Arterial Regulators Taken Up by Lymphatics

Carrie J. Shawber¹ and Jan Kitajewski^{1,2}

Abstract

We present three examples of key genes that function in arterial specification that have recently been implicated in lymphatic development; ephrinB2, FoxC2, and Notch. In arterial cell fate determination, Foxc2 regulates both Notch and Notch ligand expression. In turn, Notch signal activation in arteries drives expression of ephrinB2. It will be interesting to determine if the regulatory relationships between these pathways found in arterial development are relevant to understanding lymphatic development, that is, we ask whether arterial regulators are also key regulators of lymphatic development.

Introduction

BOTH ARTERIAL AND LYMPHATIC SPECIFICATION involve bi-potential cell fate decisions, arterial/venous and lymphatic/venous. Arteries function to hold blood tight and deliver it under pressure and force. Lymphatics collect fluid and are not subject to the high pressure of blood flow.¹ Although these two types of vasculature differ greatly in structure and function, they may utilize some common factors to promote differentiation and specialization. Here, we will discuss recent studies suggesting that pathways known to regulate arterial specification, such as Notch and ephrinB2, may have unexpected but important roles during specialization of the lymphatic vasculature.

In arterial/venous endothelial specification, it is thought that EphB4-expressing venous and ephrinB2-expressing arterial endothelial cells originate from a common progenitor.² Polarized expression of the master regulator of lymphatic endothelial specification, Prox1, is thought to initiate lymphangiogenesis, reviewed in Oliver and Alitalo,¹ that is, Prox1 in venous endothelial cells marks the initiation of the bi-potential cell fate decision that occurs to differentiate lymphatic endothelial cells from venous cells.³ Regulation of bi-potential cell fate determination steps, such as arterial/venous specification or lymphatic differentiation from veins, is often the job of the Notch signaling pathway. Thus, it is worthwhile to consider a potential function of Notch in these processes.

In the arterial/venous bi-potential cell fate decision, Notch promotes arterial endothelial differentiation, while suppressing venous specification.⁴ This activity is carried out in part through regulation of ephrinB2 expression in arterial en-

dothelium. Interestingly, this same signaling molecule, ephrinB2, has also been shown to function in remodeling of the lymphatic plexus into lymphatic collecting ducts and capillaries.⁵

Once lymphatic specification is initiated by Prox1, the lymphatic endothelial cell comes to depend on VEGF receptor 3 (VEGFR-3) and the ligands VEGF-C and VEGF-D to promote further growth.⁶ In fact, VEGFR-3 is considered the most important growth factor receptor for lymphatic development. Notch signaling is now known to be a direct regulator of VEGFR-3 expression in blood endothelial cells,⁷ leading to speculation of a similar relationship between Notch and VEGFR-3 in lymphatic endothelial cells. Notch1 and Notch4 are both expressed in some murine lymphatics, including those found in neonatal dermis.⁷ In humans, Notch1 and Notch4 are expressed in the extratumoral lymphatics of human micropapillary mammary carcinomas.⁷ Thus, the receptors that function to mediate endothelial function of Notch signaling are expressed in select normal and pathological lymphatics. Equally compelling is the finding that an activated peptide of Notch1 is present in tumoral lymphatics associated with such mammary carcinomas. Together, these data suggest that it is important to consider if Notch signaling may participate and regulate different cell fate determination steps of physiological and pathological lymphangiogenesis. Further, one might ask if Notch, a known regulator of ephrinB2, functions upstream of ephrinB2 in remodeling lymphatic vasculature.

Notch signaling is not thought to be active in venous endothelium. In fact, the COUP-TFII orphan nuclear receptor is actively involved in maintaining venous cell fate by repressing Notch signaling, thus maintaining vein identity.⁸ It

Departments of ¹Obstetrics/Gynecology and ²Pathology, Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, New York, New York.

is not currently known whether COUP-TFII functions in lymphatics and, if so, if it similarly functions to repress Notch signaling. Thus, the potential role of Notch on lymphatic development may depend on defining expression patterns in lymphatics of the factors that regulate arterial (Notch), venous (COUP-TFII), and lymphatic cell fate.

The Notch Signaling Pathway

Notch functions in an evolutionarily conserved signaling pathway that modulates cell-fate decisions (reviewed in Ref. 9). In mammals, there are four Notch genes (Notch1–4) and five ligands, Jagged1, Jagged2 and Delta-like 1, 3, 4 (Dll). Upon ligand binding, the cytoplasmic domain of Notch is released from the cell surface by a presenilin/ γ -secretase proteolytic cleavage. The cytoplasmic Notch peptide translocates to the nucleus and interacts with the CBF-1/Suppressor of Hairless/Lag-2 (CSL/*rbpsuh*) transcriptional repressor, converting it to an activator. Hairy Enhancer of Split (HES) and HES-Related (Hey) transcriptional repressors are direct targets of Notch/CSL signaling. Consistent with the Notch/CSL signaling pathway regulating the expression of blood endothelial cell genes involved in cell fate determination, mice deficient for *Notch1*, *Dll4*, *Hey1*, and *Hey2* or *CSL/rbpsuh* are embryonic lethal prior to E10.5 and display defects in angiogenesis and arterial endothelial cell specification.¹⁰ Transgenic mice that activate Notch4 within the embryonic endothelium also die in mid-gestation with defects in vascular remodeling.¹¹ Thus, the dosage of Notch activity within the endothelium is critical.

In blood endothelial cells, the Notch/CSL signaling pathway regulates cell fate decisions by regulating gene expression.¹² The downstream mediators of Notch function in endothelial cells are becoming known. Hey1 and Hey2 figure prominently in models of Notch function and are directly regulated by Notch/CSL.¹³ However, other intriguing targets of the Notch signaling pathway consist of genes that encode members of the VEGF receptor family. Notch induces VEGFR-3 directly, that is, it functions by binding and transactivating the VEGFR-3 promoter.⁷ Notch also suppresses the expression of VEGFR-2 and upregulates ephrinB2 via its induction of Hey2.¹² This complex relationship is schematized in Figure 1. How important is VEGF receptor expression for Notch function in endothelial cell fate determination

steps? This question is currently being addressed experimentally and we consider evidence for such regulation in the lymphatic vasculature.

Notch Signaling in Angiogenesis and Arterial Endothelial Specification

VEGF drives a variety of key steps in vascular development including growth of endothelium, specification of arteries and veins, and promotion of new angiogenic sprouts. During sprouting angiogenesis, VEGF promotes a new sprout from a pre-existing vessel. A role for Notch in sprouting angiogenesis is evident from analysis of retinal vasculature, where Notch limits angiogenic sprouting, reviewed in Refs. 14 and 15. The role of Notch signaling in this process is to limit further sprouting via a feedback loop involving downregulation of VEGFR-2 (Fig. 1).

VEGF also utilizes Notch signaling to drive the process of endothelial cell specification to an arterial fate; that is, Notch functions downstream of VEGF in arterial/venous (A/V) specification.¹⁶ In both zebrafish and mammals, VEGF induces the expression of Notch and Notch ligands, suggesting VEGF regulates arterial specification via activation of Notch signaling. In this setting, VEGF promotes Notch signaling which then in turn drives expression of ephrinB2 via Hey2 to promote the arterial cell fate (Fig. 1).

EphrinB2 in Lymphangiogenesis Signaling

EphrinB2 is a molecule long appreciated as a protein expressed at the onset of arterial specification and in differentiated arterial endothelium.² The arterial expression of ephrinB2 is mediated by Notch signaling.^{17,18} The discovery of new roles for ephrinB2 in lymphatic remodeling⁵ highlights the diversity of functions that can be carried out by distinct angiogenic regulators.

In mouse postnatal day 0 (P0) neonates, the dermal lymphatic vasculature is a uniform vascular plexus deep with the dermis. This lymphatic vascular plexus at this early stage of development expresses both ephrinB2 and EphB4.⁵ At P3, lymphatic endothelial cells sprouting from the plexus begin moving towards the upper dermis. Between P5 and P7, lymphatic capillaries that express EphB4 and are devoid of mural cells differentiate in the upper dermal layer. Deeper in the dermis, ephrinB2 expression is maintained in the lymphatic collecting ducts that are surrounded by α SMA-positive vascular smooth muscle cells. Thus, ephrinB2 comes to be associated with lymphatic collecting ducts and not lymphatic capillaries.

EphrinB2 can act both as a ligand for EphB4 and also as a receptor that depends on EphB4 binding to promote reverse signaling. EphrinB2 signaling (referred to as “reverse” signaling) depends on an intracellular PDZ domain.¹⁹ Deletion of the ephrinB2 PDZ domain in mice resulted in lymphatic defects, demonstrating a previously unappreciated role for ephrinB2 signaling in lymphangiogenesis.⁵ Mice lacking the PDZ domain die during the first 3 weeks of life. Analysis of the mutant mice showed a failure to remodel the primary lymphatic plexus into a network of vessels with the proper hierarchy of collecting ducts and capillaries. In addition, luminal valve formation in the lymphatic collecting ducts was defective and general hyperplasia of lymphatics was observed. Finally, new sprout formation appeared to be

FIG. 1.

aborted, displaying few proper elongated sprouts and stumps where sprouts might have attempted to initiate.

Although no similar role in lymphatic remodeling has been ascribed to the Notch pathway, as that described above for ephrinB2 signaling, one may consider if Notch is involved in regulation of ephrinB2 in the lymphatic endothelium. If this was the case, one might expect that the expression pattern whereby ephrinB2 becomes restricted to collecting ducts and absent from capillaries may involve Notch. Further analysis of this concept will depend on understanding the expression of Notch and Notch ligands in lymphatic ducts. To date, no Notch mutant mouse has been described with defects in lymphatic vasculature, however, many of the Notch mutant mice die during stages of cardiovascular development prior to the onset of lymphatic vascular development.

Notch Signaling Regulates the Balance of VEGFR-2 and VEGFR-3

During angiogenesis, endothelial cells are exposed to a variety of external stimuli, such as those provided by bFGF, VEGF, ephrins, and angiopoietins.^{20,21} Notch alters endothelial cell responsiveness by changing the expression of VEGF receptors at the cell surface of endothelial cells. Notch signaling suppressed VEGFR-2 expression, while dramatically increasing VEGFR-3 in cultured primary endothelial cells,⁷ schematized in Figure 2. Consistent with this reciprocal change in expression, HUVEC survival in response to VEGF-A was reduced when Notch signaling was activated and increased in response to VEGF-C.⁷ Thus, Notch signaling may regulate blood endothelial cell fate decisions by decreasing VEGFR-2 and increasing VEGFR-3-based signaling. *In vivo*, Notch signaling has been found to regulate the expression of VEGF receptors during postnatal retinal angiogenesis.²² In the retina of *Dll4* heterozygous mice, disruption of Notch signaling correlated with an increase in vascular

density, an increase in VEGFR-2 expression and a reduction in VEGFR-1 endothelial expression.²²

The alteration in the balance of VEGFR-2 and VEGFR-3 levels by Notch may affect the ability of VEGFR-2 and VEGFR-3 to form a heterodimeric complex.²³ The VEGFR-2/VEGFR-3 receptor complex has been suggested to maintain vascular integrity and promote angiogenesis.²⁴ In zebrafish, VEGFR-3 interacts with VEGFR-2 during morphogenesis of the intersomitic arteries.²⁵ In mice, embryonic endothelial Notch promoted VEGFR-3 expression within the developing intersomitic vessels. Thus, Notch may regulate the balance of VEGFR-2 and VEGFR-3 expression during angiogenesis. VEGFR-2 and VEGFR-3 heterodimers have also been proposed to regulate different steps of lymphangiogenesis and thus, Notch signaling may also regulate their expression levels.

Notch, a Regulator of Lymphangiogenesis?

Notch1, Notch2, and Notch4 and the Notch ligand, Dll1, are all expressed by cultured rat thoracic lymphatic endothelial cells (rLECs).²⁶ Interestingly, expression of Notch2 and Dll1 was found to be restricted to the rLEC and absent in the venous endothelial cells. Rat LEC grown in hypoxic conditions, a closer simulation of LEC physiological condition, expressed higher levels of Notch4 relative to normoxic conditions. Hey2 expression was also increased, suggesting that Notch signal activation was increased under hypoxic conditions. This correlated with a downregulation of VEGFR-2 and an increase in VEGFR-3. Under normoxic conditions, treatment with a gamma-secretase inhibitor or expression of a dominant-negative Dll-1 resulted in an increase in VEGFR-2 expression and increased LEC migration in scratch/wound assay. These data suggest that Notch functions to suppress VEGFR-2 expression in lymphatic endothelial cells, similar to blood endothelial cells. However, it is yet unclear whether Notch regulates VEGFR-3 expression in LECs.

A role for Notch in lymphangiogenesis *in vivo* has yet to be described. Within the murine dermis, both Notch1 and Notch4 are co-expressed with VEGFR-3 and the lymphatic endothelial cell marker, LYVE-1.⁷ In humans, Notch1 and Notch4 are expressed in the extratumoral lymphatic vessels of invasive mammary micropapillary ductal carcinomas. Moreover, Notch1 signaling was found to be activated with in a subset of the tumoral lymphatic endothelial cells, indicating that Notch1 is not only expressed, but is activated in tumor lymphatic vessels. In human breast cancer, VEGF-C via VEGFR-3 signal activation has been proposed to promote lymphatic endothelial cell proliferation, tumor lymphatic invasion, and tumor metastasis.²⁷ Thus, Notch1 signaling may function in tumor lymphangiogenesis and by extension breast cancer metastasis.

A Potential FoxC-Notch Signaling Axis in the Endothelium?

FoxC2 is a transcription factor of the forkhead family that functions in differentiation of lymphatic vasculature. Specifically, FoxC2 is expressed in luminal valves of lymphatic vasculature and loss of function of *FoxC2* results in failure to form valves and recruit smooth muscle cells to lymphatic collecting ducts.²⁸

FIG. 2.

Recently, it has been found that in mouse embryonic endothelial cells (MEECs), FoxC1 and FoxC2 upregulate the expression of Notch1, Notch4, and Dll4, as well as the arterial specific markers, ephrinB2 and the Notch target, Hey2.²⁹ The regulation of Dll4 occurs via direct binding of FoxC to fork-head binding sites in the Dll4 promoter. This suggests that the FoxC transcription factors function upstream of Notch to promote arterial specification. In fact, FoxC1 and FoxC2 double homozygous knockout mice exhibit arteriovenous malformations and a loss of arterial markers within the embryonic endothelium. As with ephrinB2, Foxc2 functions with Notch in arterial specification. Also like ephrinB2, FoxC2 is involved in lymphatic remodeling and maturation.

Another intriguing observation relates to the fact that *Foxc1* heterozygous/*Foxc2* homozygous double knockouts have reduced sprouting of Prox1 positive cells from the cardinal vein that was suggested to arise from reduced mesenchymal VEGF-C expression. Thus, early lymphatic specification depends to some extent on proper Foxc1 activity. However, Foxc2 was found expressed in endothelium of the cardinal vein. Could the lymphatic defects be in part due to aberrant Notch signaling as well?

Closing Comments

We present three examples of key genes that function in arterial specification that have recently been implicated in lymphatic development; ephrinB2, FoxC2, and Notch. In arterial cell fate determination, Foxc2 regulates both Notch and Notch ligand expression that in turn drives expression of ephrinB2. It will be interesting to determine if the regulatory relationships between these pathways found in arterial development are relevant to understanding lymphatic development, that is, we ask whether arterial regulators are also key regulators of lymphatic development. On the other hand, should one be asking whether key regulators of lymphatic development also function in arterial development?

Disclosure Statement

Professors Shawber and Kitajewski have no conflicts of interest or financial ties to disclose.

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Address reprint requests to:

Jan Kitajewski, Ph.D.

Irving Cancer Research Center

Columbia University Medical Center

1130 St. Nicholas Avenue

New York, NY 10032

E-mail: jkk9@columbia.edu