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Endothelial Metabolic Control of Lymphangiogenesis

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Abstract

Lymphangiogenesis is an important developmental process that is critical to regulation of fluid homeostasis, immune surveillance and response as well as pathogenesis of a number of diseases, among them cancer, inflammation and heart failure. Specification, formation and maturation of lymphatic blood vessels involves an interplay between a series of events orchestrated by various transcription factors that determine expression of key genes involved in lymphangiogenesis. These are traditionally thought to be under control of several key growth factors including vascular growth factor-C (VEGF-C) and fibroblast growth factors (FGFs). Recent insights into VEGF and FGF signaling point to their role in control of endothelial metabolic processes such as glycolysis and fatty acid oxidation that, in turn, play a major role in regulation of lymphangiogenesis. These advances have significantly increased our understanding of lymphatic biology and opened new therapeutic vistas. Here we review our current understanding of about metabolic controls in the lymphatic vasculature.

Keywords

lymphatics; endothelial cells; metabolism; glycolysis; fatty acid β -oxidation; lymphangiogenesis; growth factor signaling

1. Introduction

In vertebrates, normal organ development and function are supported by complex vascular networks composed of the blood and lymphatic vasculatures.^[1] While blood vessels form a closed circulatory network, the lymphatic vasculature is an open system that links interstitial spaces to the venous circulation.^[2] This structure underlies the key function of the lymphatic network- maintenance of the tissue fluid balance. Accumulating interstitial fluid is driven into lymphatic capillaries, transported through collecting lymphatics, and, eventually, returned to the arterio-venous system via subclavian and internal jugular veins.^[3] In addition to its involvement in fluid homeostasis, lymphatics are in integral part of the immune

defense by virtue of functioning as conduits for lymphocyte trafficking.^[4] Finally, a series of recent studies have demonstrated a link between fat metabolism and lymphatic vessels, indicating the potential therapeutic value of targeting lymphatics for treating metabolic disorders, such as obesity and atherosclerosis.^[5]

Formation of the lymphatic network is a highly stereotyped and finely controlled process (Figure 1).^[6] During early embryonic development, the majority of lymphatic endothelial cells (LECs) arise from venous endothelial cells (VECs).^[7] Recent studies using cell lineage tracing have also indicated that some of LECs in certain organs such as the heart, mesentery and skin are derived from non-venous cells (e.g., hemogenic endothelium).^[8] In mice, venous endothelium-derived LECs first form lymph sacs.^[9] This primitive lymphatic network then expands via a process involving proliferation, sprouting and migration of lymph sac LECs to form a fully branched mature lymphatic vasculature.^[7, 10] While various signaling events involved in the regulation of lymphatic fate specification and lymphangiogenesis have been extensively studied and reviewed,^[11, 12] two recent studies have identified previously unappreciated role of cellular metabolism in lymphatic vascular development, thereby establishing a functional crosstalk between metabolism and lymphatic development.^[13, 14] Here we will review the current knowledge on how metabolic processes regulate lymphatic development and function. Specifically, we will focus on the emerging roles of fatty acid β -oxidation and glycolysis in these events.

2. Molecular regulation of lymphatic differentiation and lymphangiogenesis

2.1. Transcriptional control of lymphatic fate specification: role of prospero-related homeobox 1 (PROX1).

Extensive genetic and phenotypic studies have identified the transcription factor PROX1 as the key regulator of VEC-to-LEC differentiation.^[6] In *Prox1* knockout mice, endothelial cells in anterior cardinal veins fail to express any lymphatic markers and their budding into surrounding mesenchyme is arrested, resulting in mouse embryos devoid of any lymphatic structures.^[15] *Prox1* heterozygous mutants exhibit a disorganized and mispatterned lymphatic vasculature and greatly reduced lymphatic function.^[16] Lymphatic vessels in these mice frequently lack structural integrity and demonstrate increased leakiness. Notably, most of *Prox1* heterozygous knockout mice die soon after birth and exhibit severe lymphatic dysfunction. Interestingly, those few mutants that survive to adulthood, develop late-onset obesity, which can be mitigated by restoring lymphatic-specific expression of Prox1.^[16] Finally, a deletion of a single copy of *Prox1* is sufficient to prevent development of lymphovenous valves.^[17]

Not only is *Prox1* necessary for lymphatic fate acquisition, it is also sufficient to drive the differentiation of VECs to LECs, because overexpression of PROX1 in cultured blood endothelial cells (BECs) induces a lymphatic-specific gene signature while suppressing BEC-restricted genes.^[18] Importantly, when *Prox1* is ablated globally, including in the lymphatic endothelium, LECs lose their lymphatic identity and partially acquire BEC phenotypic features, demonstrating that Prox1 activity is required for maintenance of the

LEC fate.^[19] The expression of *Prox1* is controlled by two transcription factors, Sox18 and Coup-TFII, which are expressed in cardinal veins at the time of the initial lymphatic fate specification. Both bind directly to promoter regions of *Prox1* thereby inducing its transcription.^[20] A deficiency of either Sox18 or Coup-TFII blocks *Prox1* expression and lymphatic fate specification in mice.^[20] Notably, loss-of-function mutations in *SOX18* lead to dominant or recessive hypotrichosis-lymphedema-telangiectasia syndrome in human.^[21] Ectopic activation of ERK signaling in endothelial cells induced by expression of a gain-of-function Raf1 mutant induces expression of *Sox18*, *Prox1*, and several other lymphatic marker genes, thereby leading to increased VEC-to-LEC differentiation and consequently lymphangiectasias,^[22] demonstrating that lymphatic fate specification is an ERK-controlled transcriptional program.^[11]

2.2. Growth factor signaling in lymphangiogenesis

Vascular endothelial growth factor receptor (VEGFR) signaling.—During lymphatic differentiation, PROX1 induces expression of a set of genes that play critical roles in lymphangiogenesis.^[12] One of these, *FLT4*, encodes VEGFR3, which is bound and activated by its ligand, vascular endothelial growth factor (VEGF) -C, a member of the VEGF family of growth factors.^[2] *Vegfr3* is expressed in blood vessels before development of the lymphatic system commences.^[23] Consistent with this expression pattern, global knockout of *Flt4* leads to abnormal formation of large blood vessels, fluid accumulation in the pericardial cavity and cardiovascular failure in E9.5 mouse embryos.^[23] As mammalian development progresses, the expression of *Flt4* is downregulated in the blood vasculature and gradually becomes restricted to the lymphatic vasculature.^[24] However, it remains poorly understood what molecular mechanisms shut down *Flt4* expression in blood vessels. Extensive genetic analyses have demonstrated the crucial roles played by VEGFC/VEGFR3 signaling in lymphangiogenesis and lymphatic vessel maintenance. Forced expression of *Vegfc* in transgenic mice leads to lymphatic hyperplasia without affecting the blood vasculature.^[25] Conversely, a total deletion of *Vegfc* results in failure of LEC migration out of cardinal veins and severe defects in lymphatic formation, which consequently lead to edema and prenatal death.^[26] In agreement with this finding, inhibition of *Vegfc* signaling by expression of a soluble form of VEGFR3 causes impairment of lymphatic capillary sprouting, regression of the already formed lymphatic vessels, and interstitial fluid accumulation.^[27] These defects are to a large extent phenocopied in postnatal mice with lymphatic-specific deletion of *Flt4*.^[28] The importance of VEGFC/VEGFR3 signaling in lymphatic development is also demonstrated by the fact that missense mutations that disrupt the kinase activity of human VEGFR3 cause congenital lymphedema.^[29]

Fibroblast growth factor receptor (FGFR) signaling.—FGF signaling is another major growth factor signaling pathway that regulates lymphatic development.^[12] Implantation of FGF2 in the mouse cornea stimulates robust lymphangiogenesis,^[30] which can be inhibited by anti-VEGFR3 blocking antibody treatment,^[31] suggesting a functional crosstalk between FGF2 and VEGFC signaling. Insertion of both FGF2 and VEGFC into the mouse cornea results in an additive effect in driving lymphatic formation and VEGFR3-dependent lymphatic endothelial tip cell formation seems to be required for the lymphangiogenic effect of FGF2.^[32] FGF2 binds lymphatic vessel endothelial hyaluronan

receptor-1 (LYVE1), a homolog of the hyaluronan receptor CD44, that is highly enriched in LECs.^[33] LYVE1 expression is regulated by FGF2 and disruption of the LYVE1-FGF2 interaction impaired FGF2 signaling and its function in promoting lymphangiogenesis.^[33]

Of the four FGFRs (FGF1–4) that bind various FGF family members, FGFR1 is the most prominent FGF receptor in LECs and is required for FGF2-induced LEC proliferation, migration and tube formation.^[34] Another FGFR, FGFR3, is transcriptionally controlled by PROX1 through interacting with Coup-TFII and is also involved in FGF2-dependent stimulation of LEC proliferation. Importantly, FGFR1 and R3 can compensate for each other so that lymphatic development remains unchanged when either *Fgfr1* or *Fgfr3* (but not both) is deleted in lymphatic endothelium in mice.^[13] The expression pattern is similar in the blood vasculature where FGFR1 is also the most highly expressed FGFR family member.^[35] Similar to the lymphatic vasculature, individual knockouts of either *Fgfr1*, *Fgfr3*, or a double *Fgfr1;Fgfr2* knockout do not affect developmental angiogenesis,^[13, 36] while a combination of *Fgfr1;Fgfr3* knockout disrupts normal blood vascular development as well. These results suggest a complex genetic compensation between different FGFRs in regulating vascular morphogenesis. In particular, expression of FGFR3, but not the other FGFRs, is significantly increased in FGFR1-deficient endothelial cells.^[13]

Not only is FGFR signaling critical for vascular development, it is also involved in several other vascular biological processes. Ablation of *Fgfr1* and *Fgfr2* in endothelial cells reduces injury-induced neovascularization in the skin and eye.^[36] A suppression of FGF signaling due to deletion of FGFR1 or an FGFR adaptor fibroblast growth factor receptor substrate 2 (FRS2 α), triggers endothelial-to-mesenchymal transition (EndMT) via activation of the transforming growth factor (TGF)- β pathway.^[37] Finally, FGF signaling is also required for maintenance of the adult vasculature, as suppression of normal FGF signaling input leads to disruption of adherens and tight junctions, impairment of endothelial barrier function, and, in some cases, even disintegration of the blood vasculature.^[38]

Other growth factor signaling.—In addition to FGF and VEGF, insulin-like growth factor (IGF)1, IGF2, hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGF)B are also able to stimulate lymphangiogenesis under certain circumstances.^[2] However, genetic evidence demonstrating the functional significance of these growth factor signals in lymphatic development is still lacking.

3. Role of endothelial metabolism in the development of the lymphatic vasculature.

3.1. Fatty acid β -oxidation

During the process of fatty acid oxidation (FAO), fatty acids are catabolized into acetyl-CoA, which subsequently enters the Krebs cycle, and NADH and FADH₂, which are used in the electron transport chain as co-enzymes.^[39] Thus, FAO is the major source of energy and a key regulator of redox homeostasis in most cell types and tissues.^[40] The endothelium is unique in this regard as FAO in both BECs and LECs contributes primarily to de novo nucleotide synthesis without a major impact on either energy generation or a redox balance.

[14, 41] In endothelial cells, fatty acid-derived carbons are incorporated into aspartate and uridine monophosphates that serve as precursors for nucleotide and pyrimidine nucleoside triphosphates, respectively, thereby supporting DNA replication.^[14, 41] Inhibition of FAO in the endothelium by reducing expression of its rate limiting enzyme, the carnitine palmitoyltransferase 1A (CPT1A), significantly reduces aspartate and uridine monophosphate levels, resulting in impaired synthesis of deoxyribonucleotide (dNTP) and decreased endothelial cell proliferation.^[14, 41] However, it is not clear how FAO exerts this effect when glucose and glutamine are readily available, given that both are also carbon sources for the tricarboxylic acid (TCA) cycle and acetyl-CoA.^[42]

In addition to this effect on DNA biosynthesis, FAO in LECs also plays an important role in lymphatic differentiation via PROX1-dependent regulation of VEGFR3 expression (Figure 2A).^[14] PROX1 induces *CPT1A* transcription directly and promotes FAO, which leads to increased generation of acetyl-CoA.^[14] PROX1 also interacts with P300, a lysine acetyltransferase, at the promoter region of *Vegfr3* and other PROX1-controlled genes.^[14] Histone acetylation as one type of the dynamic chromatin marks plays critical roles in several molecular processes, including regulation of gene expression.^[43] Acetyl-CoA serves as the donor substrate in the histone acetylation catalyzed by P300, which transfers an acetyl group from acetyl-CoA to lysine residues on histones.^[14] Therefore, PROX1 harnesses FAO in its induction of *FLT4* and other lymphatic-specific genes through a histone acetylation-dependent epigenetic modulation.^[14] It is unclear whether FAO impacts histone acetylation in BECs as well, as published studies indicate that in non-endothelial cells oxidative TCA cycles and fatty acid oxidation regulate histone acetylation.^[44] Consistent with the role of CPT1A-driven FAO in regulation of VEGFR3 expression, a lymphatic-specific deletion of *Cpt1a* results in defective lymphatic vessel formation and function.^[14] Inhibition of CPTA activity also suppresses pathological lymphangiogenesis, which is rescued by acetate that can be metabolized into acetyl-CoA.^[14]

3.2. Glycolysis

Both lymphangiogenesis and angiogenesis involve robust sprouting, migration, and proliferation of endothelial cells,^[1] all energy-requiring processes.^[45] Measurements of the flux rates of the four major metabolic processes in LECs (anaerobic glycolysis, and oxidation of glucose, glutamine and fatty acids) shows that the former accounts for >70% of the ATP production (Fig 2B).^[13] Similar to LECs, BECs, regardless of their origin (i.e. venous, arterial or microvascular) are also highly glycolytic and heavily rely on anaerobic glycolysis for ATP production.^[46] This endothelial reliance on anaerobic glycolysis rather than FAO for energy generation is reminiscent of the Warburg effect in cancer cells.^[47]

As all metabolic processes, glycolysis is tightly controlled by several rate-limiting enzymes including hexokinases (HKs), phosphofructokinases, and pyruvate kinases.^[45] Hexokinases are responsible for the first step in glucose metabolism by catalyzing conversion of glucose to glucose-6-phosphate.^[48] The mammalian genome encodes four major isoforms of HKs: HK1, HK2, HK3, and HK4.^[49] Although they share a few common biochemical properties, these HK isoforms show distinct enzymatic activities and are differentially regulated.^[49] HK1, HK2, and HK3 are high-affinity HKs and are inhibited by high levels of glucose-6-

phosphate, whereas HK4 (also known as glucokinase) is a low-affinity isoform whose activity is not limited by glucose-6-phosphate.^[49] HK3 activity is also inhibited by glucose at physiological concentrations.^[49] HK1 and HK2 are the two HK isozymes physically associated mitochondria.^[48] While HK1 is expressed ubiquitously in mammalian tissues, HK2 is highly expressed in tissues at early developmental stages but in adult cells it is robustly expressed in only a small number of cell types including endothelial cells and cardiomyocytes.^[49]

As already mentioned, glycolysis plays a critical role in lymphangiogenesis.^[13] EC- or LEC-specific deletion of *Hk2*, whose expression downregulation reduces glycolysis, results in retarded growth of the lymphatic vasculature in embryonic mouse skin.^[13] Glycolysis also mediates the effect of FGFR signaling in promoting lymphatic vessel formation.^[13] A recent study has shown that FGFR activation in LECs stimulates glycolysis and robustly increases the expression of HK2, but not HK1 or other rate-limiting glycolytic enzymes.^[13] Reduction of HK2 expression significantly decreases FGF2-induced glycolytic flux and the ability of endothelial cells to proliferate, migrate and sprout.^[13] Interestingly, HK2 overexpression can partially restore impaired LECs responsiveness to FGF stimulation caused by knockdown of FGFR1.^[13] FGF signaling regulates HK2 expression via control of transcription activity of MYC in a manner that has not been fully elucidated.^[13]

Collectively, these data point to a central role of HK2-driven glycolysis in regulating lymphangiogenesis (Figure 2B). However, it should be noted that impaired angiogenesis and lymphangiogenesis caused by HK2 ablation could only in part be due to its effect on ATP generation^[46] since HK2 also regulates other metabolic pathways linked to glycolysis, such as glycosylation and pentose phosphate pathway. It is likely that these metabolic processes are also involved in HK2-dependent regulation of endothelial cells' homeostasis. Metabolism aside, HK2 has also been implicated in control of apoptosis and reactive oxygen species (ROS) accumulation in many cell types, and particularly in cardiomyocytes.^[50, 51] HK2 protein is present at the outer mitochondrial membrane.^[50] In cultured cardiomyocytes, HK2 protects against cell apoptosis and mitochondrial permeability transition induced by hydrogen peroxide, and this protection depends on its association with the mitochondria.^[52] A knock out of a single copy of HK2 or disruption of mitochondria-HK2 association dramatically increases cardiomyocyte apoptosis induced by ischemia-reperfusion injury or transverse aortic constriction-caused pressure overload.^[53] It is not clear if this mechanism is operational in the endothelium as there is no data demonstrating that HK2 knockout increases ROS production. Furthermore, the whole "teleological" reason for endothelial cells relying on glycolysis instead of FAO for energy generation has to do with reduced ROS production during the former compared to the latter metabolic pathway.^[45]

Because BECs and LECs are both highly glycolytic,^[13, 46] it can be expected that enzymes and signaling components involved in glycolysis also regulate angiogenesis. Indeed, FGF signaling regulates MYC and HK2 in BECs as well.^[13] Endothelium-restricted *Myc* or *Hk2* knockout mice show reduced angiogenesis in embryonic skin and in the postnatal retina.^[13, 54] Similarly, genetic and pharmacological inhibition of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), an important enzyme in glycolysis, causes obvious angiogenic defects in mice and zebrafish.^[46, 55] Notably, partial suppression of glycolysis

reduced pathological ocular angiogenesis and leads to tumor vessel normalization and decreased cancer cell dissemination.^[55, 56]

4. Conclusions and outlook

Recent progresses have demonstrated unexpected important involvement of fatty acid oxidation and glycolysis in regulation of lymphangiogenesis. Yet our understanding of molecular mechanisms involved and the link between metabolic controls and growth factor signaling is rudimentary. New studies will be required to fully explore complex paradigms emerging in this field and to understand their significance. In particular, the ability to stimulate or inhibit lymphatic growth by selectively regulating unique endothelial metabolic checkpoints may open the possibility of developing new therapeutic applications.

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Figure 1.

Formation of the lymphatic vasculature. LECs are differentiated from a subset of venous ECs at around embryonic day (E)10 and migrate out of anterior cardinal veins to form lymph sacs, which are the primitive lymphatic vasculature structure. LECs further proliferate and migration from lymph sacs to assemble into new lymphatic vessels.

Figure 2.

FAO and glycolysis regulate lymphangiogenesis. (A) CPT1A-catalyzed FAO generates acetyl-CoA (AcCoA), which support deoxyribonucleotide (dNTP) synthesis and consequently LEC proliferation. Moreover, AcCoA is used in histone acetylation (Ac) and together with P300 and PROX1 modulates the epigenetic state and transcription of *VEGFR3*, thereby controlling LEC differentiation, migration, and proliferation. (B) Activation of FGFR1 and FGFR3 (FGFR1/R3) by FGFs increases the expression of MYC, which translocates into the cell nucleus and directly stimulates the transcription of *HK2*. HK2 drives robust glycolysis that supports lymphatic and blood vessel development.