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How glucose, glutamine and fatty acid metabolism shape blood and lymph vessel development

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ABSTRACT

Recently, endothelial cell metabolism has emerged as an essential driver and regulator of both blood and lymph vessel development. Evidence rapidly builds that metabolism is not only necessary for endothelial cell function, but moreover controls several aspects of the (lymph)-angiogenic process. So far, the best-characterized metabolic pathways to have an impact on angiogenesis are glycolysis, fatty acid oxidation and glutamine metabolism. Glycolysis regulates tip cell behavior by providing ATP, fatty acid oxidation controls stalk cell proliferation by producing nucleotide biomass, and glutamine metabolism is critical for tip and stalk cell dynamics by supporting Krebs cycle anaplerosis, protein production and redox homeostasis, and links to asparagine metabolism. During lymphangiogenesis, glycolysis and fatty acid oxidation are key metabolic pathways. Glycolysis provides energy for growing lymph vessels, while fatty acid oxidation is a critical metabolic regulator of lymphangiogenesis, in part by promoting nucleotide synthesis as well as by mediating epigenetic changes of histone acetylation, which promotes transcription of key lymphatic genes, and hence venous-to-lymphatic endothelial cell differentiation. On the whole, increasing knowledge on the metabolic landscape of endothelial cells offers a fresh impetus to future treatment possibilities of vascular related diseases.

1. Introduction

Blood and lymph vessels are vital lifelines, necessary to support the survival of large organisms. While blood vessels supply tissues with oxygen and nutrients, lymph vessels drain interstitial fluid and play an important role in lipid transport and immune surveillance (Potente et al., 2011; Aspelund et al., 2016). In both vessels, the endothelial cells lining the lumen are crucial for vessel function.

The development of blood and lymph vessels commences early during embryogenesis and is a complex, highly coordinated process, tightly controlled by multiple mechanisms including genetic and epigenetic signals (Herbert and Stainier, 2011), mechanical forces (Potente and Makinen, 2017) and post-transcriptional and post-translational modifications (Herbert and Stainier, 2011). Although long overlooked, recent evidence suggests that endothelial cell metabolism is a key regulator of different stages of blood and lymph vessel formation. In this review, we describe how endothelial cell metabolism influences blood and lymph vessel development, focusing on three major metabolic pathways, best characterized to date: glycolysis, fatty acid utilization and glutamine metabolism. We discuss the evidence on metabolic control of both prenatal and postnatal vascular development while mainly focusing on murine models of skin, retinal and brain vascular development.

2. Development of blood vessels depends on vasculogenesis and angiogenesis

During embryogenesis, the first blood vessels are formed in a process called vasculogenesis. Endothelial progenitor cells called angioblasts arise from the mesoderm to assemble into the dorsal aorta and cardinal vein and into blood islands that later fuse to form a primary capillary plexus in the yolk sac (Herbert and Stainier, 2011; Potente and Makinen, 2017; Adams and Alitalo, 2007; Goldie et al., 2008). Following vasculogenesis, this primitive vasculature further expands when new blood vessels emerge from existing ones, a process called sprouting angiogenesis (Fig. 1) (Potente et al., 2011; Potente and Makinen, 2017). As such, both vasculogenesis and angiogenesis are essential for normal blood vessel development. Angiogenesis is in-

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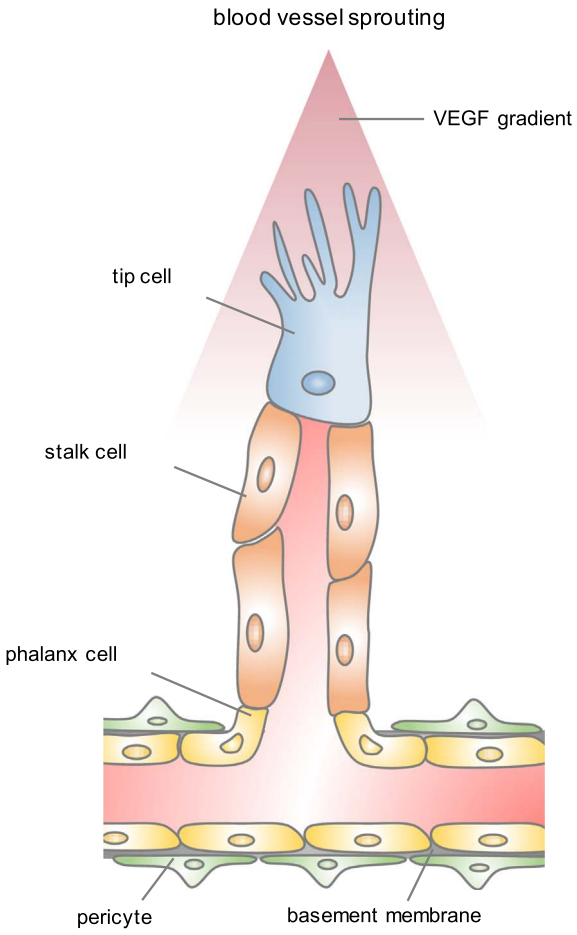

Fig. 1. Blood vessel sprouting. Schematic illustration of a sprouting blood vessel. The tip cell migrates to a VEGF gradient and is followed by proliferating stalk cells that elongate the sprout. Phalanx cells line mature (quiescent) blood vessels and are surrounded by a basement membrane and pericytes. VEGF, vascular endothelial growth factor.

itiated when proangiogenic factors like vascular endothelial growth factor (VEGF) trigger endothelial cells to change their phenotype from lining quiescent endothelial cells into 'tip' and 'stalk' cells (Potente et al., 2011). Tip cells act as the vanguard of a newly formed blood vessel and guide the sprout using lamellipodia and filopodia to migrate towards the source of the angiogenic signal. Tips cells are closely followed by proliferating stalk cells that lengthen and stabilize the sprouting vessel and create a new vessel lumen (Fig. 1) (Potente et al., 2011). Remarkably, the tip and stalk cell position in the arising vessel sprout are not fixed, as stalk cells near the tip cell dynamically compete for the tip cell position (Jakobsson et al., 2010). Therefore, to secure their tip cell position, tip cells inhibit neighbouring stalk cells by VEGF receptor 2 (VEGFR2)-induced secretion of delta like ligand 4 (Dll4), which binds to the Notch receptor on stalk cells, thereby reducing VEGFR2 signaling and inducing a non-migratory phenotype. This intriguing mechanism ensures that the endothelial cell that is exposed to the highest VEGF concentration guides the arising vessel sprout towards the source of VEGF.

After the tip and stalk cells have paved the path, mural cells consisting of pericytes and vascular smooth muscle cells are recruited and a basement membrane is deposited, leading to a mature and functional network of blood vessels (Fig. 1). In these vessels, endothelial cells switch their phenotype to quiescent endothelial cells and become so-called 'phalanx' cells (Fig. 1). Finally, after angiogenesis is completed, the vascular network can still undergo further modifications to meet the particular needs of tissues (Herbert and Stainier, 2011; Potente and Makinen, 2017; Adams and Alitalo, 2007). Although angiogenesis is abundant and essential during embryogenesis and development, later during adult life, it occurs in particular circumstances: during the menstrual cycle, upon intense muscular exercise, wound healing and in pathologic conditions such as cancer, diabetic retinopathy, inflammation, ischemic conditions and others (Kubis and Levy, 2003). We will discuss the available evidence of how metabolism regulates vessel formation, both during embryonic and postnatal development (as well in pathological conditions for reasons of completeness). However, many of the insights obtained during (lymph)-angiogenesis in disease conditions are likely also at play in embryonic development, though this awaits formal approval.

3. Metabolism of blood endothelial cells regulates angiogenesis

Like for all living cells, endothelial cell metabolism is governed by a complex network of countless metabolic pathways. Recent studies revealed the importance of some key pathways in this metabolic labyrinth in blood endothelial cells (BECs): glycolysis, fatty acid utilization and glutamine metabolism (Fig. 2) (De Bock et al., 2013b; Schoors et al., 2014, 2015; Huang et al., 2017; Kim et al., 2017). Not only are these pathways essential for endothelial cell function, they also regulate different aspects of angiogenesis (De Bock et al., 2013b; Schoors et al., 2014, 2015; Huang et al., 2017; Kim et al., 2017). In addition, migrating tip cells, proliferating stalk cells and quiescent phalanx cells each have different metabolic needs and can adjust their cellular metabolism accordingly (De Bock et al., 2013b; Schoors et al., 2014, 2015; Huang et al., 2017; Kim et al., 2017). In this section, we will describe how these metabolic pathways influence angiogenesis and how BECs can adapt their metabolism when changing phenotype. Importantly, these pathways are probably only the tip of the iceberg of what remains to be discovered. Nevertheless, these studies bring important understanding in the metabolic control of angiogenesis and therefore likely have translational potential, worthwhile to be further considered in the future.

3.1. Glycolysis regulates tip and stalk cell behavior

Although BECs are exposed to oxygenated blood, they rely on glycolysis rather than oxidative phosphorylation for energy production, independently of their subtype (arterial, venous, microvascular) (Peters et al., 2009; Parra-Bonilla et al., 2010; Krutzfeldt et al., 1990; Mertens et al., 1990). They generate up to 85% of their cellular ATP through glycolysis and die quickly when deprived of glucose (De Bock et al., 2013b; Schoors et al., 2014). Although glycolysis yields about 17-fold less ATP per mole glucose than oxidative phosphorylation, BECs prefer glycolysis for particular reasons. First, when available glucose is unlimited, glycolysis can produce more ATP in a shorter time span than oxidative phosphorylation, thereby allowing BECs to quickly sprout and form new vessels (Eelen et al., 2015). Second, by being independent of oxygen, BECs have the advantage to be able to sprout into hypoxic or even anoxic tissues as long as glucose is present. Third, glucose can shunt glycolytic intermediates into side branches of glycolysis (Fig. 2) where they can be used for the synthesis of macromolecules and generation of reducing power for redox homeostasis (DeBerardinis et al., 2008; Vander Heiden et al., 2009).

In dormant phalanx cells, glycolysis derived-energy is used for basal cellular homeostasis. During angiogenesis however, stalk and tip cells need additional biomass and energy to proliferate and migrate and therefore nearly double their glycolytic flux compared to phalanx cells (De Bock et al., 2013b). Therefore, during angiogenesis, several factors stimulate glycolysis in endothelial cells. First, vascular endothelial growth factor A (VEGFA) increases glycolysis by enhancing the uptake as well as the breakdown of glucose through the increase of glucose transporter 1 (GLUT1), lactate dehydrogenase A (LDHA) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) (Fig. 3A) (De Bock et al., 2013b; Peters et al., 2009; Parra-Bonilla

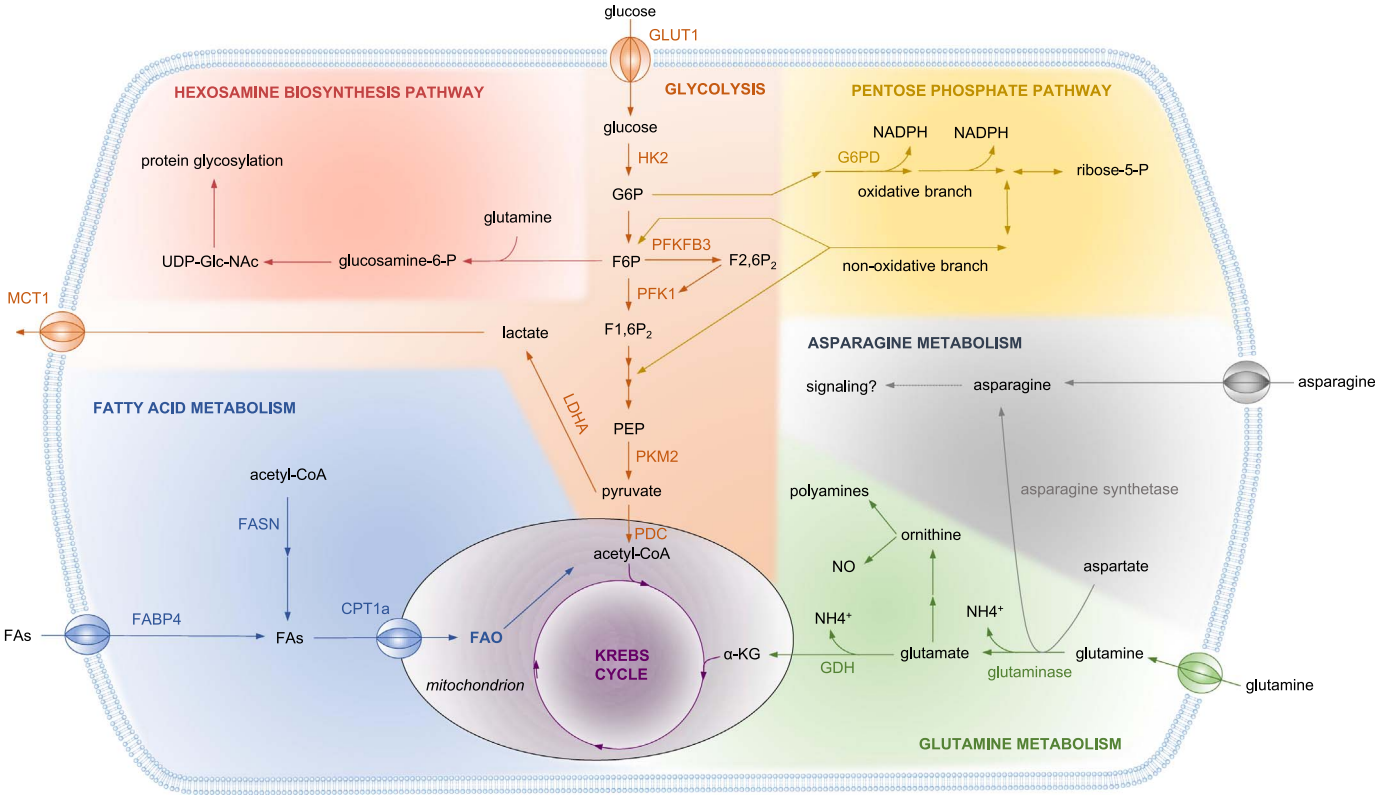

Fig. 2. Overview of metabolic pathways that control blood vessel formation. Schematic representation of the metabolic pathways that control blood vessel formation, discussed in this review. For clarity, pathways are simplified and not all metabolites and enzymes are shown. GLUT1, glucose transporter 1; HK2, hexokinase 2; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6- biphosphatase 3; $F2,6P_2$, fructose-2,6-bisphosphate; PFK1, phosphofructokinase 1; $F1,6P_2$, fructose-1,6-bisphosphate; PEP, phosphoenolpyruvate; PKM2, pyruvate kinase M2; PDC, pyruvate dehydrogenase complex; G6PD, glucose-6-phosphate dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate; ribose-5-P, ribose-5-phosphate; $NH4^+$, ammonia; NO, nitric oxide; GDH, glutamate dehydrogenase; αKG, α-ketoglutarate; acetyl-CoA, acetyl coenzyme A; FAO, fatty acid oxidation; CPT1, carnitine palmitoyltransferase 1; FAs, fatty acids; FASN, fatty acid synthase; FABP4, fatty acid binding protein 4; LDHA, lactate dehydrogenase A; MCT1, monocarboxylate transporter 1; glucosamine-6-P, glucosamine-6-phosphate; UDP-Glc-NAc, uridine diphosphate N-acetylglucosamine.

et al., 2010; Yeh et al., 2008). Second, fibroblast growth factor (FGF) enhances glycolysis by inducing the expression of hexokinase 2 (Fig. 3A) (Yu et al., 2017). Third, hypoxia stimulates glycolysis by inducing GLUT1 expression and hexokinase activity and furthermore increases PFKFB3 expression through binding of hypoxia-inducible factor 1 (HIF1) to the promotor of PFKFB3 (De Bock et al., 2013b; Obach et al., 2004; Fukasawa et al., 2004; Xu et al., 2014; Paik et al., 2017). In inflamed conditions, cytokines also upregulate glycolysis (Cantelmo et al., 2016).

PFKFB3 is an important activator of glycolysis (Yalcin et al., 2009). This enzyme is not directly part of the glycolytic pathway, but produces fructose 2,6-bisphosphate, which in turn activates phosphofructokinase 1 (PFK1), one of the key rate-controlling enzymes of glycolysis (Fig. 2). As such, PFKFB3 is an indirect regulator of glycolysis and inhibition of this enzyme decreases glycolysis moderately by 35–40% (De Bock et al., 2013b). PFKFB3-driven glycolysis is a regulator of angiogenesis as it controls the performance and phenotype of both stalk and tip cells during vascular development in the postnatal retina. Stalk cells rely on PFKFB3-driven glycolysis for proliferation (De Bock et al., 2013b). Tip cells use glycolysis-derived ATP for cytoskeletal remodeling, which is required for lamellipodia and filopodia formation and therefore for tip cell migration (De Bock et al., 2013b). Moreover, PFKFB3 (together with other glycolytic enzymes) compartmentalizes with F-actin in filopodia and lamellipodia, thereby locally and rapidly producing high amounts of ATP needed for the energy-consuming process of actin cytoskeleton remodeling, required for tip cell competition. As a consequence, PFKFB3 inhibition in tip cells impairs their competitive advantage and leads to a switch to the stalk cell phenotype, while PFKFB3 overexpression promotes the tip position and even induces tip cell behavior in BECs that are genetically predetermined to become stalk cells (via Notch-driven pro-stalk cell signaling) (De Bock et al., 2013b; Cruys et al., 2016).

This was illustrated both in mosaic endothelial cell spheroid assays in vitro as well as in zebrafish embryos, mosaically overexpressing transgenes in sprouting endothelial cells in vivo (Fig. 3B-C). In the first model, endothelial cells, in which PFKFB3 was over- or under-expressed, were mixed in mosaic endothelial cell spheroids with wild type cells, and since each cell type expressed a different fluorescent protein, its relative contribution to the tip could be analyzed (De Bock et al., 2013a, 2013b). In the second model, zebrafish embryos were generated that selectively overexpressed in endothelial cells either PFKFB3, NICD (the intracellular Notch signaling domain that promotes the stalk cell phenotype), or both, and a distinct fluorescent protein to identify the transgenic mosaic endothelial cells. Both the in vitro and in vivo analyses revealed that PFKFB3, co-expressed with NICD, overruled the stalk cell-promoting activity of NICD and converted these cells to tip cells (Fig. 3B-C) (De Bock et al., 2013a, 2013b). Conversely, silencing PFKFB3 impaired the competitive tip cell behavior of endothelial cells in mosaic spheroids (De Bock et al., 2013a, 2013b). These findings indicated for the first time that cellular metabolism is able to drive vessel sprouting and to overrule established genetic pro-stalk signals during angiogenesis.

Tip cells suppress the competition from neighbouring stalk cells by decreasing (but not extinguishing) PFKFB3 expression in stalk cells, a process mediated by Dll4-Notch signaling (Fig. 3A) (De Bock et al., 2013b). Generally, genetically silencing or pharmacologically blocking PFKFB3, either in developing zebrafish embryos, the postnatally developing mouse retinal vasculature or in mouse disease tissues reduces the migrational potential of tip cells and the proliferative potential of stalk cells, while impairing vessel branching and outgrowth

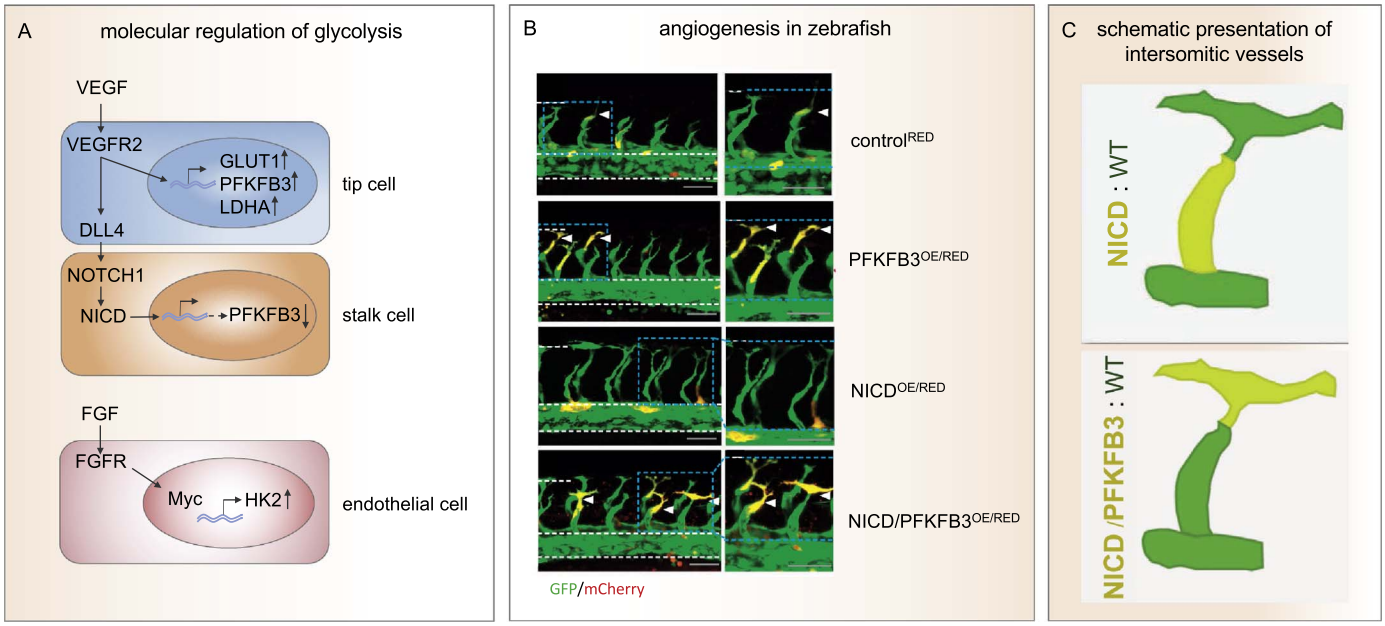

Fig. 3. Glycolysis in angiogenesis (A) VEGF induces the expression of glycolytic enzymes and PFKFB3 in the tip cell, whereas in the stalk cell, DLL4/Notch signaling reduces expression of the glycolytic enzyme PFKFB3. FGF induces hexokinase 2 in angiogenic endothelial cells via Myc signaling, more details are discussed in the text. (B) Images of intersomitic vessels from transgenic mosaic *Fli1EGFP*^{y1} zebrafish embryos, expressing only mCherryRed (control^{RED}) or coexpressing mCherryRed with either PFKFB3 (PFKFB3$^{OE/RED}$), NICD (NICD$^{OE/RED}$) or both transgenes (PFKFB3/NICD$^{OE/RED}$). PFKFB3 overexpression promotes the tip position and is able to overcome the pro-stalk activity of NICD. Arrowheads indicate ECs that reach the tip position (adapted with permission from De Bock et al. (2013b)). (C) Upper panel: Schematic illustration of a tip and stalk cell in transgenic zebrafish intersomitic vessels. In comparison to wild-type (WT) cells (green) at the tip position, cells that overexpress NICD (yellow cell) are more frequently found in the stalk cell position. Lower panel: However, when NICD-overexpressing cells also overexpress PFKFB3, they can compete again for the tip position (yellow cell). Thus, overexpression of PFKFB3 overrules the stalk-cell-inducing activity of NICD. (adapted with permission from De Bock et al. (2013a)). VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; DLL4, delta like ligand 4; GLUT1, glucose transporter 1; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6- biphosphatase 3; LDHA, lactate dehydrogenase A; NICD, NOTCH intracellular domain; FGF, fibroblast growth factor; FGF3, fibroblast growth factor receptor; HK2, hexokinase 2; OE, overexpression; WT, wild type.

(De Bock et al., 2013b; Schoors et al., 2014; Xu et al., 2014). Notably, when homozygous PFKFB3 deficient embryonic stem cells are injected in wild type blastocysts, progeny of various cell lineages lacking both PFKFB3 alleles develop normally and contribute to mosaic tissue development, but endothelial cells, lacking both PFKFB3 alleles, are outcompeted by their wild type neighbours, indicating the key importance of PFKFB3-driven glycolysis for endothelial cell survival and competitive contribution to vessel formation (De Bock et al., 2013b), and the glycolysis-addiction of endothelial cells in general.

Besides PFKFB3, also hexokinase 2 (HK2) is an essential glycolytic enzyme. It is the first rate-limiting enzyme of glycolysis and catalyzes the conversion of glucose into glucose-6-phosphate (Fig. 2). Similar to PFKFB3, HK2 activity is essential for angiogenesis and tip and stalk cell performance (Yu et al., 2017). By analogy with VEGF that regulates the expression of PFKFB3, fibroblast growth factor (FGF) is the growth factor that regulates HK2 activity. At the molecular level, FGF controls glycolysis through a Myc-dependent regulation of HK2 expression (Fig. 3A) (Yu et al., 2017). Hence, FGF stimulation elevates HK2 levels, leading to an increase in glycolysis. Myc mediates the FGF effects on HK2 expression by directly binding to the regulatory region of the HK2 gene and thereby controlling its transcription. Therefore, both the knockdown of FGF and HK2 in mice at an early embryonic developmental stage (E10.5) reduces angiogenesis in the mouse skin. Moreover, knockdown of FGF and HK2 at postnatal day 0 (P0) reduces the number of tip cells and the proliferation of stalk cells and leads to a reduced expansion of the developing retinal vasculature (Yu et al., 2017). As such, FGF controls vascular development through metabolic regulation of BECs both in the embryonic as well as in the postnatal stage. These findings are intriguing, as FGF-mediated metabolic control of angiogenesis was not yet described and this study underscores the pivotal role of FGF signaling in early vascular development.

In addition to PFKFB3 and HK2, pyruvate kinase is another key glycolytic enzyme. Pyruvate kinase catalyzes the last rate-limiting step of glycolysis, where phosphoenolpyruvate (PEP) is converted to pyruvate (Fig. 2). Pyruvate kinase is expressed as four isoforms. The M2 isoform (PKM2) is expressed in adult dividing healthy and malignant cells, and in embryonic cells. PKM2 can either exist as a dimer or tetramer (Israelsen et al., 2013; Christofk et al., 2008; Anastasiou et al., 2012). The tetramer has high affinity for PEP and drives glycolytic production of ATP. In contrast, the dimer has low affinity for PEP, thereby reducing the conversion of PEP to pyruvate and instead shunting glycolytic intermediates into glycolytic side pathways for biomass synthesis (Israelsen et al., 2013). Therefore, in highly proliferative cells, PKM2 switches to the dimer composition (Christofk et al., 2008; Hitosugi et al., 2009; Li et al., 2014). In endothelial cells, PKM2 silencing decreased endothelial cell sprouting in a spheroid assay (Boeckel et al., 2016). Notably, PKM2 interacts with the protein Jumonji C domain-containing protein 8 (Jmjd8; a member of the Jumonji C class of proteins that is important for the control of stem cell plasticity and differentiation), and knockdown of Jmjd8 also reduces endothelial cell sprouting and network formation, however the precise mechanism of how Jmjd8 regulates PKM2's activity (ATP versus biomass production) remains to be clarified (Boeckel et al., 2016). Interestingly, PKM2 is the main pyruvate kinase isoform in embryonic tissue (Gupta and Bamezai, 2010), raising the question whether PKM2 plays a role in embryonic vascular development.

Glucose transporter 1 (GLUT1) imports glucose into the cytoplasm of BECs and is essential for postnatal development of the murine brain vasculature. This evidence is deduced from insights in the pediatric neurodevelopmental disorder GLUT1 deficiency syndrome (GLUT1-DS) (Tang et al., 2017). GLUT1 is particularly abundant in endothelial cells of the brain microvasculature, where it promotes the transport of blood glucose across the blood-brain-barrier (the safeguards of the brain that allows transfer of essential nutrients and metabolites from the blood to the central nervous system, while preventing the passage of peripheral toxic molecules) into the central nervous system. In a

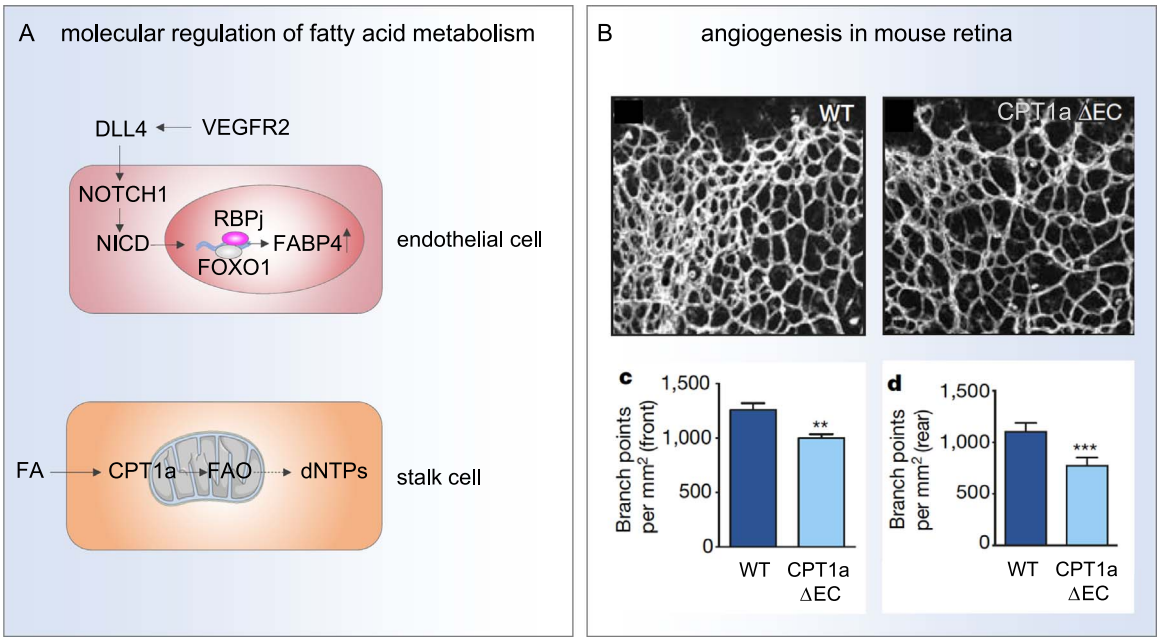

Fig. 4. Fatty acid metabolism in angiogenesis. (A) Upper panel: Signaling pathway of how VEGFR activation (by VEGF) stimulates transcription of FABP4 in endothelial cells. Lower panel: CPT1a-driven fatty acid oxidation metabolizes fatty acids to produce dNTPs for proliferation of stalk cells. See text for more details. (B) In vivo, endothelial cell-specific CPT1a knockout results in reduced angiogenesis in a mouse retina model (adapted with permission from Schoors et al. (2015)). VEGFR2, vascular endothelial growth factor receptor 2; DLL4, delta like ligand 4; NICD, NOTCH intracellular domain; FOXO1, forkhead box protein 1; FABP4, fatty acid binding protein 4; FA, fatty acid; CPT1a, carnitine palmitoyltransferase 1a; FAO, fatty acid oxidation; dNTPs, deoxyribose nucleotide triphosphates; ctrl, control; eto, etomoxir.

mouse model of GLUT1 DS (with GLUT1 haploinsufficiency), low levels of GLUT1 arrest brain angiogenesis from the second postnatal week on (Tang et al., 2017). Subsequently, this induces microvasculature rarefication and eventually causes seizures, hypoglycorrhachia, microencephaly and reduced motor performance, some of the signature abnormalities of human GLUT1 DS syndrome. Restoring the level of GLUT1 in neonatal GLUT1 DS mice rescues the phenotype completely and ensures normal development of the brain microvasculature (Tang et al., 2017). In contrast, repletion of GLUT1 in 2-week old GLUT1 DS mice only partially restores the brain microvasculature and symptoms, while repletion in 8-week old GLUT1 DS mice fails to normalize the brain microvasculature and leads to permanent brain damage (Tang et al., 2017). This indicates that brain endothelial cells critically rely on GLUT1, temporally during the early neonatal period, more than at later postnatal stages. Of note, persistent GLUT1 depletion leads to permanent brain damage that is irreversible upon GLUT1 repletion, possibly because the accumulating damage is already irreversible, when not swiftly rescued after birth. Overall, these findings highlight that GLUT1 is critical for the normal postnatal development and maintenance of the brain microvasculature (Tang et al., 2017).

Glycolysis-derived lactate is an important pro-angiogenic metabolite. Evidence for this observation derives from studies on wound healing and cancer, conditions that are characterized by high lactate production (Porporato et al., 2012; Vegran et al., 2011). Indeed, when BECs take up lactate from the bloodstream via the lactate transporter monocarboxylate transporter 1 (MCT1), this leads to inhibition of prolylhydroxylase domain 2 (PHD2), which results in HIF1α activation and initiation of angiogenesis (Vegran et al., 2011; Sonveaux et al., 2012; Milovanova et al., 2008). Moreover, BECs that are exposed to lactate, increase the expression of VEGF and other receptor tyrosine kinase ligands, thereby promoting angiogenic behavior (Milovanova et al., 2008; Kumar et al., 2007; Ruan and Kazlauskas, 2013; Hunt et al., 2007). During embryonic development of the brain, the neurogenic niche (where new neurons develop, a process called neurogenesis) is a relatively hypoxic environment that depends on the production of glucose-derived lactate to guide vascular outgrowth to support neurogenesis (Salmina et al., 2015). As such, glucose-derived lactate is a pivotal regulator of angiogenesis in early brain vasculature development. Interestingly, the expression of MCT1 in brain endothelial cells is the highest in the early neonatal period, indicating that MCT1 might be important during this stage of development (Salmina et al., 2015). Later, the expression of MCT1 gradually decreases, while GLUT1 expression simultaneously increases (Gerhart et al., 1997; Vannucci and Simpson, 2003; Mac and Nalecz, 2003; Vannucci, 1994). This highlights how the metabolic demands of the brain change during development and how different metabolites dynamically control vascular developmental in the brain. Moreover, this raises the question whether glucose-derived lactate could be important for post-implantation embryonic vascular development (Gardner, 2015).

3.2. Fatty acid utilization controls stalk cell function

While glucose is their main energy source, BECs use fatty acids for biomass production, and greatly differ from most other cell types in this characteristic feature. BECs can import, metabolize (oxidize), synthetize and export fatty acids and these processes all regulate angiogenesis. However, the relative contribution of fatty acid import, oxidation, synthesis and transport (to the surrounding tissues) to the function of both quiescent and angiogenic BECs is not yet elucidated.

BECs import fatty acids from the bloodstream into their cytoplasm either through active transport (mediated by fatty acid binding proteins (FABPs)) or via passive diffusion (Fig. 2) (Schoors et al., 2015; Wong et al., 2017). Amongst the fatty acid binding proteins, the role of FABP4 in angiogenesis is best characterized (Harjes et al., 2014). VEGFA stimulates the transcription of FABP4. By inducing DLL4 expression in endothelial cells, VEGFA promotes the paracrine activation of NOTCH signaling in neighbouring ECs and hence induces the binding of NICD to the recombination signal binding protein for immunoglobulin κJ region (RBPJκ) – a transcription factor complex – that in turn stimulates FABP4 gene transcription (Fig. 4A) (Harjes et al., 2014). Adding an extra layer of complexity, the transcription of FABP4 (both basal expression and upregulation upon VEGFA-stimulation and Notch-signaling) requires the transcription factor forkhead

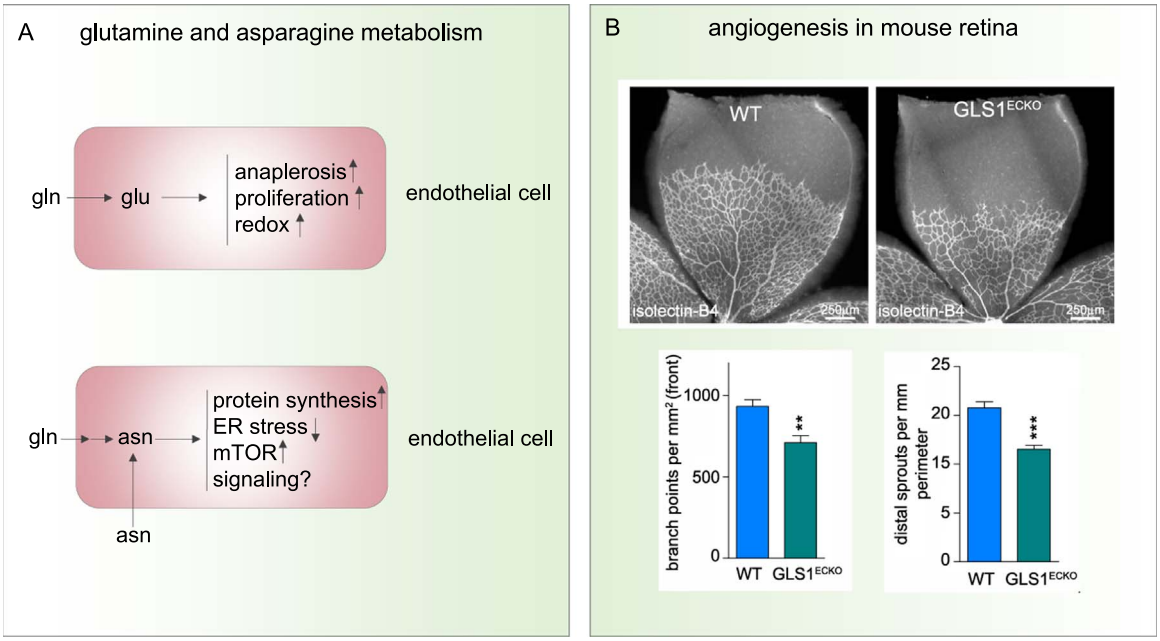

Fig. 5. Glutamine and asparagine metabolism in angiogenesis. (A) Upper panel: Glutamine is metabolized to glutamate, important for replenishment of Krebs cycle intermediates (anaplerosis), proliferation and redox homeostasis of endothelial cells. Lower panel: Asparagine, synthesized from glutamine or taken up from the blood, contributes to protein synthesis, reduces the endoplasmatic reticulum stress response, activates mTOR signaling and possibly functions as a signaling metabolite. (B) Illustration and quantification of angiogenesis in the mouse retina. Endothelial loss of glutaminase 1 (GLS1) causes vascular defects, quantified by the reduced number of branch points and distal sprouts with filopodia (adapted with permission from Huang et al. (2017)). WT, wild type; GLS1, glutaminase; ECKO, endothelial knockout; gln, glutamine; glu, glutamate; asn, asparagine; ER, endoplasmatic reticulum; mTOR, mammalian target of rapamycin.

box O1 (FOXO1), which furthermore is, as we will discuss later, a metabolic checkpoint for quiescent endothelial cells (Harjes et al., 2014). As a consequence, in vitro, BECs deficient in FABP4 proliferate, migrate and sprout markedly less (Harjes et al., 2014; Cataltepe et al., 2015; Elmasri et al., 2012, 2009).

Once fatty acids are imported into the cytosol, BECs can metabolize them via the process of fatty acid oxidation (FAO) (Fig. 2). The rate contolling enzyme of FAO, carnitine palmitoyltransferase 1 (CPT1), first transports fatty acids from the cytosol into the mitochondria where they undergo fatty acid ß-oxidation to generate acetyl coenzyme A (acetyl-CoA) (Fig. 2). Subsequently, acetyl-CoA enters the Krebs cycle, where (in conjunction with other anaplerotic substrates), this fatty acid-derived metabolite sustains the Krebs cycle for synthesis of deoxynucleotide triphosphates (dNTPs), used for DNA replication in proliferating stalk cells (Fig. 4A) (Schoors et al., 2015). Consequently, blockade of CPT1a in BECs reduces FAO, leads to a depletion of the dNTP pool, compromizes stalk cell proliferation and results in vessel sprouting defects during postnatal retinal vascular development, while leaving tip cell behavior intact (Fig. 4B) (Schoors et al., 2015). Supplementation with nucleotides rescues these defects, indicating that sprouting BECs indeed rely on FAO for nucleotide synthesis (Schoors et al., 2015). These findings are remarkable, as most other cell types primarily use carbons from glucose and glutamine for nucleotide synthesis (Schoors et al., 2015; Vander Heiden, 2013), and proliferating BECs cannot compensate for the dNTP synthesis defect upon CPT1a silencing by upregulating glutamine and glucose anaplerosis (Schoors et al., 2015). In this study, FAO only contributed minimally to ATP production (Schoors et al., 2015), but another study reported a more important contribution (Patella et al., 2015).

In addition to importing fatty acids from the bloodstream, BECs can also synthetize fatty acids themselves through fatty acid synthesis (starting from acetyl-CoA), the metabolic process coordinated by fatty acid synthase (FASN) (Fig. 2). Although still incompletely investigated, a few studies indicate the importance of fatty acid synthesis for vascular sprouting and angiogenesis (Wei et al., 2011; Browne et al., 2006; Seguin et al., 2012). Mechanistically, knockdown of FASN leads to decreased palmitoylation of endothelial nitric oxide synthase (eNOS), thereby impairing its bioavailability. This in turn, leads to displacement of eNOS away from the membrane and reduces sprouting and permeability of BECs (Wei et al., 2011). Moreover, BECs exposed to orlistat, an anti-obesity drug that blocks the metabolic function of FASN, exhibit reduced FA synthesis, proliferation and expression of VEGFR2 on the cell membrane (Browne et al., 2006). These findings have however not been mechanistically related to the tip-stalk cell model in in vivo genetic mouse models. Further studies will need to provide more insight to the contribution of FASN to the angiogenic process.

3.3. Role of glutamine metabolism in tip and stalk cell dynamics

In addition to glycolysis and FAO, glutamine metabolism has been recently studied in more detail as a regulator of angiogenesis (Huang et al., 2017; Kim et al., 2017). Glutamine is the most abundant free amino acid in the blood, and BECs from venous, arterial and microvascular origin exhibit detectable flux through glutaminase (Wu et al., 2000; Leighton et al., 1987; Lohmann et al., 1999). Glutamine is used for multiple purposes, including protein synthesis. Glutaminase, the first enzyme of glutamine catabolism, converts glutamine to glutamate and ammonia (Fig. 2) (DeBerardinis and Cheng, 2010). While ammonia-derived nitrogen functions as an important amino group donor for biosynthesis of nucleotides, hexosamine and of asparagine, glutamate is located at a metabolic crossroad. Indeed, glutamate dehydrogenase can metabolize glutamate to α-ketoglutarate (DeBerardinis and Cheng, 2010), which in turn is used as carbon source for Krebs cycle replenishment (anaplerosis) (Fig. 2) (DeBerardinis and Cheng, 2010). Glutamate-derived nitrogen is additionally used for the synthesis of non-essential amino acids in transaminase reactions converting glutamate to α-ketoglutarate, while glutamate is also a precursor of glutathione for maintenance of cellular redox homeostasis (DeBerardinis and Cheng, 2010). Alternatively, glutamate can be converted to ornithine to generate polyamines and nitric oxide (NO), both pro-angiogenic factors (Fig. 2) (Kucharzewska et al., 2010; Matsunaga et al., 2002).

Two recent studies highlighted the importance of glutamine metabolism in sprouting endothelial cells. Of all amino acids available in the culture medium, endothelial cells consumed glutamine more than any other amino acid (Huang et al., 2017). Sprouting endothelial cells rely on glutamine metabolism for protein synthesis, Krebs cycle anaplerosis and redox balance, but not for lipid synthesis (Fig. 5A) (Huang et al., 2017; Kim et al., 2017). In addition, glutamine deprivation in ECs evokes endoplasmatic reticulum stress and lowers activation of mammalian target of rapamycin (mTOR) (Huang et al., 2017). Not surprisingly therefore, in vivo inhibition of glutaminase (either by BEC specific knockout or pharmacologically) in a postnatal model of retinal vascular development reduced proliferation and migration of BECs without lowering the intracellular ATP pool (Fig. 5B) (Huang et al., 2017). Furthermore, glutamine metabolism proved essential for tip/stalk cell dynamics, as inhibition of glutaminase impeded the competitiveness of endothelial cells to obtain the tip position when using mosaic endothelial spheroids, likely by impairing endothelial cell migration (Huang et al., 2017).

Another study confirmed the importance of glutamine metabolism for endothelial cell proliferation, but apparently not for migration, and noticed a drop in cellular ATP levels upon glutamine deprivation (Kim et al., 2017). However, in this study, migration in the scratch wound assay was assessed at 24–48 h, when the wound is already largely covered with migrating cells, thus not excluding a possible migration defect at earlier time periods (as analyzed in Huang et al. (2017) study). Nonetheless, other differences, possibly related to different experimental conditions in this study (later passage endothelial cells; use of lower serum concentration; use of different media for testing glutamine-replete versus glutamine-free conditions, etc) might also have contributed to this apparent paradox. Apparent differences in migratory endothelial cell behavior were also noticed at the vascular front (number of filopodia, etc) in vivo, but this might possibly be related to the use of different Cre-drivers. Indeed, compared to VE-cadherin-Cre^{ERT2}, PDGFB- Cre^{ERT2} is known to be more efficient in inactivating floxed target genes throughout the retinal vasculature, thus also at the vascular forefront, explaining why migratory endothelial cell abnormalities were noticeable upon use of the PDGFB- Cre^{ERT2} driver (Claxton et al., 2008; Franco et al., 2013).

Notwithstanding these differences, these combined findings underscore a critical role for glutamine metabolism in angiogenesis. However, glutamine metabolism was less/not important for other endothelial functions, such as regulation of vascular tone, inflammation, or differentiation (Huang et al., 2017).

In sharp contrast to cancer cells, for which replenishment of Krebs cycle intermediates or antioxidant supplementation sufficed to rescue the phenotypes (van den Heuvel et al., 2012; Son et al., 2013), these and other single interventions failed to rescue the glutamine deprivation-induced proliferation arrest in BECs (Huang et al., 2017). In contrast, the combination treatment of asparagine with an anaplerotic carbon donor completely rescued the defects of glutamine-deprived endothelial cells, while asparagine alone partially restored endothelial proliferation upon partial glutamine starvation (Huang et al., 2017). Endothelial cells either take up asparagine from the bloodstream (if sufficiently abundant) or produce it de novo by fusing glutamine-derived nitrogen with asparate, a process catalyzed by asparagine synthetase (Fig. 2), in nutrient-limited environments, such as occurs in the tumor setting or when endothelial cells invade into avascular regions to vascularize tissues (Huang et al., 2017). Endothelial cells may additionally rely on asparagine synthetase when asparagine synthetase expression levels are elevated (upon starvation of glucose or amino acids, hypoxia, or endoplasmic reticulum stress) or when the asparagine level in the extracellular milieu is limiting (in certain cancers, upon protein limitation or unbalanced amino acid intake, during asparaginase treatment, etc) (Balasubramanian et al., 2013; Jousse et al., 2004; Scioscia et al., 1998; Ahlman et al., 1994; Newburg et al., 1975).

Asparagine rescues the proliferation defects of glutamine-deprived endothelial cells by increasing protein synthesis, diminishing the endoplasmatic reticulum stress response and reactivating mTOR signaling (Fig. 5A) (Huang et al., 2017). Hence, the activity of asparagine in endothelial cells is downstream of glutamine, explaining its crucial role in angiogenesis. For long, asparagine has been considered to only function as a precursor of protein synthesis, in contrast to the 19 other common amino acids. However, increasing evidence assigns a possible additional role to this amino acid, namely as a signaling metabolite that senses metabolic fuel reserves and availability, and coordinates cellular homeostatic responses accordingly (Zhang et al., 2014). Indeed, the findings that the intracellular asparagine level is the lowest of all non-essential amino acids in proliferating cells, and asparagine exclusively relies on glutamine as a nitrogen-donor, suggest that asparagine is a cellular rheostat that senses the stockpile of Krebs cycle intermediates and the supply of reduced nitrogen in order to maintain sufficient biosynthesis of non-essential amino acids for cell division (Zhang et al., 2014). Therefore, endothelial cells may upregulate asparagine synthesis upon amino acid and carbohydrate starvation, as asparagine might serve as a “red flag” for the cell to warn about insufficient substrate for continued cell division. Interestingly, as the carbon to nitrogen ratio is lower for asparagine than for glutamine, plants redirect the nitrogen flow to asparagine during carbohydrate starvation in order to spare carbons (Lam et al., 1994; Chevalier et al., 1996). Whether asparagine functions as a signaling metabolite in endothelial cells, and plays a similar role in endothelial cells as in plants, are outstanding questions that require future investigation (Fig. 5A).

Remarkably, endothelial cells fundamentally differ from malignant cells in their response to glutamine deprivation and their use of asparagine in mediating this response. Indeed, asparagine suppresses the glutamine deprivation-induced death, but not the proliferation defect of cancer cells (Zhang et al., 2014). In contrast, in endothelial cells, asparagine plus α-ketoglutarate rescue the proliferation defect and all other glutamine-dependent phenotypes, including the intracellular pool of the Krebs cycle intermediates, cell size, mTORC1 signaling, protein synthesis, the ER stress response, and redox imbalance (Huang et al., 2017).

3.4. Metabolism modulation co-regulates the switch from sprouting to quiescent endothelial cells

Once newly formed blood vessels adequately provide tissue with oxygen and nutrients, the sprouting process discontinues and BECs adopt a quiescent phenotype. This phenotypic switch is accompanied by metabolic alterations that are partly driven by two well-characterized transcription factors, forkhead box protein O1 (FOXO1) and Krüppel-like factor 2 (KLF2) (Fig. 6) (Wilhelm et al., 2016; Doddaballapur et al., 2015).

FOXO1 is a member of the forkhead box (FOX) family that regulates the expression of genes required for cell proliferation, growth, differentiation and longevity (Coomans de Brachene and Demoulin, 2016). FOXO1 is especially enriched in BECs and is crucial for embryonic development and vascular homeostasis (Potente et al., 2005; Hosaka et al., 2004). FOXO1 is a key metabolic checkpoint as it functions as a gatekeeper of BEC quiescence by inhibiting the proto-oncogene Myc. This inhibition results in a global reduction of both glycolysis and mitochondrial oxidative phosphorylation (Fig. 6) (Wilhelm et al., 2016). As a result, BEC-selective FOXO1 gene deletion in newborn mice leads to vascular hyperplasia and overgrowth in the developing mouse retina. Conversely, BEC specific expression of a constitutively active FOXO1 protein causes defective vessel development with hypobranching and fewer than normal BECs (Wilhelm et al., 2016). In BECs with constitutively active FOXO1, Myc overexpression restores metabolism and proliferation of BECs and repairs the induced vascular defects, illustrating that Myc is a mediator of FOXO1

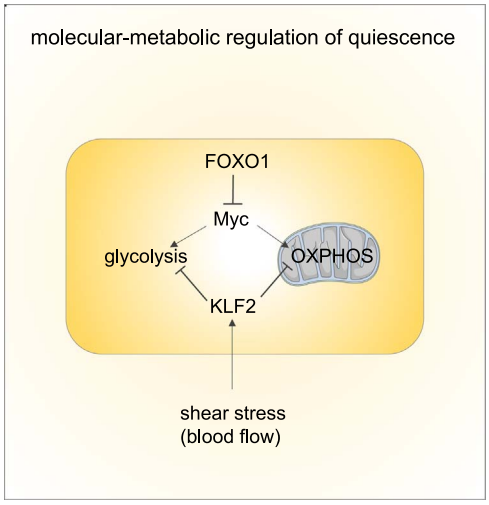

Fig. 6. Molecular-metabolic regulation of endothelial cell quiescence. The transcription factors FOXO1 and KLF2 both induce endothelial cell quiescence by regulating endothelial cell metabolism (for more details, see text). FOXO1, forkhead box protein O1; KLF2, Krüppel-like factor 2.

deficiency in BECs. Together, these findings indicate the importance of FOXO1 for angiogenesis and vessels homeostasis.

Similar to FOXO1, KLF2 overexpression decreases glycolysis and mitochondrial content in BECs, thereby inducing endothelial cell quiescence (Fig. 6) (Doddaballapur et al., 2015). Mechanistically, KLF2 reduces the expression of glycolytic enzymes such as PFKFB3, PFK1 and HK2. Interestingly, blood flow (laminar shear stress) induces KLF2, which shows how a mechanical cue influences angiogenesis in a metabolism-dependent manner (Doddaballapur et al., 2015). This raises the question whether this mechanical-metabolic interaction could also determine the arterial-venous fate of BECs during vascular development. Overall, these findings illustrate how metabolism co-regulates the switch from sprouting to quiescent endothelial cell phenotype.

3.5. Metabolites regulate angiogenic signal transduction

Besides supplying energy or building blocks, the pathways of central carbon metabolism (glycolysis, FAO, glutamine metabolism) can also generate metabolites for other purposes. Thereby, they produce signaling molecules or induce post-translational modifications that modulate activity, localization, stability, and gene expression of proteins, including angiogenic factors (Wellen and Thompson, 2012; DeBerardinis and Thompson, 2012).

Glycolysis can divert some of its intermediates into the pentose phosphate pathway (PPP) and the hexosamine biosynthesis pathway (HBP), two side branches that influence the angiogenic process (Fig. 2). The PPP produces metabolites for biomass synthesis and redox homeostasis. Indeed, the non-oxidative branch of the PPP generates ribose units for nucleotide synthesis, while the oxidative branch of the PPP produces NADPH for reactive oxygen scavenging and lipid synthesis (Fig. 2) (Riganti et al., 2012). Hence, decreasing the activity of the PPP by inhibiting its flux-generating enzyme glucose-6-phosphate dehydrogenase reduces VEGF-induced proliferation, migration and tube formation in cultured BECs (Leopold et al., 2003a). However, by generating NADPH, the PPP also contributes to the production of the angiogenic molecule nitrogen oxide (NO) (Leopold et al., 2003b). As another example of the production of metabolites involved in signaling, the HPB converts fructose-6-phosphate (F6P) into glucosamine-6-phosphate (GlucN6P) in a process that requires glutamine (Fig. 2) (Buse, 2006). GlucN6P is then metabolized to uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc in turn acts as the substrate of the final glycosylation step (Fig. 2). Posttranslational modification of proteins by glycosylation is important for EC functions and angiogenesis (Benedito et al., 2009; Laczy et al., 2009; Croci et al., 2014). Moreover, the endothelial glycocalyx layer, a gel-like layer at the luminal surface of ECs that has an atheroprotective role, consists of glycoproteins, glycolipids and glucosaminoglycans, all of which contain glycosyl side chains (Dane et al., 2015; Tarbell and Cancel, 2016). Therefore, the production of the endothelial glycocalyx layer is likely linked to glucose metabolism in endothelial cells.

Acetylation is another example of post-translational protein modification. Metabolism itself regulates acetylation, as glycolysis and the subsequent pyruvate dehydrogenase reaction (catalyzed by the pyruvate dehydrogenase complex), as well as fatty acid oxidation, can supply acetyl-CoA for acetylation (Fig. 2) (Choudhary et al., 2014). During acetylation, acetyl-CoA donates its acetyl-group to proteins – such as histones, transcriptional regulators or metabolic enzymes – thereby regulating their function (Choudhary et al., 2014). For example, acetylation of DNA histones regulates epigenetic gene control (Wong et al., 2017). Also, reversible acetylation controls key angiogenic signaling molecules such as VEGFR2. Indeed, acetylation of the intracellular domain of VEGFR2 regulates VEGFR2 phosphorylation and activation upon ligand stimulation (Zecchin et al., 2014). Another example is NICD, the acetylation of which alters it protein turnover and results in its stabilization (Guarani et al., 2011). As a consequence, endothelial cells lacking the NICD deacetylase SIRT1, show enhanced expression of Notch target genes in response to stimulation with Dll4. This promotes a stalk cell-like phenotype (Guarani et al., 2011). This demonstrates how acetylation regulates angiogenic signaling in a metabolism-responsive way.

4. Development of lymph vessels

Lymph vessel development (lymphangiogenesis) consists of various consecutive phases. First, lymphatic specification marks embryonic venous endothelial cells from the anterior cardinal vein to differentiate towards a lymphatic endothelial cell (LEC) phenotype and to express key lymphatic-enriched markers such as lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), prospero homeobox protein 1 (PROX1), fibroblast growth factor receptor 3 (FGFR3) and vascular endothelial cell growth factor receptor 3 (VEGFR3) (Banerji et al., 1999; Hong et al., 2002; Petrova et al., 2002; Shin et al., 2006; Mishima et al., 2007). Second, PROX1^{+} and VEGFR3^{+} LECs migrate from the embryonic vein, proliferate to form a capillary like structure and organize into lymphatic sacs and a primitive lymphatic plexus (Wigle and Oliver, 1999). This phase largely depends on VEGFR3-signaling, which promotes LEC migration, proliferation and survival (Karkkainen et al., 2004; Cao et al., 2012; Kubo et al., 2002; Chang et al., 2004). Third, the lymphatic vessels mature and thereby separate from the original embryonic veins and the primary lymphatic plexus expands and remodels into lymphatic capillaries and collecting lymphatic vessels (Dellinger et al., 2008). As lymphatic vessels mature, the expression of LYVE-1, PROX1 and VEGFR3 is downregulated (Sabine et al., 2012). Lymphatic maturation including valve formation is regulated by forkhead box C2 (FOXC2) and Notch1 signaling (Petrova et al., 2004; Murtomaki et al., 2014), amongst others. Recent studies show that LECs may also arise from non-venous origin as well (Buttler et al., 2006; Sebzda et al., 2006).

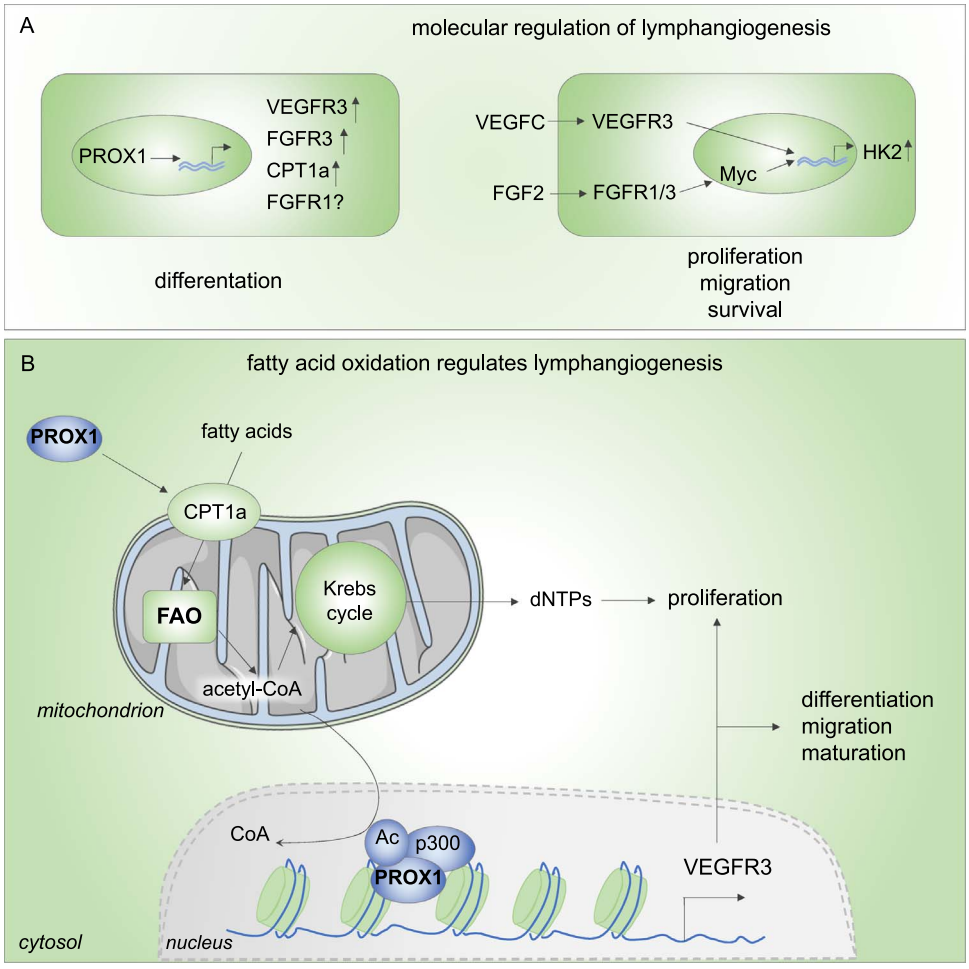

Fig. 7. Metabolism in lymphatic development. (A) Molecular regulation of lymphangiogenesis. Left panel: PROX1 governs lymphatic differentiation by inducing the expression of lymphatic-specific genes as well as CPT1a. Right panel: VEGFC and FGF2 enhance glycolysis in lymphatic endothelial cells to stimulate proliferation, migration and survival. (B) PROX1 enhances CPT1a-dependent fatty acid oxidation (FAO) to produce acetyl-CoA. In conjunction with another anaplerotic carbon source, fatty acid-derived acetyl-CoA sustains the Krebs cycle to produce nucleotides necessary for proliferation. On the other hand, FAO-derived acetyl-CoA also functions as an acetyl donor for histone acetylation by p300 (interacting with PROX1), which results in decondensation of the chromatin at PROX1 target genes such as VEGFR3. PROX1, prospero homeobox protein 1; VEGFR3, vascular endothelial cell growth factor receptor 3; FGFR3, fibroblast growth factor receptor 3; CPT1a, carnitine palmitoyltransferase 1a; FGFR1, fibroblast growth factor receptor 1; VEGFC, vascular endothelial cell growth factor C; FGF2, fibroblast growth factor 2; HK2, hexokinase 2; FAO, fatty acid oxidation; acetyl-CoA; acetyl coenzyme A; dNTPs, deoxynucleotide triphosphates; Ac, acetate; p300, histone acetyltransferase p300.

5. Metabolism of lymphatic endothelial cells regulates lymphangiogenesis

For long, general knowledge on lymphatic vessels was limited and insight into the contribution of cellular metabolism to lymphangiogenesis was non-existing. Recently however, a few studies discovered that LECs rely on both glycolysis and FAO for lymphangiogenesis. Analogous to BECs, LECs use glycolysis for energy production during proliferation and migration, and FAO for biomass production during proliferation (Yu et al., 2017; Wong et al., 2017). In addition, however, and interestingly, LECs use FAO also for the epigenetic regulation of the expression of key lymphatic markers (Wong et al., 2017). Whether there is a similar role for FAO in the epigenetic modulation of the angiogenic process remains to be determined. In this section, we will discuss how glycolysis and FAO regulate lymphangiogenesis.

5.1. Glycolysis produces energy for lymphangiogenesis

An important similarity between angiogenesis and lymphangiogenesis is the need for growth factors to induce vessel formation. Analogous to VEGFA as a keydriver of angiogenesis, FGF2 and VEGFC are regulatory growth factors of lymphangiogenesis (Yu et al., 2017; Zheng et al., 2014). Both FGF2 and VEGFC stimulate LEC proliferation, migration and survival as seen in a mouse cornea model (Cao et al., 2012; Kubo et al., 2002; Chang et al., 2004). Of interest, PROX1 stimulates the transcription of the receptors of FGF2 and VEGFC, respectively FGFR3 and VEGFR3 (Fig. 7A) (Hong et al., 2002; Shin et al., 2006; Mishima et al., 2007). Whether PROX1 also controls the transcription of FGFR1, another FGF2 receptor, is currently unknown.

By analogy with BECs, glycolysis is the main energy source for quiescent LECs as glycolysis produces > 70% of the total amount of their ATP (Yu et al., 2017). Furthermore, cultured LECs that are stimulated with FGF2 or VEGFC further increase their glycolytic flux, an effect mediated by an increase in hexokinase 2 (HK2), the first flux-controlling enzyme of glycolysis (Figs. 7B, 2) (Yu et al., 2017). In contrast, the expression of PFKFB3, one of the most important glycolytic regulators in BECs, remains unchanged upon FGF2 or VEGFC stimulation (Yu et al., 2017).

Prenatal experiments in mice with LEC-specific double knockout

for FGFR1/FGFR3 – induced at various time points during lymphatic development – result in reduced LEC migration and branching with fewer LECs in the skin. Altogether, this leads to significant edema and the appearance of blood filled capillaries, demonstrating the importance of FGFR1/FGFR3 signaling in lymphangiogenesis (Yu et al., 2017). Remarkably, single knockdown of FGFR1 in human dermal LECs (HDLECs) reduces LEC proliferation and migration, while single FGFR3 knockdown has no effect, indicating the significance of the less-studied FGFR1 for lymphatic migration and branching (Yu et al., 2017).

As mentioned above, FGF-stimulation of LECs increases HK2 activity (Fig. 7A). Therefore, FGFR1 knockdown in HDLECs decreases HK2 levels, while stimulation with FGF2 increases HK2 expression (with minimal changes in the expression of other glycolytic enzymes). Moreover, mice with LEC-specific knockout of hexokinase 2 during embryonic development display the same phenotype as FGFR1 single knockout mice. As FGF2 also induces HK2 expression in BECs, these data indicate that FGF regulation of lymphangiogenesis and angiogenesis share similar metabolic mechanisms. As in BECs, FGF-dependent control of Myc expression underlies the control of HK2 levels in LECs (Fig. 7A).

In addition to stimulating embryonic lymphatic development, FGF also stimulates adult lymphangiogenesis in a HK2-dependent manner. Indeed, in adult LEC-specific HK2 knockout mice, implanted FGF2-containing corneal pellets lead to less lymphangiogenesis than in control mice with normal HK2 levels. Overall, these findings emphasize the critical role of FGF-Myc-HK2-driven glycolysis during lymphangiogenesis.

5.2. Fatty acid oxidation regulates lymphangiogenesis

Besides FGFR3 and VEGFR3, another target gene of PROX1 is CPT1a. PROX1 elevates the transcription of CPT1a, which increases FAO in LECs during lymphangiogenesis (Fig. 7A, B) (Wong et al., 2017). This elevated FAO serves two purposes in LECs. First, similar as BECs, LECs rely on FAO-derived acetyl-CoA to sustain the Krebs cycle and deoxyribonucleotide (dNTP) synthesis for proliferation in conjunction with an anaplerotic substrate (Fig. 7B) (Schoors et al., 2015). Second, Prox1-induced FAO has a role in epigenetic regulation of lymphatic gene expression. Indeed, FAO generates acetyl-CoA that is used by the histone acetyltransferase p300 for histone acetylation at PROX1 target genes such as VEGFR3 (Fig. 7B) (Wong et al., 2017). This occurs preferentially at lymphangiogenic rather than angiogenic genes, because PROX1 interacts with p300 at these lymphangiogenic sites. This acetylation results in decondensation of chromatin, which makes the genes more accessible for PROX1, so that this transcription factor can better activate the transcription of the lymphatic genes. As such, PROX1 promotes lymphatic development by upregulating FAO and subsequently seizing the metabolites of FAO (Wong et al., 2017). These findings expand our knowledge of PROX1 as the master regulator of lymphatic development, and identify PROX1 as a “smart” *bona fide* transcription factor that highjacks metabolism to improve its own transcriptional activity.

As a result, inhibition of CPT1a (either pharmacologically or with a LEC-specific genetic knockdown) during early stages of embryonic lymphatic development results in a reduced number of $PROX1^+$ and $VEGFR3^+$ expressing endothelial cells present in and emigrating from the anterior cardinal vein, indicating the importance of FAO for lymphatic differentiation and early lymphatic development (Wong et al., 2017). Also, inhibition of CPT1a at later developmental stages results in impaired LEC migration and proliferation, which impairs lymph sac formation and causes dermal edema due to lymphatic branching defects (Wong et al., 2017). Furthermore, FAO is necessary for lymph vessel maturation and, likely, also for lymphovenous separation as inhibition of CPT1a in LECs results in subcutaneous edema (Fig. 8A), impairment of network outgrowth to the midline (Fig. 8A), and formation of a disorganized lacy mesh with fewer branches and the emergence of blood-filled lymphatic structures, a marker of incomplete lymphovenous separation (Wong et al., 2017).

Of note, LECs also rely on fatty acid synthesis for lymphangiogenesis. For instance, pharmacological inhibition of FA synthase (FASN) in LECs decreases their viability, proliferation and migration (Bastos et al., 2017). Therefore, further investigation to elaborate these findings would be of interest.

6. Translational potential

The finding that endothelial cell metabolism regulates different aspects of both blood and lymph vessel development opens up new perspectives for future therapies of numerous vasculature related diseases. For example, in a mouse model of pathological ocular angiogenesis, the inhibition of glycolysis with the PFKFB3-inhibitor 3-(3-pyridinyl)−1-(4-pyridinyl)−2-propen-1-one (3PO), of FAO with the CPT1a-inhibitor etomoxir and of glutamine metabolism with the glutaminase 1 (GLS1)-inhibitor CB-839 all independently decrease pathological neovascularization (De Bock et al., 2013b; Schoors et al., 2014, 2015; Huang et al., 2017). Similarly, CPT1-inhibition with etomoxir in a mouse cornea injury model reduces pathological lymphangiogenesis (Wong et al., 2017).

Furthermore, these insights are of high interest for the treatment of cancer, as this life threatening disease depends on blood vessel formation (Hanahan and Weinberg, 2000). Newly formed tumor blood vessels do not resemble normal blood vessels, instead, they are structurally and functionally aberrant. This creates a hostile microenvironment deprived of oxygen and nutrients, from where cancer cells attempt to escape and form distant metastases (Carmeliet and Jain, 2011; Jain, 2014). Tumor endothelial cells are more metabolically active than normal endothelial cells and have an even higher glycolytic flux (Cantelmo et al., 2016). Fascinatingly, inhibition of PFKFB3 in tumor endothelial cells results in normalization of tumor blood vessels and hence lowers metastasis, but improved chemotherapy delivery and response (Cantelmo et al., 2016). This finding is promising, as in most cancer patients, current anti-angiogenic strategies suffer from limited clinical success and new complementary treatment paradigms are much needed (Jayson et al., 2016). Another promising avenue is the use of metabolites for regenerative medicine. Indeed, supplementation of acetate (a precursor of acetyl-CoA) is able to stimulate sprouting of BECs and LECs in vitro, and restore etomoxir-impaired lymphangiogenesis in vivo (Fig. 8B) (Schoors et al., 2015; Wong et al., 2017).

7. Conclusion

Endothelial cell metabolism recently emerged as an important regulator of angiogenesis and lymphangiogenesis. In both of these developmental processes, glycolysis is the main source for energy production and drives endothelial cell migration and proliferation, while FAO generates nucleotide biomass for endothelial cell proliferation. In addition, in LECs, FAO governs the epigenetic control of key lymphatic genes involved in lymphangiogenesis, such as VEGFR3. Furthermore, in BECs, glutamine metabolism supports angiogenesis through Krebs cycle anaplerosis, protein production and redox homeostasis. Inhibition of the key enzymes of glycolysis, FAO and glutamine metabolism (respectively PFKFB3, CPT1a, GLS1) reduces neovascularization in mouse models of pathological ocular angiogenesis, indicating the translational potential of these findings. Together, these insights underscore the underappreciated role of endothelial cell metabolism in health and disease. Hopefully, these new insights can advance therapeutic opportunities to cure vascular related diseases.

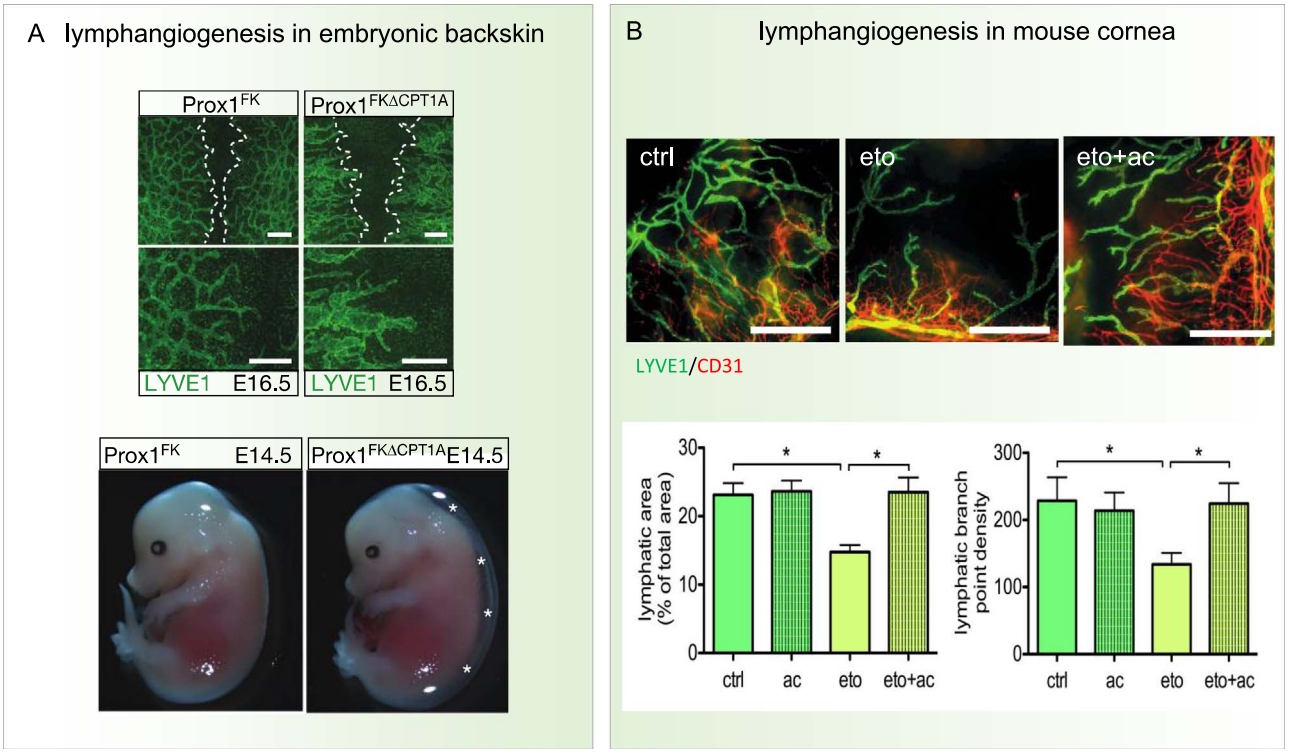

Fig. 8. Fatty acid oxidation in lymphangiogenesis in vivo. (A) Top panel: Illustration of lymphangiogenesis defects upon lymphatic endothelial loss of CPT1A. Compared to CPT1A wildtype (CPT1a^{FK}) mice, CPT1A knockout (Prox1$^{FK\Delta CP1A}$) mice exhibit impaired lymphangiogenesis in the back skin at embryonic day 14.5. Lower panel: Prox1$^{FK\Delta CP1A}$ mice also develop lymphedema under the skin (adapted with permission from Wong et al. (2017)). (B) Images and quantification of lymphangiogenesis in the mouse cornea after cauterization. The growth of LYVE1^{+} (green) lymphatic vessels is impaired upon etomoxir treatment, but the sprouting defect is rescued by acetate supplementation in vivo; the quantification of lymphangiogenesis is shown below the images (adapted with permission from Wong et al. (2017)). ctrl, control; eto, etomoxir; ac, acetate; Lyve1, lymphatic vessel endothelial hyaluronan receptor 1; CD31, cluster of differentiation 31. PROX1, prospero homeobox protein 1; LYVE1, lymphatic vessel endothelial hyaluronan receptor 1; CPT1a, carnitine palmitoyltransferase 1a; E14.5, embryonic day 14.5; E16.5, embryonic day 16.5.

Acknowledgements

We apologize for not being able to cite the work of all other studies related to this topic because of space restrictions.

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Conflicts of interest

PC declares to be named as inventor on patent applications, claiming subject matter related to the results described in this paper. The remaining authors declare no competing financial interests.

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