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Notch-mediated cellular interactions between vascular cells

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Abstract

Vessel formation and differentiation to a proper hierarchical vasculature requires a coordinated effort from endothelial and mural cells. Over the last decade Notch was identified as a key player in this process by promoting vascular arterialization and modulating endothelial tip-stalk phenotypes. Recent work has identified that Notch fine-tunes the diverse endothelial phenotypes through regulation of canonical cell-cycle and metabolism regulators, such as ERK and Myc. During arterialization, Notch signaling inhibits the cell-cycle and metabolism of endothelial cells which coincides with the acquisition of arterial identity. During angiogenesis, the same molecular machinery prevents the hypermitogenic arrest and excessive sprouting of vessels. Notch also signals in pericytes and smooth muscle cells promoting vascular coverage and maturation. Here, we will review the latest findings on how Notch signals regulate the differentiation and interactions among vascular cells during organ development and homeostasis.

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Introduction

Blood vessels are mostly known for their role delivering oxygen and nutrients to every corner of the body. To meet this demanding task, their numbers, caliber, and hierarchy need to be carefully regulated. Crafting an efficient network of vessels requires teamwork and coordinated interactions between the different cell types comprising the vasculature. In this mini-review we will discuss the role of Notch signaling in changing the transcriptional landscape and differentiation of vascular cells during development. We will focus on cell-to-cell Notch-mediated cellular interactions shown to be critical for vascular cell differentiation, proliferation, and function.

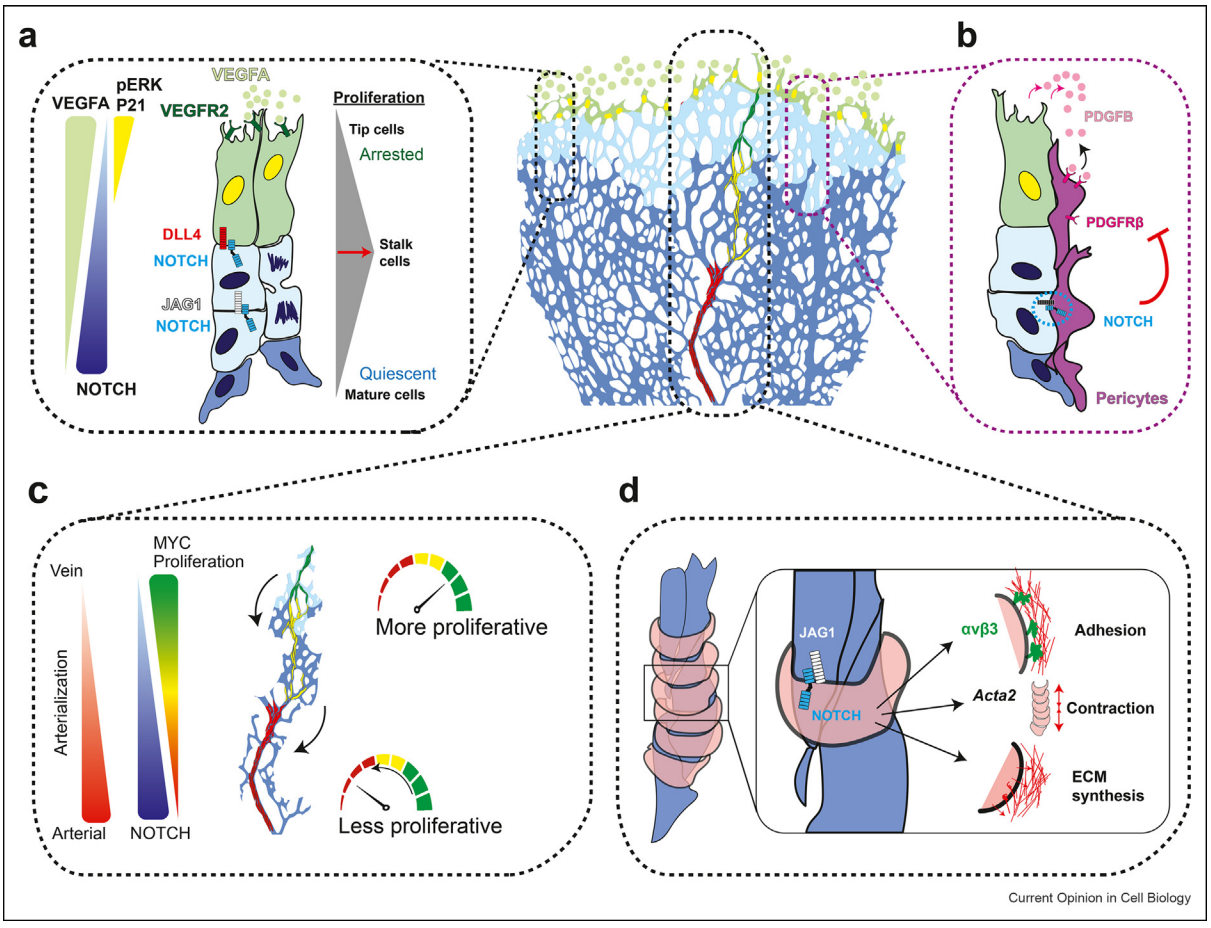
The regulation of endothelial cell-to-cell differentiation and growth by Notch signaling

The first set of blood vessels arise during development by *de novo* differentiation from angioblasts (vasculogenesis), however, most of the blood vessel growth in mammals occurs via expansion and further differentiation of pre-existing vessels, a process named as angiogenesis. Vascular endothelial growth factor-A (VEGFA) is secreted by hypoxic cells and, upon binding to its receptors (mainly KDR, VEGFR2) in endothelial cells (ECs), drives a series of cellular and molecular events that will regulate EC behavior and ultimately give rise to a new vascular plexus [1].

During angiogenesis, ECs at the leading edge of blood vessels, and therefore with the highest levels of VEGFR signaling, adopt migratory properties such as the development of filopodia and dactylopodia to drive the vessel to the source of VEGFA [2]. VEGFR2 signaling induces the expression of the most important NOTCH ligand, Delta-like 4 (DLL4), in this filopodia bearing cells (tip cells). DLL4 will, in turn, signal to the NOTCH receptors in the neighboring ECs to promote a stalk EC phenotype [3,4]. Stalk cells trail behind tip cells, support vascular lumen formation and express higher levels of the ligand JAGGED1, that is significantly weaker in Notch activation due to the strong expression of the Notch glycosyltransferases Fringes, particularly Manic Fringe, in angiogenic ECs [5]. This cell-to-cell heterogeneity in ligand expression results in differential Notch activity among tip and stalk ECs (Figure 1).

Endothelial proliferation and cell cycle dynamics are tightly regulated by the differential levels of Notch and VEGFR signaling [6–8]. At the angiogenic front, DLL4-expressing tip cells are believed to receive lower Notch signaling from adjacent JAGGED1-expressing stalk cells and are exposed to the highest levels of VEGFA. Tip cells have therefore the highest mitogenic stimulation

Figure 1



Notch signaling in vessel formation and specification. (a) Tip cells (lime green) at the angiogenic front are exposed to high levels of VEGFA acting on VEGFR2 and display low NOTCH levels, high pERK and P21 levels (yellow nuclei) which leads to cell-cycle arrest. DLL4 in the tip cells stimulates NOTCH in the stalk cells (light blue) leading to lower levels of pERK, which don't trigger P21 allowing for robust endothelial cell proliferation in response to VEGFA. Mature quiescent cells (dark blue) are exposed to very low VEGFA levels, and together with existing cell-to-cell Notch signaling results in low pERK and low cell proliferation. **(b)** Pericytes (purple) migrate along endothelial cells towards the angiogenic front following a gradient of PDGFB. Notch signaling in pericytes regulates PDGFRβ expression. **(c)** In capillaries forming arteries, MYC suppression by Notch signaling results in a decrease in metabolism and cell-cycle exit inducing arterialization. **(d)** In arteries, JAG1 signals to NOTCH receptors in vascular smooth muscle cells (pink) to promote αvβ3 expression and adhesion to the vessel wall, *Acta2* expression, contractility, and maturation, and ECM homeostasis. VEGFA: Vascular endothelial growth factor-A, VEGFR2: Vascular endothelial growth factor receptor 2, pERK: phospho-ERK, DLL4: Delta like-4, PDGFB: Platelet-derived growth factor-B, PDGFRβ: Platelet-derived growth factor receptor β, JAG1: Jagged-1, ECM: Extracellular matrix.

due to high VEGFR2 signaling and low Notch signaling, which results in the highest MAPK/ERK signaling levels [9,10]. However, paradoxically, tip cells proliferate significantly less than stalk cells. Hyperactivation of ERK signaling was shown to induce P21 and hyper-mitogenic cell cycle arrest in tip cells [8] (Figure 1).

In stalk cells, Notch signaling is activated by DLL4 expressed in the tip cells. pERK levels are relatively lower than in tip cells and are not sufficient to trigger P21-mediated cell cycle arrest. Therefore, stalk cells are the most proliferative ECs, because they have Notch signaling and are still exposed to VEGFA [8]. When DLL4-Notch signaling is blocked, or VEGFA signaling is hyperactivated, stalk cells arrest [8]. Exactly the same pharmacological or genetic manipulations in quiescent vessels or vessels experiencing significantly lower growth factor signaling induce instead EC proliferation. This highlights the existence of a bell-shaped mitogenic balance that either drives sprouting, proliferation or quiescence [7,11] (Figure 1). This gross regulation of tip-stalk differentiation by Notch/VEGFA is also fine-tuned by other signaling pathways such as the bone morphogenetic protein–activin receptor-like-kinase (BMP-ALK) pathway or the sphingosine-1-phosphate (S1P)–S1P receptor pathway, as reviewed in Refs. [1,12,13].

Once an immature network of blood vessels has been formed by vasculogenesis or angiogenesis, the immature

vascular plexus needs to be remodeled into distinct vascular segments formed by differentiated arteries, capillaries, or veins. This specialization is needed for proper vascular function. Arteries need to deliver oxygenated blood across large distances, capillaries filtrate and deliver the blood components to the majority of the organ cells and veins collect after the hypoxic blood and waste products back to circulation.

There are several molecular mediators involved in the process of arteriovenous specification such as VEGF/Notch, Transforming growth factor (TGF)-beta and COUP-TFII. For a complete review see the study by Fang et al. [14]. Similarly, a role for hemodynamic forces has also been proposed to be critical for arterial specification [15]. However, recent studies have suggested that both molecular mediators and hemodynamic flow modulate arterialization by ultimately altering endothelial cell cycle and metabolism.

Notch signaling is cell-to-cell transcriptional machinery, that rewires the transcriptional landscape of the cells, inducing cell-type and context-dependent transcriptional changes [16]. In ECs, Notch signaling is essential for the acquisition of the arterial transcriptional identity, and the mobilization and assembly of ECs into arteries, a process named as arterialization [17–20]. Recently, the process of arterialization has been associated with cell-cycle and metabolic suppression [21–23]. It was shown that arteries and surrounding capillaries have much lower cell-cycle and metabolic activity than venous ECs [22–24]. Su et al. showed that *Myc* expression is suppressed in single ECs acquiring the arterial program [23], and Luo et al. showed that Notch acts mainly by suppressing *Myc* expression and its downstream metabolic and cell cycle responses during arterialization [22]. Cells losing *Notch* or *Rbpj*, the master transcriptional regulator and critical factor mediating Notch signaling, are able to make arteries when *Myc* is deleted, questioning the essential role of *Notch*/*Rbpj* in directly regulating the expression of arterial genes. In addition, ECs with forced activation of NOTCH, are more biased to form and mobilize to arteries [22,25], but can also form normal veins, showing again that Notch signaling alone does not directly specify the arterial fate, even though its function is essential for arterialization.

Notch signaling in mural cells is essential for proper blood vessel formation and stabilization

Blood vessel formation and maturation also requires the coordination and cellular interactions between ECs and mural cells. There are several players known to modulate mural cell coverage during vessel formation [26]. Notch signaling is critical for vascular smooth muscle cells in the arterial compartment of the vessels [27–30]. The NOTCH ligand JAGGED1 in ECs signals to NOTCH receptors in mural cells to promote smooth muscle cell maturation and acquisition of contractile properties [5,31–33], adhesion to the vessel wall [34], and homeostasis of the extracellular matrix [35,36]. JAGGED1 is also a direct target of Notch signaling in smooth muscle cells, which leads to a lateral wave of JAGGED1-Notch signaling through the smooth muscle cell layers of the wider arteries to promote smooth muscle cell assembly of the arterial wall [37,38].

Notch signaling in pericytes regulates PDGFRβ levels [35,39], and when it is lost, leads to paucity of pericytes in the zebrafish brain [40] and the angiogenic plexus of the mouse retina [41]. Interestingly, loss of endothelial JAGGED1 does not alter pericyte coverage [5,34], suggesting that activation of Notch signaling in pericytes occurs in a different fashion than smooth muscle cells, in which endothelial JAGGED1 is the primary NOTCH ligand. It is also unknown if pericyte-to-pericyte Notch ligand-receptor signaling is relevant for their biology.

Recent studies using single-cell analysis of ECs from vessels with pericyte loss also highlight the importance of these cells in establishing proper arteriovenous zonation of blood vessels. ECs shift to a venous gene expression profile in the absence of pericytes [42]. How mural cells modulate endothelial functions during vessel formation and specification is an up-and-coming field thanks to the recent generation of mouse lines allowing the study of gene function and isolation of these populations [43]. Future studies will further our understanding on how endothelial cell–mural cell interactions shape the development and specification of blood vessels.

New insights on the role of Notch in vascular quiescence from single-cell transcriptomics

Sequencing and analysis of whole transcriptomes of single cells (scRNAseq) has revolutionized our understanding of cell biology. In vascular biology, the use of this method has significantly contributed to understand vascular cell heterogeneity and interactions during development, health, and disease [44,45]. Vascular cell atlases of mouse and human tissues have been generated using this technology and serve as a reference of vascular cell transcriptional heterogeneity and arteriovenous zonation [23,42,46–50]. In addition, various single-cell studies were able to reveal changes in transcriptional profiles in disease, such as in response to heart failure, or in vascular anomalies. These studies were instrumental to find treatments that reverse the observed transcriptional changes and disease [44,45,50–53].

Nevertheless, it is important to point out that in certain conditions in both organ physiology and pathology,

transcriptomic analyses may only show a part of a cell's identity, state, or function. For example, a recent publication using a combination of bulk RNAseq, protein and phospho-protein analyses of liver ECs, demonstrated that the expression of endothelial receptor tyrosine kinases (RTKs) such as TIE1, TIE2 and VEGFR2 is similar between portal (arterial) and central (venous) vessels. However, a significantly stronger tyrosine phosphorylation in the central compared to the portal vessels was observed, which may better explain the difference in the biology of these cells and their different sensitivity to RTK inhibitors [54].

Genetic- or tumor-induced activation of endothelial Notch has been shown to induce the expression of senescence markers such as p16 (encoded by *Cdkn2a*) in mouse lung to promote a pro-metastatic environment [55]. Moreover, sustained endothelial Notch activity has also been reported to influence endothelial-to-mesenchymal transition, vascular inflammation and permeability (reviewed in the study by Hasan et al. [56]).

The loss of Notch signaling in adult ECs causes vascular abnormalization and neoplasms specifically in the liver, heart, and muscle [51,57,58], but not most other organ vascular beds. This reactivation of the adult endothelium questioned the use of Anti-Dll4 in the clinics [58]. Interestingly, the vascular abnormalization observed in some organs after Dll4 targeting did not correlate with transcriptional changes [51]. Brain vessels with loss of Dll4/Notch signaling had significant transcriptional changes, but no clear phenotypic changes, whereas heart vessels had minor transcriptional changes, but significant phenotypic changes. Liver vessels with endothelial-specific deletion of *Rbpj* or *Dll4* (the main ligand activating Notch signaling in the liver vasculature) had similar transcriptomic profiles determined by bulk RNAseq, but very different single-cell states and phenotypes [51]. Key differences between these two genotypes were revealed by 3D vascular imaging, histology and scRNAseq. After *Dll4* loss in liver ECs, there is a complete loss of the quiescent arterial and venous transcriptional programs, an increase in proliferating cells, and an additional tip cell angiogenic state having higher *Myc* and expression of its downstream targets. As a result, livers with endothelial *Dll4* loss presented severe vascular abnormalization and organ pathology. In comparison, loss of *Rbpj* or *Notch1* in liver ECs also resulted in a complete loss of the arterial program, but ECs remained largely quiescent, and did not become tip cells. This correlated with the lack of major organ pathology and suggested that the activated or angiogenic tip cell state seen in *Dll4* mutants caused liver pathology. However, co-deletion of *Dll4* and *Myc* prevented most transcriptomic changes associated with deletion of *Dll4*, and prevented the appearance of angiogenic cell states, but did not prevent the vascular abnormalization and organ pathology [51]. These data suggest that angiogenic cell states correlate with organ disease but do not cause it. It also highlights that single-cell transcriptomics alone should not be used to infer vascular function or pathology. There needs to be a careful analysis and distinction between what cells are doing in a tissue (phenotype, cell—cell interactions, and signaling) from what genes those cells are expressing.

Conclusions

Notch signaling has long been identified as an essential pathway involved in the formation and differentiation of blood vessels, and yet we are still discovering the molecular and cellular intricacies involved in these processes. The advent of single-cell genetics and profiling technologies has certainly contributed to revealing the complexity of cellular heterogeneity, their signaling and interactions with an unprecedented resolution. We are also beginning to learn about the limitations of single-cell transcriptomics to fully understand a gene function or a mutant phenotype. The advance of novel functional genetics technologies, proteomics and bioinformatic tools will be key to improve our understanding of cardiovascular development and disease, and to discover novel therapeutic targets.

Author contributions

H.C., S.M., I.G-G, and R.B. wrote and edited the manuscript. I. G-G. prepared illustrations.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data availability

No data was used for the research described in the article.

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