable and fertile.[58] However, PS1 and PS2 double-knockout die between E9.5 and E10.5. Vascular anomalies in the double-mutant embryos included a lack of organization of vitelline vessels, a "blistered" looking yolk sac, the absence of blood circulation, and an enlarged pericardial sac. The similarities to the Notch knockout mice are consistent with an essential requirement for the presenilins in Notch signaling.[58]

The degradation of Notch, resulting in the inactivation of this signaling pathway, is also vital to the proper maintenance of blood vessels. Mice lacking a component of an stem cell factor–type ubiquitin ligase, Fbw7 (also known as Sel-10), fail to regulate levels of NICS.[59,60] These mice die between E10.5 and E11.5, with defects in vascular remodeling in the brain and yolk sac and the absence of major veins. It is anticipated that Fbw7 regulates other proteins in addition to Notch, thus it is not entirely surprising that some of these abnormalities (absence of major veins) do not phenocopy Notch inactivation. Furthermore, based on its function, inactivation of Fbw7 should lead to a Notch gain-of-function phenotype; in fact, Fbw7-null mice showed increased levels of Notch1, Notch4, and Hey1 (HERP2), suggesting that effective protein turnover and proper levels of Notch are required for the integrity and formation of vessels.[60]

Inactivation of downstream targets of Notch signaling also can lead to embryonic lethality and vascular defects reminiscent of Notch-deficient mice. Disruption of the basic helix–loop–helix transcription factor Hey2 revealed a role in heart development, although the majority of the knockout mice survived until a week after birth.[61–63] Double-knockouts of Hey1 and Hey2 (also known as HERP, HESR, HRT, and CHF) showed embryonic lethality between E9.5 and E11.5 and suggested some redundancy between these genes. Hey1/Hey2 double-null mice display vascular defects typical of a disruption in Notch signaling, such as hemorrhage, defects in arterial/venous specification, enlarged pericardial sacs, heart abnormalities, lack of vessel remodeling, and enlarged vessels in the embryo and yolk sac, as well as a failure to organize vessels in the placental labyrinth.[64,65] These results indicate the requirement of Hey1 and Hey2 in transducing the Notch signal within the vascular compartment.

As is discussed in more detail later in this review, the EphB4 receptor and corresponding ligand, ephrinB2, are important for arterial/venous specification.[66–68] EphrinB2 was found to be expressed in arteries, whereas EphB4 was located in veins, and the inactivation of either gene resulted in early embryonic lethality with similar vascular abnormalities.[69–71] The link between EphB4/ephrinB2 and Notch was

provided by Lawson et al in experiments performed in zebrafish. This work demonstrated that Notch acts upstream of EphB4/ephrinB2 and is necessary for the expression of artery-specific genes and the subsequent repression of venous-specific genes in arteries.[72] Mice deficient for EphB4 die around E9.5 and have fusion in the cranial vessels, in the branches of the anterior cardinal vein, and in the intersomitic vessels. Vessels of the yolk sac are also not remodeled from the primitive plexus into the hierarchal vascular branches.[71] Although ephrinB2-null mice die slightly after EphB4 mutant mice, between E10.5 and E11.5, their phenotype is almost identical, with defects in both arteries and veins.[69,70]

In summary, the importance of Notch signaling in vascular morphogenesis has been highlighted by the severity of the phenotypes resulting from genetic ablation targeting receptors, ligands, and regulatory molecules of this pathway.[4,34,73] The common theme is that inactivation of the Notch signaling pathway prevents the transition from the primitive vascular plexus to the hierarchical progression of a defined highly branched network of arteries, capillaries, and veins (Figure 1). The cellular events that participate in this transition include sprouting angiogenesis, selective regression of vascular segments and maintenance of others, incorporation of smooth muscle/pericyte in larger vessels, and progressive differentiation of arterial, venous, and capillary phenotypes. As is discussed below, the contribution of Notch signaling to sprouting, arterial specification, and endothelial cell proliferation has provided some mechanistic explanations for the outcome of the loss-of-function experiments.

Adult neovascularization is also altered by deregulated Notch signaling. A number of recent reports have indicated that suppression of Notch signaling either genetically or pharmacologically (presenilin inhibitors or Dll4 antibodies) leads to increased endothelial proliferation with excessive vascular branching.[74–77] Resulting vessels, however, display defective maturation with either reduced or absent lumen. The outcome is the expansion of a nonfunctional vascular network that, although extensive in endothelial number, does not support tissue perfusion and tumor expansion.[75–77] In contrast, activation of Notch signaling in tumors by overexpression of Jag1 has been shown to promote angiogenesis and stimulate tumor growth.[78]

Expression Mapping of Receptors and Ligands in the Vasculature

Notch receptors are expressed in both the endothelium and in vascular smooth muscle.[4,34,73,79] Notch1 and Notch4 have been detected in endothelial cells. Notch1 is also frequently observed in arterial smooth muscle cells during development and in the adult (Figure 2).[4,73,79] Notch3 expression appears specific to vascular smooth muscle.[80–83] Whereas most analyses have highlighted the preponderance of Notch receptors and ligands in the arterial vascular tree, several publications have also indicated the presence of Notch ligands and receptors in the capillary network, as well as in veins (albeit with much lower frequency).[45,82–89]

Notch ligands are present in both endothelial and smooth muscle cells. Varying levels of Jag2 have been reported in the endothelium, both in the embryo and in adults, before and after arterial injury.[81,90] In contrast, Dll3 has not been detected in the vasculature. The expression of these ligands appears dynamic and can differ both temporally and spatially. During early development, the first ligand to be expressed in a robust manner is Dll4, followed by Jag1.[45,79] The timing and location of Dll4 expression most closely mimics that of Notch1. The phenotype of the Dll4 knockout mice is more severe than that of Notch1 knockout mice, and it resembles the Notch1/Notch4 double knockout,[32,40–42] suggesting a requirement for Dll4 as a ligand for both receptors. Transcripts for Dll4 have been observed in most capillary beds at midgestation,[83] and, in the retina, Dll4 expression highlights the cells at the end of capillary sprouts (tip cells) (Figure 2).[45,74,91] At later developmental stages, Dll4 segregates to the arterial components of the vascular tree,[45,92] and it is generally considered a marker of arterial cell phenotype.[66] In the arteries, Dll4 is expressed in the endothelium, although lower levels can also be detected in smooth muscle cells.[45] Dll4 is re-expressed at times of neoangiogenesis, including in certain models of cancer growth.[75–77]

Jag1 is not as prevalent in capillaries as Dll4, and its distribution is complementary rather than overlapping with that of Dll4. In the retina, Jag1 is excluded from tip cells, but it is clearly present in stalk cells.[45] During vascular remod-

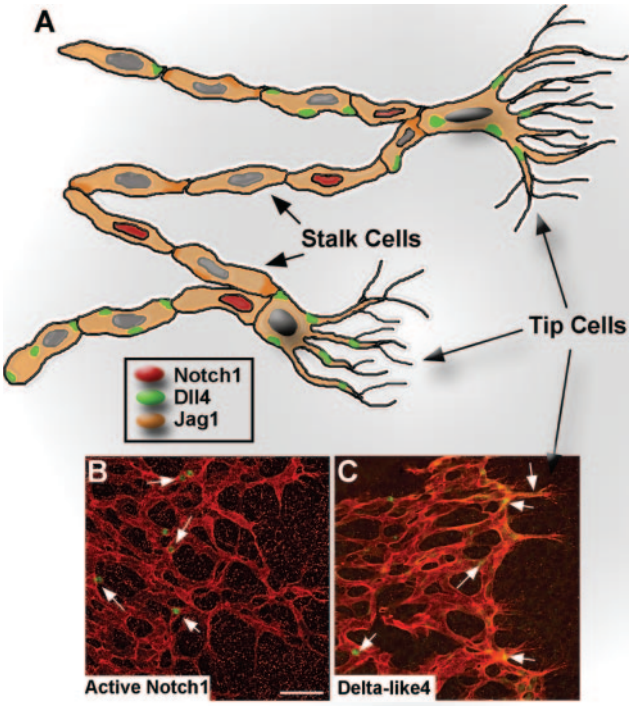

Figure 2. Active Notch signaling during angiogenesis. A, Schematic showing the relative distribution of active Notch1 (red), Delta-like4 (Dll4) (green), and Jagged1 (Jag1) (orange) in tip and stalk cells of angiogenic sprouts.[45] B, The pattern of active Notch (green, arrows) (Val1744 antibody; Cell Signaling Technology) in the developing vasculature of postnatal day 7 mouse retinas, stained with platelet endothelial cell adhesion molecule (PECAM) (red) (CD31; Pharmingen), is scattered throughout the plexus and in stalk cells. C, In contrast, Dll4 expression (green) (mDll4 antibody; R&D Systems) at postnatal day 7 solely marks the tip cells at the leading edge of the vascular front but is also found throughout the homogeneous vascular plexus before remodeling (arrows). Scale bar, 50 μm (B and C).

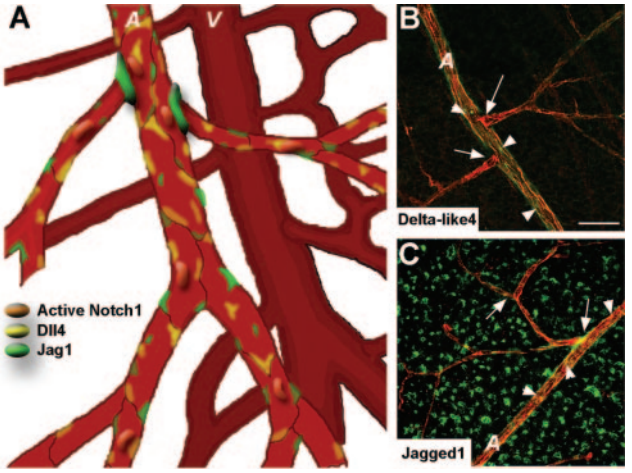

Figure 3. Distinct patterns of Delta-like4 and Jagged1 may indicate specific and nonoverlapping roles in the remodeling of vessels. A, Schematic representation of a medium-size artery (A) and vein (V) showing distribution of Notch1, Dll4, and Jag1. B, Immunostaining of postnatal day 15 mouse retina stained with PECAM (red) and Dll4 (green) reveals the presence of Dll4 in the endothelium (arrowheads), but a lack of Dll4 at the branching points (arrows). C, Whole-mount immunocytochemistry of a postnatal day 15 retina showing the distribution of PECAM (red) and Jag1 (green) (PCR8; gift from Gerry Weinmaster, University of California, Los Angeles). Branch points (arrows) tend to be frequently labeled with Jag1 at this stage. Colocalization with PECAM indicates that some of Jag1 staining is endothelial (arrowheads), but some is also smooth muscle (arrows). Scale bar, 50 μm (B and C).

eling, Jag1 expression was noted in both the endothelium and smooth muscle cell layer.[45,83] Interestingly, Jag1 was not detected in visceral smooth muscle.[83] In addition, Jag1 was found associated with branching of medial arteries in the retina, a site where Dll4 was clearly absent.[45] Figure 3 illustrates the relative distribution of Dll4 and Jag1 in differentiated arteries at stages that immediately follow remodeling in the retina (postnatal day 15 in the mouse). The subtle differences in timing, location of expression, and loss-of-function phenotypes for these 2 Notch ligands (Dll4 and Jag1) suggest that these receptors are not functionally overlapping and support the notion that each might convey independent signals to accomplish events associated with vascular remodeling. Although this statement requires further experimental support, it is intriguing that examples have emerged indicating that Notch ligands might confer distinct signals through Notch1, even in identical cells. For example, during T-cell differentiation, the activation and proliferation of T helper cells is differentially regulated by Jag1, Dll1, and Dll3.[93] Therefore, it is conceivable that, as in immune cells, Notch signal transduction in endothelial cells could differ depending on the nature of the ligand.

Dll1 expression in the endothelium is found in both arteries and veins of midgestational embryos.[44] In the retina, Dll1 is also detected through the vascular plexus, but, unlike most Notch-related proteins, Dll1 is retained in veins after remodeling.[45] In adults, Dll1 has been detected in arterial endothelium, but it is absent in the endothelium of veins and capillaries.[47]

Although the distribution of receptors and ligands is important, a faithful map of Notch activation will aid in obtaining a more comprehensive understanding of when and where Notch is relevant. This has become possible by the development of neoepitope antibodies. Because Notch requires proteolytic cleavage for its activation, the exposure of this cleavage site has been explored for the generation of antibodies that recognize cleaved (ie, active) Notch. We used this antibody to localize active Notch signaling during retinal development and found that Notch activation is more frequent in the plexus before hierarchic remodeling, and in the stalk cells of a vascular sprout.[45] In addition, active Notch1 was also detected, albeit less frequently, in fully differentiated arterial vessels.[45] Interestingly, active Notch is also identifiable at later stages of development, in neonates, and in the adult (Figure 4). These findings clearly highlight the significance of the Notch signaling pathway during all stages of life.

Tip Versus Stalk: The Contribution of Notch in Endothelial Cell Fate

During angiogenic expansion, vascular sprouts are guided by the migration of tip cells in response to graded distributions of matrix-bound VEGF.[94,95] Tip cells are the most terminal cells in a vascular sprout and display morphological and functional features distinct from stalk cells. For example, tip cells migrate but do not proliferate in response to VEGF, whereas stalk cells proliferate in response to this growth factor. The combined effect of migration by tip cells and proliferation by stalk cells results in vascular growth.[95] These observations have revealed cellular differences within an actively growing capillary sprout (ie, tip versus stalk cells) and raise a crucial question: what determines tip/stalk cell fate? The question is important, as tip cells comprise a key cellular determinant of angiogenesis.

A relationship between Notch signaling and endothelial tip cells was first established by expression analysis. In situ hybridization of developing retinas demonstrated the prevalent expression of Dll4 transcripts in tip cells.[91] This was also supported by visualization of Dll4 protein.[45,74] Notch1 is frequently absent in tip cells, but is prominently expressed in stalk cells that are in close proximity to the tip cell (Figure 2).[45] Together the results suggest that Dll4 expression in the tip cell signals to Notch1 in the adjacent stalk cells. Recently, many groups have independently demonstrated that the Dll4-Notch1 signaling axis does indeed coordinate fate specification at the end of angiogenic sprouts.[74–77,96–99]

Genetic and pharmacological inactivation of Dll4-Notch leads to the formation of a highly branched and dense vascular network.[74–77,96–99] In the absence of Notch signaling, endothelial cells display excessive cellular extensions (filopodia) analogous to tip-like cells.[74–77,96–99] Nonetheless, the vascular structures formed by these endothelial cells are often not fully lumenized, generating nonproductive vessels that are inefficient in delivering oxygen to target tissues.[75–77] Combined, the findings indicate that both permissive and suppressive signals within the growing sprout are required for the formation of an effective vascular network.

Notch in Arterial/Venous Specification

Accumulating experimental data provide evidence that arterial and venous identity is established early in development

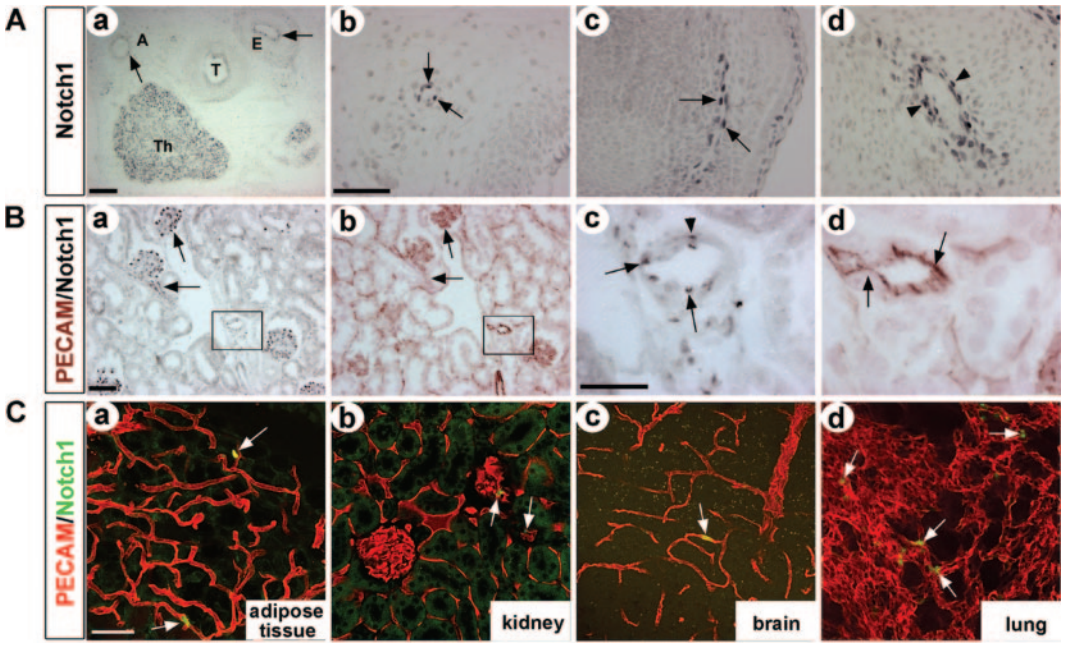

Figure 4. Identification of active Notch at late fetal stages and in the adult vasculature. In all panels, active Notch1 was identified by immunohistochemistry using an antibody that recognizes a neoepitope exposed once the receptor has been cleaved (ie, activated) (Val1744 antibody). A, Immunohistochemistry of active Notch revealed by peroxidase in mouse sections at E14.5. Antigen retrieval was used to expose the neoepitope. Aa, Notch1 activity is widespread at this developmental stage, as shown in the thymus (Th), trachea (T), esophagus (E), and large arteries (A). Ab through Ad, Endothelial (arrows) and smooth muscle cells (arrowheads) showed active Notch in a salt-and-pepper pattern. B, Active Notch (Ba and Bc) is shown in black and PECAM staining in adjacent sections (Bb and Bd) is shown in red. Ba and Bc, In the kidney of adult mice, active Notch1 was detected in the glomeruli and vessels (arrows). Bb, Adjacent kidney sections were stained with an endothelial marker (PECAM) (arrows). Bc and Bd, Higher magnification of boxes from (Ba and Bb), respectively. Endothelium (arrows) and smooth muscle cells (arrowhead) are indicated. C, Immunofluorescence of adult tissue vibratomed and stained for cleaved Notch (green) and PECAM (red). Ca, Vasculature in adipose tissue. Cb, kidney. Cc, brain. Cd, Lung showed detectable active Notch levels (arrows) in the endothelium. Scale bars: 100 μm (Aa), 50 μm (Ab through Ad, Ba and Bb, Ca through Cd), 25 μm (Bc and Bd).

through the contribution of specific transcription factors. Arterial fate is acquired by the combined effect of the forkhead transcription factors Foxc1 and Foxc2 and VEGF signaling.[72,100,101] Simultaneous inactivation of both Foxc1 and Foxc2 results in arterial/venous shunts and a lack of arterial markers, whereas upregulation of either transcription factor results in increased expression of Dll4, Notch, and ephrinB2.[100] In contrast, vein identity is regulated by the orphan nuclear receptor COUP-TFII through the repression of Notch1.[102]

Supporting the concept of Notch1 signaling in arterial specification, Dll4 mutants showed a lack of arterial markers and activation of venular markers in the aorta.[40–42] Furthermore, as mentioned previously, a study of double mutants for the downstream genes, Hey1 and Hey2, in mice has shown reduction in arterial markers in the mutant aortas and lethality by E9.5.[64,65] In zebrafish, a mutant of gridlock (the only Hey-related gene in zebrafish) shows coarctation of the dorsal aorta.[103] Suppression of gridlock expression abolishes arterial markers and expands contiguous regions of the vein.[104] Importantly, overexpression of gridlock does not drive expression of arterial markers in veins, implying that this gene acts to repress venous fate rather than actively promote arterial specification.[104] The contribution of gridlock is still controversial, as others have suggested that Notch imparts arterial specification in a manner that is independent from that of gridlock.[101]

Combined, the data from zebrafish and mouse show both consistencies and differences in arterial/venous specification. The zebrafish data would indicate that a venular phenotype is the "default" state and that expression of Notch represses the vein phenotype. Thus, the absence of Notch would result in expression of vein markers in arteries. By contrast, the expression of COUP-TFII is required to repress Notch and initiate the "venous" genetic program in mice, indicating that the arterial phenotype is the default state. Nonetheless, experiments in these 2 model organisms agree that genetic predetermination through Notch contributes to the specification of the arterial fate.

The endothelium of arteries (aorta, carotid artery, vitelline artery, umbilical artery) exhibit a different genetic expression profile (including Notch 1, neuropilin1, and ephrinB2)[69,105] than veins (EphB4 and neuropilin2).[69,105] This distinction is set up before significant hemodynamic flow and thus indicates a genetic program that distinguishes endothelial cells, at least in the large vessels of the embryo.[69,106] Interestingly, recent data in the chick have clearly demonstrated that blood flow can alter this "genetic predisposition" of vessel identity.[66,106] Alteration of blood flow was followed by a change in the expression profile and the de novo expression of arterial genes within venous tracts. The findings are also in concert with a large body of evidence in mouse and humans that demonstrates the ability of vessels to adapt to flow volume and hemodynamic forces.[107–110] Collectively, the data gath-

ered thus far indicate that although endothelial cells appear to respond to predetermined arterial or venous patterns (based on their location in the embryo), there is a certain level of plasticity with respect to local cues imposed by physical forces, such as hemodynamics. Future studies combining hemodynamic and genetic alterations will likely elucidate the links between these 2 parameters at the cellular level.

The Notch Pathway and Hereditary Cardiovascular Pathologies

In addition to its contribution to early vascular morphogenesis and arterial-fate specification, the Notch signaling pathway also impacts vascular homeostasis. In fact, the connection between Notch and the vasculature was first recognized when mutations in members of the pathway were found to be responsible for certain late-onset hereditary vascular anomalies in humans. CADASIL is a disease manifested in the mid-30s and characterized by strokes, migraines, and progressive dementia.[111] CADASIL has been linked to mutations in Notch3, resulting in progressive degeneration of the smooth muscle layer surrounding cerebral and skin arterioles.[112,113] Histologically, the arteriopathy shows destruction of the medial layer (smooth muscle layer) of arteries and substitution of these layers with connective tissue, leading to fibrosis and narrowing of the lumen.[114] Although the initial characterization was focused on brain arteries, the disease affects small-medial arteries systemically.[113] Before complete fibrosis of the media, deposits of a nonatheromatous, nonamyloidotic nature are noted and visualized because of their granular osmiophilic features under electron microscopy.[113] The molecular explanations for these cellular outcomes are still under investigation; however, the current model suggests that unpaired cysteine residues in the mutated epidermal growth factor repeats of Notch3 result in abnormal conformation and accumulation of the ectodomain of the receptor at the cell surface.[115] This is consistent with immunocytochemical findings demonstrating high levels of Notch3 extracellular domain in the granular deposits, also referred to as "GOMs" (granular osmiophilic materials), located in the cytosol of smooth muscle cells in small arteries.[80,116]

Recently, a mouse model for CADASIL has been developed. The transgenic mouse consists of the mutant form of the Notch3 coding region (Arg90Cys) under control of a smooth muscle–specific promoter SM22.[117] The authors have reported alterations in cell–cell and cell–matrix adhesion, followed by the appearance of granular deposits and cell death.[117] This is consistent with previous studies showing that activation of Notch3 provides cytoprotection by increasing the levels of c-FLIP, a caspase inhibitor that blocks apoptosis mediated by FasL.[118] Nonetheless, a more complete interpretation of these data in the context of the disease is complicated by the fact that Notch3 is classically a very poor activator of the standard downstream signaling targets, such as Hes1. Other investigators, however, have shown that in smooth muscle cells, Notch3 is able to activate Notch target genes, suggesting that perhaps contextual differences between cell types are important to consider.[119–121] Recently, Notch3 has been shown to strongly activate Hes5 reporters in combination with a Zinc-finger transcription factor.[122]

Evaluations of the mutations found in CADASIL have shown a broad spectrum in ligand-binding and ligand-induced signaling through the canonical pathway. This lends credence to the interpretation that CADASIL mutations may be gain-of-function instead of loss-of-function mutations, something that is still under debate.[123,124]

AGS is an autosomal dominant disorder that has been attributed to mutations in Jag1.[125,126] Patients with AGS exhibit abnormally formed blood vessels, arterial stenosis, and heart disease, in addition to hepatic lesions and skeletal defects.[127–129] In approximately 70% of the cases, patients are either haploinsufficient or have truncations in the Jag1 gene.[125,130] Sequence analysis of several Jag1 mutations has revealed little additional information as to the spectrum of phenotypes observed. In an evaluation of 230 AGS patients, it was determined that roughly 4% displayed deletion of the entire Jag1 gene. The large majority (49%) had protein-truncating mutations (frameshift and nonsense); 9% had splicing mutations; 9% showed missense mutations and 31% of the patients did not show Jag1 mutations.[130] Other studies have tried to correlate the type of mutation with the specific clinical presentation of the disease, but this has proven difficult to tease out. In fact, that heterozygous mice for the Jag1 allele do not display any of the abnormalities associated with AGS,[43] indicating that haploinsuficiency of Jag1 alone is unlikely to be responsible for this complex disorder. More recently, animal models have indicated that Notch2 might be an important modifier gene in AGS. In particular, double transgenic mice that are heterozygous for Jag1 and carry a Notch2 hypomorphic allele display congenital anomalies in the liver, heart, and kidney that are consistent with the abnormalities in AGS.[131] Interestingly, patients with clinical manifestations of AGS, but who were negative for Jag1 mutations, were further evaluated for mutations in Notch2. The investigators found Notch2 mutations in 2 families and identified 5 affected individuals.[132] This information has clarified differences between the clinical and genetic aspects of the disease and has opened new possibilities for therapeutic exploration.

Notch and Vascular Homeostasis

Notch activity continues beyond the period of vascular morphogenesis and can be detected in adult tissues. As discussed previously, we have found evidence that Notch1 is active in nonpathological adult vessels, both in endothelial cells and vascular smooth muscle cells (Figure 4). Others have also noted significant upregulation of Notch1 and Jag1 after vascular injury.[81] This information, together with the late-onset incidence of CADASIL, suggests that Notch1 signaling might play a role in vascular homeostasis.

Notch activation in the vasculature occurs through 4 potential mechanisms: (1) neighboring endothelial cells (homotypic trans-activation); (2) the same endothelial cell (homotypic *cis* activation/inhibition); (3) smooth muscle cells (heterotypic); and (4) via interaction with the microfibrillar proteins (MAGP1 and MAGP2) that are often associated with fibrillin in elastic fibrils (Figure 5). Expression of Notch receptors and ligands (Jag1 and Dll4) has been identified in both the endothelial cell and the smooth muscle of medial-

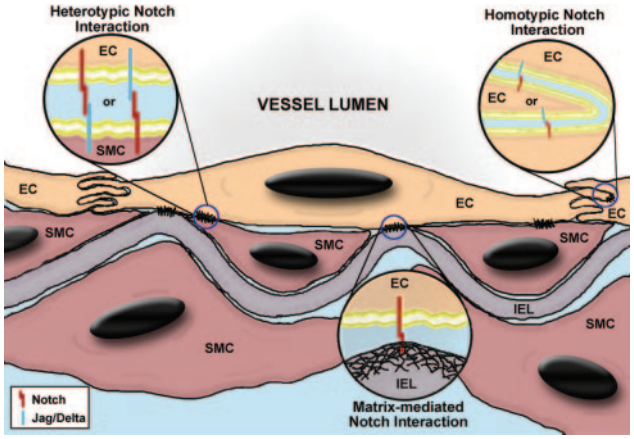

Figure 5. Notch signaling in the adult vascular wall. Drawing highlights various modes by which Notch can be activated. Expression of both receptors and ligands in endothelium (EC) and smooth muscle (SMC) at different stages of development can result in heterotypic Notch activation (shown in the top left inset). Alternatively, Notch receptor/ligand interaction can occur in a homotypic manner both in *cis* or *trans* in neighboring endothelial cells (seen in the top right inset). Interestingly, MAGP1 and MAGP2, often present on microfibrils of elastic tissue such as the inner elastic lamina (IEL), can result in the dissociation of the Notch1 extracellular domain and the activation of downstream signaling (seen in lower inset).[138]

size arteries.[32,45,73,81] The significance of each of these interactions is still unclear; however, specific inactivation of Notch ligands and receptors in a cell-specific manner will shed light into understanding their function.

The contribution of Notch1 to vascular homeostasis still remains to be fully understood. One possibility is a role in endothelial cell survival. Activation of Notch in endothelial cells that have been deprived of serum has been shown to prevent apoptosis.[133] Therefore, Notch might be important in suppressing cell death mediated by various insults in vivo. Pertinent to this point, activation of Notch4 prevents apoptosis in endothelial cells exposed to lipopolysaccharide, through upregulation of Bcl2 (an antiapoptotic factor) and by blocking c-Jun N-terminal kinase activation.[134] A second possibility is that Notch is required in adult vessels to retain the differentiated, nonproliferative state of the endothelium. Activation of Notch1 has been shown to block endothelial cell proliferation in a cell autonomous manner.[135] This process requires the initial blockade of $p21^{CIP1}$ upregulation and the blockade of pRb phosphorylation by cyclin D-cdk4 complexes.

Notch-dependent downregulation of $p21^{CIP1}$ suppresses the translocation of cyclinD-cdk4 to the nucleus, affecting entry in to S-phase and reducing proliferation. Supporting these data, confluence of endothelial cells is associated with activation of Notch and downregulation of $p21^{CIP1}$. Inhibition of Notch1 in confluent cultures results in pRb phosphorylation and increased levels of $p21^{CIP1}$.[135] Although these studies are compelling, if Notch were to be required to maintain constant endothelial quiescence in vivo, one would anticipate high levels of active Notch1 in adult vessels. Although the current data show that this is not the case (Figure 4), it is possible that Notch expression is highly dynamic and that technical limitations of the current probes prevent a complete examination of the transient dynamics of Notch signaling.

The third possibility, albeit not exclusive of the previous 2, is a constitutive role for Notch in the maintenance of the arterial phenotype. Data from several laboratories have provided validity to the concept that the intrinsic differences between arteries and veins are functionally important and likely to prevent the development of pathology.[136,137] Thus, the expression profile of arterial genes enables these cells to sustain the intermittent high pressure of the blood. In contrast, venous endothelial cells are molecularly more receptive to leukocyte interactions. These inherent genetic differences are maintained in arteries and veins. However, as previously discussed, a vein can become "arterialized" when exposed to arterial hemodynamic flow,[109] indicating a certain degree of plasticity in differentiated endothelium. It is possible that Notch may act as a mechanosensor and molecular regulator for this plasticity.

Interactions of Notch with microfibrillar proteins MAGP1 and MAGP2 have been noted and may provide yet another source of regulation in vascular tissues.[138] MAGP1 and MAGP2 are secreted proteins associated with elastic microfibrils. Both MAGP proteins can interact with the epidermal growth factor–like repeats of Notch and activate Notch1 signaling in vitro (Figure 5).[138] Binding of Notch1 to MAGP2 activates Notch reporter constructs.[138] The finding holds important implications for the activation of the intramolecular heterodimeric Notch receptor within the vascular compartment. It is tempting to speculate that the physical intermittent pressure sustained by endothelial cells in an "arterial" site could itself activate Notch by literally "pulling and ripping off" the extracellular domain of Notch and thereby activating proteolytic release of NICD to impose an arterial-specific gene profile. Consequently, the activation of Notch would not necessarily be cell-dependent, but site-dependent. The concept of "pulling" the Notch extracellular domain of the heterodimer has been recently established for the standard Notch ligands in a manner that does not involve proteolysis.[139]

As discussed previously, activation of Notch1 has been thought to initiate transcription of genes associated with arterial fate specification, such as ephrinB2. It will be interesting to expand this list and test the viability of this hypothesis using arterial/venous shunts with genetically modified mice.

Making Sense of Molecular Findings: Future Challenges

Although the transduction of the canonical Notch signal is remarkably simple, ie, there are no intermediates (secondary messengers), this pathway impacts multiple and fundamental aspects of vascular development and physiology. Future directions in this area of research would likely focus on the dissection of the molecular nuances to explain this diversity. Furthermore, elucidation of the intersections between Notch and other pathways, particularly VEGF, will be a factor in clarifying their combined contribution to vascular morphogenesis and homeostasis. In particular, this will show us how

cooperative, sequential, and/or antagonistic these signaling pathways are, both spatially and temporally in blood vessels.

We have previously made reference to the importance of "context" in which Notch signaling takes place. It is becoming increasingly apparent that the state of differentiation of the cell (tip, stalk, or fully differentiated), location within the vascular system (artery, vein, or capillary), and physiological status (flow and/or shear stress) are all likely to impact the array of signals conveyed by Notch.

In addition, evaluation of Notch signaling in other systems also indicates complexities that have yet to be examined in detail within the vascular compartment. Some of these are outlined below.

Cis and *Trans* Interactions

Endothelial cells express both receptors (Notch1 and Notch4), as well as ligands (Dll1, Dll4, and Jag1). As shown for other cell types, cell-autonomous activation might convey signals that differ from those that are of a non–cell-autonomous nature, even when using the same receptor pair. Usually, *cis* interactions have been found to be inhibitory rather than activating.[9,10]

Ligand Diversity/Specificity

Until recently, the prevailing view of Notch signaling was one where binding of any ligand was sufficient for structural changes exposing cleavage sites in Notch and initiating the proteolytic cascade that ultimately led to generation of NICD. The concept that different ligands might convey an alternative cascade of signaling in the same cell has been brought to light only recently.[93,140,141] Furthermore, not all ligand-binding interactions might be "productive," ie, result in Notch activation.[142] In the vasculature, ligand-specific signaling has not been demonstrated. However, the exclusive expression patterns displayed by Jag1 and Dll4 in recently remodeled arterioles provide a platform in which to ask these questions more directly.

Intracellular Trafficking

Similar to other cell-surface receptors, recent findings on the process of endocytosis, followed by endosomal sorting of Notch receptors, have highlighted bidirectional interplay between activation and membrane-transport networks.[143,144] These trafficking events can provide a tighter spatial and temporal control to the signaling events.

The impact of Notch pathways genes in vascular morphogenesis and function has brought considerable attention to this pathway in vascular cells. In a relatively short time, the field has gained a great appreciation for the multiple contributions of Notch in vessels. The vast array of reagents available for studying Notch signaling, originally developed in other model systems, has greatly accelerated this work. Further mechanistic understanding of Notch function will be gained from a combination of sophisticated cell biological analysis, together with validation in the multiple animal models already established. This information will allow the manipulation of the Notch signaling pathway with the objective of therapeutic exploration through management of vascular growth and function.

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Disclosures

None.

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