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Notch signal integration in the vasculature during remodeling

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Abstract

Notch signaling plays many important roles in homeostasis and remodeling in the vessel wall, and serves a critical role in the communication between endothelial cells and smooth muscle cells. Within blood vessels, Notch signaling integrates with multiple pathways by mechanisms including direct protein-protein interaction, cooperative or synergistic regulation of signal cascades, and co-regulation of transcriptional targets. After establishment of the mature blood vessel, the spectrum and intensity of Notch signaling changes during phases of active remodeling or disease progression. These changes can be mediated by regulation via microRNAs and protein stability or signaling, and corresponding changes in complementary signaling pathways. Notch also affects endothelial cells on a systems level by regulating key metabolic components. This review will outline the most recent findings of Notch activity in blood vessels, with a focus on how Notch signals integrate with other molecular signaling pathways controlling vascular phenotype.

Keywords

Notch signaling; endothelial cell; smooth muscle cell; microRNA; metabolism

1. Introduction

Notch signaling is a primary mediator of cell fate, differentiation, and intercellular communication in virtually all tissues. In this issue of *Vascular Pharmacology*, work from the Lilly lab expands upon the concept of Notch-mediated communication between vascular endothelial cells and smooth muscle cells, which is critical during vascular development and the pathogenesis of vascular disease. This work continues to expand our knowledge of endothelial and smooth muscle cell communication. This review will focus on recent concepts of Notch signal integration in the postnatal blood vasculature. We refer the reader to recent reviews on the related topics of Notch signaling in embryonic vascular

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development [1, 2], differentiation and function of vascular cells [3, 4], and an excellent comprehensive book on Notch signaling [5].

2. Vascular quiescence to activation - overview

Most blood vessels in adult organisms have a very low rate of cellular proliferation. Quiescent endothelium expresses Delta-like1 (DLL1), DLL4, Jagged1, Notch1 and Notch4. Vascular smooth muscle cells in the homeostatic condition express primarily Jagged1 and Notch3. Once the vasculature achieves its mature conformation and function, constant communication between vascular cells is required to maintain homeostasis, and to respond to external stimuli such as cytokines, hormones, changes in blood flow or shear stress, inflammation, and mechanical trauma. In particular, endothelial cell communication with vascular smooth muscle cells is mediated via Notch signaling on adjacent cells; however, unique mechanisms including paracrine signaling via exosomes are emerging, and will be discussed.

The endothelium is a primary mediator of homeostasis, forming a contact-inhibited monolayer with tight cellular junctions. DLL4 is highly expressed in arterial endothelial cells, and plays a critical role in maintaining endothelial cell quiescence. One tissue-specific exception to DLL4/Notch-mediated endothelial quiescence was recently reported in postnatal long bones, where Notch activity promoted endothelial proliferation in columnar and arched vessels in metaphyseal growth plates, and was responsible for osteoblast maturation and bone deposition in a paracrine manner [6]. Circulating bone morphogenetic proteins (BMP), BMP9 and BMP10 are additional quiescence signals that promote homeostasis in endothelial cells (Figure 1). Integration of Notch signaling with BMP signaling will be detailed below. With an intact endothelium, the smooth muscle cells also remain in a quiescent, contractile state, with high expression of smooth muscle cell markers. Highlights of the interaction between endothelium and smooth muscle cells via Notch signaling will be described.

The levels and expression patterns of Notch proteins and their ligands drastically change upon injury or pathological disease progression. These changes support the concept that Notch signaling is requisitioned during vascular remodeling, and also underscore the non-redundant roles of Notch ligands and receptors. Cardiovascular diseases are associated with dysregulation of Notch signaling, and specific genetic mutations have been mapped to *JAG1* or *NOTCH2* (Alagille syndrome, pulmonary artery stenoses, tetralogy of Fallot, cardiac septal defects, and coarctation of the aorta). In addition, mutations in *NOTCH1* are associated with tetralogy of Fallot and aortic valve abnormalities. These mutations have been comprehensively reviewed [7]. Recently, mutations in *NOTCH1* have also been linked to Adams-Oliver Syndrome, characterized by scalp aplasia cutis and terminal transverse limb defects, secondary to vasculopathy [8].

Several human vascular pathologies are associated with alterations in Notch signaling activity. Here we provide a few examples that include novel signaling interactions (Figure 1). It is well known that Notch signaling regulates the processes of angiogenesis and arteriogenesis following ischemic injury or during tumorigenesis. Recent insight has linked

cellular metabolism with angiogenesis, and exciting developments in this area will be discussed. In addition, interacting proteins such as synaptojanin-2 binding protein have the potential to regulate Notch signaling during angiogenesis via direct proteinprotein interaction, and specific microRNAs are being discovered as regulatory factors affecting the Notch pathway.

Notch dysregulation also occurs during the pathogenesis of pulmonary arterial hypertension. Pulmonary arterial hypertension involves hyperproliferation of smooth muscle cells of the pulmonary arterioles that leads to decreased vessel lumen size and vessel elasticity, and increased pulmonary vascular resistance. Notch3 levels are increased under hypoxic conditions leading to pulmonary hypertension [9], suggesting a role in disease progression. Indeed, mice homozygous for Notch3 deletion are resistant to pulmonary hypertension, and inhibition of Notch by gamma secretase inhibitor can reverse the hypertensive phenotype in wild type mice [10]. Disease progression in pulmonary arterial hypertension is also associated with impaired BMP signaling and the activation of miR-145. It is interesting to consider the possibility that mutations leading to loss of BMP signals could lead to compensatory increases in Notch signaling. In addition, we previously showed that miR-145 is a transcriptional target of Notch in smooth muscle cells, and thus Notch activation would be predicted to further increase miR-145 levels, whose upregulation is also associated with BMPR2 mutation [11]. Associations of Notch signaling with microRNAs will be addressed in a later section.

Recent work has also highlighted the role of Notch signaling in inflammatory vascular diseases, such as atherosclerosis, where DLL4/Notch signaling has been implicated in macrophage and foam cell accumulation within the lesion [12]. In addition to chronic inflammation, Notch signaling also affected metabolic parameters such as insulin resistance and development of fatty liver [13]. Given the multiple cell types involved and unique activities of Notch on each, the role of Notch in inflammatory vascular diseases is complex. While Notch activity in endothelial and mural cells is necessary for maintenance of endothelial barrier function and smooth muscle contractile phenotype, multiple reports have demonstrated that suppression of Notch also inhibits inflammation, inflammatory cytokine release, atherosclerotic plaque formation, and activated macrophage infiltration [12, 14, 15]. It is important to distinguish between the function of Notch signaling in the different cell types involved during vascular remodeling and lesion formation, and also the timing of Notch activation in each lineage during disease progression. Activation of Notch on inflammatory cells exacerbates pathological lesion formation, but Notch activity in endothelial cells is necessary for prevention of remodeling. Further, inflammatory cytokines suppress endothelial Notch, leading to upregulation of adhesion molecules that recruit inflammatory cells [16, 17]. Notch signaling in bone marrow endothelial and stromal cells has also been linked to inhibition of a proinflammatory circuit involving suppression of miR-155 and subsequent NF κ B-mediated myeloproliferative disease [18]. Another microRNA involved in inflammatory disease is miR-126, which regulates endothelial cell proliferation during atherogenesis and vascular repair. The mechanisms of activity of these microRNAs in the vasculature will be described below. Finally, complications of arteriovenous malformations are involved in multiple vascular diseases, and specific novel signal interactions with Notch in this context will be discussed further.

3. Endothelial cell and smooth muscle cell communication

Communication between endothelial cells and mural cells (smooth muscle cells or pericytes) occurs either by direct contact or by soluble or secreted factors, including ions that convey membrane potential information. Direct contact between endothelial cells and mural cells must overcome the distance imposed by the basal lamina and the extracellular matrix of the basement membrane, whose thickness varies based on vessel type and maturity. Myoendothelial gap junctions [19] and microprojections [20] may serve as conduits for cell contact mediated signaling, including juxtacrine Notch ligand-receptor interaction. There is strong evidence for the importance of heterotypic Notch signaling between endothelial and mural cells for proper vessel formation and integrity. Jagged1 signaling from endothelial cells to mural cells induces the contractile phenotype of differentiated smooth muscle cells [21], specifically via the induction and activation of Notch3 [22]. Reciprocal signaling from mural to endothelial cells is also important for proper postnatal angiogenesis [23]. Contact-mediated Notch signaling between endothelial and mural cells can also reinforce maturation signals by enhancing integrin-mediated binding to the basement membrane and activation of matrix-bound signaling peptides, such as endothelial-derived von Willebrand Factor [24]. The study by Lin et al. in this issue has explored these interactions using co-culture of human endothelial cells with vascular smooth muscle cells. Contact-mediated activation of Notch in smooth muscle cells was specific to co-culture with endothelial cells, which led to increased contractile protein expression, collagen secretion, and decreased proliferation. Both Notch2 and Notch3 in smooth muscle cells were mediators of the endothelial cell-induced differentiated phenotype, but Notch2 was specifically required for the suppression of cell proliferation. Thus, contact-mediated activation of Notch signaling plays an important role in cell and vessel maturation, survival, and homeostasis, and different Notch receptors may have specific roles in these processes.

Notch signaling between cells within the blood vessel microenvironment can also be transmitted via non-contact mediated signals, for example by membrane bound ligands on exosomes. Exosomal DLL4 has been shown to activate Notch signaling in endothelial cells to induce tip cell retraction [25], similar to the endogenous juxtacrine activity of DLL4 in angiogenic sprouting endothelial cells. By contrast, an earlier report showed cis-inhibition activity by exosomal DLL4, wherein exosome-incorporating endothelial cells decreased Notch and enhanced tip cell sprouting [26]. Exosome-mediated Notch signaling between endothelial and mural cells has not yet been reported.

4. Notch pathway crosstalk with other signaling networks in the vasculature

There are many reports of crosstalk between Notch signaling and other pathways within the vasculature. Due to the multi-step nature of Notch signaling, there are numerous levels of potential crosstalk. These include direct binding of nuclear transcription factors to one another or to shared promoter elements; cytoplasmic protein interactions; enzymatic modification such as ubiquitination; transmembrane and membrane-bound protein interactions, and binding of components in the extracellular environment such as extracellular matrix-bound and soluble proteins. We define crosstalk to include relationships

wherein Notch signaling components bind directly to other proteins, or which share binding targets and cooperatively activate or diametrically antagonize one another. It is outside of our scope to discuss all instances of crosstalk between Notch and other proteins/pathways in vascular cells, and we refer the reader to recent excellent reviews that cover additional areas, including Hedgehog signaling as an upstream activator of Notch expression and vascular arterialization [27], VEGF and Notch interaction in endothelial cells [28], and Wnt/Sox 17 in arterial identity [29].

4a. Proteins directly enhancing or suppressing Notch activity in the vasculature

Post-translational regulation by protein interactions can enhance Notch signaling in both endothelial cells and mural cells. Synaptojanin-2 binding protein (Synj2bp) was identified as an enhancer of Notch signaling in endothelial cells [30]. Synj2bp, a mitochondrial outer membrane protein, interacts with the PDZ domains of DLL1 and DLL4. The activity of Synj2bp was anti-angiogenic, leading to decreased endothelial cell proliferation and migration. The mechanism of Synj2bp could partially be linked to activation of Notch signaling by stabilization of DLL4 ligand. In an angiogenic vessel, Synj2bp co-expression with DLL4 in a stalk cell could promote Notch signaling to suppress the development of tip cells. Indeed, suppression of Synj2bp in endothelial cells transplanted *in vivo* led to increased vascular density, similar to the suppression of DLL4 in an angiogenesis context. Non-canonical activation of Notch was also induced by estradiol in endothelial cells, especially in cooperation with DLL4 ligand stimulation, potentiating Notch expression of arterial genes and vessel stability [31]. In vascular smooth muscle cells, positive regulation of Notch was demonstrated in conjunction with angiotensin-2 (AngII) signaling. AngII rapidly promotes gamma secretase activity to cleave and activate Notch1, resulting in smooth muscle cell migration, proliferation, and neointima formation [32]. The effects of AngII were reversed by treatment with gamma secretase inhibitor.

Notch activity can also be negatively regulated via interacting proteins. EGFL7 is an endothelial cell-derived secreted protein that directly binds the extracellular domain of Notch and inhibits its activation. EGFL7 activity thus promotes vascular sprouting in an angiogenic context [33]. However, as vessels mature, EGFL7 expression is progressively restricted to veins and capillaries, where Notch maintenance of arterial fate is not needed [34]. Interestingly, nested within the EGFL7 gene is the intronic miR-126 gene, whose regulatory activity on Notch signaling will be described below. Inhibition of Notch1 can also occur in endothelial cells by direct binding to FKBP1a, a peptidylprolyl isomerase. Loss of FKBP1a led to hyperactive Notch signaling in the endocardium, causing hypertrabeculation and left ventricular heart disease [35]. Pathological inhibition of Notch signaling in cerebral microvascular mural cells can occur via von Willebrand Factor (vWF). vWF, when secreted by endothelial cells abluminally, can bind to and inhibit activation of Notch3, resulting in decreased expression of smooth muscle cell genes and vascular instability [36].

4b. Notch crosstalk with TGF β

Notch crosstalk with the TGF β pathway has been widely reported, comprehensive reviews are available [37, 38], and recent findings will be discussed here. In endothelial cells,

crosstalk between Notch and TGF β is often associated with endothelial-to-mesenchymal transition (EnMT) and pathological fibrosis. EnMT in corneal endothelial cells was inhibited by gamma secretase inhibitor, DAPT, suggesting that Notch signaling is a contributing factor [39]. Interestingly, EnMT was also inducible by treatment of the corneal endothelial cells with TGF β , and it was also inhibited by DAPT, further implicating direct interaction and interdependence of the Notch and TGF β pathways for this physiological process.

In a mouse model of chronic kidney disease, neointima formation and inflammatory cell invasion, secondary to arteriovenous fistulas, was attributed to crosstalk between Notch and TGF β signaling. Increased TGF β 1 during uremia corresponds to activated Notch in endothelial cells and subsequent barrier dysfunction [40]. Not only were Notch ligands sufficient to elicit the same effect, but TGF β -induced changes in phenotype were blocked with Notch inhibition or knockout of RBPJ. In cerebrovascular endothelial cells, direct binding of RBPJ to SMAD4 was shown to be responsible for cooperative, Notch-dependent expression of N-cadherin, which mediates endothelial cell-pericyte association. Loss of SMAD4 decreased the expression of N-cadherin, causing pericyte loss, vascular instability and intracranial hemorrhage [41].

4c. Notch crosstalk with BMP in the vasculature

Notch crosstalk with BMP signaling was recently reviewed in an excellent article that focused on regulation of oscillatory gene expression [42]. In endothelial cells, BMP signaling is generally thought to cooperate with Notch signaling to induce quiescence and promote vessel maturation and arterialization. Recent reports have highlighted this cooperative crosstalk between DLL4/Notch1 and the BMP9-ALK1-SMAD1/5 pathway. Endothelial-specific knockout and knockdown of SMAD1 and SMAD5 abrogate the ability of DLL4/Notch1 to properly coordinate tip and stalk angiogenic phenotypes, leading to hypersprouting vessels *in vitro* and embryonic lethality *in vivo* [43]. Inhibition of BMP9/ALK1 signaling resulted in similar consequences in a postnatal retinal angiogenesis model, where ALK1-Fc ligand trapping of circulating BMP9 induced hypersprouting, arteriovenous malformations, and decreased Hey1/2 expression. Additionally, inhibition of Notch activity with DAPT could be overcome with addition of excess BMP9, indicating that the cooperative signals between the pathways can be compensatory and fine-tuned [44]. The ability of Notch to compensate for loss of ALK1 signaling was also demonstrated in an *in vitro* model of hereditary hemorrhagic telangiectasia, where expression of the Notch target gene and arterial-specific marker EphrinB2 was analyzed. Decreased expression of EphrinB2 secondary to loss of ALK1 was reversed by co-culture with cells expressing DLL4 [45]. The converse was also demonstrated in an *in vivo* model of arteriovenous malformations in the matrix gla protein (MGP) knockout mouse. Since MGP is a BMP inhibitor, excess BMP and Notch target gene activation were associated with AVM development. The pathology was reversed after crossing the MGP null mice onto a Jag1/2 heterozygous background [46], thus demonstrating that cooperative signaling is required for AVM development in this model. In mural cells, BMP cooperation with Notch has been implicated in vascular calcification [47]. Stimulation with BMP2 and Notch cooperatively activates the expression of the osteogenic marker MSX2, and NotchICD complexed with SMAD1 on the MSX2 promoter. Finally, Notch1, BMP2, and MSX2 proteins were co-

localized in atherosclerotic plaques from human vessels, suggesting cooperation in the pathogenesis of human disease progression.

5. Notch signal integration with cell metabolism

Vascular cell metabolism is of increasing interest, and several studies have focused on the role of metabolic changes in endothelial cells with respect to angiogenesis. The seminal study by De Bock et al. demonstrated that the primary method of endothelial metabolism and ATP generation is via anaerobic glycolysis, with glucose oxidation and mitochondrial respiration only activated during endothelial cell stress [48]. Minimizing oxygen consumption is consistent with the primary function of endothelial cells to deliver oxygen to tissues. This study showed that the most highly expressed glycolytic enzyme in endothelium is PFKFB3, a secondary fructose-6-phosphate kinase, whose product (fructose 2,6 biphosphate) is an allosteric activator of the primary phosphofructokinase-1 glycolytic pathway. In this way, PFKFB3 levels could be regulated in endothelial cells without completely inhibiting glycolysis, which would cause cellular stress and apoptosis. While DLL4 suppressed the expression and activity of PFKFB3, the enzyme was upregulated in sprouting endothelial cells, induced by VEGF and FGF cytokines, and concentrated in the distal ends of filopodial tips where kinetic demand for ATP was high, and extracellular pO_2 low. The increased glycolytic flux induced by PFKFB3 overexpression was sufficient to drive endothelial angiogenesis independent of VEGF signaling, and was sufficient to overcome Notch signaling, which would normally promote a stalk cell phenotype. Inhibition of PFKFB3 induced quiescence, even in the absence of Notch activation. Thus, glycolysis is a driver of angiogenesis, independent of activating and inhibitory signaling pathways. Schoors et al. demonstrated the therapeutic potential of regulating PFKFB3 in endothelial cells using the small molecule inhibitor of PFKFB3, 3PO [49]. Multiple *in-vivo* models of pathologic angiogenesis were successfully reversed using 3PO-mediated inhibition of glycolysis, including mouse models of choroidal neovascularization, psoriasis and colitis.

Sawada et al. showed that pathologic suppression of metabolism in endothelial cells was mediated by the PPAR γ coactivator protein, PGC1 α [50]. This protein induces expression of various metabolic genes, driving thermogenesis in brown fat [51] and mitochondrial biogenesis in cardiomyocytes and skeletal muscle [52, 53]. PGC1 α induced the expression and activation of Notch1 and potentially inhibited endothelial sprouting, migration and angiogenesis. Transgenic expression of PGC1 α mimicked phenotypes of endothelial dysfunction secondary to diabetes, and vessel repair and wound healing were severely impaired. Consistent with these finding, knockout models of PGC1 α rescued endothelial cell migration, angiogenesis, and diabetic endothelial dysfunction.

Although the role of metabolism and metabolic effector pathways has been thoroughly investigated in vascular smooth muscle cells, the interaction of Notch with the metabolic state of smooth muscle cells has not been investigated. However, some evidence suggests that Notch may have an impact on smooth muscle cell metabolism. In patients with the R133C CADASIL mutation, smooth muscle cells had reduced proliferation and an increased number of dysfunctional mitochondria [54]. The mitochondrial morphology was highly irregular, with decreased mitochondrial coupling or connectivity and decreased membrane

potential. Thus, Notch3 signaling may contribute to normal smooth muscle cell metabolism by maintaining mitochondrial health.

Recent data have also implicated Notch signaling in various aspects of metabolism, although these pathways have not been addressed in vascular cells. In a human chronic myelogenous leukemia cell line, expression of the Notch1 intracellular domain was found to modify the mitochondrial proteome, leading to changes in genes involved in glutamine catabolism and the respiratory electron transport chain [55]. In addition, adipose tissue-specific inactivation of Notch1 led to the browning of white adipose tissue, leading to improved glucose metabolism and elevated energy expenditure via Ucp-1 [56]. These types of systemic metabolic changes mediated by Notch activity in endocrine organs such as adipose tissue are predicted to affect vascular homeostasis and remodeling.

6. Notch interaction with microRNAs

Many microRNA have been implicated in the regulation of angiogenesis and cardiovascular diseases [57, 58], but limited studies have identified specific microRNAs that regulate Notch ligand and receptor transcripts in endothelial cells or mural cells. One exception is DLL4 transcript, which is directly targeted by the miR-30 family [59, 60] as well as miR-27b [61] in endothelial cells. miR-30b and miR-30c directly bind to a conserved sequence in the DLL4 3' UTR and decrease DLL4 protein in endothelial cells. Further, in zebrafish, expression of a miR-30 mimic suppressed DLL4 and led to hypersprouting and branching of vessels, similar to the DLL4 loss of function phenotype [59, 60]. DLL4 transcript is also targeted by miR-27b, and thus the levels of miR-27b can regulate vascular sprouting and arterial-venous identity [61]. Outside of the vasculature, particularly in cancer cells, multiple microRNAs have been validated to directly target and suppress the production of Notch ligands or receptors (Table 1). It will be of interest in future studies to determine if these interactions are also regulating Notch availability in the vessel wall, especially in conditions of vascular remodeling or tumor angiogenesis, when Notch components are dynamically regulated.

In addition to directly targeting transcripts of Notch pathway genes, microRNAs can also work upstream to alter ligand and receptor levels via indirect mechanisms. For example, delivery of miR-126 to injured mouse myocardium in transplanted mesenchymal stem cells increases secretion of VEGF and FGF2 and upregulates expression of DLL4, resulting in enhanced functional ischemic angiogenesis [62]. The hypoxia-induced miR-210 also appears to promote ischemic angiogenesis, possibly via upregulation of Notch1 as observed in human umbilical vein endothelial cells and rat models of ischemic stroke [63]. Specific targets of these microRNAs and mechanisms for their regulation of DLL4 and Notch1 in these contexts have yet to be defined.

MicroRNAs may also target Notch regulatory proteins, thereby affecting Notch signaling. One example is miR-126, which targets the non-canonical Notch ligand DLK1 [64]. In endothelial cells, expression of a miR-126 (miR-126-5p) mimic decreased DLK1 protein levels, and studies with a DLK1 3'UTR reporter confirmed miR-126 regulation via its predicted binding site. DLK1 functions as a Notch inhibitor, and Schober et al. have

described the physiological consequences of miR-126 targeting of DLK1 in vascular repair and atherosclerosis [64]. Loss of function studies showed that mice lacking miR-126 on an ApoE null background had impaired endothelial cell recovery after injury, and increased atherosclerotic lesions. This effect was reversed when DLK1 was silenced, showing that the miR-126 null phenotype required increased DLK1. The proposed mechanism of miR-126 suppression of atherosclerosis is by promoting endothelial proliferation, creating a “reserve” of cells that can quickly repopulate areas of luminal denudation, suppressing sclerotic lesion formation. Although miR-126 may target multiple transcripts in the vasculature, increased DLK1 in endothelial cells after vascular injury corresponded to decreased Notch1 activation *in vivo*. As described above, Notch signaling has complex, cell-specific roles in atherogenesis and other inflammatory vascular diseases.

Notch as a transcriptional activator has been confirmed for only a few microRNA gene targets in the vasculature. The miR-143/145 cluster, a known regulator of vascular smooth muscle cell phenotype [65], is a direct downstream transcriptional target activated by Jagged1/Notch signaling via RBPJ (CBF1) consensus binding sites [66]. The miR-143/145 cluster is involved in vascular remodeling following injury, and mediates phenotypic changes in vascular smooth muscle cells [67, 68]. Of note, miR-143/145 is also activated by serum response factor/myocardin transcriptionally via the CArG box [65], and this activity is modified by TGF β and BMP [69]. TGF β increases myocardin levels and BMP4 induces nuclear translocation of MRTF-A. Thus, miR-143 and miR-145 are examples of microRNAs that are regulated coordinately through multiple signal pathways. The miR-223 promoter was activated by Notch1ICD or Notch3ICD in Jurkat cells, and both receptors were immunoprecipitated in a complex with a promoter fragment containing an RBPJ consensus-binding site [70]. Although this has not been confirmed in vascular cells, miR-223 has been implicated in arterial remodeling [71].

Notch signaling can also suppress microRNA levels. In zebrafish, miR-221 was characterized as an essential angiogenic regulator that is negatively regulated by active Notch signaling [72]. In this case, loss of Notch led to hyper-angiogenesis and increased miR-221 levels, and knockdown of miR-221 rescued the loss of Notch phenotype. Although it is not clear whether Notch signaling regulates miR-221 gene transcription, miR-221 levels were decreased by Notch signaling. There is also some evidence that Notch signaling may lead to transcriptional repression of microRNAs. miR-155 is a flow-sensitive microRNA associated with inflammation and atherosclerosis, and has been described either as pro- or anti-inflammatory in the vasculature [73, 74]. Loss of function studies demonstrated that RBPJ downstream of Notch signaling repressed miR-155 and inflammation in bone marrow endothelial and stromal cells [18]. In this study, Notch/RBPJ appeared to act as a transcriptional repressor, which can be further validated with mutational analysis of the RBPJ consensus site in the miR-155 promoter. Alternatively, since the miR-155 promoter has multiple potential binding sites for the Hes/Hey family of transcriptional repressors, canonical Notch/RBPJ activation of Hes/Hey could be a second mechanism for repression of miR-155 transcription. The activity of miR-155 on inflammation and atherosclerosis appears to be temporally regulated, with basal expression of miR-155 inhibiting pathogenic inflammatory stimuli, which later becomes pro-inflammatory and detrimental after

establishment of mature disease. Because Notch dysregulation is associated with atherogenesis, it will be of interest to determine whether Notch regulation of miR-155 plays a role in the inflammatory phenotype during atherosclerosis. Given the roles of these microRNAs in inflammation, angiogenesis, and vascular pathology, continued understanding of Notch interaction with microRNAs has implications for vascular therapeutics.

7. Conclusions

Balanced Notch signaling is required in endothelial and mural cells for proper blood vessel function. Too much or too little Notch activation causes cellular dysfunction and failure of vessel function or responsiveness. The potency of Notch signaling and the pathological consequences of imbalanced signaling requires careful regulation, which is aided by interacting proteins that fine-tune its activity. Identification of the regulators, binding partners, and targets of Notch in the vasculature will lead to better understanding of related vascular pathologies and strategies for restoration of vascular homeostasis.

Abbreviations

AngII	angiotensin II
AVM	arteriovenous malformation
BMP	bone morphogenetic protein
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Sub-cortical Infarcts and Leukoencephalopathy
DLL	delta-like
EGFL7	EGF-like domain 7
MGP	matrix Gla protein
PGC1α	peroxisome proliferator activated receptor gamma coactivator 1 alpha
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase3
RBPJ	recombination signal sequence-binding protein J κ
Synj2bp	synaptojanin-2 binding protein
TGFβ	transforming growth factor beta

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Figure 1. Notch signaling in vascular homeostasis and remodeling

In the mature vasculature, the endothelium is a quiescent monolayer with extensive cell adhesions that extend to underlying smooth muscle cells. Quiescence signals include BMP9/BMP10 activation of ALK1, and DLL4 activation of Notch. Normal expression of endothelial cell Jagged1 activates Notch3 in smooth muscle cells to maintain their differentiated, contractile phenotype. Vascular activation under conditions of stress, injury, or disease progression is accompanied by changes in Notch ligand and receptor expression in the vessel wall. These changes regulate cellular proliferation, cell identity, cell function, and overall phenotype.

Table 1

Validated miRNA-Notch target interactions compiled from PubMed, miRTarbase [120], and TarBase [121]. Included are direct target interactions confirmed by strong experimental evidence as defined in most cases by 3'UTR assay with wild type and mutant sequence, and qPCR and Western blot analysis after expression of the microRNA mimic or antagomir. By these criteria, there is no strong experimental evidence validating predicted miRNA target interactions for mammalian Jagged2.

Notch1	Cell Context	Jagged1	Cell Context
miR-10b	glioma [75]	miR-21	dendritic cells [76], breast cancer [77]
miR-30	podocytes [78], dendritic cells (miR-30b) [79], acute myeloid leukemia (miR-30c) [80]	miR-34a	dendritic cells [76], osteoblasts [81], renal epithelial cells [82], cervical carcinoma and choriocarcinoma [83]
miR-34a	glioma, medulloblastoma [84], glioblastoma multiforme [85], renal epithelial cells [82], renal cell carcinoma [86], bladder carcinoma [87], colon cancer [88], breast and cervical carcinoma and choriocarcinoma [83, 89], mouse macrophage [90], keratinocytes and HeLa cells [91]	miR-34b	breast cancer [92]
		miR-124	kidney epithelial and glioblastoma cells [93], neural progenitor cells (miR-124a) [94]
		miR-193a	bone marrow stromal cells [95]
miR-34b	osteoblasts [96], neuroblastoma [97]	miR-199a	myogenesis [98]
miR-34c	osteoblasts [96], neuroblastoma [97]	miR-199b	HEK-293, ovarian cancer cells [99]
miR-139	colorectal cancer [100]	miR-200	multiple cancer cell lines [103]
miR-146a	glioma [101], dendritic cells [102]	miR-214	kidney epithelial and glioblastoma cells [93]
miR-146b	myoblasts [104]		
miR-200	neuroblastoma [97], nasopharyngeal carcinoma (miR-200b) [105]	Jagged2	
miR-326	glioma [106]	no strong experimental validation	
miR-449	myeloid leukemia [107], airway mucociliary epithelial cells [108]		
Notch2		DLL1	
miR-34a	glioma, medulloblastoma [84]	miR-34a	choriocarcinoma [109], medulloblastoma [110]
miR-107	glioma [111]	miR-449	airway mucociliary epithelial cells [108]
miR-326	glioma [106]		
Notch3		DLL3	
miR-1	prostate cancer [112], colorectal cancer [113]	miR-18a	glioma initiating cells [114]
miR-150	T-cell development [115]		
miR-206	HeLa cells, mouse fibroblasts [116]	DLL4	
Notch4		miR-27b	endothelial cells [61]
miR-34c	breast carcinoma [117]	miR-30a	endothelial cells [60]
miR-181c	gastric carcinoma [118]	miR-30b	endothelial cells [59]
miR-302a	melanoma [119]	miR-30c	endothelial cells [59]