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COMMENTARY

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Angiogenesis revisited – role and therapeutic potential of targeting endothelial metabolism

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ABSTRACT

Clinically approved therapies that target angiogenesis in tumors and ocular diseases focus on controlling pro-angiogenic growth factors in order to reduce aberrant microvascular growth. Although research on angiogenesis has revealed key mechanisms that regulate tissue vascularization, therapeutic success has been limited owing to insufficient efficacy, refractoriness and tumor resistance. Emerging concepts suggest that, in addition to growth factors, vascular metabolism also regulates angiogenesis and is a viable target for manipulating the microvasculature. Recent studies show that endothelial cells rely on glycolysis for ATP production, and that the key glycolytic regulator 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) regulates angiogenesis by controlling the balance of tip versus stalk cells. As endothelial cells acquire a tip cell phenotype, they increase glycolytic production of ATP for sprouting. Furthermore, pharmacological blockade of PFKFB3 causes a transient, partial reduction in glycolysis, and reduces pathological angiogenesis with minimal systemic harm. Although further assessment of endothelial cell metabolism is necessary, these results represent a paradigm shift in anti-angiogenic therapy from targeting angiogenic factors to focusing on vascular metabolism, warranting research on the metabolic pathways that govern angiogenesis.

KEY WORDS: Angiogenesis, Anti-angiogenesis therapy, Glycolysis, Metabolism

Introduction

The vasculature is the physiological transport system that evolved to traffic oxygen, nutrients, growth factors, waste and immune cells throughout the body. At first, primitive invertebrates only required hollow, acellular tubes for slow biotransport to meet their metabolic needs (Muñoz-Chápuli et al., 2005). However, as organisms became more complex, blood vessels adapted by acquiring a lining of endothelial cells to support the rapid transport required by high metabolic demands. Metabolically stressed cells signal quiescent endothelial cells to initiate angiogenesis, a process that is defined as the growth of new blood vessels from existing vasculature in order to vascularize the tissue (Fraisl et al., 2009; Murray and Wilson, 2001). Although the links between metabolic demand and vascular development are apparent, how endothelial cells change their own metabolism between quiescent and active states remains unclear.

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Under physiological or non-pathological conditions, endothelial cells remain quiescent as 'phalanx' cells under a balance of pro- and anti-angiogenic factors. When pro-angiogenic factors dominate, endothelial cells quickly switch to angiogenic phenotypes that are categorized as either migratory tip cells or proliferating stalk cells (Adams and Alitalo, 2007; Potente et al., 2011). Tip cells sprout from existing vasculature and navigate towards the angiogenic signal (Gerhardt et al., 2003). Stalk cells proliferate behind the tip cell and extend the vascular lumen as sprouts elongate. Tip and stalk cell differentiation is tightly regulated by a feedback system involving vascular endothelial growth factor (VEGF) and Notch, as well as other genetic control signals. VEGF induces endothelial tip cell mobilization and expression of delta-like ligand 4 (DLL4). DLL4-mediated activation of Notch signaling in neighboring cells suppresses their expression of VEGF receptor 2 (VEGFR2) and, thereby, promotes the stalk cell phenotype (Jakobsson et al., 2010). Endothelial cells dynamically switch phenotypes during angiogenesis, depending on their fitness as the tip cell (Jakobsson et al., 2010). By ensuring that the most suitable endothelial cells are in the tip and stalk positions, the DLL4–Notch feedback system promotes efficient sprouting and mediates vascular growth patterns (Bentley et al., 2009). However, metabolic requirements of endothelial cells during angiogenesis are under-investigated, and whether mechanisms of endothelial cells (such as Notch signaling) differentially regulate their metabolic state remains unclear. This Commentary discusses the current understanding of endothelial cell metabolic pathways and highlights glycolysis as a regulator of endothelial cell phenotype during angiogenesis (Fig. 1).

Endothelial metabolic pathways

Glycolysis

Endothelial cells generate most of their energy through glycolysis, with a glycolytic rate similar to that of highly glycolytic tumor cells (De Bock et al., 2013a) (Fig. 1). Compared with other healthy cell types, endothelial cells display a high glycolytic flux while quiescent. When activated for migration or proliferation, endothelial cells double their glycolytic flux (De Bock et al., 2013b). Meanwhile, immune-cell glycolytic flux is minimal during quiescence, but increases 20–30 fold upon activation (Wang et al., 2011a). Facilitated diffusion by glucose transporters allows endothelial cells to take up glucose without expending energy. Following VEGF stimulation, endothelial cells increase expression of their primary glucose transporter, glucose transporter type 1 (GLUT-1; also known as SLC2A1), by activation of phosphoinositide 3-kinase (PI3K)–AKT signaling (Yeh et al., 2008). Reducing GLUT-1 expression in endothelial cells decreases uptake of glucose in brain and heart tissue, implying that vascular glucose uptake influences parenchymal glucose metabolism (Huang et al., 2012). Despite their immediate

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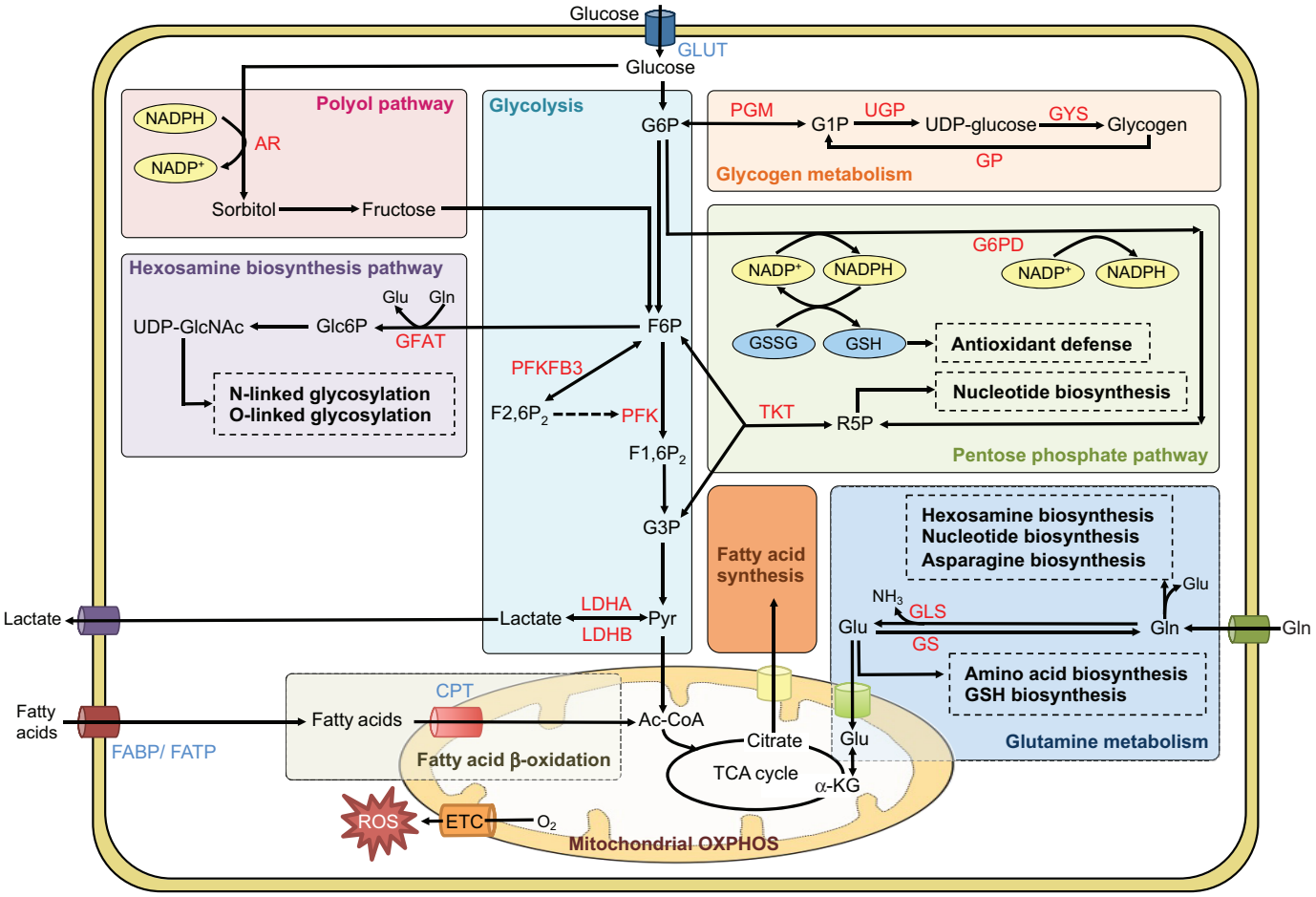

Fig. 1. Endothelial cell metabolism. A simplified map of the known metabolic pathways in endothelial cells and the enzymes relevant for this Commentary. Dashed arrow indicates allosteric activation. α-KG, α-ketoglutarate; Ac-CoA, acetyl coenzyme A; AR, aldolase reductase; CPT, carnitine palmitoyltransferase; ETC, electron transport chain; F1,6P$_2$, fructose-1,6-bisphosphate; F2,6P$_2$, fructose-2,6-bisphosphate; F6P, fructose-6-phosphate; FABP, fatty acid binding protein; FATP, fatty acid transport protein; G1P, glucose-1-phosphate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; GFAT, glutamine fructose-6-phosphate amino-transaminase; Glc6P, glucosamine-6-phosphate; Gln, glutamine; GLS, glutaminase; Glu, glutamate; GP, glycogen phosphorylase; GLUT, glucose transporter; GS, glutamine synthetase; GSH, glutathione; GSSG, glutathione disulfite; GYS, glycogen synthase; LDH, lactate dehydrogenase; OXPHOS, oxidative phosphorylation; PFK, phosphofructokinase; PFKFB3, phosphofructokinase-2/fructose-2,6-bisphosphatase isoform 3; PGM, phosphoglucomutase; Pyr, pyruvate; R5P, ribose-5-phosphate; ROS, reactive oxygen species; TKT, transketolase; TCA, tricarboxylic acid; UDP-GlcNAc, uridine diphosphate-N-acetylglucosamine; UDP-glucose, uridine diphosphate glucose; UGP, UDP-glucose phosphorylase.

proximity to oxygen, endothelial cells generate >85% of their ATP by anaerobic metabolism of glucose into pyruvate through glycolysis (Culic et al., 1997; De Bock et al., 2013b). Reducing glycolytic activity by 80% with 2-deoxy-D-glucose (2DG) induces endothelial cell death, indicating the necessity of glycolysis for endothelial function (De Bock et al., 2013b; Merchan et al., 2010). Although endothelial cells rely mainly on glycolysis, they also reserve the capacity for oxidative metabolism and mitochondrial respiration under conditions of stress or when glycolysis is compromised (Dranka et al., 2010; Krützfeldt et al., 1990; Polet and Feron, 2013).

Endothelial cell metabolism is dictated in part by environmental and molecular signals. Although all blood vascular endothelial cell identities (arterial, venous, microvascular) are highly glycolytic *in vitro*, arterial endothelial cells are comparatively less glycolytic and more oxidative, whereas microvascular-derived endothelial cells are relatively more glycolytic and proliferative. However, the *in vivo* relevance of these endothelial cell subtype-dependent findings requires further study (De Bock et al., 2013b; Parra-Bonilla et al., 2010). Interestingly, endothelial cells under pathological conditions exhibit characteristics of the Warburg effect – which is commonly associated with hyperproliferative cells – whereby glycolysis increases and oxidative phosphorylation decreases (Delgado et al., 2010; Fijalkowska et al., 2010; Metallo and Vander Heiden, 2013; Mullen and DeBerardinis, 2012). Upon activation by factors such as VEGF, endothelial cells increase expression of GLUT-1 as well as that of glycolytic enzymes, such as lactate dehydrogenase-A (LDH-A) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3), among others (De Bock et al., 2013b; Parra-Bonilla et al., 2010; Peters et al., 2009; Yeh et al., 2008). By contrast, DLL4–Notch signaling, which suppresses VEGFR2 expression and vascular branching, also decreases glycolytic flux in endothelial cells *in vitro* by downregulating PFKFB3 (De Bock et al., 2013b) (Fig. 2). PFKFB3 generates fructose-2,6-bisphosphate, an allosteric activator of phosphofructokinase-1 (PFK-1), itself a rate-limiting step in glycolysis. In line with findings that endothelial cells rely on glycolysis, silencing PFKFB3 in endothelial cells impairs

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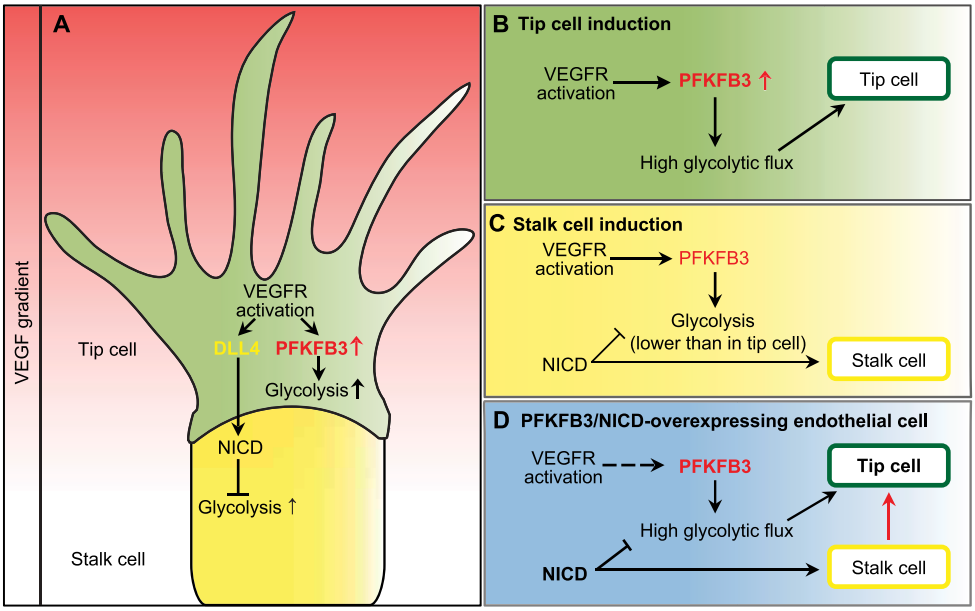

Fig. 2. Metabolic differences in tip and stalk cells. (A) The metabolic dynamic of tip (green) and stalk (yellow) cells whereby VEGF (red background) activates VEGFR (presumably VEGFR2) signaling in tip cells to upregulate PFKFB3 levels and increase glycolysis. Meanwhile, VEGFR activation also increases expression of the Notch ligand Delta-like 4 (DLL4) to initiate Notch signaling in the neighboring stalk cells. There, the Notch 1 intracellular domain (NICD) reduces PFKFB3-driven glycolysis so that stalk cells have lower levels of glycolysis than tip cells. (B) VEGFR signaling increases PFKFB3 protein expression, triggering an increase in glycolysis and induction of the tip cell phenotype. (C) Reduced VEGFR signaling together with increased Notch signaling through NICD results in less PFKFB3-driven glycolysis in stalk cells compared with tip cells. (D) Overexpression of PFKFB3 increases glycolytic flux to induce the tip cell phenotype, even when the potent 'pro-stalk cell signal' NICD is also overexpressed (shown by bold text). Thus, PFKFB3 overexpression overrides the stalk cell-inducing activity of Notch signaling to promote the tip cell phenotype (red arrow). Font sizes and arrow widths indicate relative differences. Dashed arrows indicate low activity.

proliferation, migration and sprouting *in vitro*. In mice, inactivation of the *Pfkfb3* gene in endothelial cells also impedes angiogenesis (De Bock et al., 2013b). Thus, PFKFB3 is a mediator of VEGF–Notch-driven angiogenesis.

Although glucose oxidation can produce ten times more ATP per unit of glucose as compared with glycolysis, endothelial cell preference for glycolysis in angiogenic processes is in line with their role in neovascularization (Harjes et al., 2012). Their reliance on anaerobic generation of ATP enables endothelial cells to proliferate, and to migrate in hypoxic environments where angiogenesis is stimulated and oxidative metabolism would be impaired. Accordingly, glucose availability is not a rate-limiting factor because its concentrations are sufficient in interstitial tissue whereas those of oxygen drop away from blood vessels (Buchwald, 2011; Gatenby and Gillies, 2004). Although lack of glucose enhances endothelial cell vulnerability to hypoxia, when sufficiently high concentrations of glucose are present, glycolysis can generate as much ATP as glucose oxidation because glycolysis rapidly produces ATP (Locasale and Cantley, 2011; Mertens et al., 1990). Rapid ATP production through compartmentalized or local glycolysis also enables endothelial cells to quickly adapt filopodia and lamellipodia during angiogenesis and migration (De Bock et al., 2013b; Lamalice et al., 2007). In addition, glycolytic products and intermediates are shunted into other metabolic pathways, and serve as essential macromolecule precursors required for normal cell function (see below). Furthermore, glycolysis produces fewer reactive oxygen species (ROS) and, thus, less oxidative stress – which might be advantageous for endothelial cells as residents of a highly oxygenated location.

Pentose phosphate pathway

The glycolytic intermediate glucose-6-phosphate (G6P) is oxidized in the pentose phosphate pathway (PPP) to produce NADPH and ribose-5-phosphate (R5P) in part for redox homeostasis and nucleotide biosynthesis (Fig. 1). NADPH is an important factor involved in the synthesis of lipids and nitric oxide (NO), and in the conversion of oxidized glutathione disulfide (GSSG) to glutathione (GSH), which is a crucial redox buffer; R5P is used for biosynthesis of nucleotides. The PPP has oxidative (oxPPP) and non-oxidative (non-oxPPP) branches that, respectively, produce NADPH and ribulose-5-phosphate (Ru5P; an intermediate to R5P), and R5P only. Cells can modulate oxPPP or non-oxPPP flux for redox control or cell division according to their needs (Anastasiou et al., 2011; Vander Heiden et al., 2011).

Normally, only 1–3% of all glucose consumed by cells passes through the PPP. Under oxidative stress, however, cells shunt glucose through the oxPPP to maintain GSH concentrations that are high enough to combat ROS damage (Dobrina and Rossi, 1983; Jongkind et al., 1989; Krützfeldt et al., 1990; Spolarics and Spitzer, 1993; Spolarics and Wu, 1997; Vizán et al., 2009). Glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme that catalyzes the entrance of G6P into the oxPPP. Overexpression of G6PD in endothelial cells increases NADPH and NO production, and protects endothelial cells from oxidative damage (Leopold et al., 2003b). Relevant in diabetes, hyperglycemia inhibits G6PD activity by increasing protein kinase A-mediated phosphorylation of G6PD. This reduction in G6PD function incapacitates the ROS-scavenging machinery and causes cell death (Zhang et al., 2000). Restoring G6PD activity

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under hyperglycemia recovers endothelial cell redox homeostasis (Zhang et al., 2012).

In endothelial cells, increasing G6PD expression also promotes VEGF-induced angiogenic functions, possibly by increasing NO availability, whereas knockdown of G6PD dampens their response to VEGF stimulation, possibly owing to reduced tyrosine phosphorylation of VEGFR2 (Leopold et al., 2003a). VEGF-stimulated, G6PD-deficient endothelial cells also display decreased NO production, proliferation, migration and tube formation, indicating that the metabolic status can regulate endothelial cell activation (Leopold et al., 2003a; Vizán et al., 2009). As part of bi-directional signaling, VEGF stimulation increases G6PD activity near the plasma membrane (Pan et al., 2009; Vizán et al., 2009). However, G6PD deficiency in diabetic patients encourages retinal vasculopathy, presumably because increased non-toxic levels of ROS induce angiogenesis (Cappai et al., 2011; Okuno et al., 2012). Thus, modulation of G6PD activity could be a strategy to prevent or promote angiogenesis in a context-dependent manner. Inhibition of transketolase, the bottleneck of the non-oxPPP, also reduces endothelial cell viability and migration (Vizán et al., 2009). Still, how endothelial cells coordinate flux through the oxPPP and non-oxPPP during angiogenesis remains uncharacterized.

Hexosamine biosynthesis pathway

The hexosamine biosynthesis pathway (HBP) is another side branch of glycolysis and produces substrates for protein glycosylation – a post-translational modification important in cell signal transduction (Fülöp et al., 2007) (Fig. 1). The rate-limiting HBP enzyme glutamine-fructose-6-phosphate aminotransaminase (GFAT, also known as GFPT1), catalyzes the conversion of the glycolytic intermediate fructose-6-phosphate into glucosamine-6-phosphate (Glc6P). Glc6P is converted into uridine-diphosphate-N-acetylglucosamine (UDP-GlcNAc) in three additional steps. The GlcNAc group of UDP-GlcNAc is used in both O- and N-linked protein glycosylation reactions (Laczy et al., 2009). Posttranslational glycosylation is important for proper protein function including activity, structure and localization. Although the HBP as well as increased protein glycosylation are implicated in diabetes-induced dysfunction of endothelial cells (Blake and Trounce, 2013), the exact role of the HBP in sprouting angiogenesis is not well established. Supplementing endothelial cell growth medium with glucosamine increases protein glycosylation and reduces endothelial cell migration and tube formation. In addition, glucosamine impairs sprouting from cultured aortic rings (Luo et al., 2008). Additional evidence that the HBP is important for angiogenesis comes from the observation that overexpression of O-GlcNAcase, which reduces glycosylation, increases migration and tube formation. It has been postulated that increased glycosylation of Akt mediates the effect of glucosamine on migration (Luo et al., 2008).

However, glycosylation is a common post-translational modification and other proteins are probably also involved. For instance, glycosylation of the Notch receptor alters responsiveness to the Notch ligands DLL4 and Jagged1 (Benedito et al., 2009). VEGFR2 is also heavily glycosylated, and it has been suggested that glycosylation is important for VEGFR2 retention at the cell membrane (Markowska et al., 2011). In tumors that show refractory responses to anti-VEGF treatment, glycosylation of VEGFR2 has been implicated in perpetuating VEGFR2-dependent angiogenic activity that is facilitated by binding of galactin-1 but independent of the VEGF ligand (Croci et al., 2014). Angiogenesis and tumor growth decreased in mice that lack glycosyltransferase, an enzyme responsible for endothelial VEGFR2 glycosylation in refractory tumors (Croci et al., 2014). Together, these data indicate that the HBP is important for angiogenesis, but the mechanisms and physiological function of protein glycosylation in endothelial cells remain to be further elucidated.

Polyol pathway

As previously mentioned, high glucose levels can impair endothelial cell redox homeostasis in part by reducing G6PD levels (Zhang et al., 2000; Zhang et al., 2012). Hyperglycemia also pushes the excess glucose through the polyol pathway where aldose reductase expends NADPH to reduce glucose into sorbitol (Fig. 1). Flux through the polyol pathway decreases NADPH levels, which are necessary to maintain GSH levels for redox homeostasis, and leads to ROS accumulation. The increase in ROS presumably contributes to aberrant angiogenesis in diabetic retinopathy. Blockade or genetic deficiency of aldose reductase reverses the excessive angiogenesis associated with vascular retinopathy (Fu et al., 2012; Obrosova and Kador, 2011). Interestingly, aldose reductase inhibitors also decrease VEGF-stimulated angiogenesis, suggesting a role for polyol pathway flux in normal angiogenic functions (Tammali et al., 2011).

Oxidative phosphorylation in mitochondria

Mitochondria are highly active and dynamic organelles that constantly change their morphology, fuse, divide and move in response to physiological stimuli (Makino et al., 2010). There is increasing evidence that mitochondrial morphology is influenced by metabolic perturbations (Pangare and Makino, 2012). Diabetes and high glucose levels induce mitochondrial fragmentation that is associated with endothelial dysfunction, apoptosis and reduced oxygen consumption, emphasizing the important role for mitochondria in endothelial cells (Makino et al., 2010; Shenouda et al., 2011; Trudeau et al., 2010). However, endothelial cells do not seem to rely on mitochondrial OXPHOS or aerobic metabolism for the generation of ATP (De Bock et al., 2013a). Endothelial cells even display the Crabtree effect – downregulating respiration under high glucose concentrations (Koziel et al., 2012). Mitochondria, the organelles in which OXPHOS occurs, take up only 4% of the intracellular space witin endothelial cells, compared with 28% in hepatocytes (Blouin et al., 1977), despite their immediate access to high levels of oxygen from the blood (Locasale and Cantley, 2011). This might be because high rates of endothelial cell respiration would be counterproductive to oxygen delivery to surrounding tissues, in addition to the advantages that glycolysis provides during angiogenesis (see above).

Although mitochondria in endothelial cells are fully functional and coupled to ATP synthesis (Dranka et al., 2010; Koziel et al., 2012) (Fig. 1), oxidative pathways only contribute a small portion of the total ATP production (Culic et al., 1997; De Bock et al., 2013b; Quintero et al., 2006; Spahr et al., 1989). This implies that the flux through the electron transport chain is kept in check. One hypothesis is that NO, derived from the endothelial NO synthase (eNOS), keeps basal mitochondrial respiration at a low level by inhibiting complex IV (cytochrome *c* oxidase) (Brown and Cooper, 1994; Clementi et al., 1998), the terminal acceptor in the electron transport chain (Davidson and Duchen, 2007). However, regulation of mitochondrial activity in endothelial cells remains difficult to understand. Mitochondrial reserve capacity is still maintained, allowing for upregulation of OXPHOS in times of high metabolic

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demand or stress. Endothelial cells may also depend on OXPHOS for their physiological function in differing vascular beds. In an *in vitro* model of the blood–brain barrier, a highly specialized vascular bed, endothelial cells upregulate genes of enzymes from the tricarboxylic acid cycle in response to shear stress while downregulating glycolytic genes, implying a possible functional switch to OXPHOS for this specialized niche (Cucullo et al., 2011).

Mitochondrial biogenesis is in part regulated by angiogenic signaling factors. VEGF has been shown to activate one of the master regulators of mitochondrial biogenesis and oxidative metabolism, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α; encoded by the gene *PPARGC1A*), leading to increased mitochondrial biogenesis in endothelial and other cell types (Arany et al., 2008; Wright et al., 2008). PGC-1α expression in heart and muscle cells induces angiogenesis, implying that PGC-1α stimulates vascularization and prepares ischemic tissues for oxidative metabolism that is permitted by improved oxygenation. Intriguingly, induction of PGC-1α in endothelial cells by high levels of glucose mediates cellular dysfunction and impairs angiogenesis (Sawada et al., 2014). These observations highlight the complexities of cell-type-specific metabolism but also indicate the important role of PGC-1α in tissue vascularization.

Despite their seemingly minor role in energy production, mitochondria are essential for endothelial cell survival and several other physiological functions (Groschner et al., 2012; Zhang and Gutterman, 2007). Mitochondria are the main source of ROS in most cell types, including endothelial cells. Emerging as an essential endothelial signaling molecule at low, non-toxic levels, mitochondrial ROS are mainly produced in the form of superoxide anions by the electron transport chain complexes I and III (Zhang and Gutterman, 2007). Superoxide anions can be transformed to hydrogen peroxide by superoxide dismutases and into peroxynitrite through a reaction with NO (Li and Shah, 2004). Low levels of hydrogen peroxide exert angiogenic signaling effects, whereas high levels of both hydrogen peroxide and peroxynitrite have been associated with dysfunction of endothelial cells in cardiovascular disease (Cai, 2005; Hink et al., 2001; Ruiz-Ginés et al., 2000; Zanetti et al., 2002). Hemodynamic forces lead to both cytoplasmic and mitochondrial ROS production in endothelial cells (Ali et al., 2004; Chatterjee and Fisher, 2014; Raaz et al., 2014). The cytoplasmic and mitochondrial ROS pathways are tightly intertwined (Dikalov et al., 2014; Nazarewicz et al., 2013), and mediate shear-stress-induced blood vessel dilation, vascular permeability and angiogenesis (Liu et al., 2003; Pearlstein et al., 2002). In addition, low levels of ROS are known to induce angiogenesis partly through upregulation of VEGF and activation of VEGFR2 signaling (Chua et al., 1998; Wang et al., 2011b).

Mitochondria contain up to 25% of endothelial cell Ca^{2+}, implying that Ca^{2+} is essential for mitochondrial function. On one hand, mitochondria regulate Ca^{2+} homeostasis in endothelial cells, including restoration of the endoplasmic reticulum Ca^{2+} stores necessary for normal cellular function (Malli et al., 2003; Wood and Gillespie, 1998). Ca^{2+} binds to a range of signaling molecules, receptors and transporters, modulating their activity (Groschner et al., 2012). On the other hand, mitochondrial ATP production *in vitro* is directly related to Ca^{2+} levels inside mitochondria (Denton and McCormack, 1990), implying that Ca^{2+} homeostasis is vital for mitochondrial respiration. Indeed, an increase in intracellular Ca^{2+} boosts mitochondrial ATP production by increasing mitochondrial enzyme activity (Kluge et al., 2013). However, the functional interplay between mitochondrial function and Ca^{2+} signaling and homeostasis in endothelial cells is left unclear by a paucity of studies.

Mitochondria also play a role in triggering endothelial cell apoptosis associated with vessel regression as a crucial balance to endothelial cell proliferation during angiogenesis (Mallat and Tedgui, 2000). They mediate programmed cell death in response to intracellular stress or extracellular signals (e.g. activation of death receptors) by releasing mitochondrial proteins such as cytochrome *c* into the cytosol (Kluge et al., 2013; Vander Heiden et al., 1997; Vaux, 2011); these proteins are bound by caspases, triggering apoptosis (Lee et al., 2012). Much is known about mitochondria function in multiple cell types and the same might hold true for endothelial cells, but mitochondrial function during angiogenesis is under-investigated.

Fatty acid β-oxidation

Fatty acid β-oxidation (FAO) can produce high amounts of ATP (Fig. 1). Theoretically, one mole of palmitate can generate a maximum of 129 moles of ATP, compared with 38 moles of ATP produced by the oxidation of 1 mole of glucose (Vo et al., 2004). Endothelial cells take up free fatty acids (FFAs) that are circulating in the blood bound to albumin or in the form of triglycerides as part of very-low-density lipoproteins (Jeppesen and Kiens, 2012). Interestingly, fatty acid uptake and transport across the endothelial cell layer is regulated by the angiogenic growth factor VEGF-B. Although its role in physiological angiogenesis is unclear (Carmeliet et al., 2012), VEGF-B signals through VEGFR1 and induces the upregulation of fatty acid transport proteins 3 and 4 (FATP3 and FATP4, respectively) in endothelial cells (Hagberg et al., 2012). Moreover, VEGF-A (commonly referred to as simply VEGF but only one protein of the VEGF family) is an important inducer of FABP4 (Elmasri et al., 2009), which binds cellular long-chain fatty acids and potentiates fatty acid uptake. FABP4 deficiency has been shown to attenuate endothelial cell proliferation (Elmasri et al., 2009) and angiogenesis (Ghelfi et al., 2013) . Together, these data imply that endothelial cells play an important role in fatty acid uptake for internal use and/or for transport to peripheral tissues. However, when human aortic endothelial cells are stimulated with VEGF-A or VEGF-B, FAO is unaffected (Reihill et al., 2011). The acquired fatty acids are possibly transported across the endothelium to fuel perivascular cells (Carmeliet et al., 2012). Endothelial cells themselves are also capable of FAO and a small amount of the total ATP is produced in this manner (Dagher et al., 1999; Dagher et al., 2001; De Bock et al., 2013a; Héliès-Toussaint et al., 2006), although the physiological role of FAO may be context-dependent and remains debated. Whether fatty acid catabolism influences endothelial cell capacity for branching angiogenesis is unknown.

The rate-limiting step in FAO is the transfer of long chain acyl-CoA fatty acids into the mitochondria by carnitine palmitoyltransferase 1 (CPT1) to form fatty acylcarnitines (Fu et al., 2013). This step is inhibited by malonyl-CoA, which is produced from acetyl-CoA by acetyl-CoA carboxylase (ACC) (Dagher et al., 2001). Induction of the kinase AMPK (encoded by the gene *PRKAA2*), which is a sensor for the cellular AMP:ATP ratio, decreases the activity of ACC and, thereby, stimulates FAO in endothelial cells (Dagher et al., 1999; Dagher et al., 2001; Mount et al., 2008), linking cellular energy status with FAO. FAO flux in endothelial cells also responds to glucose (Knapp et al.,

2012). In the absence of glucose, cultured endothelial cells favor FAO over other metabolic pathways (Dagher et al., 2001), suggesting that its function of compensatory energy production is limited under conditions in which ATP is generated from glucose. Notably, highly increased glucose levels also increase endothelial cell FAO *in vitro* (Koziel et al., 2012), possibly because glycolysis is impaired under such hyperglycemic conditions (Giacco and Brownlee, 2010). Alternatively, FAO has been involved in protection against oxidative stress (Pike et al., 2011). Indeed, although not confirmed in endothelial cells, FAO-derived acetyl-CoA entering the tricarboxylic acid cycle is converted to malate and citrate, metabolites that are the respective substrates for the NADPH-producing malic enzyme and for isocitrate dehydrogenases 1 and -2 (Jeon et al., 2012). The resultant NADPH might replenish GSH, which is a strong ROS scavenger and forms one of the main cellular defenses against oxidative stress (Jongkind et al., 1989; Sekhar et al., 2011). Although evidence shows that FAO is important for endothelial cell function, the specific mechanisms that FAO mediates during angiogenesis remain undetermined.

Amino acid metabolism

Amino acid metabolism in endothelial cells has not been studied in detail, and its role in angiogenesis remains unclear. Glutamine has been the primary focus because it inhibits NO production through eNOS and is the most important nitrogen donor (Wu et al., 2001). Endothelial cells take in glutamine through the amino acid transporter SLC1A5 and system N family of amino acid transporters (Mann et al., 2003). Intracellular glutamine is either transported out of the cell by SLC7A5 in exchange for other non-essential amino acids or converted into glutamate by glutaminase (GLS) and glutamine amidotransferases (GATs) (Mouilleron and Golinelli-Pimpaneau, 2007; Nicklin et al., 2009) (Fig. 1). GLS converts glutamine into glutamate in a deamidation reaction that releases the γ-nitrogen as ammonia (NH_3). Glutamate can be used in several metabolic pathways, including conversion into α-ketoglutarate for subsequent anaplerotic refueling of the Krebs cycle, non-essential amino acid biosynthesis and GSH biosynthesis (DeBerardinis and Cheng, 2010).

Glutamine-derived carbons that enter the Krebs cycle can be used as an alternative energy source in glucose-depleted tissues. Under normal conditions, ATP production through glutamine metabolism is limited compared with that of glucose (De Bock et al., 2013b). However, when oxidative stress impairs metabolic enzymes and glycolysis, glutamine-derived ATP production rapidly increases (Hinshaw and Burger, 1990). Blocking the conversion of glutamine into glutamate by GLS knockdown inhibits proliferation and induces a senescence-like state in endothelial cells (Unterluggauer et al., 2008). GATs also produce NH_3 from glutamine but, instead of being released, the NH_3 is transferred onto another molecule. These reactions are essential for various processes, including hexosamine biosynthesis, nucleotide biosynthesis and asparagine synthesis, many of which are essential to cell function (Mouilleron and Golinelli-Pimpaneau, 2007), although their importance in endothelial cells specifically remains to be explored. To maintain sufficient glutamine levels during nutrient starvation, endothelial cells can produce glutamine from glutamate using glutamine synthetase (Häberle et al., 2005). Deficiency of glutamine synthetase is linked to vascular disease in diabetic patients, but the role of glutamine synthetase in endothelial cell function remains to be determined (Qi et al., 2013).

Glycogen metabolism

Glycogen is a hydrated, branched, spherical glucose polymer formed in four steps from the glycolysis intermediate G6P (Fig. 1). G6P is converted into G1P through phosphoglucomutase, and then G1P is converted into UDP-glucose through UDP-glucosepyrophosphorylase. UDP-glucose is the activated form of glucose and is used by glycogen synthetase to add glucose to non-reducing ends of glycogen or glycogenin (Roach et al., 2012). Endothelial cells have the capacity to divert a fraction of glucose into the glycogen synthesis pathway and store glycogen for later use (Amemiya, 1983; Artwohl et al., 2007; Vizán et al., 2009). Indeed, glucose deprivation results in complete depletion of glycogen stores, indicating that endothelial cells metabolize glycogen to sustain glycolysis during glucose deprivation (Vizán et al., 2009). Although the physiological role of glycogen metabolism is not well studied, its importance is demonstrated by the observation that pharmacological inhibition of glycogen catabolism reduces endothelial cell viability and migration (Vizán et al., 2009).

Endothelial cell metabolism: a paradigm shift in anti-angiogenesis therapy?

Metabolic regulation of angiogenesis

Significant work on angiogenesis has focused on genetic and molecular signals that regulate endothelial dynamics and activation (Augustin et al., 2009; Eilken and Adams, 2010; Potente et al., 2011). During angiogenesis, endothelial tip cells migrate and guide vascular sprouts in response to VEGF–VEGFR2 signaling. Endothelial stalk cells, which trail tip cells and are induced by DLL4–Notch signaling, proliferate and elongate the sprout. Notably, even though Notch signals promote the proliferative stalk cell phenotype *in vivo*, they reduce endothelial cell proliferation *in vitro*. This apparent paradox can be explained by findings that the anti-proliferative activity of Notch is overruled by other (e.g. Wnt) pro-proliferative signals (Phng et al., 2009).

Recent studies indicate that tip and stalk cells have different metabolic phenotypes, suggesting that angiogenesis is regulated by metabolic mechanisms in addition to genetic and molecular signals (De Bock et al., 2013b). When cells enter S-phase, they increase glycolysis (Almeida et al., 2010); accordingly, VEGF increases glycolysis in proliferating endothelial cells by upregulating PFKFB3 levels. PFKFB3 knockdown reduces endothelial cell proliferation *in vitro* and the number of sprouting retinal vessels *in vivo*, indicating that PFKFB3 regulates stalk cell behavior (De Bock et al., 2013b) (Fig. 2). PFKFB3-driven glycolysis is also essential for tip cell functions, including migration, formation of lamellipodia and filopodia, and tip cell competitiveness. Indeed, when tested in mosaic endothelial cell spheroids containing an equal mixture of control and transgenic endothelial cells *in vitro*, PFKFB3-overexpressing cells are more competitive in reaching the tip cell position, whereas PFKFB3-silenced cells show the opposite behavior, indicating that tip cells need a high rate of glycolysis (De Bock et al., 2013b) (Fig. 2). Transgenic zebrafish experiments yielded similar results *in vivo* (De Bock et al., 2013b).

As discussed above, DLL4–Notch signaling reduces endothelial cell proliferation, a finding that is in line with its inhibitory effect on PFKFB3-driven glycolysis *in vitro* (De Bock et al., 2013b) (Fig. 2). This effect is presumably overruled *in vivo* by other pro-proliferative signals, which allows glycolysis in stalk cells to mediate proliferation. However, the above-mentioned mosaic competition findings also suggest that glycolytic flux is

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higher in tip than stalk cells, and that high glycolysis levels are required for endothelial cells to compete for the tip position in the vascular sprout. This is further supported by mosaic transgenic zebrafish experiments, showing that activation of Notch through overexpression of Notch 1 intracellular domain (NICD) drives transgenic endothelial cells into a stalk cell position but, importantly, that overexpression of PFKFB3 and NICD in endothelial cells promotes an identity switch from stalk to tip cells, while still promoting the tip cell phenotype (De Bock et al., 2013b) (Fig. 2D). This is remarkable, given that Notch is the most potent pro-stalk cell signal known to date.

It is noteworthy that overexpression or inhibition of PFKFB3 signaling does not affect the expression of genes associated with sprouting, indicating that PFKFB3 does not affect angiogenesis by regulating the expression of sprouting-modulatory genetic signals, but rather that PFKFB3-driven glycolysis directly modulates vessel sprouting (De Bock et al., 2013b) (Fig. 2B). Overall, these studies show that endothelial cell metabolism, in parallel with genetic signals, mediate angiogenesis.

Tip cells dynamically extend lamellipodia and filopodia that are composed of actin networks as they migrate during angiogenesis (Fraccaroli et al., 2012). Actin cytoskeleton remodeling requires large amounts of ATP, coupling actin polymerization with ATP hydrolysis (Pollard, 2007; Romero et al., 2007; Yogurtcu et al., 2012). However, endothelial lamellipodia and filopodia are devoid of mitochondria (De Bock et al., 2013b). As endothelial cells migrate, glycolytic enzymes appear in lamellipodia, particularly in F-actin-enriched membrane ruffles where ATP is also produced (De Bock et al., 2013b). Actin-binding sites also enhance glycolytic enzyme activity (Real-Hohn et al., 2010), allowing rapid and localized generation of ATP and thus forming energy hotspots. This explains why PFKFB3 knockdown inhibits lamellipodia formation in endothelial cells *in vitro*, and impairs filopodia formation *in vivo* (De Bock et al., 2013b).

Other glycolytic metabolites also play a role in the regulation of angiogenesis, partly by acting as signaling molecules. Extracellular lactate can be taken up by endothelial cells through the monocarboxylate transporter 1 (MCT-1) and, subsequently, inhibits the activity of the oxygen-sensing prolyl-hydroxylase domain protein 2 (PHD2, also known as EGLN1), whose absence leads to activation of the hypoxia-inducible transcription factor HIF-1α and pro-angiogenic signaling (Végran et al., 2011). In the context of cancer, high lactate concentrations upregulate HIF-1α and, thereby, induce tumor angiogenesis by increasing VEGF concentrations (De Saedeleer et al., 2012; Hunt et al., 2007). Metabolic signals can also regulate Notch signaling in endothelial cells. The NAD^+-dependent deacetylase activity of SIRT1 deacetylates NICD, thereby reducing the stability of NICD, and promoting vascularization (Chalkiadaki and Guarente, 2012; Guarani and Potente, 2010). SIRT1 deficiency results in stalk cell phenotypes by decreasing NICD destabilization (Potente et al., 2007). Similarly, SIRT1 regulates vessel branching by deacetylating FOXO1, a transcription factor that inhibits endothelial angiogenic activity (Eijkelenboom and Burgering, 2013). FOXO1 deficiency causes abnormal vascular branching that leads to embryonic lethality, demonstrating that metabolic signals can intricately regulate vascular branching (Furuyama et al., 2004; Oellerich and Potente, 2012).

Endothelial cell metabolism as a target in anti-angiogenesis therapy

Traditional anti-angiogenic therapies for cancer aim to starve tumors by inhibiting vascular growth signals, such as VEGF, to cut off their blood supply (Ferrara and Kerbel, 2005; Folkman, 1971). However, tumors often adapt to anti-angiogenic therapies with compensatory mechanisms that are problematic to treat. Moreover, eliminating the tumor blood supply can lead to a more-harsh and hostile tumor environment, promoting cancer cell invasion and metastasis (Carmeliet and Jain, 2011). Thus, new anti-angiogenic approaches are necessary to arrest the complexities of cancer biology (Goel et al., 2011). Recent discoveries about the role of endothelial cell metabolism in angiogenesis suggest that altering their metabolism is a viable anti-angiogenic strategy to treat tumors.

Indeed, the aforementioned findings indicate that PFKFB3-driven glycolysis is necessary for optimal stalk cell proliferation and tip cell migration (De Bock et al., 2013b). Interestingly, glycolysis modulates angiogenesis directly, without a reliance on genetic signals, therefore making it an attractive and relevant therapeutic target (Fig. 2). In contrast, both VEGF and Notch signaling regulate PFKFB3 expression and glycolytic flux in endothelial cells, indicating that glycolysis regulators are downstream of angiogenic signals and glycolysis is the 'engine' driving vessel sprouting in response to genetic signals (De Bock et al., 2013b). Also, because endothelial cells are 'addicted' to glucose and have very high glycolysis levels, endothelial metabolism might be an attractive target through which to modulate angiogenesis.

Recent studies show that pharmacological blockade of PFKFB3 with 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) causes a partial and transient reduction in glycolysis, and reduces angiogenesis in health and disease (Schoors et al., 2014a; Schoors et al., 2014b) (Fig. 3). Endothelial cells treated with 3PO *in vitro* display a 30–40% decrease in glycolysis that is similar to that resulting from silencing PFKFB3 expression, but this decrease is reversible when 3PO is removed. Furthermore, oxygen consumption and energy balance remain unchanged in PFKFB3-inhibited endothelial cells (Schoors et al., 2014b). 3PO decreases proliferation, migration and sprouting of endothelial cells, but increases the number of quiescent endothelial cells. In contrast, 2-deoxy-D-glucose (2DG) treatment almost completely inhibits glycolysis and is toxic to endothelial cells, but has yielded minimal success as a monotherapy (Raez et al., 2013; Tennant et al., 2010).

Treatment with 3PO *in vivo* reverses excessive vascular growth caused by genetic ablation of Notch or VEGFR1 in mice without causing significant endothelial cell death (Schoors et al., 2014a; Schoors et al., 2014b). Furthermore, 3PO prevents pathological angiogenesis associated with choroid neovascularization, retinal vasculopathy, skin inflammation and inflammatory bowel disease (Schoors et al., 2014b). Because minimally glycolytic cells are not (or are less) sensitive to 3PO, and because $<2.5\%$ of the total ATP in the body is produced by glycolysis, systemic effects of the 3PO treatment are minimal (Schoors et al., 2014a; Schoors et al., 2014b). Cancer cells and endothelial cells that undergo angiogenesis are, however, highly glycolytic, suggesting the potential of using 3PO as a tool to reduce endothelial cell (and cancer cell) hyperactivity and tumor-associated angiogenesis without destroying quiescent blood vessels. Indeed, systemic treatment with 3PO in the aforementioned models of pathological angiogenesis did not alter the vasculature of quiescent vessels (Schoors et al., 2014a).

Previous anti-glycolysis therapies for cancer attempted to block glycolysis completely and permanently, but concerns about the toxicity of circulating drug concentrations limited their

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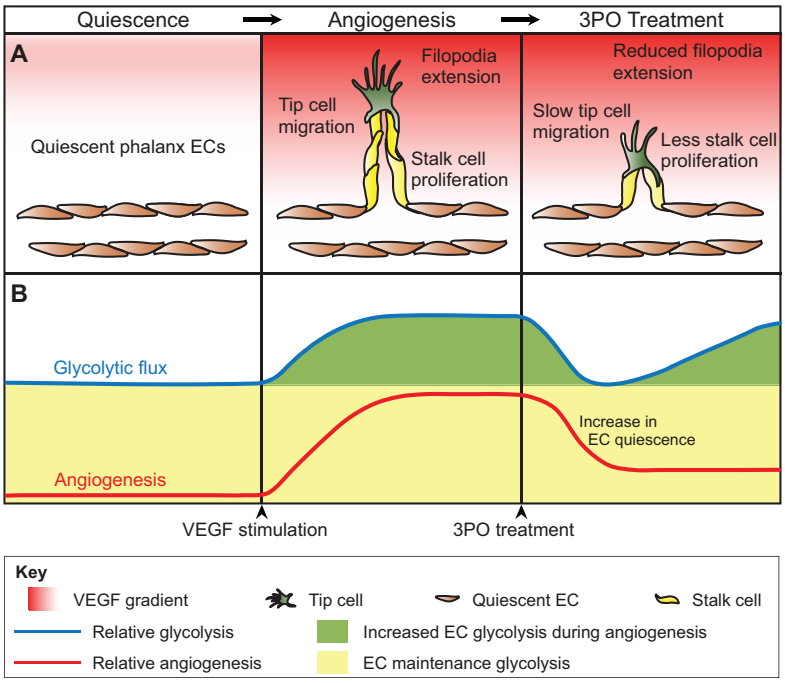

Fig. 3. Endothelial cell metabolism as a target for anti-angiogenesis treatment. Endothelial cell (EC) activity during quiescence, angiogenesis and after 3PO treatment (A), with corresponding relative changes in glycolytic flux in endothelial cells and in vessel sprouting activity (B). Quiescent endothelial cells maintain a steady glycolytic flux for ATP production. Endothelial cells increase PFKFB3-driven glycolysis that is necessary for migration, filopodia extension and proliferation during angiogenesis. Treatment with 3PO partially and transiently reduces glycolysis, sufficient to hinder endothelial angiogenic activity. Because PFKFB3-driven glycolysis is necessary for endothelial cell proliferation and migration (green), PFKFB3 blockade inhibits vessel sprouting by impairing angiogenic functions and promoting endothelial cell quiescence, but without affecting glycolysis for endothelial cell maintenance (yellow).

exploitation. (Granchi and Minutolo, 2012). The incomplete and transient decrease in glycolysis by PFKFB3 blockade with 3PO, however, induces endothelial cell quiescence without causing extensive damage, highlighting a paradigm-shifting strategy in anti-angiogenic treatment (Fig. 3). Because PFKFB3 blockade only reduces the hyper-glycolysis needed for endothelial cells to sprout, but does not affect the residual glycolysis necessary for baseline homeostasis, this strategy targets sprouting vessels more selectively, without affecting healthy pre-established vessels and evoking a compensatory metabolic rescue adaptation. Although continued work on PFKFB3 blockade as an anti-angiogenic therapy for cancer is necessary, these studies exhibit the potential and novelty of targeting abnormal vascular-cell metabolism associated with pathological vasculature.

Conclusions and future outlooks

Although the relationship between endothelial metabolism and angiogenesis has only recently been explored, understanding the functional importance of endothelial cell metabolic pathways holds significant therapeutic potential. The recent studies showing that pharmacological inhibition of a glycolytic enzyme (in this case, PFKFB3) can lead to positive therapeutic outcomes demand further exploration of the endothelial cell metabolome. Considering that PFKFB3 is just one metabolic enzyme involved in endothelial cell function, many questions about endothelial metabolism remain unanswered – questions regarding regulatory mechanisms (of glycolysis and other metabolic pathways), relative metabolic differences between endothelial cells and other cell types, metabolic regulation of endothelial function and the metabolic landscape of other vascular cell types, such as pericytes. Furthermore, the variety of molecules involved in cell metabolism, combined with the complexities of metabolic pathways, offer a multitude of targets for manipulating the microvasculature. Metabolic flux analysis, the use of transgenic animals and computational systems biology will be instrumental to determine their clinical relevance, and could lead to future angiogenic therapies that alter vascular metabolism.

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Competing interests

The authors declare no competing interests.

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