ATVB In Focus
Smooth Muscle Cells

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Notch Signaling in Vascular Development

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Abstract—Notch signaling is an extremely conserved and widely used mechanism regulating cell fate in metazoans. Interaction of Notch receptors (Notch) with their ligands (Delta-like or Jagged) leads to cleavage of the Notch intracellular domain (NICD) that migrates into the nucleus. In the nucleus, NICD associates with a transcription factor, RBP-Jk. The NICD–RBP-Jk complex, in turn, upregulates expression of primary target genes of Notch signaling, such as hairy and enhancer of split (HES) and HES-related repressor protein (HERP) transcriptional repressors. Recent evidence has demonstrated that the Notch pathway is involved in multiple aspects of vascular development, including proliferation, migration, smooth muscle differentiation, angiogenic processes, and arterial–venous differentiation. In this brief review, we focus on ligands, receptors, and target genes of Notch signaling in the vascular system and discuss (1) tissue distribution; (2) gain- and loss-of-function studies; and (3) the role of Notch components in human diseases involving the vascular system. **(*Arterioscler Thromb Vasc Biol*. 2003;23:543-553.)**

Key Words: notch signaling ■ smooth muscle differentiation ■ angiogenic processes ■ vascular system

Formation of the vascular system is one of the earliest and most important events during embryogenesis in mammals. During the early stages of vascular development, the de novo formation of blood vessels occurs from a dispersed population of mesodermally derived endothelial cell (EC) precursors, angioblasts. Angioblasts first differentiate and assemble into a reticulum of homogeneously sized primitive blood vessels, or the primary vascular plexus, in a process termed vasculogenesis. This primary vascular plexus is then remodeled by the process of angiogenesis, which involves sprouting, bridging, and intussusception, to generate both the large and small vessels of the mature vascular system. During angiogenesis, endothelial channels are covered by multiple layers of smooth muscle cells (SMCs) in large vessels and by single pericytes around small vessels to provide structural support and stability for the vascular walls. A number of different intercellular signaling pathways have been implicated in the control of vasculogenesis and angiogenesis. These pathways include the vascular endothelial growth factor (VEGF) pathway, the transforming growth factor-β pathway, fibroblast growth factor pathway, platelet-derived growth factor pathway, the Angiopoietin/Tie receptor pathway, the ephrin/Eph receptor pathway, and many other pathways. Recent studies have added the Notch signaling pathway to this list.[1–8]

The evolutionarily conserved Notch signaling pathway controls cell fate in metazoans through local cell–cell interactions.[9–11] Notch signaling dictates cell fate and critically influences cell proliferation, differentiation, and apoptosis.[12] In vertebrates, receptors, ligands, and other components of Notch signaling are expressed in various organs from all three germ lines, including vessels (Table 1). Mutations of Notch receptors and ligands in mice lead to abnormalities in many tissues, including the vascular system (Table 2). Human

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TABLE 1. Tissue Distribution of Components of Notch Signaling in Vascular System in Vertebrates

	Systemic Artery	Pulmonary Artery	Vein
Ligand			
Delta like 1	ND[102], descending aorta (−)[33]		Subcardinal vein (−)[33]
Delta like 3	ND[101], descending aorta (−)[33]		Subcardinal vein (−)[33]
Delta like 4	Dorsal aorta, intersomitic vessels, internal carotid A (**EC**)[49], choroid plexus, brain, kidney, lung, umbilical cord (**EC**)[110], umbilical artery (**EC**), capillaries[33]		Primary head vein (−)[49] Umbilical cord (−)[110] Umbilical vein (−)[33]
Jagged1	ND[98], descending aorta, aortic arch, (**EC/SMC**)[33], aorta, intervertebral A, ductus arteriosus, coronary A (**EC/SMC**)[43], aorta, splenic A, hepatic A, iliac A[37], cranial blood vessels[50], aorta, dorsal A, coronary A, mesenteric A, renal A, adrenal A[38], small to medium-sized penetrating A in brain (**SMC**)[86], aorta[44,85], carotid A[29], branchial blood vessels (**EC**)[39], hepatic A (**EC**)[28], capillaries in brain (−)[86]	Pulmonary A[33,37,38,43,44], fine and large vessels (**EC**)[27], arterioles[38], capillaries (−)[43]	Subcardinal vein (−)[33] *Portal vein (+)*[37] *Portal vein (**EC**) (+)*[28] *Hepatic vein (**EC**) (+)*[28] *cardinal vein (+)*[38] *vitelline veins (+)*[39]
Jagged2	ND[101], a major artery supplying a limb[84], carotid A[29], descending aorta[33]		subcardinal vein (−)[33]
Receptor			
Notch1	ND[94,97,100], dorsal A, aortic branches, intersomitic vessels (**EC**)[96], intersegmental A, dorsal aorta (**EC**)[95], a major artery supplying a limb[84], branchial blood vessels (**EC**)[39], descending aorta[33], carotid A[29], coronary A (**EC**)[27], aorta (**EC/SMC**)[26]	Fine and large vessels (**EC**)[27]	Vena cava (−)[33]
Notch2	Choroid plexus[93,99], aorta[26], descending aorta (−)[33]	Pulmonary A[26]	Vena cava (−)[33]
Notch3	ND[93], descending aorta[33], aorta[26,85], **SMC** of vessels[90], vessels in kidney, brain, skeletal muscle, lung and urinary bladder (**SMC**)[41], renal A, capillaries (**pericytes**)[41], aorta, small-to-medium-sized penetrating A in brain (**SMC**)[86], capillaries in brain (−)[86]	Pulmonary A[26]	subcardinal vein (−)[33] *veins in brain (+)*[41] veins in brain (−)[86]
Notch4	Dorsal aorta, aortic tract (**EC**)[42], intersomitic vessels, dorsal aorta[49], aorta (**EC**)[26], descending aorta, capillaries[33]	Pulmonary A, capillaries[42]	*cardinal vein (+)*[42] subcardinal vein (−)[33]
Effector			
HES1	ND[91]		
HES5	ND[92]		
HES7	ND[89]		
HERP1	Dorsal aorta, aortic arch A, aortic sac[45], aorta, mesenteric A (**SMC**)[30], Dorsal aorta[36]		axial vein (−), cardinal vein (−)[36]
HERP2	Dorsal aorta, aortic arch A, aortic sac[45]		
HERP3	Dorsal aorta, aorta[45], abdominal aorta (**SMC**)[90]	pulmonary A[45]	

ND indicates not described; (−), no expression in the tissue; *(+), expression was observed in the tissue*, **EC**, endothelial cell (layer); **SMC**, smooth muscle cell (layer); A, artery.

diseases, such as Alagille syndrome (AGS) and cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), which show abnormalities in the cardiovascular system, are caused by mutations of the Notch ligand Jagged-1 and the receptor Notch-3, respectively.[13–15] Such findings clearly demonstrate a crucial role of the Notch pathway in vascular development.

Interaction of Notch receptors (Notch 1 to 4) with their ligands (Delta like 1, -3, -4, Jagged-1 and -2) leads to cleavage of the transmembrane Notch receptor, giving rise to the Notch intracellular domain (NICD) that migrates into the nucleus (Figure 1).[16,17] In the nucleus, NICD associates with a transcription factor, RBP-Jk (also known as CSL for *C*BF1/*S*u(H)/*L*ag-1)[9–11,16,17] and activates transcription from the RBP-Jk DNA binding site. The NICD–RBP-Jk complex upregulates expression of primary target genes of Notch signaling, such as hairy and enhancer of split (HES)-1, -5, -7 and more recently isolated HES-related repressor protein (HERP)-1 to -3 in mammals.[18,19] The HES and HERP families are basic helix–loop–helix-type transcriptional repressors and appear to act as Notch effectors by negatively regulating expression of downstream target genes (Figure 1).[20–23] Thus, many ligands, receptors, and effectors are involved in this pathway.

In this review, we focus on various ligands, receptors, and effectors of Notch signaling in the vascular system. Studies on tissue distribution of those components should provide clues as to which Notch components are crucial for vascular development. We next describe the vascular phenotypes of mice deficient for Notch components as well as other gain- and loss-of-function studies, the results of which delimit their functions in vascular development. In addition, we also discuss the effects of Notch pathway mutations in human diseases (AGS and CADASIL), which exhibit abnormalities in the vascular system.

TABLE 2. Phenotype of Mice Deficient for Components of Notch Pathway

Gene Disrupted	Lethality	Vascular Phenotype	References
Ligand			
Delta like 1	E12	Hemorrhage	52
Delta like 3	Perinatal	ND	109
	Viable	ND	87
Jagged1	E11.5	A large hemorrhage adjacent to the optic vesicle, lack of obvious large vessels in the yolk sac, failure to remodel the primary plexus in the yolk sac, less intricate network and a reduced diameter of vessels in the head	50
Jagged2	Perinatal	ND	108
	Viable	ND	107
Receptor			
Notch1	E11.5	Lack of large vitelline blood vessels in the yolk sac, disorganized, confluent vascular plexus in the yolk sac, defect of the main trunk of the anterior cardinal vein, lack of intersomitic vessels, the collapsed dorsal aortae	49
Notch2	E11.5	ND	106
	Perinatal	Widespread hemorrhage near the surface of the skin, no capillary tuft of mature glomeruli (majority), capillary aneurysm-like structure of glomeruli (minority), numerous capillaries emanating from an aberrant bulbous structure, at the terminus of the hyaloid artery	53
Notch4	Viable	Normal development	49
Notch1/Notch4	E9.5	More severe than Notch1 null mutant	49
Effector			
HES1	Perinatal	ND	21
HES5	Viable	ND	102
HES7	Perinatal	ND	88
Hey2 (HERP1)	Perinatal	No vascular abnormality	46, 54

Tissue Distribution of Notch Pathway Components

The vascular system comprises several different kinds of vessels classified into three groups—arteries, veins, and lymphatics—with separate subdivisions into large vessels, small vessels, and capillaries (Table 1). These vessels primarily consist of ECs, supporting cells (SMCs and pericytes), and surrounding matrix. A number of reports have described the tissue distribution of various Notch components and closely evaluated distribution in the vessels from several species, including human, rat, mouse, chicken, and zebrafish. We summarize these observations in Table 1. Although the expression level of Notch components is likely dynamic during development and therefore making it difficult to detect transient expression, the data suggest that of all the known Notch components, mainly three ligands (Dll-4, Jagged-1, and Jagged-2), three receptors (Notch-1, -3, and -4) and three effectors

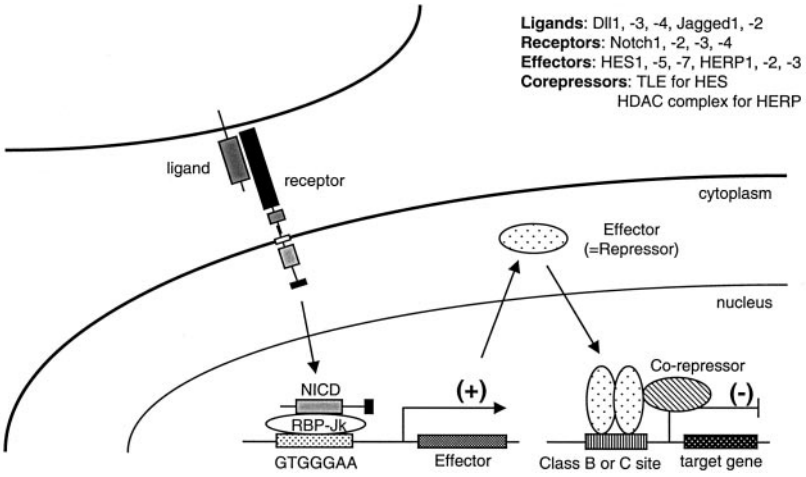

Figure 1. Brief overview of Notch signaling pathway. See the text and the references[19] for details.

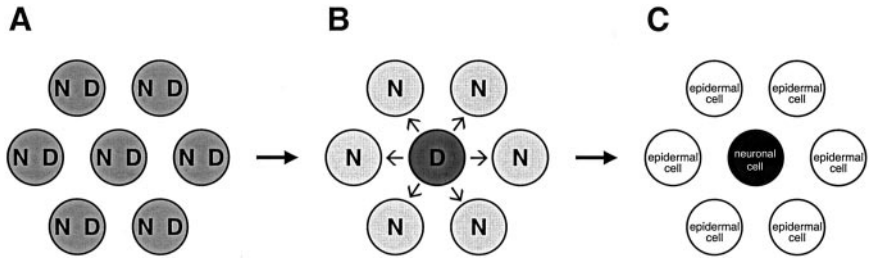

Figure 2. Basic model of lateral specification. Notch signaling is best known for lateral inhibition or lateral specification, which is exemplified by *Caenorhabditis elegans* gonadogenesis and *Drosophila* sensory organ development. In the case of *Drosophila* sensory organ development, lateral interaction occurs within a population of equivalent cells (neuronal precursor cells, A), which express both Notch receptor (N) and its ligand Delta (D). Once a small difference is created between equivalent cells, a cell that has obtained a specific fate with Delta expression prevents surrounding cells from assuming the same cell fate through cell–cell interaction (B). In the surrounding cells, Delta expression is suppressed whereas Notch expression is enhanced (B). Then, the Notch signaling pathway represses expression of proneural genes, the acaete–scute complex, via its target gene E(spl), resulting in blocking the cells from a neuronal cell fate. Thus, this process generates two different cell types; Delta expressing cells become neuronal cells whereas Notch expressing cells become epidermal cells (C). In the case of vascular development, there are several cases of two distinct subpopulations, such as (1) ECs vs SMCs; (2) artery vs vein; (3) pulmonary vs systemic vessels; and (4) large vessels vs capillaries and Notch signaling might contribute to establishing some of these cases. Thus far, several studies have revealed that, indeed, the Notch pathway is strongly involved in arterial–venous differentiation (see the text for details).

(HERP-1, -2, and -3) are involved in the vascular system. One key function of the Notch pathway is to determine the identity of distinct cell subpopulations from bipotential precursor cells, a process known as lateral specification or lateral inhibition (Figure 2).[9–11] Such cell fate control activity of Notch is exemplified by determination of T lymphoid versus B lymphoid cells and pancreatic exocrine versus endocrine cells in mammals.[24,25] Therefore, we proceed on the supposition that the Notch pathway might also contribute to establishing two distinct subpopulations at different steps of vasculogenesis and angiogenesis, such as (1) EC versus SMC/pericyte; (2) artery versus vein; (3) pulmonary versus systemic vessels; and (4) large vessels versus capillaries. We describe the tissue distribution of Notch components from this point of view. We also describe the expression of Notch components in cultured cells although they do not always precisely reflect events during in vivo differentiation.

Notch signaling plays a critical role, in addition to normal animal development, in the pathological conditions of different tissues. Vascular injury experimentally caused by balloon for instance, results in altered gene expression of Notch signaling components. Such potential roles of Notch in vascular pathology are also discussed.

ECs or SMCs/Pericytes?

Expression of Dll-4, Notch-1, and Notch-4 seems to be limited to the EC layer, whereas that of Notch-3 and HERP-3 appear to be confined to the SMC layer (see Table 1). Only one report shows that Notch-1 is expressed in the aorta throughout the EC and the SMC layers.[26] Although Jagged-1 is likely to be expressed in both EC and SMC layers (Table 1), several groups have reported that expression of Jagged-1 is observed only in the EC of some arteries (Table 1), pulmonary vessels,[27] and portal and hepatic veins.[28] One report showed that Jagged-2 is expressed in EC.[29] In neural crest-derived Monc1 cells that can differentiate into smooth muscle cells, CHF1 (HERP-1) turns positive after induction of differentiation.[30] Consistent with this, HERP-1 is expressed in an aortic smooth muscle–derived cell line and induced in it by ligand stimulation.[31] Hesr1 (HERP-2) expression is observed in purified human capillaries by reverse transcription polymerase chain reaction (RT-PCR) and is induced during in vitro capillary-like network formation.[32] HERP-2 is also expressed in an aortic smooth muscle-derived cell line and is also induced by ligand stimulation.[31] Expression of Notch-1 through 3 is also detected in an aortic smooth muscle-derived cell line at least by RT-PCR.[31] Taken together, Jagged-1 and Notch-3 may be the only ligand and receptor in the Notch pathway that play an important role in VSMC lineage whereas several ligands (Dll-4, Jagged-2) and receptors (Notch-1, -4) are involved only in EC lineage. Jagged-1 seems to be the only ligand that is expressed in both ECs and SMCs. In terms of target genes, HERP-1 and HERP-3 may be SMC-specific effectors in the vascular system whereas HERP-2 may play some role in both ECs and SMCs. By targeting one of several distinct Notch pathways, Notch signaling might contribute to defining EC and SMC cell populations.

Arteries or Veins?

Arteries and veins are morphologically, functionally, and molecularly very different. However, little is known about how this distinction is established during vascular development. The tissue distribution of Notch components leads us to speculate that the Notch pathway may play a crucial role in defining artery versus vein. Villa et al[33] recently reported that Notch-1, Notch-3, Notch-4, Dll-4, Jagged-1, and Jagged-2 are all expressed in arteries but are not expressed in veins. Expression of DeltaC as well as Notch5, a Notch ligand and a receptor of zebrafish, is also restricted to the dorsal aorta.[34,35] A putative Notch effector, gridlock (zebrafish homologue of HERP-1), is selectively expressed in the dorsal aorta, but not in the axial or cardinal veins.[36] Thus, many Notch components show arterial specific expression. However, there are several exceptions for vascular distribution of the Notch components. Expression of Jagged-1 was observed in portal,[28,37] hepatic,[28] cardinal,[38] and vitelline veins.[39] Transcripts of Jagged-1 were also upregulated in human umbilical vein endothelial cells (HUVECs) exposed to fibrin.[40] Expression of Notch receptors has also been found in some venous components: Notch-1 and Notch-2 in HUVECs,[40] Notch-3 in veins in brain,[41] and Notch-4 in the cardinal vein.[42] Thus, although the tissue distribution of most Notch components

and a further functional analysis of arterial differentiation (see below) strongly suggest its pivotal role in defining arteries, the possibility remains that the Notch pathway might also play some roles in venous development.

Pulmonary or Systemic Vessels?

Expression of Jagged-1 in the pulmonary artery has been well studied because AGS, which is caused by mutation of Jagged-1, shows major abnormalities in pulmonary arteries.[14,15] Jagged-1 is expressed in the pulmonary artery and arterioles[27,33,37,38,43,44] but not in capillaries,[43] which is in agreement with the pulmonary phenotype of AGS (see below). Taichman et al[27] have reported EC-specific expression of Jagged-1 as well as Notch-1 in the pulmonary vessels. Although Loomes et al[26] reported that two other receptors, Notch-2 and Notch-3, are expressed in the pulmonary artery, Taichman et al[27] concluded that Notch-2 and Notch-3 are diffusely expressed throughout the lung but not specifically in the vessels. HERP-3 is expressed in the pulmonary artery.[45] Histological sections from heterozygous Hey2$^{lacZ/+}$ (HERP-1) mice stained for expression of the lacZ reporter gene with the Xgal reaction showed strong staining in the pulmonary arteries.[46] We also observed strong expression of HERP-1 and HERP-2 in the lungs of adult rats by Northern blot analysis (unpublished data). These findings, along with the phenotype of AGS, indicate that Notch signaling is also involved in the development of pulmonary vasculature.

Large Vessels or Capillaries?

There are only a few reports showing expression of Notch components in capillaries. Villa et al[33] showed that Notch-4 is the only receptor and Dll-4 is the only ligand expressed in capillaries among all receptors and ligands they examined. Expression of Notch-4 was also observed in capillaries in lung.[42] Notch-3 was shown to be expressed in pericytes of capillaries.[41] Rat brain microvessel endothelial cells (RBE4) express Notch-1, -3, -4, and Jagged-1.[47] An RT-PCR study by Henderson et al[32] revealed that HERP-2 transcripts were detected in capillary–ECs prepared from adipose tissue. Further extensive studies are required to determine the precise tissue distribution of Notch components in capillaries because there appears to be a discrepancy in expression found in vivo and in vitro.

Expression After Vascular Injury

Lindner et al[29] have studied expression of Jagged-1, Jagged-2, and Notch-1 through 4 before and after balloon catheter denudation of the rat carotid artery. The findings of these authors was limited to observations of only the inner face of the artery, not its cross section, because they used en face preparations for in situ hybridization. Although expression of only Jagged-1, Jagged-2 and Notch-1 was observed in intact ECs, all of the many other Notch components they examined in their study were strongly induced in injured ECs. Importantly, all the 6 Notch components they examined were strongly expressed in injured SMCs after denudation of ECs, which is in sharp contrast with normal SMCs, which express only Jagged-1 and Notch-3.[33] In contrast with this report, Wang et al[48] found that in SMC of rat carotid arteries transcripts (determined by RT-PCR) for Notch-1, -2, and -3 as well as HERP-1, -2, and -3 were coordinately downregulated after balloon injury. They extracted RNA from the artery after removing the layers of intima and adventitia, thereby they consider their result to reflect events in the SMC layer. The Lindner study[29] did not compare expression levels in injured SMC with those in normal SMC, which may explain the discrepancies in these reports.

Specificity Among Ligands, Receptors, and Effectors

Based on their tissue distribution (Table 1), there might be a specific relation between Jagged-1-Notch-3-HERP-1/HERP-3 in SMCs, but direct evidence is lacking. Similarly, using an aortic smooth muscle-derived A10 cell line that expresses Notch-1, Notch-2, and Notch-3 transcripts, we reported that distinct ligands, Dll-1 and Jagged-1, induced transcripts of both HERP-1 and HERP-2.[31] These experiments did not reveal any specific relation among Notch ligands, receptors, and effectors. However, it is clear that not all the Notch components are equally expressed in vascular tissues and, rather, only selected sets of Notch components are implicated in angiogenesis. Clarifying the relationships among ligands, receptors, and effectors is one of the key issues whose clarification is required to understand how the Notch pathway might be implicated in establishing different vessel types (eg, artery versus vein) and vascular cell types (ie, ECs versus SMCs).

Gain- and Loss-of-Function Studies

Phenotype of Knockout Mice

Mice homozygous for null mutations of several components of the Notch pathway, including Notch-1, Notch-1 plus Notch-4, and Jagged-1, resulted in embryonic lethality with vascular remodeling defects (Table 2).[49,50] Vasculogenesis proceeded normally in these mutants whereas the next step, angiogenesis, was disrupted, suggesting that Notch signaling plays a more important role in angiogenesis. Although Notch-4–deficient mice were viable and fertile,[49] embryos homozygous for double mutations of Notch-1 and Notch-4 displayed a more severe phenotype in angiogenesis than Notch-1 single mutant embryos.[49] These findings suggest a more important role of Notch-1 than Notch-4, as well as their redundant function in angiogenesis. A lack of vascular morphogenesis in mice homozygous for a processing-deficient allele of Notch-1 also underscores its role in vascular development.[51] Although no expression of Dll-1 and Notch-2 was detected in large vessels (Table 1), both Dll-1–deficient and Notch-2–hypomorphic mice embryos show hemorrhage,[52,53] possibly resulting from poor development of vascular structures. In Notch2-hypomorphic mice, mutant kidney glomeruli lack the normal capillary tuft, and the hyaloid vasculature of the eye was also affected. Along with the observation that even Notch-2 was induced in injured arteries despite a lack of its expression in normal arteries,[29] both Dll-1 and Notch-2 may thus be crucial factors in the vascular system, at least in a context-dependent manner. Recently it was reported that Hey2 (HERP-1)-deficient mice did not exhibit any overt vascular phenotype, such as aortic coarctation, seen in a grl (a homologue of HERP1) mutant of

zebrafish, suggesting that unlike Zebrafish, HERP1 function might be compensated by other factors in the vascular system in mice,[46,54] including, of course, other HERP isoforms. Finally, mutation of the presenilin1 gene, which is involved in the processing of Notch intracellular domain, produced a complex phenotype and showed intracranial hemorrhage, also supporting the idea that Notch pathway regulates vascular development.[55,56] Whether arterial–venous differentiation is disrupted and whether functions of ECs and SMCs are affected in the mutant mice described above remain to be answered. Because other Notch components, such as Dll-4, HERP-2, and HERP-3, which might have a different/similar/redundant role, are also expressed in the vasculature, analyses of mice with targeted disruption of these genes are awaited.

Transgenic Mice

EC-specific expression of an activated form of Notch-4 driven by the flk1-promoter led to embryonic lethality with abnormal vessel structure and patterning,[57] whose phenotype is similar to that seen in Notch-1– and Notch-1/Notch-4–deficient mice.[49] The similar vascular phenotypes observed in both the transgenic (gain-of-function) and the knockout mice (loss-of-function) suggest that there may be a window of appropriate Notch expression levels for proper development of the embryonic vasculature. This study also suggested that the Notch pathway is essential at least in development of ECs. However, it is critical to be cautious in interpreting data involving the consequences of unregulated overexpression of the NICD because it can lead to a physiologically irrelevant result by interacting with other pathways and by inappropriately enhancing its own pathway.[12]

Notch in Arterial–Venous Differentiation

The role of the Notch pathway in arterial–venous differentiation has been studied elegantly in zebrafish. Zebrafish Notch5 (previously referred to as Notch-3) is expressed within the dorsal aorta (DA) but not in veins.[35] EC-specific expression of an activated form of Notch5 can repress expression of a venous-specific marker, flt4. Embryos lacking Notch activity as a consequence of overexpression of a dominant negative form of XSu(H), the *Xenopus* homologue of RBP-Jk, also fail to express arterial-specific markers, such as ephrinB2a and Notch5, within the DA. In the zebrafish mutant mindbomb (mib), which displays hallmarks of the neurogenic phenotype typical of defective Notch signaling, expression of several arterial-specific markers, including Notch5, ephrinB2, and DeltaC, was disrupted whereas expression of some venous markers, such as flt4 and rtk5, appeared in the DA.[35] Taken together, Notch signaling appears to be required for arterial–venous differentiation and repression of venous fate.

The gridlock mutation (grl^{m145}), originally isolated in a large-scale chemical mutagenesis screen for developmental mutations of the zebrafish, shows selective disturbance of assembly of the aorta.[36] The *grl* gene (HERP-1 homologue) is strongly expressed in the dorsal aorta but not in the axial vein (Table 1). The grl^{m145} mutation changes the stop codon to Gly and extends the protein by 44 amino acids at its carboxyl terminus, resulting in the phenotype. Injection of wild-type grl RNA in the mutant restores a normal phenotype,[36] indicating that grl^{m145} is a loss-of-function mutation. More interestingly, gridlock is required for arterial–venous differentiation during embryonic vascular development.[58] Reduction in grl by antisense oligonucleotides ablates regions of the artery and expands continuous regions of the vein whereas overexpression of grl diminishes the vein without increasing the artery. Taken together, these observations suggest that the normal action of grl is to repress venous fate rather than to instructively generate arteries. Although Zhong et al[58] have reported that grl is downstream of Notch, Lawson et al[35] suggested that grl does not function as a downstream target gene of Notch, at least in the vascular system. How grl regulates the process of arterial–venous differentiation and what ligands and receptors are upstream of grl are major issues to be addressed. In sharp contrast with the phenotype of the grl mutant, however, mice homozygous for a Hey2 (HERP-1) null mutation did not show any obvious abnormalities in the vascular system.[46,54] Although the reasons for different phenotypes between the mutants of two species are unknown, a lack of mouse HERP-1 might be compensated by other factors that zebrafish do not have or, during evolution, mouse HERP-1 might have acquired other functions distinct from that of zebrafish.

Recent studies suggest a complex signaling cascade responsible for establishing arterial cell fate.[59] ECs in zebrafish embryos lacking Shh (Sonic hedgehog) activity fail to undergo arterial differentiation, as defined by the expression of artery-specific markers, such as ephrinB2a, whereas injection of mRNA encoding Shh can induce ectopic vascular expression of ephrinB2a.[60] Although embryos lacking Shh fail to express VEGF within their somites, exogenous addition of VEGF in the embryo can rescue vascular ephrinB2a expression. Furthermore, VEGF is unable to rescue arterial marker gene expression in embryos lacking Notch function whereas exogenous Notch activity can induce arterial differentiation in the absence of VEGF signaling. Such studies suggest that VEGF acts downstream of Shh and upstream of the Notch pathway to determine arterial cell fate.[60] Mukouyama et al[61] reported that in mice peripheral nerves provide a template that determines the organotypic pattern of blood vessel branching and arterial differentiation in the skin, via local secretion of VEGF from sensory nerve fibers, Schwann cells, or both. Although these studies suggest that VEGF induces arterial differentiation by linking Shh with Notch signaling, further investigation is required to clarify the precise mechanism of arterial–venous differentiation by VEGF since veins may also be exposed to similar levels of VEGF.

In Vitro Studies

In vitro experiments have also provided us insight on roles of the Notch pathway in vascular development. Some groups suggested a negative role of Notch signaling in angiogenesis whereas others proposed a positive role. Zimrin et al[40] reported that expression of the Jagged-1 gene was induced in HUVECs exposed to fibrin, which induced circular structures of HUVECs in vitro. Interestingly, the addition of an antisense oligonucleotide to Jagged-1 enhanced invasion and tube formation during fibroblast growth factor–induced angiogenesis,[40] suggesting that Jagged-1 negatively regulates

this process. Maciag and colleagues[29,62,63] used a secreted form of the extracellular domain of Jagged-1 (soluble Jagged-1) as a dominant-negative effector, although the physiological effects of soluble ligands are highly controversial. NIH3T3 cells stably expressing soluble Jagged-1 form chord-like structure, which is a critical component of lumen formation mediated by ECs and epithelial cells.[63] In addition, intradermal injection of such cells into the flank of nude mice resulted in formation of tissue masses with prominent angiogenesis,[63] suggesting a positive role of soluble Jagged-1 in angiogenesis. Because soluble Jagged-1 seems to act as a dominant negative,[62] the transmembrane Jagged-1/Notch pathway may function to negatively control angiogenesis. Consistent with this notion, Leong et al[64] recently reported that activated Notch-4 in EC in vivo may inhibit angiogenesis partly by promoting β1-integrin-mediated adhesion to the underlying matrix. In contrast, expression of Jagged-1 or activated Notch-4/int3 in cultured brain ECs induced microvessel-like structures with morphological and biochemical properties similar to brain endothelial microvessels,[47] which leads to conclusions opposite those described above. The soluble Jagged-1 protein described above inhibited cell–matrix interaction, focal adhesion formation, and cellular migration of NIH3T3 cells while increasing cell–cell contacts. These findings suggested that endogenous Jagged-1/Notch may act to maintain cell interaction with the matrix and to activate the migratory ability of cells, possibly by decreasing cell–cell contacts. The authors suggested that this function of Notch might be active in regenerating ECs and SMCs after vascular injury to facilitate their migration.[29] However, one should be careful in interpreting such data because the function of Notch signaling is likely dependent on cell context and because the activity of the soluble ligand has not been studied in vascular-derived cells.

Expression of mouse CHF1 (HERP-1) was induced during differentiation of neural crest-derived Monc1 cells into vascular smooth muscle cells.[30] Expression of mRNAs for both HERP-1 and HERP-2 was induced in cultured SMCs by stimulation of Notch ligands, such as Dll-1 and Jagged-1.[31] Such studies suggest that HERPs may have a role in VSMC as Notch effectors. In EC, Hesr1 (HERP-2) mRNA expression was induced during EC tube formation, a well-characterized in vitro angiogenic process, and gain- and loss-of-function studies showed that Hesr1 (HERP-2) was involved in proliferation, migration, and network formation of ECs.[32]

Collectively, these findings from in vivo and in vitro studies indicate that the Notch pathway is involved in multiple aspects of vascular development, including in vitro proliferation and migration of ECs, smooth muscle differentiation and endothelial tube formation, as well as in vivo angiogenic processes and arterial–venous cell fate determination. Because loss- and gain-of-function studies in the Notch pathway sometimes appear to yield similar results and because comparable experiments designed for similar purposes exhibited opposite results, we should remain cautious about the interpretations of the findings. Nevertheless, it is likely to be true that Notch signaling has an essential role in vascular development.

Human Diseases

Alagille Syndrome (AGS)

AGS, first described in 1975,[65] is an autosomal-dominant disorder characterized by neonatal jaundice and a paucity of intrahepatic bile ducts. Features that often accompany this syndrome include congenital heart defects; abnormal vertebrae and decreased interpediculate distance in the lumbar spine; retinal pigmentary changes and posterior embryotoxion; and a typical faces consisting of broad forehead, pointed mandible, deep-set eyes, and a bulbous tip of the nose. The disease has a wide range of expression, ranging from an apparently normal phenotype to severe cases.[66] Some patients exhibited only cardiovascular defects, such as tetralogy of Fallot and pulmonic stenosis.[67] In terms of congenital heart diseases seen in AGS, peripheral pulmonary stenosis is the most common defect, but other structural cardiovascular defects, such as tetralogy of Fallot, atrial and ventricular septal defects, pulmonic valve stenosis, and coarctation of the aorta, are also present.[37] Mutations in the Jagged-1 gene at 20p12 have been identified in AGS patients.[14,15] These are inactivating mutations, generally leading to premature truncation of the Jagged-1 protein. The AGS phenotypes are consistent with the tissue distribution of Jagged-1 in the cardiovascular system (Table 1). An extensive survey of the types and frequency of Jagged-1 mutations in AGS patients revealed that patients with large deletions encompassing the entire Jagged-1 gene had the same phenotype as patients with intragenic Jagged-1 mutations, suggesting that haploinsufficiency for the Jagged-1 gene was the primary cause of AGS.[68,69] However, an animal model for AGS, or mice heterozygous for Jagged-1 null allele, exhibited only anterior chamber eye defects but did not exhibit other phenotypes associated with AGS in humans.[50] Recently, a more representative mouse model of AGS was generated. Mice doubly heterozygous for the Jagged-1 null allele and a Notch-2 hypomorphic allele exhibited developmental abnormalities characteristic of AGS. They exhibited jaundice; growth retardation; impaired differentiation of intrahepatic bile ducts; and defects in heart, eye, and kidney development.[44] Heart defects include narrowing of the pulmonary artery, atrial, and ventricular septal defects; right ventricular hypoplasia; and an overriding aorta that are reminiscent of tetralogy of Fallot.[44] These results strongly suggest that the Notch-2 gene acts as a genetic modifier of Jagged-1 mutations in mice. However, the hypothesis that the Notch-2 allele may influence the phenotypes of AGS patients remains to be addressed.

Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL)

CADASIL is an inherited vascular dementia caused by mutations in Notch-3 on chromosome 19p13.[13] Although the CADASIL mutations do not affect normal vascular development, they cause a defect in vessel homeostasis. The disorder most commonly manifests with transient or permanent ischemic strokes, which occur at a mean age of 45 years (range 27 to 65 years).[70,71] Most of these strokes are classic lacunar infarcts that arise in the absence of hypertension or any other recognized vascular risk factors. Pathologically, the arteri-

opathy underlying the disorder is neither atherosclerotic nor amyloid.[72–74] It involves a thickening of the vessel wall and narrowing of the lumen in the small arteries penetrating the white matter. In the thickened walls, vascular smooth muscle cells (VSMCs) degenerate, marked secondary fibrosis occurs, and granular osmophilic deposits (GOMs), detected by electron microscopy, accumulate in the basal membrane of VSMCs.[72–75] GOMs have not been characterized biochemically, and their origin remains unsolved.[73] VSMCs appear to progressively lose a part of their cytoplasm, leading to their destruction.[73] That VSMCs are the major site of pathological changes seen in CADASIL is in full agreement with tissue distribution of Notch-3 in SMC (Table 1). Pathological changes, including destruction of VSMCs and the presence of GOMs, have also been found in SMCs of small arteries in muscle and skin biopsies in patients, indicating that CADASIL is a systemic vascular disease,[72,73,75] although vascular complications appear to be limited to the brain for unknown reasons.[76] More than 95% of CADASIL cases are the result of stereotypical missense point mutations within the epidermal growth factor–like repeats of Notch-3, leading to the addition or loss of a cysteine residue.[13,77,78] As a result, instead of the normal even number of 6 cysteines, the mutated epidermal growth factor repeat contains an uneven number, either 5 or 7 cysteines. It is possible that the alteration from an even to an uneven number of cysteines affects the formation of sulfur bridges and, thereby, the three-dimensional structure of the extracellular part of Notch-3.[79] In rare CADASIL cases, small deletion mutants and even a mutant not involving a cysteine residue have been reported.[77,80] How such mutations cause CADASIL has been addressed as well. Notch-3 undergoes a proteolytic cleavage, leading to a 210-kDa extracellular fragment and a 97-kDa intracellular fragment. In CADASIL patients, a dramatic and selective accumulation of the 210-kDa Notch-3 cleavage product was observed at the cytoplasmic membrane of VSMC, in close proximity to, but not within, the GOM.[41] The accumulated Notch extracellular fragment is readily detected by skin biopsy immunostaining with high sensitivity (96%) and high specificity (100%), which is considered useful for diagnosis.[81] In an in vitro study, Notch-3 protein with the CADASIL mutations is normally expressed on the cell surface, suggesting that the mutations do not disrupt receptor maturation.[82] Also, the ability of the CADASIL mutants to bind to Delta1 was not disrupted when compared with that of wild-type Notch-3.[82] These results strongly suggest that CADASIL mutations act downstream of ligand binding, presumably through impaired clearance of the Notch-3 ectodomain from the cell surface. Wang et al[83] have recently proposed that Notch-3 may play a role in VSMC survival by inducing c-FLIP, a primary inhibitor of the Fas ligand signaling pathway. Disturbances of the clearance of the Notch-3 extracellular fragment may lead to defective signaling in VSMC and even lead to their degradation.[79]

Conclusions and Future Directions

We have reviewed here the accumulated evidence that the Notch pathway is involved in multiple aspects of vascular development. Expression analyses revealed that the major components of the Notch pathway in the vascular system consist of three ligands (Dll-4, Jagged-1, and Jagged-2), three receptors (Notch-1, -3, and -4), and three target genes (HERP-1, -2, and -3; Table 1). Although some components are detected in veins and venous-derived cell lines, the major sites of their expression are arterial ECs and/or SMCs (Table 1), suggesting a central role in arterial differentiation. Indeed, the Notch pathway is essential for arterial–venous differentiation in zebrafish. Also, the bulk of data from in vivo and in vitro studies have demonstrated that the Notch pathway positively and negatively regulates vascular development, especially angiogenesis, as well as differentiation of ECs and SMCs.

However, our review also found a number of apparent inconsistencies and problems in these studies. The tissue distribution of specific Notch components is not always consistent with vascular phenotypes of mice deficient for them. For instance, Notch-4–deficient mice do not exhibit any obvious vascular phenotype despite strong Notch-4 expression in arterial ECs (Tables 1 and 2). Although expression of Dll-1 and Notch-2 has not been detected in vessels, targeted disruption of them in mice caused hemorrhage (Tables 1 and 2). Nor is the expression of Notch components after vascular injury, consistent with their expression in intact vascular systems. Recent studies in zebrafish have suggested a crucial role of the Notch pathway in arterial–venous differentiation. However, Hey2 (mouse homologue of grl)-deficient mice have no obvious vascular abnormalities. Such findings lead us to conclude that the involvement of Notch in vascular development is poorly understood and that there remain many issues to be addressed. Is there a specific relation between ligands, receptors, and target genes in the vascular system? Are there any other Notch components involved in the vascular system? What components function at what steps in what cells? How does the Notch pathway play a role in differentiation of ECs and/or SMCs? How does the Notch pathway regulate arterial–venous differentiation? Does Notch regulate vascular development positively or negatively? Does the Notch pathway really play no role in the development of veins? Does Notch play any role in vasculogenesis or angiogenesis in pathological situations such as neoplasm, inflammation, wound repair, diabetic retinopathy, and formation of collateral vessels in ischemic diseases?

Given that Notch has a significant role in vascular development, further understanding of Notch function in the vascular system, in combination with knowledge about other pathways in vascular development, might lead to development of new therapeutic and diagnostic strategies for Notch-related diseases and the plethora of serious diseases that involve de novo formation of blood vessels.

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